UNIVERSIDADE ESTADUAL DE MARINGÁ
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PÓS-GRADUAÇÃO EM BIOLOGIA COMPARADA

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Estudos citogenéticos e do DNA mitocondrial em espécies de Pimelodidae (Siluriformes): um enfoque na taxonomia e sistemática do grupo

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Orientador: Prof. Dr. Vladimir Pavan Margarido
Co-Orientador: Profa. Dra. Carla Simone Pavanelli

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Local de defesa: Sala 208, Bloco G80, *campus* da Universidade Estadual de Maringá.

Dedico este trabalho a minha mãe, minha maior inspiração.

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Estudos citogenéticos e do DNA mitocondrial em espécies de Pimelodidae (Siluriformes): um enfoque na taxonomia e sistemática do grupo.

RESUMO

Pimelodidae é uma família de peixes endêmica e amplamente distribuída na região Neotropical. Com 114 espécies as relações filogenéticas entre seus gêneros ainda não são totalmente compreendidas. Com o objetivo de gerar dados que possam auxiliar no entendimento das relações dentro desta família, foram realizados estudos citogenéticos e análises moleculares em dez espécies de três gêneros de Pimelodidae que ocorrem ao longo das bacias hidrográficas brasileiras. Os exemplares foram coletados no rio Piquiri, bacia do Alto rio Paraná; no rio Iguaçu, jusante às Cataratas do Iguaçu na bacia do Médio rio Paraná; no rio Iguaçu, bacia do Baixo rio Iguaçu; no rio Ijuí, bacia do Alto rio Uruguai; no rio Uruguai, bacia do baixo rio Uruguai; no rio Miranda, bacia do Alto rio Paraguai e no Lago Catalão, na bacia Amazonica. Buscando compreender as relações filogenéticas envolvendo *Iheringichthys* e *Bergiaria*, foram realizados estudos citogenéticos e do DNA mitocondrial em três populações de *Iheringichthys labrosus*, uma população determinada como *Iheringichthys* cf. *syi* e uma população de *Bergiaria westermanni*. Estudos citogenéticos básicos e moleculares foram realizados em *Pimelodus pantaneiro* do rio Miranda, bacia do Alto rio Paraguai e em *Pimelodus* cf. *blochii* da bacia Amazônica, além do mapeamento de sequências repetitivas U2 snDNA em seis espécies de *Pimelodus*. As análises mostraram a presença de 2n=56 cromossomos em todas as espécies, exceto em 4 exemplares de *Pimelodus* cf. *blochii*, reforçando a hipótese de número diplóide basal para a família. As AgRONs, confirmadas pela FISH-DNAr 18S, foram localizadas na região terminal de um par de cromossomos nas espécies de *Iheringichthys* e *Pimelodus*, exceto nos cariomorfos B e C de *Pimelodus* cf. *blochii* em que múltiplos cístrons de DNAr 18S foram observados, sendo essa uma condição nova para as espécies de Pimelodidae estudadas até o momento, pois todas as outras espécies possem esses sítios em apenas um par de cromossomo. O padrão de distribuição de heterocromatina encontrado é semelhante ao observado em outros Pimelodidae, com maiores divergências em *Pimelodus* cf. *blochii*, apresentando divergencias específicas entre as espécies permitindo sua caracterização, consistindo em um importante marcador. A localização das sequências de DNAr 5S em *Iheringichthys* permitiu diferenciar *I. labrosus* de *I.* cf. *syi*, podendo ser utilizado como marcador taxonômico. As análises de máxima verossimilhança e Bayesiana agruparam *B. westermanni* e *I.* cf. *syi* em um clado distinto das populações de *I. labrosus*. As diferenças citogenética e moleculares entre essas espécies sugerem que *I.* cf. *syi* deve ser realocado em *Bergiaria* ou que todas estas espécies devem fazer parte do mesmo gênero, reforçando a necessidade de uma revisão taxonômica das espécies destes gêneros. A localização do U2 snDNA foi observada em um par de cromossomos na região pericentromérica em todas as espécies de *Pimelodus*, aqui estudadas, demostrando uma provável manutenção do padrão de dispersão desses gene no genoma dessas espécies. Em *P. pantaneiro* o DNAr 5S foi encontrado em dois pares de cromossomos. As análises citogenéticas em *Pimelodus* cf. *blochii* mostram a ocorrência de três cariomorfos (A, B e C) com variações na localização e no número de sítios de DNAr 5S e 18S. Os dados citogenéticos e moleculares de *Iheringichthys* e *Bergiaria* possibilitam a melhor compreensão da história evolutiva ocorrida entra suas espécies e sugerem um novo arranjo para esses grupos. A descrição da organização cariotípica de *Pimelodus pantaneiro* e o mapeamento da sequências repetitivas U2 snDNA, pela primeira vez em espécies de *Pimelodus*, fornecem

importantes informações sobre a organização genômica desse grupo. Os resultados do presente estudo revelam dados que contribuem para o conhecimento da história evolutiva das espécies de Pimelodidae, permitem estabelecer e compreender as relações filogenéticas dentro dos grupos e auxiliam na identificação das espécies, fornecendo bases para planos de manejo e conservação adequados.

PALAVRAS-CHAVE: cyt b, DNA mitocondrial, FISH-DNAr, relações evolutivas, U2 snDNA.

Cytogenetic and mitochondrial DNA studies in Pimelodidae (Siluriformes) species: a focus on group taxonomy and systematics.

ABSTRACT

Pimelodidae is an endemic and widely distributed fish family in the Neotropical region. With 114 species, phylogenetic relationships among their genera are not yet fully understood. In order to generate data that could help in understanding the relationships within this family, cytogenetic studies and molecular analyzes were performed on ten species of three genera of Pimelodidae that occur along the Brazilian watersheds. The specimens were collected in the Piquiri River, Upper Parana River Basin; on the Iguaçu River, downstream of the Iguaçu Falls in the Middle Paraná River Basin; on the Iguaçu River, Lower Iguaçu River Basin; on the Ijuí River, Upper Uruguay River Basin; on the Uruguay River, the Uruguay River basin; on the Miranda River, the Upper Paraguay River Basin and on the Catalão Lake, in the Amazon basin. In order to understand the phylogenetic relationships involving *Iheringichthys* and *Bergiaria*, cytogenetic and mitochondrial DNA studies were performed in three populations of *Iheringichthys labrosus*, one population determined as *Iheringichthys* cf. *syi* and one population of *Bergiaria westermanni*. Basic and molecular cytogenetic studies were performed in *Pimelodus pantaneiro* of the Miranda River, Upper Paraguay River basin and in *Pimelodus* cf. *blochii* of the Amazon basin, besides the mapping of repetitive sequences U2 snDNA in six species of *Pimelodus*. The analyzes showed the presence of 2n = 56 chromosomes in all species, except in four specimens of *Pimelodus* cf. *blochii*, reinforcing the hypothesis of basal diploid number for the family. The AgNORs, confirmed by FISH-rDNA 18S, were located in the terminal region of a pair of chromosomes in the *Iheringichthys* and *Pimelodus* species, except in the B and C caryomorphs of *Pimelodus* cf. *blochii* in which multiple cistrons of 18S rDNA were observed, a new condition for the Pimelodidae species studied so far, since all other species have these sites in only one pair of chromosomes. The pattern of heterochromatin distribution found is similar to that observed in other Pimelodidae, with greater divergences in *Pimelodus* cf. *blochii*, presenting specific divergences among the species allowing its characterization, consisting of an important marker. The location of the 5S rDNA sequences in *Iheringichthys* allowed differentiating *I. labrosus* from *I.* cf. *syi*, and can be used as a taxonomic marker. The maximum likelihood and Bayesian analyzes grouped *B. westermanni* and *I.* cf. *syi* in a distinct clade of the populations of *I. labrosus*. The cytogenetic and molecular differences between these species suggest that *I.* cf. *syi* must be reallocated in *Bergiaria* or that all these species should be part of the same genus, reinforcing the need for a taxonomic revision of the species of these genera. The location of U2 snDNA was observed in a pair of chromosomes in the pericentromeric region of all *Pimelodus* species studied, demonstrating a possible maintenance of the dispersion pattern of these genes in the genome of these species. In *P. pantaneiro,* 5S rDNA was found on two pairs of chromosomes. Cytogenetic analyzes in *Pimelodus* cf. *blochii* show the occurrence of three caryomorphs (A, B and C) with variations in the location and number of 5S and 18S rDNA sites. The cytogenetic and molecular data of *Iheringichthys* and *Bergiaria* provide a better understanding of the evolutionary history of their species and suggest a new arrangement for these groups. The description of the karyotypic organization of *Pimelodus pantaneiro* and the mapping of the repetitive sequences U2 snDNA, for the first time in species of *Pimelodus*, provide important information about the genomic organization of this group. The results of

the present study reveal data that contribute to the knowledge of the evolutionary history of the Pimelodidae species, allow to establish and understand the phylogenetic relationships within the groups and to assist in the identification of the species, providing the basis for adequate management and conservation plans.

KEYWORDS: cyt b, DNA mitochondrial, DNAsn U2, evolutionary relations, FISH-DNAr.

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1. Introdução

Os peixes constituem o grupo mais numeroso e diversificado dos vertebrados, com cerca de 32.000 espécies divididas em 85 ordens e 536 famílias, apresentam uma enorme diversidade em sua morfologia, fisiologia, biologia e habitats ocupados (NELSON, 2006; 2016). Mais de 4.000 espécies já foram descritas para a região neotropical (NELSON, 2006), entretanto essa diversidade não é totalmente conhecida, pois, somente da Ordem Siluriformes estima-se que existam cerca de 1.120 espécies ainda não descritas nessa região (OTA et al., 2015). Essa grande diversidade é decorrente de fatores históricos e ecológicos, resultado de milhões de anos de evolução desde a ruptura da Godwana até o presente (RIBEIRO, 2006).

A maior parte da diversidade dos peixes de água doce e de outros organismos aquáticos da região Neotropical é decorrente de alterações na dinâmica dos rios e nas bacias hidrográficas da América do Sul durante o Cretáceo Superior e o Cenozóico. Estas águas continentais foram, ao mesmo tempo, os agentes e o produto do processo evolutivo. No neotrópico rupturas nos sistemas de drenagem, com a formação de novas divisões nas bacias hidrográficas, promoveram eventos de vicariância e divergência alopátrica, e dessa forma propiciaram a diversificação biótica. O rompimento de divisões de bacias hidrográficas e a junção dos sistemas de drenagem promoveram a mistura e o enriquecimento de biotas. A emergência de novas terras promoveu a expansão da fauna aquática (LUNDBERG, 1998).

Na região Neotropical os peixes de água doce ocupam uma ampla diversidade de hábitats aquáticos continentais, estão presentes em lagos Alpinos, corredeiras torrencias nos Andes, planícies de inundação de rios baixos e savanas, estuários sazonalmente inundados, florestas alagadas e canais profundos de grandes rios de baixa altitude, canais subterrâneos e cavernas no escudo brasileiro (REIS et al., 2016). O conhecimento da ictiofauna desta região é essencial para o desenvolvimento de ações que possam contribuir para modelos de preservação e manejo nestes sistemas aquáticos neotropicais (VARI; MALABARBA, 1998).

Historicamente, a identificação e descrição das espécies de peixes baseia-se, fundamentalmente, em caracteres morfológicos, tanto externos, como o padrão corporal, as nadadeiras e o padrão de pigmentação, quanto internos, como as características do esqueleto e dos órgãos, além de outros caracteres (STRAUSS; BOND, 1990). Porém, em alguns casos os aspectos morfológicos podem ter seu uso limitado na delimitação de espécies devido a ocorrência de pequenas diferenças entre as espécies (TELETCHEA, 2009). As espécies crípticas são um bom exemplo da complexidade da diversidade biológica e da delimitação das espécies. Segundo Bickford et al. (2007), o verdadeiro número de espécies biológicas,

possivelmente, é maior do que a contagem atual, pois a especiação nem sempre é acompanhada de mudanças morfológicas, sendo assim a delimitação de espécie crípticas baseada em caracteres puramente morfológicos estaria subestimando a real diversidade. Essas dificuldades desencadearam a busca por novos métodos para ajudar a identificar as espécies de peixes (TELETCHEA, 2009).

Os estudos citogenéticos buscam analisar e explicar a estrutura e o comportamento cromossômico, que garantem a conservação, transmissão e ordenação da informação genética no desenvolvimento dos organismos, além de estudar os seus mecanismos de controle, variação, as consequências genéticas e as implicações evolutivas (LACADENA, 1996). A citogenética convencional é um poderoso e indispensável marcador para a caracterização e identificação das espécies de peixes (CIOFFI et al., 2018). Em conjunto com a outras ferramentas, como dados de morfologia, biogeografia, comportamento e genética molecular, a citogenética possibilita maior conhecimento da história evolutiva dos organismos (OLIVEIRA et al., 2009).

O uso de marcadores genéticos foi expandido, consideravelmente, na última década. Associados aos métodos tradicionais taxonômicos podem ajudar a clarear as relações, principalmente entre espécies que não são distinguíveis morfologicamente, mas que são geneticamente distintas, ou seja, podem prover valiosas informações para identificação das espécies, complementando os dados taxonômicos (TELETCHEA, 2009).

2. Revisão Bibliográfica

2.1. O Sistema Hidrográfico Brasileiro

O Brasil possui a maior rede hidrográfica do mundo, bem como uma das maiores diversidades de peixes de água doce. Existem cerca de 2.500 espécies descritas para o território brasileiro; porém, com o incremento de estudos e levantamentos em locais ainda não explorados, novas espécies devem ser descritas (GRAÇA; PAVANELLI, 2007).

O Ministério do Meio Ambiente brasileiro por meio do Conselho Nacional de Recursos Hídricos – CNRH, a partir da resolução nº32, de 15 de Outubro de 2003 instituiu a Divisão Hidrográfica Nacional, estabelecendo doze regiões Hidrográficas, sendo elas: Amazônica, Tocantins/Araguaia, Atlântico Nordeste Ocidental, Parnaíba, Atlântico Nordeste Oriental, São Francisco, Atlântico Leste, Atlântico Sudeste, Paraná, Uruguai, Atlântico Sul e Paraguai (CNRH, 2003).

Estudo sobre a relação entre a ictiofauna de água doce da região Neotropical realizado por Albert e Carvalho (2011), a partir de 32 clados de peixes, mostra que as bacias do Baixo rio Paraná e do Baixo rio Uruguai possuem grande semelhança ictiofaunística entre elas e com a bacia do rio Paraguai. Já a ictiofauna do Alto rio Paraná é mais semelhante à dos rios Paraíba do Sul, Iguaçu, São Francisco e Parnaíba. A ictiofauna da bacia do Alto rio Uruguai apresenta relação com a ictiofauna das bacias do rio Amazonas e Tocantins-Araguaia, já o Baixo rio Uruguai possui maior semelhança com as bacias do Baixo rio Paraná e com a bacia do rio Paraguai. Esta similaridade entre a fauna do Alto rio Uruguai e a bacia Amazônica possivelmente é resultado de extinções que ocorreram em outras porções da bacia do Prata, sendo um dos poucos casos de semelhança significativa na fauna de bacias não contíguas (ALBERT; CARVALHO, 2011). Os principais fatores que influenciaram a composição e as semelhanças entre as bacias hidrográficas brasileiras foram isolamento geográfico, evolução progressiva, diferenciação local e trocas de fauna (MENEZES, 1972).

2.2. A bacia Amazônica

A bacia Amazônica é a maior rede hidrográfica da Terra, com uma área de drenagem de mais de 6 milhões de km², desde as nascentes nos Andes Peruanos até sua foz no ceano Atlântico, possui 63,88% da sua extensão em território brasileiro (MMA, 2006; ANA, 2012).

Essa bacia drena territórios da Colômbia, do Equador, do Peru, da Bolívia e do Brasil (REIS et al., 2016).

No Brasil, a bacia Amazônica ocupa uma área de 3.870.000 km², o que representa 45% do território nacional. Caracterizada pela grande disponibilidade hídrica abrange sete estados: Acre, Amazonas, Rondônia, Roraima, Amapá, Pará e Mato Grosso, e tem como seus principais rios de destaque Purus, Juruá, Xingu, Solimões, Madeira, Negro e Guaporé (ANA, 2015). A bacia tem ao Norte e ao Sul o Escudo das Guianas e o Escudo Central Brasileiro, respectivamente, com áreas de cobertura vegetal de floresta úmida tropical e outras com cerrado, entre esses dois escudos tem-se a planície fluvial Amazônica coberta em sua maioria por floresta tropical úmida (FIZOLA et al., 2009).

Na bacia Amazônica uma imensa diversidade ictiofaunística é observada, com 2411 espécies distribuídas em 57 famílias e 525 gêneros, sendo que 111 gêneros e 1089 espécies são endêmicas dessa bacia e muitas outras ainda devem ser descritas com a ampliação dos estudos nessa região (REIS et al., 2016). A planície alagável amazônica é uma região de terras úmidas que oscila entre períodos de condições terrestres ou aquáticas. A inundação anual pelo rio Amazonas conecta a planície de inundação aos canais dos rios e lagos permanentes e durante os períodos de pouca água aos planaltos não inundados. Nas proximidades da cidade de Manaus as cheias formam um ciclo anual que cria uma fase terrestre e uma fase aquática, que são as estações secas e chuvosas (JUNK, 2001).

O lago Catalão está localizado na região de confluência dos rio Negro e rio Solimões próximo a cidade de Manaus na planície alagável amazônica, é inundado periodicamente por ambos os rios, recebendo grande quantidade de água e sedimentos. Essa conexão varia ao longo do ano, alternando períodos de ligação com pelo menos um dos rios e isolamento periódico de ambos os sistemas (BRITO et al. 2014).

2.3. A bacia do rio Paraná

A bacia do rio Paraná abrange parte do Distrito Federal e dos estados do Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Santa Catarina e São Paulo, incluindo as bacias do rio Paraná e do rio Paraguai (MALFATTI et al., 2018). Drena uma área de 879.873 km² o que representa 10% do território brasileiro. É dividida em curso superior, da sua origem na confluência dos rios Grande e Paranaíba até a barragem de Itaipu; curso médio, ao longo das fronteiras da Argentina e do Paraguai, com confluência no rio Paraguai próximo a Corrientes (Argentina); curso inferior, da vasta planície aluvial até o sul de Rosário e o curso

delta, a partir da confluência do rio Carcarana até o estuário do rio de La Plata (STEVAUX, 2000).

Por ser uma região que apresenta alta densidade populacional, intensiva atividade agrícola e pecuária, centros industriais e barragens hidrelétricas, são poucas as áreas que ainda não sofreram influência humana na bacia do rio Paraná (AGOSTINHO et al., 2007). A intensa utilização de produtos químicos na agricultura e a eliminação da vegetação ripária têm contribuído para a diminuição da qualidade da água dos principais afluentes do rio Paraná (AGOSTINHO et al., 1995). Essa bacia, apresenta grande destaque no contexto nacional por estar situada na região de maior desenvolvimento econômico do pais, além de possuir a maior demanda por recursos hídricos, principalmente devido ao uso industrial (ANA, 2015).

No Alto rio Paraná, entre o Porto Primavera e a barragem de Itaipu, incluindo as áreas adjacentes existem 211 espécies, de 126 gêneros, 41 famílias e 10 ordens (OTA et al., 2018). Entretanto, este número de espécies ainda pode ser subestimado, pois apesar da ictiofauna do Alto rio Paraná ser uma das mais conhecidas e melhor estudadas da América do Sul, a descoberta de novas espécies apresenta-se em crescimento contínuo.

O rio Piquiri é um afluente de margem esquerda do rio Paraná, possui uma área de drenagem de 24.156 km² que corresponde a 12,1% do estado do Paraná (ARAÚJO et al., 2018). Com 485 km de extensão este rio nasce no Terceiro Planalto, na região centro-sul do estado, no município de Campina do Simão com foz no rio Paraná (SEMA, 2013). Seus principais afluentes de margem direita são os rios Cantu, Goio-Bang e Goioerê e o rio do Cobre na margem esquerda. A altitude ao longo da bacia varia de 410 a 990 metros (PEREIRA; SCROCCARO, 2010). A ictiofauna desse rio é extremamente rica, com 152 espécies catalogadas, distribuídas em 8 ordens, 31 famílias e 89 gêneros possuí espécies raras, endêmicas, ameaçadas e migratórias, além da provável ocorrência de espécies ainda desconhecidas pela ciências (CAVALLI et al., 2018).

A bacia do Baixo rio Paraná se estende a partir da Usina Hidroelétrica de Itaipu até a conexão com o rio Paraguai. Esta região também recebe águas do rio Iguaçu, que tem sua comunidade ictiofaunística dividida pela presença das Cataratas do Iguaçu. A porção que ocorre acima das quedas (montante) é denominada bacia do Baixo rio Iguaçu e a porção abaixo das quedas (jusante) bacia do Baixo rio Paraná. Trabalhos no trecho jusante às Cataratas do Iguaçu são escassos, por essa região ser área de preservação ambiental fiscalizada por órgãos federais, o que dificulta o acesso, sendo que este trecho pode ser representado pela ictiofauna das bacias do rio Paraná e Paraguai (PAIZ, 2013).

O rio Iguaçu é formado pelos rios Iraí e Atuba, no município de Curitiba, na divisa com Pinhais. Cobre uma superfície de 70.800 km2 e é maior rio totalmente paranaense. Possui 1320 km de curso cruzando os três planaltos até desaguar no rio Paraná. Seus principais afluentes são os rios Iraí, Atuba, Passaúna, Barigui, Verde, Passa Dois, da Várzea, Chopim, Palmital, Cavernoso, Adelaide, Gonçalves Dias, Castro Alves, Ampére e Silva Jardim. A bacia do rio Iguaçu drena aproximadamente 70.800 km², nela estão presentes as maiores quedas em volume de água do planeta que despencam em uma profunda fenda de erosão, formando 272 saltos, com cerca 72 metros de desnível, e volume médio de 1.551 m³/s em Foz do Iguaçu (PEREIRA; SCROCCARO, 2010). A bacia do rio Iguaçu está dividida em três Unidades Hidrográficas, Baixo Iguaçu, Médio Iguaçu e Alto Iguaçu (Resolução 49/2006/cerh/pr). Segundo estudo realizado por Ingenito et al. (2004), a ictiofauna do rio Iguaçu possui 84 espécies; entretanto, estudos mostram que somente para a porção do baixo rio Iguaçu já foi registrada a ocorrência de 106 espécies (BAUMGARTNER et al., 2012)

2.4. A bacia do rio Paraguai

A bacia hidrográfica do rio Paraguai possui 363.446 km² de área, ocupando 4,3% do território nacional e abrangendo parte dos estados do Mato grosso do Sul e Mato Grosso (ANA, 2015). Essa bacia é tributária da bacia do rio Paraná que somadas a bacia do rio Uruguai constituem a bacia do rio da Prata, que se estende por 3.190.000 km² e ocupa territórios do Brasil, Bolívia, Paraguai, Uruguai e Argentina (PEREIRA et al., 2014). Com nascente na Serra dos Parecis, no Mato Grosso, percorre um percurso de 28.582 km desde a nascente até sua foz na Argentina. Banha margens exclusivamente brasileiras em 1.300 km e compartilha suas margens com a Bolívia por 48 km e com o Paraguai por 322 km. Divide-se em Pantanal (36% da bacia) e Planalto Paraguai. Seus principais rios são o Paraguai, Taquari, São Lourenço, Cuiabá, Itiquira, Miranda, Aquidauana, Negro, Apa e Jauru (ANA, 2015).

Essa região conquistou posição de destaque por abrigar uma das maiores extensões úmidas continuas do planeta, o Pantanal Mato-grossense. Foi declarado como Patrimônio Nacional pela constituição Federal de 1988 e Patrimônio Natural da humanidade em 2002 pela UNESCO (MMA, 2006). A ictiofauna dessa bacia apresenta grande diversidade de espécies, tendo sido catalogadas 257 espécies por Froehlich et al. (2017) para a região do Alto Paraguai.

A bacia hidrográfica do rio Miranda faz parte da bacia do Alto rio Paraguai e é uma das mais importantes do Mato grosso do Sul. Abrange uma área de 44.740,5 km² com uma

população de aproximadamente 1.131.000 habitantes ao longo dos 23 municípios que fazem parte dessa bacia (PEREIRA et al., 2014). O rio Miranda, tem sua nascente nos limites do município de Ponta Porã, Guia Lopes da Laguna e Jardim, e sua foz no rio Paraguai. Seus principais contribuinte de margem direita são o rio Aquidauana, com 684 km de extensão, ribeirão Taquarussu com 147,89 km e o rio Dois Irmão com 177.06 km (IMASUL, 2015). A bacia do rio Miranda possui 143 espécies catalogadas, de sete ordens e 30 famílias, sedo Characiformes e Siluriformes as ordens predominantes e Characidae e Loricariidae as famílias com o maior número de espécies (FERREIRA et al., 2017).

2.5. A bacia do rio Uruguai

O rio Uruguai é formado pela confluência dos rios Pelotas e do Peixe, com 2200 km de extensão divide os estados do Rio Grande do Sul e de Santa Catarina e o Brasil da Argentina e do Uruguai, tendo sua foz no rio da Prata (ANA, 2015). A bacia abrange uma área de 365.000 km², o que representa 11,8% da superfície total da bacia do Prata, está localizada 42% no Brasil, 41,1% no Uruguai e 16,4% na Argentina. Com 1.600 km de extensão e vazão média de 5.500 m³/segundo, o rio Uruguai tem como principais afluentes os rios Negro e Cuareim (LABORDE et al., 2008). A região hidrográfica do Uruguai pode ser dividida em Alto, Médio e Baixo rio Uruguai. A porção Alto e Médio é delimitada pelo Salto do Yucumã e a porção Médio e Baixo pelo Salto Grande na divisa do Uruguai com a Argentina (SILVA, 2011).

Cerca de 3,8 milhões de pessoas vivem na porção brasileira da região hidrográfica do rio Uruguai, que abrange 384 municípios. Esta região concentra importantes atividades agroindustriais e reconhecido potencial hidrelétrico. Possui clima subtropical com chuvas ao longo de todo o ano, mas com maior concentração no período de maio a setembro (PAIM; ORTIZ, 2006). Os estudos sobre a composição da ictiofauna desta bacia são escassos. Em uma breve revisão bibliográfica Hahn e Câmara (2000) levantaram 251 espécies nesta bacia, entretanto estima-se que esse número seja ainda maior.

A bacia hidrográfica do rio Ijuí possui área de 10.849 km², o seu principal rio, de mesmo nome, possui extremo potencial hidrelétrico ainda pouco explorado. O uso do solo é marcado pelo cultivo de soja (BRASIL, 2006). Ferreira et al. (2011) identificaram a presença de 77 espécies de peixes em estudo de levantamento em três Pequenas Centrais Hidrelétricas (PCHs) na porção do alto rio Ijuí. As famílias com maior número de representantes foram Loricariidae, Characidae e Cichilidae.

Estudo sobre a relação entre a ictiofauna de água doce da região Neotropical realizado por Albert e Carvalho (2011), a partir de 32 clados de peixes, mostra que a composição de espécies do Alto e Baixo rio Uruguai são consideravelmente distintas.

2.6. Considerações em Siluriformes e Pimelodidae

Os peixes da ordem Siluriformes são popularmente conhecidos como “bagres”, apresentam cerca de 3.090 espécies, divididas em 478 gêneros e 36 famílias (FERRARIS, 2007). Possuem o corpo nu ou coberto por placas ósseas, apresentam acúleo nas nadadeiras peitorais e dorsais, sendo que em algumas espécies está associado a uma glândula de veneno, podendo causar ferimentos graves. Geralmente apresentam quatro pares de barbilhões sensitivos (NELSON, 2006). São os mais diversos e amplamente distribuídos dentro do grupo Ostariophysi, sendo encontrados na América do Sul, América do Norte, Eurásia e África. A maioria das espécies é de água doce, embora existam algumas famílias, como Auchenipteridae e Pangasiidae, que possuem representantes de regiões de estuário, e Ariidae e Plotostidae com espécies marinhas (PINNA, 1998).

Pimelodidade é uma família de peixes de água doce, endêmica da região neotropical, que possui 114 espécies válidas, sendo 8 descritas nos últimos dez anos (NELSON et al., 2016; ESCHMEYER; FONG, 2018). As espécies dessa família apresentam uma grande diversidade de formas, cores e em sua história natural, sendo abundante e diversificados nas grandes bacias cis- Andinas e com menor diversidade nas bacias ao longo da costa caribenha (LUNDBERG et al., 2012). Morfologicamente, as espécies dessa família, representam um modelo quase arquetípico de um bagre, com o corpo nu (sem placas ósseas externas), a nadadeira adiposa grande e três pares de barbilhões longos. A coloração do corpo pode variar de cinza uniforme até padrões bem elaborados de listras, pintas e manchas escuras e claras. A maior parte das espécies de Pimelodidae possui hábitos carnívoros, com algumas espécies que representam predadores de topo de cadeia alimentar, enquanto outras consomem frutos ou são onívoras. As espécies de *Pimelodus*, em certas épocas do ano, podem formar grandes cardumes e muitos Pimelodidae possuem estratégia reprodutiva sazonal com desova durante a fase de enchente dos rios (ROCHA; ZUANON, 2013).

Pimelodinae, Heptapterinae e Pseudopimelodinae constituíam subfamílias dentro de Pimelodidae até que estudos filogenéticos realizados por Pinna (1998) revelaram que estas subfamílias possuem maior proximidade com outros grupos da ordem Siluriformes do que entre eles mesmos, sendo então elevadas à categoria de família, sendo denominadas

Pimelodidae, Heptapteridae e Pseudopimelodidae (PINNA, 1998). Apesar de vários estudos sobre as relações filogenéticas entre as espécies de Pimelodidae terem sido realizados (LUNDBERG et al., 1991; LUNDBERG; AKAMA, 2005; HARDMANN; LUNDBERG, 2006; LUNDBERG et al., 2011; 2012), muitas relações ainda não são totalmente esclarecidas.

Entre os 32 gêneros de Pimelodidae, *Pimelodus* é considerado o mais numeroso e diversificado, com 36 espécies válidas (ESCHMEYER; FONG, 2018). No entanto, essas espécies tem sido agrupadas sem um caractere exclusivo e, possivelmente, não representam a diversidade real do gênero que é considerado polifilético. O estudo das relações filogenéticas em Pimelodidae reforça a não monofilia de *Pimelodus* ao agrupar suas espécies representativas em quatro clados contendo outros gêneros, porém o incremento dos estudos certamente provocará a divisão dessas espécies em gêneros monofiléticos (LUNDBERG et al., 2011).

Outro gênero que possui problemas filogenéticos é *Iheringichthys.* Com três espécies, *Iheringichthys labrosus*, localidade-tipo rio de La Plata; *Iheringichthys megalops* descrita a partir de um único exemplar coletado no rio Paraguai; e *Iheringichthys syi*, recentemente descrita no reservatório de Jupiá, bacia do alto rio Paraná, possui questões não resolvidas com *Bergiaria*. *Bergiaria* é representada por duas espécies de pequeno porte, *Bergiaria platana* descrita na bacia do rio Paraná, e *Bergiaria westermanni* endêmica da bacia do rio São Francisco. A classificação dessas espécies em gêneros distintos tem sido questionada, e Rocha (2012), a partir de análises morfológicas, sugeriu que *Bergiaria* seja considerada sinônimo de *Iheringichthys*.

2.7. Estudos citogenéticos com ênfase em Pimelodidae

Estudos citogenéticos em Pimelodidae estão disponíveis para 35 espécies (revisão em GIRARDI et al., 2018). Esses dados mostram que apenas seis possuem número diploide diferente de 56 cromossomos, sendo elas *Calophysus macropterus*, 2n=50 (RAMIREZ-GIL et al., 1998), *Pimelodus fur*, 2n=54 (GARCIA; MOREIRA-FILHO, 2008), *Pinirampus pirinampu*, 2n=50 (VASCONCELOS; MARTINS-SANTOS, 2000), *Luciopimelodus pati*, 2n=50 (SÁNCHEZ ET AL., 2000) e *Megalonema platanum*, 2n=54 (SÁNCHEZ et at., 2000; CARVALHO et al., 2011) e, a mais recentemente estudada, *Pimelodus blochii* com 2n=58 cromossomos (FONSECA et al., 2018).

Nos Siluriformes, em muitas espécies estudadas citogeneticamente, cromossomos Bs tem sido encontrados, incluindo membros das famílias Callichthyidae, Heptapteridae,

Loricariidae, Pimelodidae, e Trichomycteridae (CARVALHO et al., 2008). Lui et al. (2009) relatou a presença desses elementos em Auchenipteridae (*Parauchenipterus galeatus*) e Takagui et al. (2017) em Doradidae (*Platydoras armatulus*, *Pterodoras granulosus* e *Ossancora punctata*. Em Pimelodidae eles foram encontrados em *Bergiaria westermanni* (DIAS; FORESTI, 1993; MALIMPENSA et al., 2018), *Iheringichthys labrosus* (CARVALHO; DIAS 2005; CARVALHO et al., 2004; VISSOTO et al., 1999), *Megalonema platanum* (CARVALHO et al., 2011), *Pimelodus ortmanni* (BORIN; MARTINS-SANTOS, 2004; GIRARDI et al., 2018) e *Pimelodus* sp. (BORIN; MARTINS-SANTOS, 2004). Esses cromossomos supranumerararios tem sido encontrado em muitos grupos de animais e plantas, são elementos adicionais e dispensáveis que estão presentes em alguns indivíduos de algumas espécies (CAMACHO, 2005). Originários dos cromossomos do complemento A, intra ou interespecífico, com os quais não se recombinam, além de não apresentarem comportamento meiótico regular, os cromossomos Bs, são amplamente distribuídos, estando presentes em cerca de 15% dos eucariotos, sendo encontrados em fungos, plantas e animais, tanto vertebrados quanto invertebrados (CAMACHO, 2005; BEUKEBOOM, 1994). A origem desses cromossomos e a existência ou não de funções são questões que intrigam os citogeneticistas desde a descoberta desses elementos nos cariótipos. Com o advento da citogenética molecular, após o desenvolvimento da técnica de Hibridização *in situ* por fluorescência (FISH), muitas sequencias presente nos cromossomos do complemento A tem sido mapeadas e descritas nos cromossomos Bs, tais como a presença de DNAr 18S e genes de histonas (H1) em *Astyanax fasciatus* e *Astyanax paranae* (SILVA et al., 2016), DNA repetitivo As51 em *Astyanax scabripinnis* (MESTRINER et al., 2000) e *Astyanax fasciatus* (SILVA et al., 2016), e DNA microssatélite em *Characidium alipioi* (SERRANO et al., 2017), entre outros. Em *Bergiaria westermanni*, DNAr 18S e sequencias microssatélites $(GATA)_n$ foram observadas nos cromossomos Bs, confirmando a provável origem a partir dos cromossomos do complemento A (MALIMPENSA et al., 2018).

O bandamento C, em Pimelodidae, evidencia um padrão de poucas regiões heterocromáticas ao longo dos cariótipos. Essas marcações são, em sua maioria, pálidas e distribuídas nas regiões dos centrômeros e telômeros e algumas nas regiões intersticiais e pericentroméricas, para algumas espécies permite caracteriza-las e distinguir das demais devido a presença de marcações especificas (CARVALHO et al., 2004; RIBEIRO et al., 2008, TRECO et al., 2009; MORAES-NETO et al., 2011).

Os DNAs repetitivos são classificados em elementos intercalados ou organizados em tandem (CIOFFI; BERTOLLO, 2012). Essas sequências são comuns no genoma dos

eucariotos e caracterizam-se por ampla heterogeneidade e diversidade de famílias repetidas, além de compreenderem uma grande porção do genoma de muitas espécies (CHARLESWORTH et al., 1994). Os elementos intercalados são representados pelos elementos transponíveis (TEs) que são amplamente distribuídos ao longo do genoma, enquanto as sequências organizadas em tandem incluem as famílias multigênicas, tais como os RNAs ribossomais (RNAr) e os genes das histonas, e os satélites, micro e minissatélites. Essas sequências, junto com as menos repetidas, que incluem baixo número de cópias por sequência e DNAs com pouca repetição, constituem a arquitetura do genoma nuclear (CIOFFI; BERTOLLO, 2012).

As famílias multigênicas apresentam grande diversidade, tanto no número de constituintes quanto na organização genômica (BUENO et al., 2013). São compostas por centenas e milhares de cópias de genes, incluindo as famílias dos genes de RNAr, das histonas (CIOFFI; BERTOLLO, 2012) e dos snRNAs (RNAs *small nucleares*). Os snRNAs (U1, U2, U4, U5 e U6), em conjunto com mais de 200 proteínas formam o spliceossomo, que consiste na união de ribonucleoproteínas e é responsável pelo processamento do precursor do RNAm. Os snRNAs são altamente conservados e desempenham papel central em todos os aspectos da reação de processamento do RNA (VALADKHAN, 2005), entretanto, estudos sobre a distribuição dessas sequências nos peixes são escassos. Estudos moleculares focados nos genes e nas sequências de DNA estão, em sua maioria, restritas a sequências repetitivas, como DNAs satélites, elementos transponíveis (TEs) e DNAr. Os poucos estudos de mapeamento dos genes de U2 snRNA mostram um padrão de conservação dessas sequências ao longo do genoma dos peixes, com sítios majoritariamente em um par de cromossomos e com poucos relatos de associação com DNAr 5S ou 18S.

O mapeamento dos DNAs repetitivos são pouco explorado, tanto em Pimelodidae, quanto em Siluriformes, apresentando a maior quantidade de dados sobre os DNA ribossomais 5S e 18S. Pimelodidae possui um padrão de DNAr 18S simples na região dos telômeros e DNAr 5S com sítios variáveis quanto ao número e a localização (GIRARDI et al., 2018). Em *Bergiaria westermanni*, foi realizado o mapeamento citogenético de U2 snDNA, Histonas (H1, H3 e H4), sequencias $(GATA)_n$ e teloméricas, sendo o primeiro estudo sobre a organização dessas sequências no genoma de Pimelodidae. O U2 snDNA foi identificado na região intersticial de um par de cromossomos metacêntricos em localização sintênica ao DNAr 5S e as sequências histônicas (MALIMPENSA et al., 2018). H1, H3 e H4 também foram observadas em mais um par de cromossomos. As sequências $(GATA)_n$ estiveram

dispersas ao longo dos cromossomos do conjunto A e B e as sondas teloméricas não revelaram sinais de recombinação cromossômica (MALIMPENSA et al., 2018).

2.8. Estudos moleculares com ênfase em Pimelodidae

Existe, atualmente, uma grande variedade de métodos baseado no DNA para o uso na identificação das espécies de peixes, eles variam no alcance da aplicação, na complexidade e nos custos. Entre os marcadores de DNA o uso de genes mitocondriais, principalmente do citocromo *b*, tem sido amplamente usados. Os fatores que favorecem o uso desses genes são: o grande número de cópias do DNA mitocondrial nas células, o que torna mais fácil sua amplificação em relação ao genoma nuclear; por ser um genoma pequeno e circular, aproximadamente 16 kb; sua herança materna em muitas espécies animais; ausência de recombinação e por evoluir muito mais rapidamente que o DNA nuclear, permitindo assim a diferenciação e identificação de espécies proximamente relacionadas. O gene citrocromo *b* é muito utilizado em estudos de filogenia, ele apresenta regiões conservadas possibilitando a amplificação do DNA de muitas espécies com o uso de primers “universais”, e regiões com grande variabilidade o que torna possível identificar espécies próximas (TELETCHEA, 2009).

Os estudos moleculares nos Siluriformes, têm focado na resolução de relações filogenéticas complexas com maior nível taxonômico, geralmente entre ou dentro das famílias (VERGARA et al., 2008). Sullivan et al. (2013), utilizando sequências nucleares *rag1* e *rag2* e DNA mitocondrial 12S e 16S, estudaram as relações entre o grupo Pimelodoidea; os resultados suportam o monofiletismo de Heptateridae, Pimelodidae e Pseudopimelodidae.

Alguns estudos têm caracterizado as relações evolutivas entre espécies do mesmo gênero, e em Pimelodidae estes estudos vêm ganhando impulso ao longo dos anos, embora escassos. Renesto et al. (2000), através do uso de isoenzimas, evidenciaram a existência de duas espécies de *Pimelodus* no rio Iguaçu. Hardmann; Lundberg (2006) estudaram as relações entre as espécies do grupo "phractocephaline" a partir das sequências do citocromo *b*, *ND1* e *rag2*. Vergara et al. (2008) fizeram uma análise filogeográfica em *Pimelodus albicans* utilizando sequências do citocromo *b* do DNA mitocondrial. Torrico et al. (2009), a partir de sequências de citocromo *b* e da região controle do DNA mitocondrial, descreveram a filogenia molecular de *Pseudoplatystoma*. Bignotto et al. (2009) analisaram as relações entre *Pseudoplatystoma corruscans* e *Pseudoplatystoma reticulatum* da bacia do rio Paraná, com

sequências de DNA mitocondrial. Ferreira et al. (2014) analisaram as relações entre quatro espécies de *Pimelodus* utilizando análises citogenéticas, RFLP (Polimorfismo no Comprimento de Fragmentos de Restrição) e DNA mitocondrial. Estudos sobre as relações filogenéticas em Pimelodidae foram realizados por Lundberg et al. (1991), Lundberg e Akama (2005), Hardmann e Lundberg (2006), Lundberg et al. (2011; 2012), entretanto muitas questões entre e intra gêneros ainda permanecem incertas.

2.9. Citogenética e genética molecular como ferramentas para auxiliar a taxonomia

A taxonomia é fundamental para o conhecimento da biodiversidade. Além de ser necessária, direta ou indiretamente para muitas disciplinas, é crucial para a conservação da biodiversidade, pois antes de proteger os táxons é necessário conhece-los e também porque ações de conservação não podem ser desenvolvidas para espécies não descritas. Problemas na delimitação taxonômica podem comprometer a pesquisa cientifica nas mais diversas áreas do conhecimento ao subestimar ou superestimar o número de espécies (ELY et al., 2010).

A taxonomia tradicional se baseia na identificação das espécies com base em caracteres morfológicos, entretanto essa abordagem, nem sempre é suficiente para identificar espécies crípticas ou com pequenas diferenças morfológicas (TELETCHEA, 2009). Nesse sentido, a integração de ferramentas de outras áreas do conhecimento, como a citogenética e a genética molecular, pode ser fundamental para o real conhecimento da biodiversidade e a correta implementação de medidas de conservação.

A análise integrada da citogenética e da genética molecular tem demostrado ser uma excelente abordagem para a resolução de problemáticas envolvendo os peixes. Essa união de ferramentas tem sido crucial para a identificação de novas espécies ou complexos de espécies como é o caso de *Hypostomus ancistroides* (ROCHA-REIS et al., 2018), para a compreensão das relações filogenéticas dentro das famílias (DUARTE et al., 2018; NIRCHIO et al., 2018), para a identificação de espécies crípticas como *Eigenmannia* (ARAYA-JAIME et al., 2017) e *Ancistrus* (PRIZON et al., 2017) e na determinação de novos gêneros (RAMIREZ et al., 2016).

Considerando a carência de dados citogenéticos e moleculares e a problemática taxonômica e sistemática em alguns gêneros de Pimelodidae, a utilização integrada da citogenética e da genética molecular visa contribuir para a resolução destes conflitos, bem

como fornecer dados que auxiliarão na compreensão da história evolutiva desse importante grupo de peixes neotropicais.

3. Referências

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CAPÍTULO 2

Contributions molecular and karyotipic for the identification and classification of *Bergiaria westermanni* Lutken, 1874 and *Iheringichthys* Eigenmann & Norris 1900 species from São Francisco, Paraná and Uruguay river basins

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Contributions molecular and karyotype for the identification and classification of *Bergiaria westermanni* Lutken, 1874 and *Iheringichthys* Eigenmann & Norris 1900 species from São Francisco, Paraná and Uruguay river basins

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Running headline: Cytogenetics and molecular in *Bergiaria* and *Iheringichthys*

ABSTRACT

There are more than 100 Pimelodidae species, including three in the genus *Iheringichthys* and two in the genus *Bergiaria*, although the validity of these genera has been questioned. Analyses cytogenetic the four populations the *Iheringichthys* were carried and molecular markers (cytocrome b) were performed in *Bergiaria westermanni* and *Iheringichthys* species with the aim of identifying chromosomal and molecular markers that may contribute to the identification of these species, as well as the differentiation of the two genera. All *Iheringichthys* have 2n=56 chromosomes, with formula 32*m*+8*sm*+12*st*+4*a* in *I.* cf. *syi*, and 32*m*+8*sm*+10*st*+6*a* in *I. labrosus*. The two *Iheringichthys* species could be distinguished based on the distribution of their heterochromatin, with a larger quantity being found in the centromeric regions of *I.* cf. *syi*. NORs (Ag-18S rDNA) were simple all species and located in terminal region of the long arm. The 5S rDNA cistrons were observed in three chromosome pairs in *I.* cf. *syi* (interstitially pair 1, pericentromérica pair 18 and telomeric pair 25) and in one pair of chromosomes in and *I. labrosus* (terminally on the long arm of pair 28). The Maximum Likelihood and Bayesian analysis grouped *Bergiaria westermanni* and *I.* cf. *syi* in distinct clade the *Iheringichthys labrosus*. The differences cytogenetic and cyt b sequence, in present work, suggest that *Iheringichthys* cf. *syi* may belong to *Bergiaria* or that all these species should be allocated in the same genus and reinforcing the need for a thorough review of the taxonomy of the species of this genus.

Keywords: 5S rDNA; 18S rDNA; cyt b; karyotype macrostructure, molecular taxonomy.

INTRODUCTION

The Pimelodidae is a family of fish endemic to the Neotropical region, with 114 valid species distributed in 32 genera (Nelson et al. 2016; Eschmeyer and Fong 2018). While a number of studies have focused on the phylogenetic relationships among the pimelodid species (Lundberg et al. 1991; Lundberg and Akama 2005; Hardmann and Lundberg 2006; Lundberg et al. 2011; 2012), the phylogeny of the family is still not yet fully understood.

The pimelodid genus *Iheringichthys* has three species: *Iheringichthys labrosus*, whose type locality is the River Plate, *Iheringichthys megalop* which was described from a single specimen collected from the Paraguay River, and *Iheringichthys syi*, described more recently from material collected in the Jupiá reservoir in the upper Paraná River basin. *Bergiaria* is represented by two small-bodied species, *Bergiaria platana*, found in the Paraná River basin, and *Bergiaria westermanni*, which is endemic to the basin of the São Francisco River. The classification of these species in distinct genera has been questioned, and Rocha (2012) has suggested that *Bergiaria* is a junior synonym of *Iheringichthys*, based on morphological analyses.

Cytogenetic data on *Iheringichthys* are available only for *I. labrosus* from the upper Paraná River basin, including the Jurumirim (Vissoto et al. 1999) and Capivara reservoirs (Carvalho and Dias 2005), on the Paranapanema River, the Tibagi River (Carvalho et al. 2004), and the Guaraúna River, a tributary of the Ivaí (Ribeiro et al. 2008). These studies recorded a diploid number of 56 chromosomes with different karyotype formulae, which indicates the existence of a species complex and reinforces the need for a taxonomic review of *I. labrosus*. The *I. labrosus* populations have simple nucleolus organizer regions (NORs) in the terminal region of the long arm of one subtelocentric chromosome pair, with larger amounts of heterochromatin in the telomeric and centromeric regions, and B chromosomes being observed in most populations (Vissoto et al. 1999; Carvalho et al. 2004; Carvalho and Dias 2005; Ribeiro et al. 2008; Sánchez et al. 2014).

Fluorescent *in situ* hybridization (FISH) was used to map the 5S and 18S rDNA in *I. labrosus* populations from the Capivara reservoir, on the Paranapanema River (Carvalho and Dias, 2007) and the Tibagi River (Carvalho et al. 2010). These studies found 5S rDNA in the terminal region of a pair of *st-a* chromosomes and the interstitial region of a pair of *st* chromosomes, respectively, with the 18S rDNA coinciding with the AgNORs. The differences between populations in the location of the 5S rDNA in the cistrons also indicate

the existence of distinct taxonomic units, and the need for a review of the evidence from these localities.

In *Bergiaria*, the available cytogenetic data are restricted to *B. westermanni*, which has 56 chromosomes and simple NORs, located in the terminal region of the long arm of one acrocentric pair, correspondent the rDNA 18S, and rDNA 5S in the interstitial region of two pairs of chromosomes metacentric (Dias and Foresti, 1993; Malimpensa et al. 2018).

Basic and molecular cytogenetic studies were conducted in two *Iheringichthys* species and mitochondrial DNA analysis were performed in *Iheringichthys* and *B. westermanni*, in search of chromosomal or/and molecular markers that may contribute to the identification of the species, and the differentiation of the genera.

MATERIALS AND METHODS

Sampling

Specimens of *Bergiaria westermanni* (São Francisco River basin), *Iheringichthys* cf. *syi*, called by Gabriel Deprá, (upper Paraná River basin) and *Iheringichthys labrosus* (lower Paraná River basin, downstream from the Iguaçu Falls, and middle Uruguay River basin) analyzed were deposited in the Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura – (NUP), of the Universidade Estadual de Maringá, Maringá, Brazil. The collecting sites and voucher number are summarized in Table I. The number of specimens used by molecular analysis, and voucher sequences in GenBank where in the table II. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (License Number: Protocol 13/09 – CEEAAP/Unioeste).

Cytogentics studies

Dates cytogenetic in *Bergiaria* were published in Malimpensa et al. (2018). All specimens of *Iherigichthys* were anesthetized and sacrificed by an overdose of clove oil according to Griffiths (2000). Chromosome preparations were obtained from cells of anterior region of kidney by technique proposed by Bertollo et al. (1978). AgNORs were revealed by silver impregnation according to Howell and Black (1980) and C-banding followed Sumner (1972), with modifications suggested by Lui et al. (2012). Physical mapping of 5S rDNA and 18S rDNA was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel et

al. (1986) and modifications suggested by Margarido and Moreira-Filho (2008), using DNA probes obtained from *Leporinus elongatus* (=*Leporinus obtusidens* according to Britski et al. 2012) (Martins and Galetti, 1999) and from *Prochilodus argenteus* (Hatanaka and Galetti 2004), respectively. Probes were labeled by nick translation method with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche®). Detection of signals was performed with antidigoxigenin-rhodamine (Roche®) for probe of 5S rDNA and amplified avidin-FITC with biotinylated anti-avidin (Sigma-Aldrich) for probe of 18S rDNA, with the chromosomes counterstained with 4', 6-diamidino-2-phenylindole (DAPI, 50 µg/mL). Metaphases were photographed using a BX 61 epifluorescence microscope, coupled with Olympus DP 71 digital camera (Olympus America, Inc.) with the Olympus DP Controller software 3.2.1.276. Chromosomes were classified and organized in accordance with Levan et al. (1964) in metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*) and acrocentric (*a*).

DNA Extracion, amplification and sequencing

Extraction of total DNA from hepatic tissue preserved in 100% ethanol was performed with the GenElute ™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following manufacturer's recommendations and by method of Sambrook et al. (2001).

The genomic DNA was quantified on a 0.8% agarose gel using the comparison with the molecular weight of the Low DNA Mass Ladder (Invitrogen Life Technologies) marker and the nano spectrophotometer NanoK (Kasvi). After quantification, the DNA was diluted to a concentration of 10 ng / µl.

For amplification do gene mitochondrial cytochrome B (cytB) the primers were used AnosCytBF and AnosCytBR (Ramirez and Galetti 2015). This region was amplified through a PCR reaction containing 4 µL the reaction buffer 5X, 1 µL $MgCl_2$ (25mM), 0,4 µL dNTP Mix (10 mM), 0,4 µL of each primer,4 µL DNA (10 ng/µL), 0,16 µL Taq Polymerase e 9,64 µL de H_2O Mili-Q. The reaction PCR conditions were: 94°C (4 minutes), 5 cycles de 92°C (30 seconds), 50°C (30 seconds), 72°C (1 minute and 30 seconds), followed by 40 cycles de 92ºC (30 seconds), 53º (30 seconds), 72ºC (1 minute e 30 seconds) and one cycle de 72ºC (10 minutes) and 4ºC. The final PCR products were purified using the Wizard Kit SV Gel and PCR Clean-up System (Promega), according to the manufacturer's guidelines. Sequencing reactions were made using BigDye® Terminator v3.1 Cycle Sequencing Kit with capillary races**.** Sequencing Analysis software 5.3.1 was used for the analysis of the sequences using the Base Caller KB.

Phylogenetic analysis

Sequences were edited used BioEdit (Hall 1999) and aligned using MUSCLE algorithm (Edgar 2004) implemented on Mega X (Kumar et al. 2018). The index of substitution saturation (Iss) was estimed by DAMBE 7.0.28 (Xia 2018). The best fit model was choice based in AICc used software jModelTest2 (Darriba et al. 2012) in CIPRES (Miller et al. 2010). The evolutionary history was inferred by Maximum Likelihood method and Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with discrete Gamma and rate variation model allowed for some sites to be evolutionarily invariable, using 1000 replicates of bootstrap (Felsenstein 1985). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). Bayesian Inference (BI) analysis was performed using BEAST (Suchard et al. 2018) package software with 10 million replicates sampling one tree every 1000 generations. After examining the log likelihood scores and ensuring convergence and stationarity (>200 ESS) with Tracer v1.7.1 (Rambaut et al. 2018), 10% of trees were discarded as burn-in in TreeAnnotatorv1.8.4 (Drummond et al. 2012). The remaining trees were using to construct a maximum credibility tree in TreeAnotator. The trees were visualized and edited in FigTree v1.4.3 (Rambaut 2016).

RESULTS

Cytogenetics

Iheringichthys cf. *syi* – Piquiri River, upper Paraná River basin

The diploid number of this species was also 56 chromosomes (32*m*+8*sm*+12*st*+4*a*) [Fig. 1(a)]. Between one and three B chromosomes (micro-chromosomes) were observed in a given individual (but were absent in one case), with intra- and inter-individual variation (Table III). The AgNORs were located in the terminal region of the long arm of a pair of subtelocentric chromosomes, pair 24 [Fig. 1(a), box]. The C banding highlighted pale heterochromatin in the region of the centromeres of almost all the chromosomes, with more conspicuous heterochromatin being found in the telomeric region of the short arm in pairs 13 and 14, and the long arm in pair 23, in both telomeres in pairs 7 and 9, in both telomeres and the pericentromeric region of the long arm of pair 22, the subterminal region of the long arm of pairs 26 and 27, while the B chromosomes are entirely heterochromatic [Fig. 1(b)]. The 18S rDNA was located on the pair of subtelocentric chromosomes (pair 24), corresponding to the AgNORs, and the 5S rDNA to the region interstitial of the long arm of the acrocentric

chromosomes, pair 1, in the region pericentromeric of the long arm of the submetacentic chromosomes, pair 18 and in the region terminal of the short arm of the subtelocentric chromosomes, pair 25 [Fig. 1(c)].

Iheringichthys labrosus – Iguaçu River, lower Paraná River basin

The diploid number recorded in this population was 56 chromosomes (32*m*+8*sm*+10*st*+6*a*) [Fig. 1(d)]. The AgNORs were located in the telomeric region of the long arm of a pair of subtelocentric chromosomes, that is, pair 24 [Fig. 1(d), box)]. The C banding revealed pale heterochromatin in the region of the telomeres of almost all the chromosome pairs, with more conspicuous heterochromatin being observed in the pericentromeric region of the short arm in pairs 2 and 19, in both telomeres in pairs 6, 7, 12 and 14, the interstitial region of the long arm of pair 21, the terminal region of the long arm of pairs 24 and 28, and in the subterminal region of the long arm dos pairs 23 and 25 [Fig. 1(e)]. The 18S rDNA was located in the terminal region of the subtelocentric pair (24), corresponding to the AgNORs, while the 5S rDNA was found in the terminal region of the long arm of the acrocentric chromosomes, pair 28 [Fig. 1(f)].

Iheringichthys labrosus – Ijuí River, middle Uruguay River basin

The diploid number recorded in this population was also 56 chromosomes, with a formula of 32*m*+8*sm*+10*st*+6*a* [Fig. 1(g)]. The AgNORs were located in the telomeric region of the long arm of a pair 24 (subtelocentric) [Fig. 1(g), box)]. The C banding revealed pale heterochromatin in the region of the telomeres of almost all the chromosome pairs, with more conspicuous heterochromatin being observed in the pericentromeric region of the short arm in pair 2, in both telomeres in pairs 6, 7, 12 and 14, the interstitial region of the long arm of pair 21, the terminal region of the long arm of pairs 24 and 28, and in the subterminal region of the long arm dos pairs 23, 24, 26 and 28, and in the subterminal region of the long arm of pair 25 [Fig. 1(h)]. The 18S rDNA was located in the subtelocentric pair (24), corresponding to the AgNORs, while the 5S rDNA was found in the terminal region of the long arm of the acrocentric pair 28 [Fig. 1(i)].

Iheringichthys labrosus – Uruguay River, lower Uruguay River basin

The diploid number recorded in this population was also 56 chromosomes, with a formula of 32*m*+8*sm*+10*st*+6*a* [Fig. 1(j)]. The AgNORs were located in the telomeric region of the long arm of a pair 24 (subtelocentric) [Fig. 1(j), box)]. The C banding revealed pale

heterochromatin in the region of the telomeres of almost all the chromosome pairs, with more conspicuous heterochromatin being observed in the pericentromeric region of the short arm in pair 2, in both telomeres in pairs 7, 9, 14 and 15, the interstitial region of the long arm of pair 19, the terminal region of the long arm of pairs 24, 27 and 28, and in the subterminal region of the long arm dos pairs 23, 24, 26 and 28 [Fig. 1(k)]. The 18S rDNA was located in the subtelocentric pair (24), corresponding to the AgNORs, while the 5S rDNA was found in the terminal region of the long arm of the acrocentric pair 28 [Fig. 1(l)].

Molecular

The aligned data matrix consisted of 25 sequences of the cytochrome b gene, containing 886 nucleotides. Of these, 791 sites were conserved, 95 variants, 85 informative of parsimony and 10 singleton. The estimation Iss showed that the data did not show saturation. The maximum likelihood analysis (ML) produced a tree with three clades, the first formed by the out group (*Pimelodus maculatus*), the second grouping *Bergiaria westermanni* and *Iheringichthys* cf. *syi* and the third with the populations of *Iheringichthys labrosus* (Fig. 2). These branches presented bootstrap values ≥ 95. The tree of maximum credibility of Bayesian inference (Fig. 3) presented the same arrangement as that of ML, between the groups.

DISCUSSION

The diploid number of 56 chromosomes found in the studied species is typical of most pimelodids. Among the 34 pimelodid species that have been karyotyped, only five have alternative diploid numbers. *Calophysus macropterus*, *Pinirampus pirinampu* and *Luciopimelodus pati* all have 2n=50 chromosomes (Ramirez-Gil et al. 1998; Vasconcelos and Martins-Santos 2000; Sánchez et al. 2000). While *Megalonema platanum* and *Pimelodus fur* have 2n=54 chromosomes (Sánchez et al. 2000; Carvalho et al. 2011; Garcia and Moreira-Filho, 2008), and *Pimelodus blochii* (Fonseca et al. 2018) have 2n=58 chromosomes. These data support the hypothesis that the 56-chromosomes arrangement is a plesiomorphic trait in the Pimelodidae (Moraes-Neto et al. 2011; Girardi et al. 2018).

The karyotype formula of *Iheringichthys* cf. *syi* was different of *I. labrosus* populations analyzed here, although it is consistent with the formula recorded in *I. labrosus* from the Tibagi River (Carvalho et al. 2004). Different studies of *I. labrosus* have described diverging karyotype formulae (Table IV), which may indicate that they may refer to one or more undiagnosed species of *Iheringichthys*, given that they were analyzed prior to the

description of *I. syi*. One example of a potentially erroneous species diagnosis can be found in the case of *Iheringichthys* from the Piquiri River, which has been identified as *I. labrosus* in previous surveys (Gubiani et al. 2006), although the species captured recently in this river has been identified as *I*. cf. *syi* by Deprá, G. The composition of the fish fauna of the Piquiri River is somewhat distinct from that of the rest of the upper Paraná basin, and includes endemic species (Pavanelli 2006). However, species that were previously classified as endemic to the Piquiri basin have also been found in the Ivaí River basin, *e.g. Apareiodon vladii* (Viana et al. 2013).

The presence of B chromosomes was observed in *Iheringichthys* cf. *syi* in the form of microchromosomes, which varied considerably between species in their frequency and maximum number. Microchromosomes were observed in 62% of the metaphases analyzed in *Iheringichthys* cf. *syi*, with no more than three per cell, although they were absent in one of the seven individuals analyzed (Table III). These chromosomes supranumerary have been found in most groups of animals and plants (Camacho et al. 2000). The B chromosomes are additional and dispensable elements that are present in some individuals of some species (Camacho 2005).

They originate from complement chromosomes A, with which they do not recombine, besides not having a regular meiotic behavior, are widely distributed, being present in about 15% of eukaryotes, being found in fungi, plants and animals, both vertebrates and invertebrates (Camacho 2005; Beukeboom 1994). In the Siluriformes, must of the species that have been analyzed in cytogenetic studies have been found to have supernumerary chromosomes, including members of the families Callichthyidae, Heptapteridae, Loricariidae, Pimelodidae, and Trichomycteridae (Carvalho et al. 2008). Lui et al. (2009) also recorded the presence of these in the Auchenipteridae (*Parauchenipterus galeatus*).

In the Pimelodidae, these chromosomes have been observed in *Bergiaria westermanni* (Dias and Foresti 1993; Malimpensa et al. 2018), *I. labrosus* (Carvalho and Dias 2005; Carvalho et al. 2004; Vissoto et al. 1999), *Megalonema platanum* (Carvalho et al. 2011), *Pimelodus ortmanni* (Borin and Martins-Santos 2004; Girardi et al. 2018) and *Pimelodus* sp. (Borin and Martins-Santos 2004). Many sequences present on complement chromosomes A have been found in these Bs chromosomes, such as the presence of 18S rDNA and histone genes (H1) in *Astianax fasciatus* and *Astianax paranae* (Silva et al. 2016), As51 repetitive DNA in *Astianax scabripinnis* (Mestriner et al. 2000) and *Astianax fasciatus (Silva et al. 2016*) and microsatellite DNA in *Characidium alipioi* (Serrano et al. 2017), among others. In *Bergiaria westermanni* 18S rDNA and microsatellite $(GATA)_n$ sequences were observed on

the Bs chromosomes, confirming the probable origin of these chromosomes from complement A (Malimpensa et al 2018).

The study populations of *I. labrosus* and *I.* cf. *syi* presented simple NORs (Ag and rDNA 18S), as all the Pimelodidae species studied up until now. Terminal NORs in long arm has been found in studies of populations *I. labrosus* (Carvalho and Dias 2007; Carvalho et al. 2010) and *Bergiaria* (Dias and Foresti 1993; Malimpensa et al. 2018). The *Iheringichthys* populations studied here shared the presence of most conspicuous heterochromatin in terminal region and pericentromeric position. However, *Iheringichthys syi* concentrates a greater amount of heterochromatin in centromere, little observed in *Iheringichthys labrosus*. The specific details of the quantity and distribution of the heterochromatin permitted the differentiation of *I.* cf. *syi* and the *I. labrosus* populations.

The use of FISH to locate the rDNA sites is a promising approach for evolutionary and taxonomic studies of fish (Moraes-Neto et al. 2011). Differences in the location of the 5S rDNA may provide an important chromosomal marker for the characterization and differentiation of species, as shown in *Parodon* (Vicente et al. 2001), *Pimelodus* (Garcia and Moreira-Filho 2008), and *Pimelodella* (Garcia and Almeida-Toledo 2010). The number of these cistrons varies considerably among different pimelodid taxa, with a simple arrangement being found in some species (Swarça et al. 2009; Moraes-Neto et al. 2011; Gonçalves et al. 2014), and a multiple configuration in others (Moraes-Neto et al. 2011; Garcia and Moreira Filho 2008; Sczepanski et al. 2013). This suggests that these sequences evolved independently in the different pimelodid genera. These cistrons were found in the same region in *Bergiaria* (Malimpensa et al. 2018) and *I.* cf. *syi*, diverging from that observed in *I. labrosus* populations. In *Bergiaria* and *I.* cf. *syi* 5S rDNA cistrons were observed in a pericentromeric and interstitial position, whereas in *I. labrosus*, they were found in a terminal position. This difference in the configuration of the DNAr sites confirms that the specimens the *Iheringichthys* represent distinct species, and that the location of these sequences can be used as a marker to distinguish *I. labrosus* and *I.* cf. *syi*. Studies of the location of these sequences in populations identified as *I. labrosus* from the Tibagi River identified differences in their location similar to those recorded in the present study (Carvalho and Dias 2007; Carvalho et al. 2010), which suggests the existence of two *Iheringichthys* species in the upper Paraná basin, as well as the possibility that some specimens identified previously as *I. labrosus* are in fact *I.* cf. *syi*.

Maxima likelihood and Bayesian analyzes using cyt b produced similar trees, evidencing that *Iheringichthys* is a non monophyletic group when grouping *Iheringichthys* cf.

syi in the same clade as *Bergiaria westermanni* and in a distinct group of *Iheringichthys labrosus*, with the branches presenting high support values. This represents strong evidence of the genetic difference between these species. Cytogenetic differences in populations of *Iheringchthys labrosus* has been evidenced in populations of the Upper Paraná River basin (Table IV). Which suggests that the determination of the species of *Iheringichthys labrosus* needs to be revised for these populations, for more than one species of this genre possibly can be found in other rivers of this basin, since several specimens were identified as *Iheringichthys labrosus* before the description of *Iheringichthys syi*.

Based on morphological analyses, Rocha (2012) proposed that *Bergiaria* should be considered synonym of *Iheringichthys*. The *Iheringichthys* and *Bergiaria* populations were similar in the macrostructure of their karyotypes, including large numbers of meta–submetacentric chromosomes, simple NORs in the terminal region of the long arm, and heterochromatin distributed in the region of the centromeres and telomeres. As the majority of pimelodid species shares these characteristics, they cannot be used to justify the inclusion of *Iheringichthys* and *Bergiaria* in a single genus. However, *Bergiaria westermanni* also has multiple 5S rDNA sites, a condition found only in *Iheringichthys* cf. *syi*. Moreover, molecular analyzes using cyt b evidence the close phylogenetic proximity between *Bergiaria westermanni* and *Iheringichthys* cf. *syi*, which were united in a separate clade of *Iheringichthys labrosus.*

Our findings confirm at least two *Iheringichthys* species in the basin of the Paraná River and reinforce the need for a taxonomic review. As well as confirming the potential of integrative cytogenetic and molecular studies for the identification and classification of species, and understanding the evolutionary history of different fish groups. Overall, then, cytogenetic and molecular analyzes show two perspectives, the first one that *Bergiaria* and *Iheringichthys* constitute a single genus, called *Iheringichthys*; and the second that *Bergiaria* and *Iheringichthys* are distinct genera, so *Iheringichthys* cf. *syi* should be reallocated in *Bergiaria*. Taxonomic review may also be important for a more detailed understanding of the relationships between *Iheringichthys* and *Bergiaria*, and between these genera and the other groups of pimelodids. In addition, cytogenetic and molecular analyzes in *Iheringichthys syi* are crucial to determine if the specimens studied here are actually of this species, or represent a new species.

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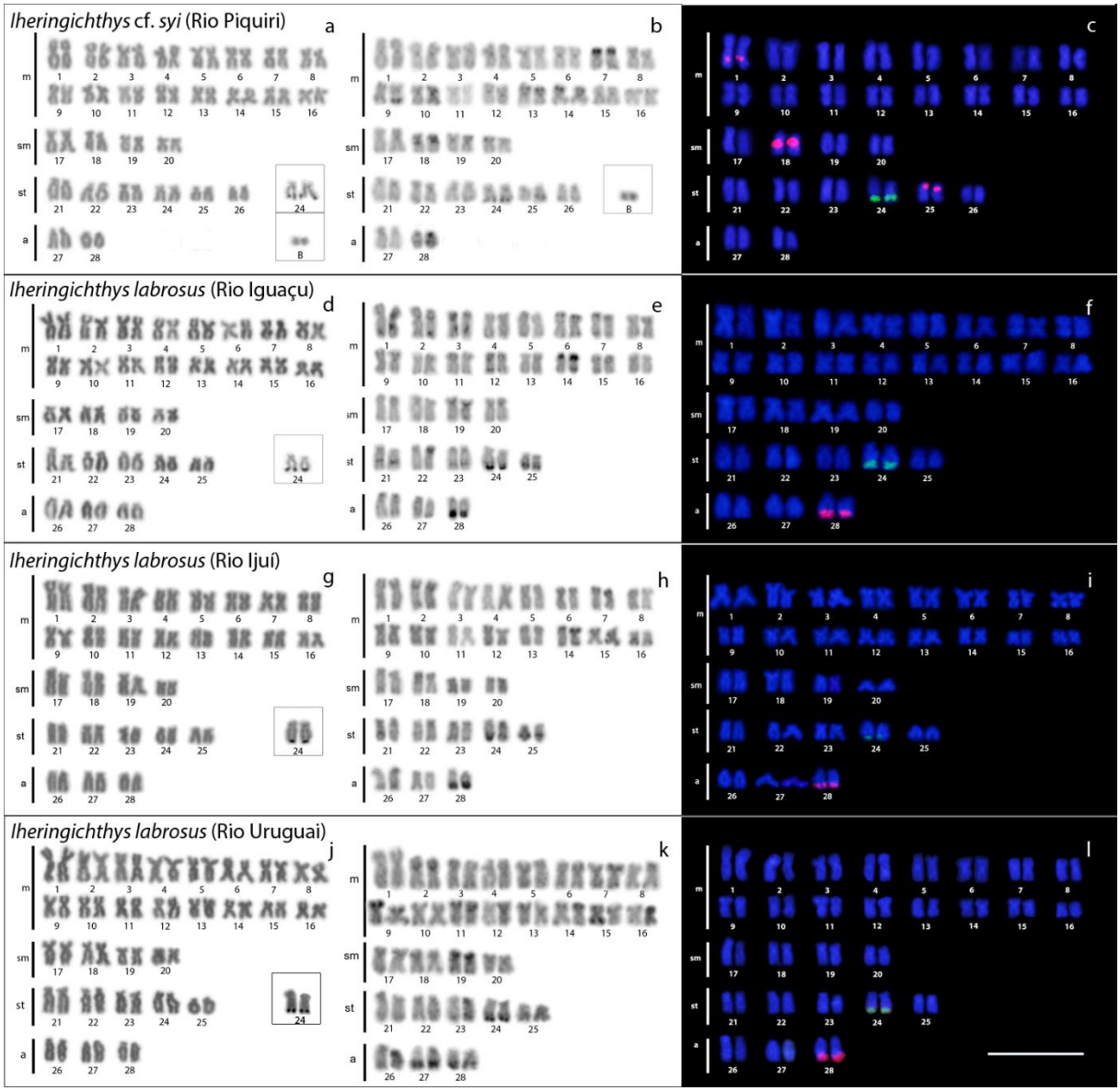

Fig. 1 Karyotypes Giemsa-stained (a, d, g, j), C-banded (b, e, h, k) and after fluorescence in situ hybridization with 5S rDNA and (rodamine, red) and 18S rDNA probes (FITC, green) (c, f, i, l). AgNORs pairs and B chromosomes are in box. Bar represents 10 μm.

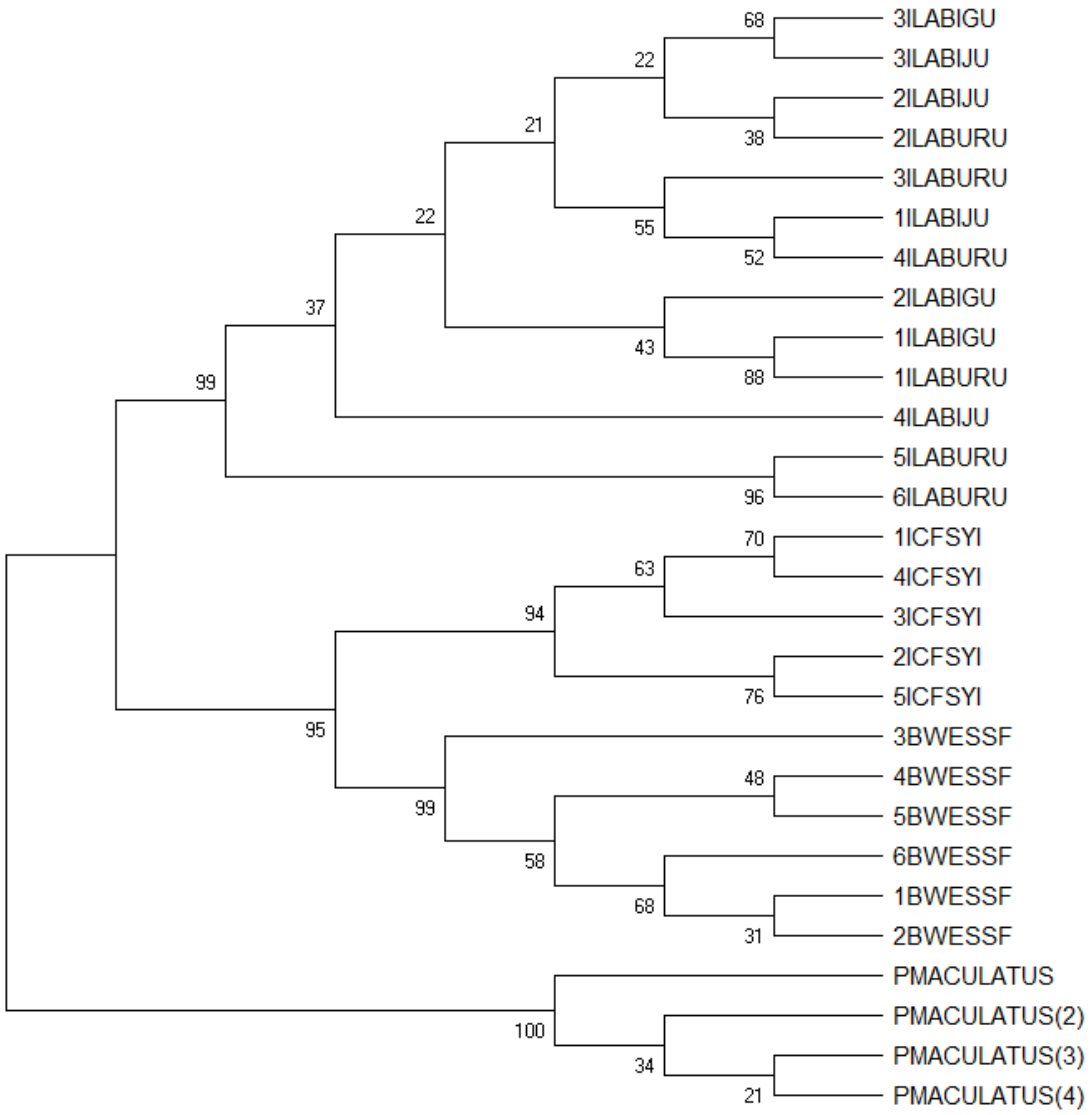

Fig. 2 The tree genic with cyt b sequences using the Maximum Likelihood (ML) within *Bergiaria westermanni* (BWESSF), *Iheringichthys* cf. *syi* (ICFSYI) and *Iheringichthys labrosus* from Iguaçu River (ILABIGU), Ijuí River (ILABIJU), Uruguay River (ILABURU) and out-group *Pimelodus maculatus* (PMACULATUS). Values of bootstrap are show.

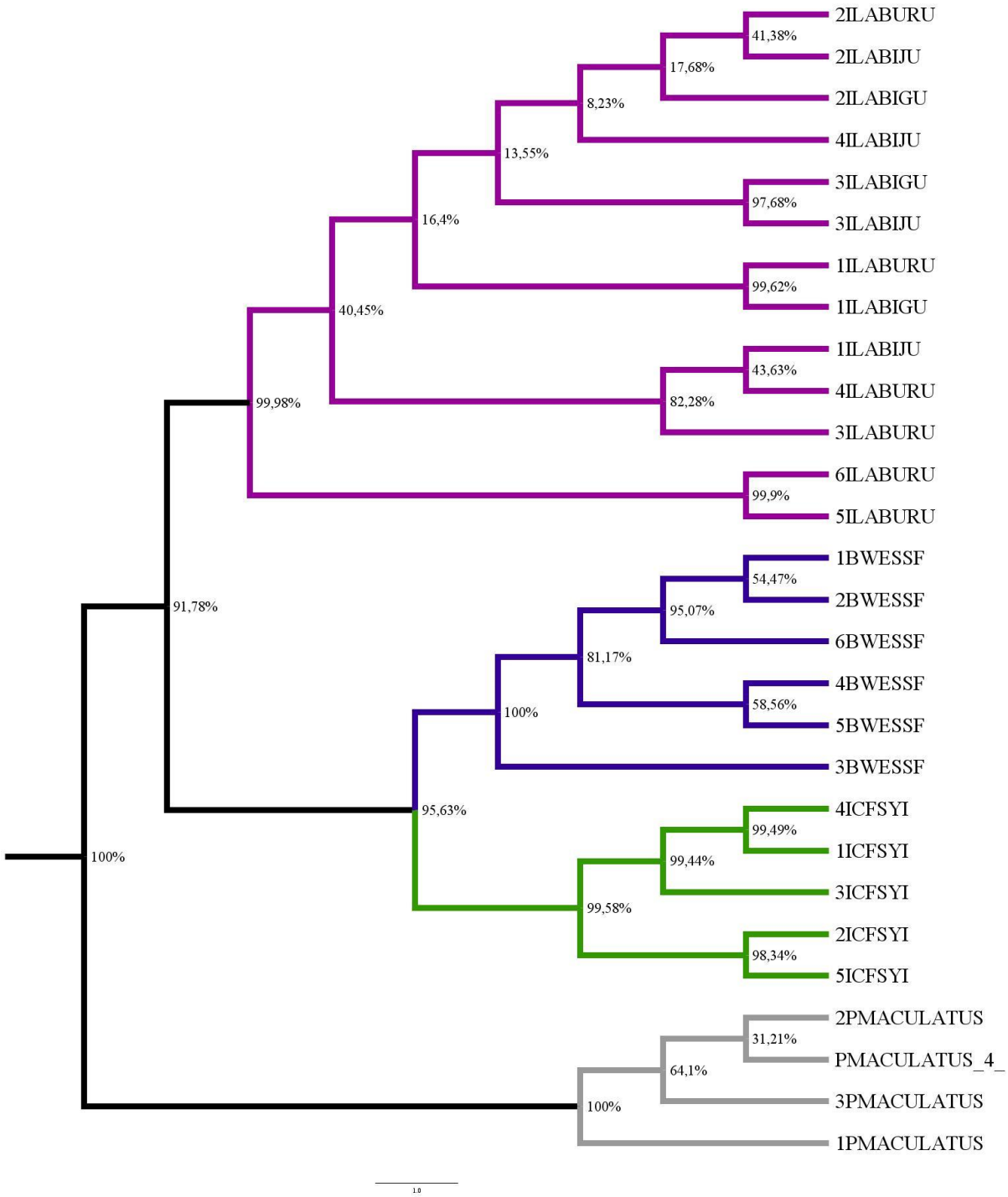

Fig. 3 Bayesian analysis with cyt b sequences within *Bergiaria westermanni* (BWESSF), *Iheringichthys* cf. *syi* (ICFSYI) and *Iheringichthys labrosus* from Iguaçu River (ILABIGU), Ijuí River (ILABIJU) Uruguay River (ILABURU) and out-group *Pimelodus maculatus* (PMACULATUS). Posterior probability are show.

Table 1 Collection sites of the populations of *Iheringichthys* and *Bergiaria*, geographic coordinates, number of analyzed individuals per sex, and voucher number.

Species	Locality	Geografhic Coordinates	♂	♀	NUP
Bergiaria westermanni	São Francisco River, MG São Francisco	28°20'19"S/45°58'52"W	2	13	18792, 18793
Iheringichthys cf. *syi*	Piquiri River, PR Upper Paraná	24°56'54"S/52°35'49"W	1	6	14937, 14942, 17276, 18041
*Iheringichthys labrosus**	Iguassu River, PR Lower Paraná	25°39'02"S/54°27'25"W	0	3	17268, 17272, 17273
Iheringichthys labrosus	Ijuí River, RS Middle Uruguay	28°18'06"S/53°53'33"W	14	6	14902, 17262
Iheringichthys labrosus	Uruguai River, RS Lower Uruguay	29°44'58"S/57°05'29"W	6	12	18107

*Population downstream from the Iguassu Falls.

Table 2 Number accession sequences in GenBank of the *Bergiaria* and *Iheringichthys* specimens.

Specime	**Species**	**Locality**	**GenBank**
2261	*Iheringichthys* cf. *syi*	Piquiri River	MK383330
2272	*Iheringichthys* cf. *syi*	Piquiri River	MK383331
2278	*Iheringichthys* cf. *syi*	Piquiri River	MK383332
2822	*Iheringichthys* cf. *syi*	Piquiri River	MK383333
2810	*Iheringichthys* cf. *syi*	Piquiri River	MK383334
2521	*Iheringichthys labrosus*	Iguassu River	MK383335
3157	*Iheringichthys labrosus*	Iguassu River	MK383336
3081	*Iheringichthys labrosus*	Iguassu River	MK383337
3009	*Iheringichthys labrosus*	Ijuí River	MK383338
3198	*Iheringichthys labrosus*	Ijuí River	MK383339
3210	*Iheringichthys labrosus*	Ijuí River	MK383340
3214	*Iheringichthys labrosus*	Ijuí River	MK383341
3738	*Iheringichthys labrosus*	Uruguai River	MK383342
3758	*Iheringichthys labrosus*	Uruguai River	MK383343
3769	*Iheringichthys labrosus*	Uruguai River	MK383344
3770	*Iheringichthys labrosus*	Uruguai River	MK383345
3781	*Iheringichthys labrosus*	Uruguai River	MK383346
3796	*Iheringichthys labrosus*	Uruguai River	MK383347
21449	*Bergiaria westermanni*	São Francisco River	MK383348
21450	*Bergiaria westermanni*	São Francisco River	MK383349
21451	*Bergiaria westermanni*	São Francisco River	MK383350
21452	*Bergiaria westermanni*	São Francisco River	MK383351
21453	*Bergiaria westermanni*	São Francisco River	MK383352
21455	*Bergiaria westermanni*	São Francisco River	MK383353
3194	*Pimelodus maculatus*	Ijuí River	MK383354
3195	*Pimelodus maculatus*	Ijuí River	MK383355
3197	*Pimelodus maculatus*	Ijuí River	MK383356

Table 3 Frequency of B chromosomes in *Iheringichthys* cf. *syi* from Piquiri River.

Specimens	Sex	Number of B chromosomes/cells				Analyzed cells	Cells with B
		0	1	2	3		
2033	♀	2	18	-	-	20	90.00
2261	♀	3	16	3	-	22	86.36
2272	♀	37	4	1	-	42	11.90
2275	♀	14	-	-	-	14	0.00
2278	♀	7	-	6	7	20	65.00
2810	♂	1	9	4	-	14	92.86
2822	♀	2	40	3	-	45	95.55
Total (%)		66 (37.29)	87 (49.15)	17 (9.60)	7 (3.96)	177 (100.00)	

Table 4 Cytogenetic data available for *Bergiaria* and *Iheringichthys.*

Species	Locality	Basin	Chromosome formulae	B	NORs	18S rDNA	5S rDNA	Ref
B. westermanni	São Francisco River	SF	44*m/sm* + 14 *st/a*	0-4	q ter *a*			1
B. westermanni	São Francisco River	SF	28*m* + 14*sm* + 10*st* + 4*a*	0-4	q ter a	q ter a	q inter *m*	2
I. labrosus	Tibagi River, Londrina	UP	32*m* + 8*sm* + 6*st* + 10*a*	0-3	q ter *st*	q ter *st*	q inter *st*	3
I. labrosus	Tibagi River, Capivara Reservoir	UP	26*m* + 12*sm* + 6*st* + 12*a*	0-1	q ter *st*	q ter *st*	q ter *st-a*	4
I. labrosus	Jurumirim River	UP	22*m* + 18*sm*+10*st* + 6*a*	0-2				5
I. labrosus	Guaraúna River, Ponta Grossa	UP	14*m* + 32*sm* + 4*st* + 6*a*	-	q ter *sm*			6
I. labrosus	Paraná River, Argentina (7 localities)	LP	42*m*/*sm* + 14*st*/*a*	-	q ter *st*			7
I. labrosus	Iguaçu River, Foz do Iguaçu	LP	32*m* + 8*sm* + 10*st* + 6*a*	-	q ter *st*	q ter *st*	q ter *a*	8
I. labrosus	Ijuí River, Ijuí	MU	32*m* + 8*sm* + 10*st* + 6*a*	-	q ter *st*	q ter *st*	q ter *a*	8
I. labrosus	Uruguai River, Uruguaiana	LU	32*m* + 8*sm* + 10*st* + 6*a*	-	q ter *st*	q ter *st*	q ter *a*	
*I.*cf. *syi*	Piquiri River, Nova Laranjeiras	UP	32*m* + 8*sm* + 12*st* + 4*a*	0-3	q ter *st*	q ter *st*	q inter a, q peri st and p ter st	8

1- Dias and Foresti (1993); 2- Malimpensa et al 2018; 3- Carvalho et al. (2004; 2010); 4- Carvalho and Dias (2005; 2007); 5- Vissotto et al. (1999); 6- Ribeiro et al. (2008); 7- Sánchez et al. (2014); 8- Here q: long arm, p: short arm, *m*- metacentric, *sm*- submetacentric, *st* – subtelocentric, *a* – acrocentric, ter: terminal, inter: interstitial, peri: pericentromeric,UP: upper Paraná River basin; LP: lower Paraná River basin; MU: middle Uruguay River basin.

ANEXO 1

Instructions for Authors

EDITORIAL PROCEDURE

Unsolicited review manuscripts must fall within the aims and scope of the journal and should not exceed 33 pages in manuscript length (including figures, tables and literature). Invited editorials, book and conference reviews, letters to the editor, and selected announcements are published at the discretion of the editorial staff and the publisher. Ideas on topics appropriate for RFBF special issues (SI) must first be presented in outline form to the Editor in Chief for consideration for publication. Contributors should feel free to discuss subject and content with the Editor in Chief at any time.

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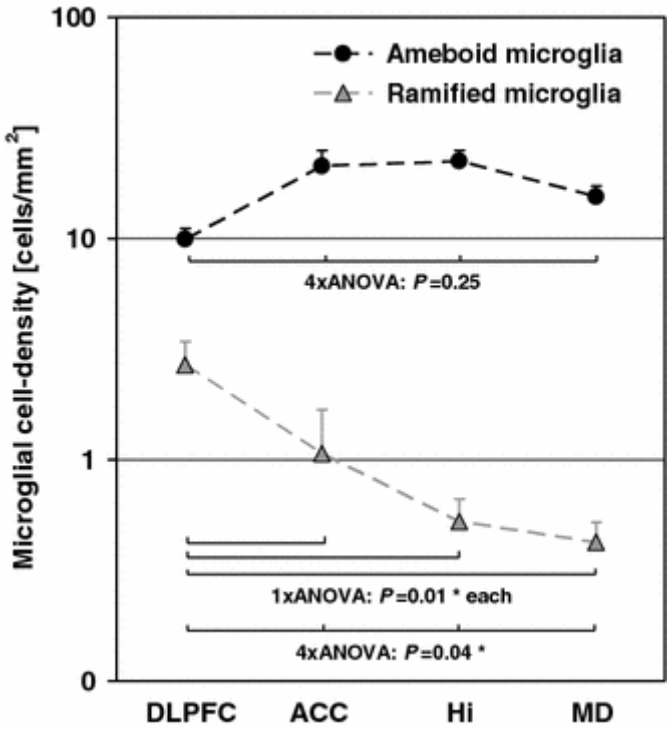

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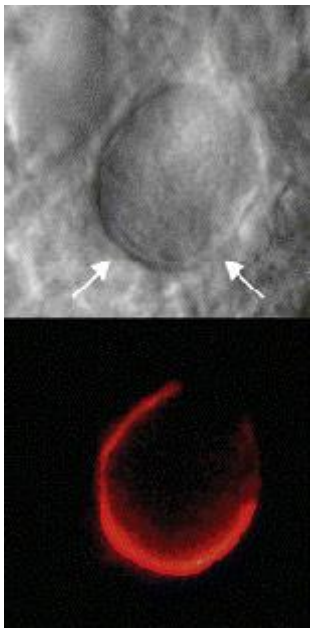

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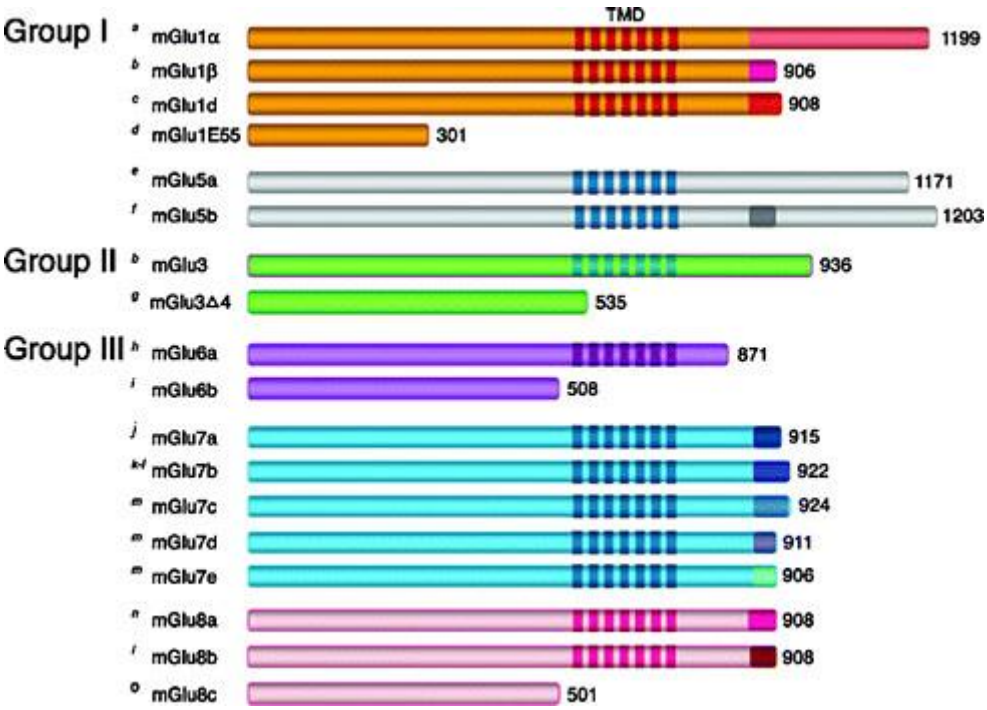

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CAPÍTULO 3

Caracterização citogenética de *Pimelodus pantaneiro* (Siluriformes: Pimelodidae) e mapeamento do DNA repetitivo U2 snDNA em espécies de *Pimelodus*.

Artigo em preparação elaborado e formatado conforme as normas para publicação científica no periódico *Journal of Fish Biology*.

Caracterização citogenética de *Pimelodus pantaneiro* (Siluriformes: Pimelodidae) e mapeamento do DNA repetitivo U2 snDNA em espécies de *Pimelodus*.

Running head: DNA repetitive em *Pimelodus*

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Caracterização citogenética de *Pimelodus pantaneiro* (Siluriformes: Pimelodidae) e mapeamento do DNA repetitivo U2 snDNA em espécies de *Pimelodus*.

Resumo: As famílias multigênicas apresentam grande diversidade de constituintes, sendo formadas por DNA repetitivo em tandem, incluindo os DNAr, os snRNA, entre outros. Os DNAr são amplamente estudados nos peixes, ao contrário dos snRNAs que são menos explorados mas têm se mostrado bastante promissores para compreender a organização genômica do DNA dos peixes e auxiliar no entendimento da evolução cromossômica nesse grupo de vertebrados. Pimelodidae é uma família endêmica da região Neotropical que possui grupos com relações incertas, como é o caso do gênero polifilético *Pimelodus*. No presente trabalho, estudos citogenéticos básicos (Giemsa e Bandamento-C) e moleculares (DNAr 5S e 18S) foram realizados em *Pimelodus pantaneiro* que apresentou 2n=56 cromossomos, formula cariotípica composta por 22m+16sm+10st+8a, heterocromatina pálida na região dos centrômeros da maioria dos cromossomos e conspícuas na região do braço curto do par 21. O DNAr 18S foi localizado nos telômeros do braço curto, possivelmente do par 21 e o DNAr 5S foi evidenciado em 2 pares de cromossomos, sendo um em sintenia com o DNAr 18S e o outro cístron na região pericentromérica do braço curto de um par de cromossomos submetacêntricos. O mapeamento do gene U2 snDNA também foi realizado em *P. absconditus*, *P. britskii*, P. *maculatus*, *P. microstoma*, *P. ortmanni* e *P. pantaneiro*, sendo evidenciado um par portador dessa sequência em todas as espécies. Os resultados revelam um padrão conservado do número diploide de *Pimelodus* e da localização dos sítios de DNAr U2 snDNA, permitindo estabelecer relações entre as espécies de Pimelodidae, além de fornecer novos dados sobre a distribuição e organização genômica das sequencias multigênicas, contribuindo para o melhor conhecimento da história evolutiva desse grupo.

Palavras chave: citotaxonomia, DNAr 5S, DNAr 18S, DNA repetitivo, Pimelodidae.

Introdução

Os DNAs repetitivos são classificados em elementos intercalados ou organizados em tandem (Cioffi & Bertollo, 2012). Essas sequências são comuns no genoma dos eucariotos e caracterizam-se por ampla heterogeneidade e diversidade de famílias repetidas, além de compreenderem uma grande porção do genoma de muitas espécies (Charlesworth *et al*., 1994). Os elementos intercalados são representados pelos elementos transponíveis (TEs) que são amplamente distribuídos ao longo do genoma, enquanto as sequências organizadas em tandem incluem as famílias multigênicas, tais como os RNAs ribossomais (RNAr) e os genes das histonas, e os satélites, micro e minissatélites. Essas sequências, junto com as menos repetidas, que incluem baixo número de cópias por sequência e DNAs com pouca repetição, constituem a arquitetura do genoma nuclear (Cioffi & Bertollo, 2012).

As famílias multigênicas apresentam grande diversidade, tanto no número de constituintes quanto na organização genômica (Bueno *et al*., 2013). São compostas por centenas e milhares de cópias de genes, incluindo as famílias dos genes de RNAr, das histonas (Cioffi & Bertollo, 2012) e dos snRNAs (RNAs *small nucleares*). Os snRNAs (U1, U2, U4, U5 e U6), em conjunto com mais de 200 proteínas formam o spliceossomo, que consiste na união de ribonucleoproteínas e é responsável pelo processamento do precursor do RNAm. Os snRNAs são altamente conservados e desempenham papel central em todos os aspectos da reação de processamento do RNA (Valadkhan, 2005), entretanto, estudos sobre a distribuição dessas sequências nos peixes são escassos. Estudos moleculares focados nos genes e nas sequências de DNA estão, em sua maioria, restritas a sequências repetitivas, como DNAs satélites, elementos transponíveis (TEs) e DNAr. Os poucos estudos de mapeamento dos genes de U2 snRNA mostram um padrão conservado dessas sequências ao longo do genoma dos peixes, com sítios majoritariamente em um par de cromossomos e com poucos relatos de associação com DNAr 5S ou 18S (Yano *et al*., 2016).

Pimelodus é o gênero mais diversificado e numeroso dentro de Pimelodidae, com 36 espécies descritas (Eschmeyer *et al*., 2018). É conhecido por ser um grupo polifilético e agrupar espécies sem um caracter exclusivo definido. *Pimelodus pantaneiro* é uma espécie encontrada na bacia do Alto rio Paraguai, na região do Pantanal (Eschmeyer *et al*., 2018), tendo sido por muito tempo identificada como *Pimelodus maculatus.* Possui estudos citogenéticos básicos que relatam a ocorrência de 2=56 cromossomos, AgRONs na região telomérica do braço longo e heterocromatina na região dos centrômeros e em um par de cromossomos com forte marcação intersticial (Souza *et al*., 2003).

Estudos sobre DNAs repetitivos são pouco descritos, tanto para *Pimelodus*, quanto para Pimelodidae, apresentando a maior quantidade de dados sobre o DNAr 18S e 5S, que possui um padrão de DNAr 18S simples na região dos telômeros e DNAr 5S com sítios variáveis quanto ao número e a localização (Girardi *et al*., 2018). Mapeamento citogenético de U2 snDNA está restrito a *Bergiaria westermanni* com identificação na região intersticial de um par de cromossomos metacêntricos em localização sintênica ao DNAr 5S (Malimpensa *et al*., 2018).

Devido as relações incertas em Pimelodidae, principalmente em *Pimelodus*, o mapeamento dos genes de U2 snRNA pode fornecer valiosas informações sobre a organização do genoma desses peixes e ser um importante marcador para auxiliar na elucidação das relações filogenéticas entre os grupos de Pimelodidae. No presente estudo foram realizadas análises citogenéticas básicas e moleculares em *Pimelodus pantaneiro* e o

mapeamento do U2 snDNA em seis espécies de *Pimelodus*, buscando contribuir para o aumento das informações sobre a organização genômica das espécies de *Pimelodus*.

Metodologia

Seis espécies de *Pimelodus* de diferentes bacias hidrográficas brasileiras foram coletadas e analisadas, o número de indivíduos, os locais de coleta e o voucher do depósito estão listados na Tabela 2. Em *Pimelodus pantaneiro* técnicas citogenéticas básicas (Giemsa e Banda C) e moleculares (DNAr 5S, 18S e snU2 DNA) foram realizadas. Nas demais espécies de *Pimelodus* o mapeamento da sequência snU2 DNA foi realizado. Esse estudo seguiu as recomendações do Guia de cuidado e uso de animais de laboratório, aprovada pelo comitê de ética em pesquisa no uso de animais da Universidade Estadual do Oeste do Paraná (Número da licença: Protocolo 13/09- CEEAAP/ Unioeste). Os espécimes foram anestesiados e eutanasiados por overdose de óleo de cravo (Griffiths, 2000). As preparações cromossômicas foram obtidas de células da porção anterior dos rins (Bertollo *et al*., 1978). Para observar as regiões heterocromáticas foi utilizada a técnica de bandamento C (Sumner, 1972) com coloração por iodeto de propídio (Lui *et al*., 2012). O mapeamento físico dos DNAr 5S e 18S foi realizado por Hibridização *in situ* fluorescente (FISH) (Pinkel *et al*., 1986), com modificações (Margarido & Moreira-Filho, 2008), utilizando as sondas de *Megaleporinus elongatus* (= *Leporinus elongatus*) (Martins & Galetti-Junior, 1999) e *Prochilodus argenteus* (Hatanaka & Galetti-Junior, 2004) respectivamente. A sonda DNAsn U2 foi preparada usando PCR com *primers* descritos por Bueno *et al.* (2013) (U2F, 5′-ATC GCT TCT CGG CCT TAT G-3′; e U2R, 5′-TCC CGG CGG TAC TGC AAT A-3′) e DNA total extraído do tecido hepático de *Astyanax altiparanae* preservado em etanol 100% realizado com o *Kit GenElute™ Mammalian Genomic DNA Miniprep* (Sigma-Aldrich) seguindo recomendações do fabricante.

As sondas foram marcadas por nick translation com digoxigenina-11-dUTP (DNAr 5S) e biotina-16- dUTP (DNAr 18S) (Roche®) e a detecção dos sinais foi feita com antidigoxigenina-rodamina (Roche®) para as sondas de DNAr 5S e U2 snDNA e avidina-FITC com amplificação por Anti-avidina-biotinilada (Sigma-Aldrich) para a sonda de DNAr 18S, os cromossomos foram contra corados com 4’,6-diamidino-2-fenilindol (DAPI, 50 μg/mL). As metáfases foram fotografadas utilizando o microscópio de epifluorescência BX 61 com câmera digital Olympus DP 71 (Olympus America, Inc.) e com o software DP Controller 3.2.1.276. Os cromossomos foram organizados e classificados de acordo com

Levan *et al*. (1964). Dados citogenéticos básicos e a localização de DNAr 5S e 18S das espécies de *Pimelodus* aqui estudadas, exceto *Pimelodus pantaneiro*, foram publicados por Girardi *et al*. (2018).

Resultados

Pimelodus pantaneiro apresentou 2n=56 cromossomos e fórmula cariotípica composta por 22m+16sm+10st+8a (Fig. 1a). O bandamento C evidenciou regiões heterocromáticas pálidas na região dos centrômeros da maioria dos cromossomos e regiões conspícuas na região do braço curto do par 21 (Fig. 1b). O DNAr 18S foi localizado nos telômeros do braço curto, possivelmente o par 21. O DNAr 5S foi evidenciado em 2 pares de cromossomos, sendo um em sintenia com o DNAr 18S e o outro cístron na região pericentromérica do braço curto de um par de cromossomos submetacêntrico (Fig. 2). Todas as espécies de *Pimelodus* aqui estudadas apresentaram cístrons de U2 snDNA em um par de cromossomos na região pericentromérica do braço curto (Fig. 3).

Discussão

Estudos citogenéticos em Pimelodidae tem evidenciado manutenção do número diploide basal de 2n=56 cromossomos, com ocorrência de redução do número diploide em espécies do grupo *Calophysus* (2n= 50 ou 54 cromossomos) (Ramirez-Gil *et al*., 1998; Vasconcelos & Martins-Santos, 2000; Swarça *et al*., 1999; Sanches *et al*., 2010; Carvalho *et al*., 2011) e *Pimelodus fur* (2n=54 cromossomos) (Garcia & Moreira-Filho, 2005), e com aumento do número de diploide em *Pimelodus blochii*, com 2n=58 cromossomos (Fonseca *et al*., 2018). O cariótipo de *Pimelodus pantaneiro* do rio Miranda foi descrito por Souza *et al*. (2003) (citado como *Pimelodus maculatus*), nossos resultados confirmam o mesmo número diploide e a fórmula cariotípica, porém a localização dos sítios de DNAr 18S descritos aqui pela primeira vez, diferem das AgRONs evidenciadas por Souza *et al*. (2003). É possível que essa variação seja decorrente da incorreta identificação dessa espécie de *Pimelodus*, que, segundo Souza-Filho & Shibatta (2007), possui padrões de coloração e caracteres morfométricos parecidos com *Pimelodus mysteriosus*, também encontrada na bacia do rio Paraguai, sendo possível diferenciar essas duas espécies principalmente pelo comprimento do barbilhão maxilar e mentoniano externo, que são mais longos em *P. mysteriosus*. A ausência

dos números de depósito dos exemplares estudados por Souza *et al*. (2003) impossibilitam a revisão dessas espécies e de seus dados citogenéticos.

A localização do DNAr 5S em *Pimelodus panteiro* é descrita aqui pela primeira vez e essa espécie exibiu cístrons na região pericentromérica de dois pares de cromossomos, sendo observado em um deles sintenia com o DNAr 18S. Os dados de mapeamento dessa sequência em Pimelodidae mostram a ocorrência de sítios simples e múltiplos, com variação na localização e com casos de sintenia em *Pimelodus britskii* (Moraes-Neto *et al*., 2011, Girardi *et al*., 2018), *Pimelodus blochii* (Fonseca *et al*., 2018) e *Pimelodus maculatus* (Girardi *et al*., 2018). Nos peixes o arranjo mais comum dos genes DNAr 5S e 18S é em diferentes cromossomos (Martins & Galetti-Junior, 2001; Martins & Wasko, 2004). Segundo Martins & Galetti-Junior (1999) a sintenia entre esses genes pode causar interferências disrruptivas, o que explicaria o arranjo desses genes em cromossomos separados ser o mais comum entre os vertebrados.

Esse estudo é o primeiro a fazer o mapeamento dos cístrons de U2 snDNA em espécies de *Pimelodus*. A ocorrência desses sítios em apenas um par de cromossomo foi observada em todas as espécies, estando localizadas na região pericentromérica do braço curto. Em Pimelodidade a localização dessa sequência em um par de cromossomos também foi observada em *Bergiaria westermanni* (Malimpensa *et al*., 2018), única espécie com dados desse marcador na família, o que pode indicar um padrão conservado de localização do U2 snDNA nesse grupo, porém estudos de mapeamento dessa sequência em maior número de espécies são necessários para validar essa suposição. Cístrons simples tem sido identificados em várias espécies de peixes nas diferentes ordens, como nos Batrachoidiformes (Merlo *et al*., 2012a; Úbeda-Manzanaro *et al*., 2010), Characiformes (Silva *et al*., 2015; Piscor *et al*., 2016; Piscor *et al*., 2018; Santos *et al*., 2017; Serrano *et al*., 2017**;** Ponzio *et al*., 2018), Cyprinodontifornes (Araya-Jaime *et al*., 2017a), Gadiformes (Garcia-Souto *et al*., 2015), Siluriformes (Malimpensa *et al*., 2018; Supiwong *et al*., 2013), entre outros, sendo o padrão predominante entre as espécies estudadas. O mapeamento do U2 snDNA em peixes tem demonstrado que a ocorrência de sítios na posição intersticial/pericentromérica é a mais comum (Yano *et al*., 2016), possivelmente a organização conservada dessas sequencias em regiões internas dos cromossomos representa alguma vantagem, da mesma forma que ocorre com o DNAr 5S (Martins & Galetti Jr 2001).

Sintenia do DNAr 18S e U2 snDNA foram evidenciadas em nove das 50 espécies/populações de peixes analisadas e a associação do DNAr 5S e U2 snDNA foi observada e, demonstrando assim, que sítios independentes desses genes prevalecem na

organização genômica dos peixes. Segundo Yano *et al*. (2016) a presença de heterocromatina nesses sítios pode contribuir para esses variados arranjos no genoma dos peixes, devido a conhecida ocorrência de rearranjos nas regiões heterocromáticas. Nos Siluriformes as duas condições são observadas (DNAr 18S ou DNAr 5S sintênico ao U2 snDNA), mas devido a carência de espécies estudadas com esses marcadores ainda não é possível estabelecer relações sobre sua distribuição nesse grupo.

Os dados mostram que a presença de U2 snDNAr em um par de cromossomos representa um padrão comum em *Pimelodus* e Pimelodidae até o momento, além de ser o mais evidenciado nas espécies de peixes, refletindo uma característica conservada. O mapeamento das sequências repetitivas de DNAr 5S, 18S e U2 snDNA mostra a manutenção no número de sítios de DNAr 5S e U2 snDNA, enquanto variações no DNAr 5S são comuns em Pimelodidae, possibilitando assim estabelecer relações entre os grupos dessa família utilizando marcadores citogenéticos.

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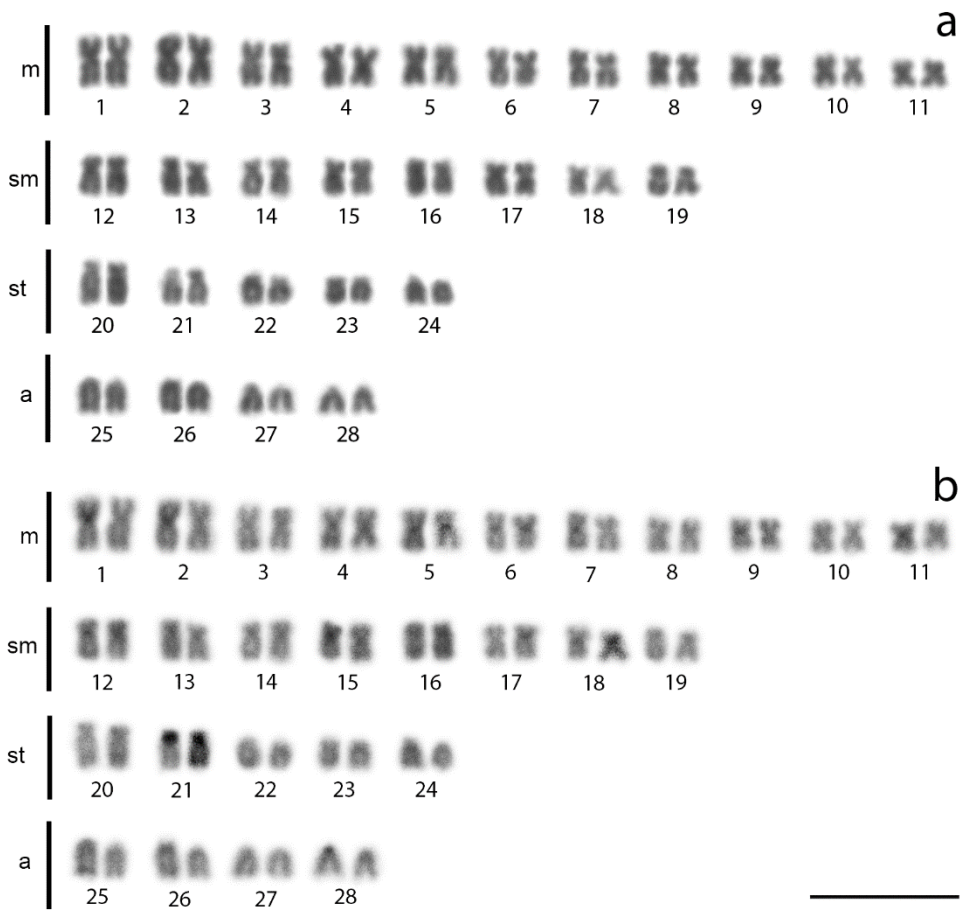

Figura 1. Cariótipos de *Pimelodus pantaneiro* corado com Giemsa (a) e C-bandado (b). Barra representa 10 μm.

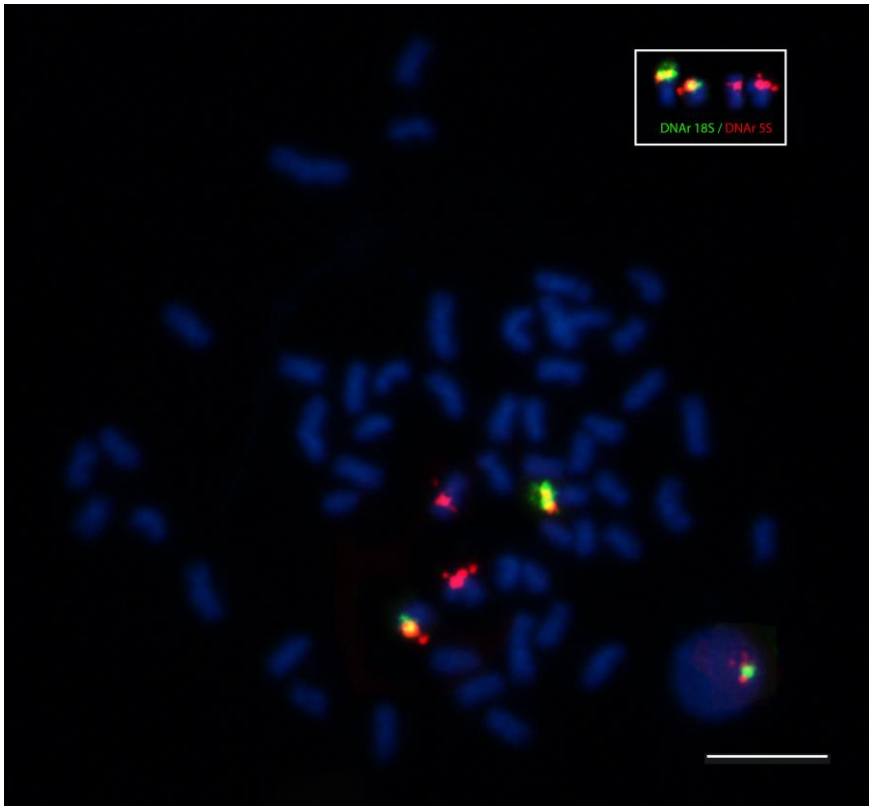

Figura 2. Metáfase de *Pimelodus pantaneiro* após a hibridização *in situ* fluorescente com sondas de DNAr 5S (rodamina, vermelho) e sonda de DNAr 18S (FITC, verde). Barra representa 10 μm.

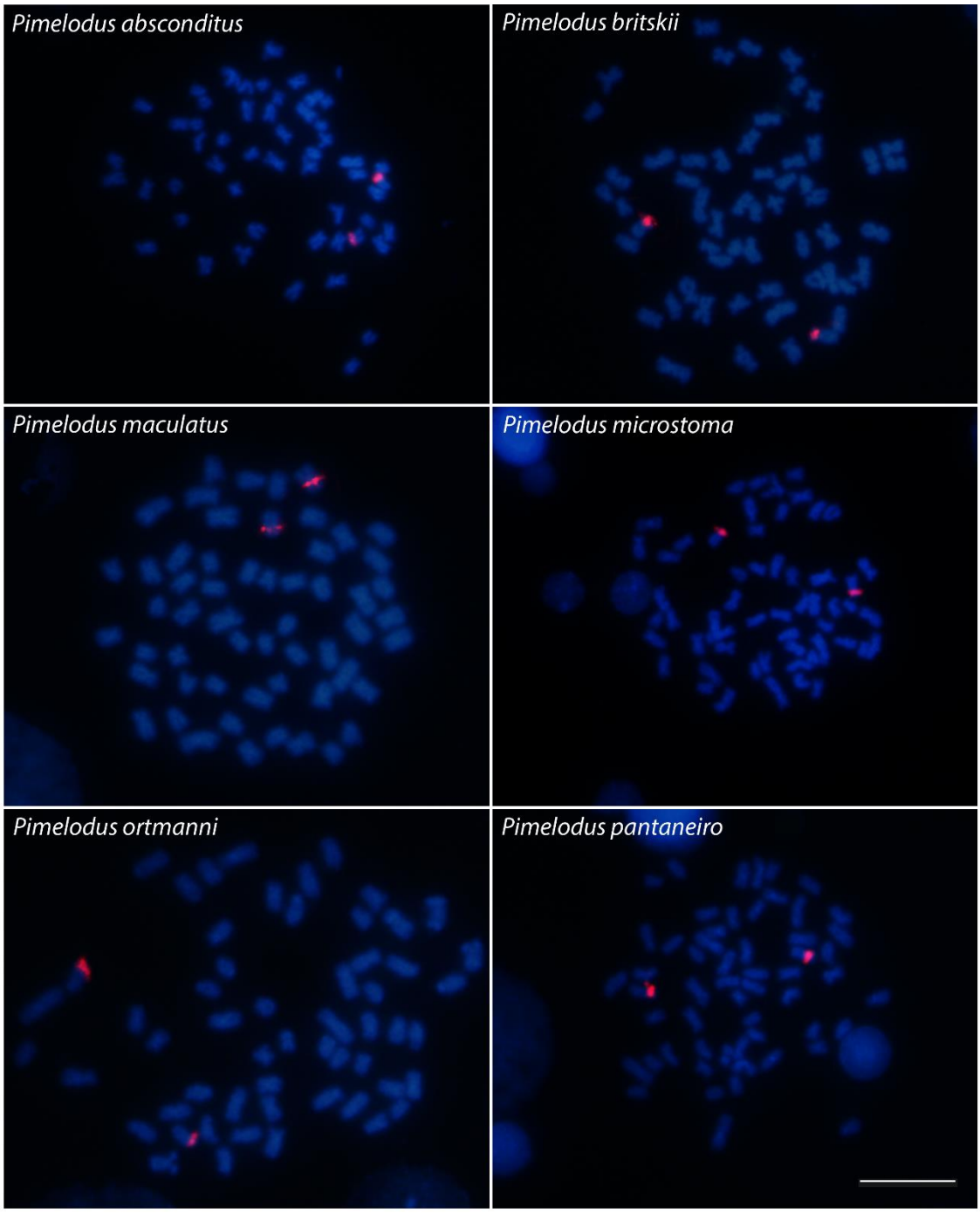

Figura 3. Metáfases de *Pimelodus* após a hibridização *in situ* fluorescente com sondas de U2 snDNA (rodamina, vermelho). Barra representa 10 μm.

Tabela 1. Dados citogenéticos de U2 snDNA em Pimelodidae. D: disperso; p: braço curto; q: braço longo; -: ausência; +: presença.

Espécie	Sítios	Posição	Referência
Bergiaria westermanni	2	Intersticial	Malimpensa *et al.* (2018)
Pimelodus absconditus	2	Pericentromerica p	Presente estudo
Pimelodus britskii	2	Pericentromerica p	Presente estudo
Pimelodus maculatus	2	Pericentromerica p	Presente estudo
Pimelodus microstoma	2	Pericentromerica p	Presente estudo
Pimelodus ortmanni	2	Pericentromerica p	Presente estudo
Pimelodus pantaneiro	2	Pericentromerica p	Presente estudo

Tabela 2. Espécies de *Pimelodus* estudadas. N= número de espécimes estudados. ♂ = macho, ♀ = fêmea, ? = indeterminado, NUP= número do voucher da Coleção Ictiológica do Nupélia.

Espécie	**Localidade/ Bacia**	**Coordenada Geográfica**	**N**	**NUP**
Pimelodus absconditus	Rio Ijuí Alto rio Uruguai	28°18'06.3"S5 3°53'33.6"O	17♂;6♀;1?	17259, 17264
Pimelodus britskii	Rio Iguaçu Baixo rio Iguaçu	25°37'13.2"S5 4°23'29.2"O	2♂;8♀;1?	17260, 17265, 17266, 17269
Pimelodus maculatus	Rio Ijuí Alto rio Uruguai	28°18'06.3"S5 3°53'33.6"O	1♂;3♀	17263
Pimelodus microstoma	Rio Piquiri Alto rio Paraná	24°56'54.0"S5 2°35'49.0"O	4♂;9♀	14938
Pimelodus ortmanni	Rio Iguaçu Baixo rio Iguaçu	25°37'13.2"S5 4°23'29.2"O	5♂;4♀;1?	17261, 17267, 17270, 17271
Pimelodus pantaneiro	Rio Miranda Alto rio Paraguai	19º34'38.0"S57 º01'07.0"O	14♂;3♀	19287

CAPÍTULO 4

Cryptic diversity in the species complex *Pimelodus blochii* (Siluriformes: Pimelodidae) from the Amazon basin.

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Cryptic diversity in the species complex *Pimelodus blochii* (Siluriformes: Pimelodidae) from the Amazon basin.

Running head: Cryptic diversity in *Pimelodus blochii*

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Using basic (Giemsa, C-banding) and molecular (FISH-DNAr 5S and 18S) cytogenetic techniques, exemplars of *Pimelodus* cf. *blochii* collected on Lake Catalão were studied. Three distinct caryomorphs, differentiable by the heterochromatin pattern, location and number of 5S and 18S rRNA sites were observed. This cytogenetic diversity in *P*. cf. *blochii*, compared to the relatively conserved pattern in Pimelodidae, indicates the existence of cryptic species being called *P*. *blochii* in the Amazon Basin. Cytogenetic studies contribute to a better classification of these species and the understanding of phylogenetic relationships in *Pimelodus*.

KEYWORDS

Cytotaxonomy, 5S rDNA, 18S rDNA, cryptic species.

Pimelodus is considered the most numerous and diverse genus of Pimelodidae, with 36 valid species (Eschmeyer & Fong, 2018). However, these species have been grouped without an exclusive character and possibly do not represent the actual diversity of the genus that is considered polyphyletic. The study by Lundberg *et al*. (2011) on the phylogenetic relationships in Pimelodidae reinforces the non-monophyly of *Pimelodus* by grouping its representative species into four clades containing other genera and the increment of the studies will certainly cause the division of these species into monophyletic genera. *Pimelodus blochii* Valenciennes 1840 is widely distributed in the Amazon, Corantijn, Essequibo and Orinoco river basins (Eschmeyer *et al*., 2018). Known for having color variations and morphometry, showed in a study conducted by Lundberg *et al*. (2011) levels of genetic divergence between specimens of populations of the Amazon, Orinoco and Essequibo populations, as large as that observed between well-established species of Pimelodidae, in addition to a specimen of Essequibo being grouped as lineage sister of *Pimelodus argenteus* Perugia 1891. This demonstrates the diversity of this species and reinforces the need for revision in the specimens identified as *P. blochii* along the South American basins.

The cytogenetic data valid for Pimelodidae are restricted to 36 species, with a diploid number ranging from 50 chromosomes in *Calophysus macropterus* (Lichtenstein 1819) (Ramirez-Gil *et al*., 1998), *Luciopimelodus pati* (Valenciennes 1835) (Sanchez *et al*., 2010), and 58 chromosomes in *Pimelodus blochii* (Fonseca *et al*., 2018). *Pimelodus* concentrates the largest number of karyotype species, with a higher incidence of 2n = 56, simple RONs in the long arm, and the few available data of 5S rDNA show single or multiple cistrons and at least one pair with interstitial, pericentromeric or subterminal marking (Girardi *et al*., 2018). Variation in the diploid number among *Pimelodus blochii* populations has been described. In the population of the Tapajós River 2n = 56 chromosomes, and in the lower Amazon River

and in the Trombetas river 2n = 58, variations in the karyotype formula and in the location of the cístrons of 5S and 18S rDNA (Fonseca *et al*., 2018).

Basic and molecular cytogenetic analyzes were performed on six *Pimelodus* specimens identified by Jansen Zuanon as *Pimelodus* cf. *blochii* (1 male and 5 females), collected at Lake Catalão, at the confluence of the Negro and Solimões Rivers (3º09'47 "S, 59º54'29" W) in the city of Manaus, AM, Brazil. This study was carried out in accordance with the recommendations of the Guide to Care and Use of Laboratory Animals, approved by the ethics committee on animal research at the State University of Western Paraná (License number: Protocol 13 / 09- CEEAAP / Unioeste) . The specimens were anesthetized and euthanized by Griffiths (2000). Chromosomal preparations were obtained by Bertollo *et al*. (1978).

The C-band technique by Sumner (1972) with propidium iodide staining was used (Lui *et al*., 2012). The physical mapping of 5S and 18S rDNA was performed by fluorescence *in situ* hybridization (FISH) (Pinkel *et al*., 1986), with modifications (Margarido *et al*., 2008) using the *Megaleporinus elongatus* (= *Leporinus elongatus*) (Valenciennes 1850) probes (Martins & Galetti-Junior, 1999) and *Prochilodus argenteus* Spix & Agassiz 1829 (Hatanaka & Galetti-Junior, 2004) respectively. The probes were nick-labeled with digoxigenin-11-dUTP (rDNA 5S) and biotin-16-dUTP (RNA) and the detection of the signals was done with antidigoxigenin-rhodamine (Roche®) for the probe 5S rDNA and avidin-FITC with Anti-avidin-biotinylated (Sigma-Aldrich) amplification for the 18S rDNA probe, the chromosomes were stained with 4 ', 6-diamidino-2-phenylindole (DAPI, 50 µg / ml).

The metaphases were photographed using the BX 61 epifluorescence microscope with Olympus DP 71 digital camera (Olympus America, Inc.) and DP Controller software 3.2.1.276. The chromosomes were organized and classified according to Levan *et al.* (1964).

This is the first cytogenetic study in *P. blochii* of this locality and its description aims to increase the knowledge of the diversity of Pimelodidae and to provide data that aid in the elucidation of the taxonomic problem involving this species and its phylogenetic relationships.

Three distinct caryomorphs were found. Cariomorph A (two ♀) consists of 2n = 56 and a karyotype formula of 32m + 12sm + 8st + 4a (Figure 1a). The C-banding showed pale heterochromatic regions in the region of the centromere and telomeres and some conspicuous bands in the pericentromeric and telomeres region (Figure 1b). 18S rDNA was located on the short arm of the subtelocentric chromosome pair 24 and the 5S rDNA in two pairs of chromosomes, one adjacent / intercalary syntenic to the 18S rDNA and the other in the pericentromeric region of the submutacentric chromosome pair 20 (Figure 1c).

The Cariomorph B (2 ♀ and 1 ♂) has 2n = 58 chromosomes and the karyotype formula 22m + 6sm + 6st + 24a (Figure 1d). The C-banding showed conspicuous heterochromatic regions in the telomere region of most acrocentric chromosomes (Figure 1e). 18S rDNA was located on the short arm on pairs 15, 16 and on a homologue of pair 1, and on the long arm of a pair of homologue 20. 5S rDNA was evidenced in 16 chromosomes, six in syntenic position with 18S rDNA, and the other cistrons in the telomeres of the short arm of pairs 19, 22, 25 and 26 and the pericentromeric region of the long arm of pair 21 (Figure 1f).

Cariomorph C (1 ♀) presents 2n = 58 chromosomes and karyotype formula 10m + 4sm + 24st + 20a (Figure 1g). The C-banding showed conspicuous heterochromatic regions in the pericentromeric and telomeric regions of most chromosomes, being absent only in the metacentric chromosomes (Figure 1h). 18S rDNA was located in the telomeres of the long arm of pair 6, the short arm of pairs 7 and 8 and a homologue of pair 17. DNAr 5S was evidenced in 16 chromosomes, 6 in syntenic position with the 18S rDNA in pairs 6 , 7 and 8,

and the other cistrons in the telomeres of the short arm of pairs 15, 16, 23, 27, in a homolog of pair 28 and in the long arm region of a homolog of pair 14 (Figure 1i).

The three caryomorphs (A, B and C) distinguishable by the karyotype formula, heterochromatin distribution and localization of 5S and 18S rDNA were observed in the sympatric specimens identified as *P*. cf. *blochii.* Numerical and structural chromosome variations have been reported in other populations of *Pimelodus blochii* with different patterns of body pigmentation, but distinct karyotypes are also observed in specimens with the same staining pattern (Fonseca *et al*., 2018). These different cariomorphs suggest that *Pimelodus blochii* constitutes a group of cryptic species. Examples of this cryptic diversity are common in neotropical fish, such as *Astyanax fasciatus* (Cuvier 1819) (Pazza *et al*., 2006, Ferreira-Neto *et al*., 2012), *Erythrinus erythrinus* (Bloch & Schneider 1801) (Ciofii *et al*., 2010), *Hoplias malabaricus* (Bloch 1794) (Bertollo *et al*., 2000; Blanco *et al*., 2010a; Blanco *et al*., 2010b), *Synbranchus marmoratus* Bloch 1795 (Utsunomia *et al*., 2014), among others. Among the populations of *P.* cf. *blochii* studied there were no intermediate caryomorphs, indicating the absence of hybridization events between them, corroborating the hypothesis that these karyotypic forms represent cryptic species.

The occurrence of 2n = 56 chromosomes (cariomorph A) is suggested as an ancestor for Pimelodidae because it is present in basal genera and in most of the species studied (Moraes-Neto *et al*.,2011, Girardi *et al*., 2018), which is also the most observed in species of *Pimelodus*. However, the presence of 2n = 58 chromosomes in the B and C caryomorphs is an uncommon condition within the family, and until the exclusive moment of *P. blochii*, with reports in the populations of the Low Amazon River and the Trombetas River (Fonseca *et al*., 2018). This variation, possibly, originated from a centric fission and inversions during the evolutionary process, which is supported by the large number of acrocentric chromosomes found in these individuals with 2n = 58. Centric fission events have been suggested to explain

the increase in diploid numbers in genera of Neotropical fish such as *Hypostomus* (Alves *et al*., 2012) and *Potamorhina* (Feldberg *et al*., 1993).

Pimelodidae presents the pattern of simple NORs (Ag- and 18S rDNA) in the terminal region (Girardi *et al*., 2018; Swarça *et al*., 2007). In specimens with caryomorph A, the location of the 18S rDNA follows this pattern. However, individuals with cariomorph B and C presented a new condition for Pimelodidae species by evidencing multiple cistrons of 18S rDNA. In Heptateridae and Pseudopimelodidae, sister groups of Pimelodidae, the presence of simple RONs is also predominant among the species analyzed (Gouveia *et al*., 2018) and multiple sites, confirmed by FISH, are observed only in *Heptapterus mustelinus* (Valenciennes 1835) (Gouveia *et al*., 2018; Vissoto *et al*., 1999), *Pimelodella* sp. (Garcia & Almeida-Toledo, 2010) and *Rhyacoglanis pulcher* (Boulenger 1887) (=*Pseudopimelodus pulcher*) (Gouveia *et al*., 2015). This demonstrates that this is a derived condition for these families and that it arose independently throughout the evolution of these groups.

The location of the RONs in the basal genera and in most species of Pimelodidae are observed in the short arm. However, in *Pimelodus* the location in the long arm prevails with the exception of *P. argenteus* (Souza *et al*., 2003), *P. blochii* (Fonseca *et al*., 2018), *Pimelodus ornatus* Kner 1858 (Borin & Martins-Santos, 2002) and *Pimelodus mysteriosus* Azpelicueta 1998 (Souza *et al*., 2003). In a phylogenetic study, involving the first three species mentioned, none of them were grouped in the same clade as *Pimelodus maculatus* Lacepède 1803, a type species of the genus, which reinforces the polyphyletic character of *Pimelodus*. In specimens with caryomorph A these sequences were also located on the short arm. However, specimens of the B and C caryomorphs presented RONs (DNAr 18S) in both arms, different from the populations studied by Fonseca et al. (2018). The existence of dispersion mechanisms such as centric fission, pericentric inversions, action of transposable elements and the presence of heterochromatin associated with 18S rDNA sites are used to

explain the variation in location and number of these sites in *Hypostomus* (Bueno *et al*., 2014), and possibly one or more of these mechanisms are also responsible for this diversity of 18S rDNA cistrons found in *Pimelodus* cf. *blochii*.

Heterochromatin showed a distinct distribution for each of the caryomorphs described here. Cariomorph A followed the pattern observed for Pimelodidae, with mostly pale bands, and few conspicuous markings on the centromere, telomeres and pericentromeric region. Individuals with 2n = 58 had more conspicuous heterochromatic blocks than those normally found in Pimelodidae species. In the specimens of caryomorph B one of the homologues of pair 1 presented the long heterochromatic arm and several pairs with band in the telomere region. In the caryomorph C, heterochromatin blocks were observed involving the region of the centromere and telomeres of the short arm in almost all acrocentrics, and the absence of banding-C in the metacentric chromosomes allows characterization of this individual. Thus, the location of heterochromatin in these individuals is an efficient marker to distinguish these sympatric specimens, which may represent distinct species.

There are few studies involving 5S rDNA in Pimelodidae. Among the *Pimelodus* species, variation in the number of sites is common and the location in the long arm is predominant (Girardi *et al*., 2018). In *P.* cf. *blochii*, caryomorph A presented 2 pairs with 5S rDNA cistrons, one on the short arm in the telomere region and the other on the long arm in the pericentromeric region. While in the specimens with 2n = 58 (caryomorphs B and C) a large amount of 5S rDNA sites was observed, most of them in the telomere region of the short arm. This shows that *P.* cf. *blochii* has a distinct pattern from that observed in the other species of *Pimelodus* studied so far, which may indicate a different evolutionary history.

Considering the distinct caryomorphs observed in the *P.* cf *blochii* specimens of Catalão Lake, compared to the relatively conserved cytogenetic pattern of Pimelodidae, as for the diploid number, the location of the 18S rDNA and the heterochromatin distribution, it is

possible to infer that these sympatric specimens represent species cryptic. In addition, cytogenetic, taxonomic and molecular studies in the populations of *P. blochii* along the rivers of South America will allow the resolution of the problem involving this complex group, besides helping to understand the evolutionary relations in *Pimelodus* and the correct classification of the species of this group polyphyletic genus.

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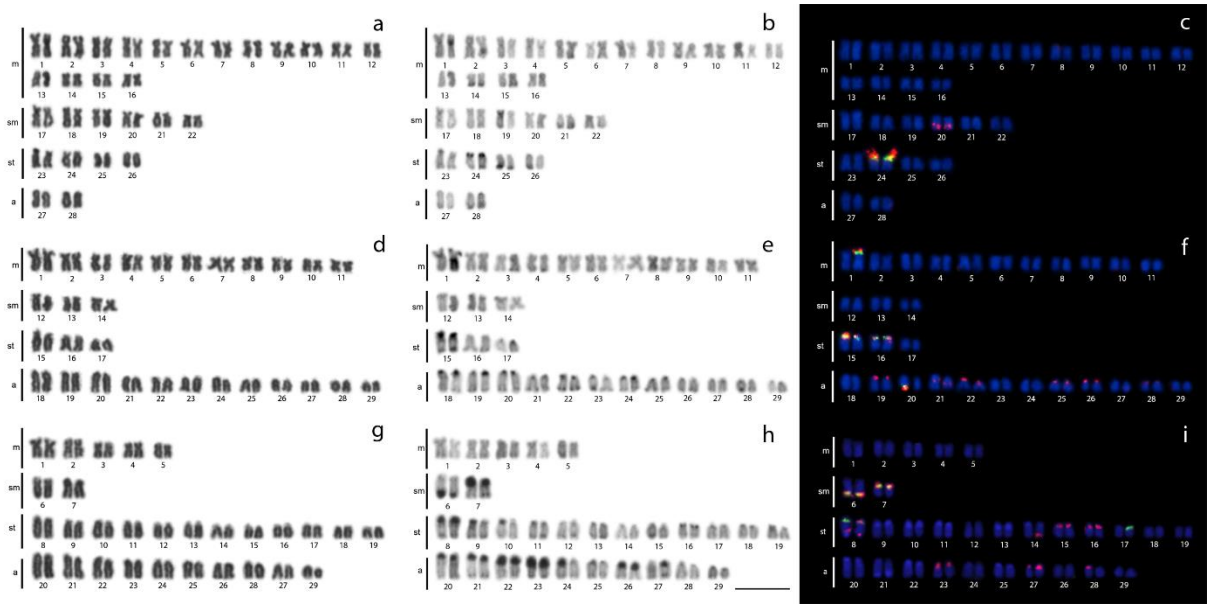

FIG. 1. Giemsa, C Band and FISH. Caryomorph A (a, d, g), B (b, e, h) and C (c, f, i), respectively. Bar represents 10 μm.

ANEXO II

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INSTRUCTIONS FOR AUTHORS (Updated February 2018)

Thank you for your interest in the J*ournal of Fish Biology* (*JFB*). We look forward to handling your submission.
To allow swift consideration of the work, *JFB* has relaxed some aspects of manuscript formatting until a manuscript is resubmitted following review and provisional acceptance for publication. So, please carefully follow these instructions to avoid unnecessary delay and possible rejection of your paper, especially as some instructions are new. The review process itself remains unchanged.

Contents

1. AIMS AND SCOPE

The aim of *JFB* is to publish exciting, high quality science that addresses fundamental questions in fish biology. All submissions must be original and not simultaneously submitted to another journal.

We publish four categories of papers:

An Original Research Article: This contains new biological insight into any aspect of fish biology, particularly those that report results and ideas of interest and value for our wide international readership. Hence, the novelty of the content of manuscripts should have relevance beyond a particular species or place in which the work was carried out.

A Brief Research Communication: This covers any subject within the scope of *JFB*, but should be confined to a single topical point or issue of progress, such as an unusual occurrence, an interesting observation, a timely finding or an important technical advance. Again, relevance beyond the species or locality under consideration is needed.

A Review Article: This is a concise, critical and creative article that synthesizes and integrates available knowledge, and that stimulates topical debate and new research. Authors should submit a synopsis (two pages maximum) of their paper to an Associate Editor for consideration.

A Comment to the Editor: A brief comment on a recently published research paper in *JFB* may be submitted for publication to the Editor-in-Chief. If accepted, it will be sent to the original authors to provide an opportunity for a **Reply** that will be published along with the comment. The following topics are usually **not considered** for publication in *JFB*:
- Commercial fishery stock assessment.
- Basic studies on diet, reproduction, aquaculture techniques, new aquaculture species or toxicology for a single species or a narrow geographic area, unless they have broader significance/interest.
- New markers, unless they are accompanied by detailed work focusing on their usage and addressing relevant biological questions (e.g. population structuring, parentage and genetic mapping).

Special Issues of *JFB* are also published. These regular issues usually comprise either submissions on emerging topics that are specially commissioned by the Editorial Team, or key contributions presented at the FSBI Annual Symposium.

2. SUBMISSION PROCESS

A submission to *JFB* implies that the content has not been submitted for publication elsewhere or previously published except as either a brief abstract in the proceedings of a scientific meeting/symposium or in a MSc/PhD thesis. *JFB* allows for the submission of articles previously available as preprints on servers provided they are non-commercial (such as ArXiv, bioRxiv, etc). Authors may also post the submitted version of their manuscript to non-commercial servers at any time. If the article is accepted for publication in *JFB*, authors will be requested to update any pre-publication versions with a link to the final published article.

All categories of manuscripts are submitted online at http://jfb.edmgr.com, where a user ID and password are assigned on the first visit. Full instructions and support are available on this site. The manuscript text (with pagination, line numbering and a legible 12 pt font size) is uploaded as a text file (not as a .pdf). Separate files for any Tables (text files) and Figures (ESP files) are uploaded to the website independently. During submission, authors must identify an appropriate subject area ('Select Section/Category section) to assign a handling editor and suggest potential referees ('Suggest Reviewers' section). Suggested reviewers are expected to be established experts in the field and be independent of the research under discussion, including the source of funding and the authors' institutions. We strongly recommend that authors use an ORCID iD (a unique author identifier) to help distinguish your work from that of other researchers (for more details visit: https://authorservices.wiley.com/author-resources/Journal-Authors/submission-peer-review /orcid.html). If you experience difficulty with your submission, please contact the Managing Editor at: journaloffishbiology@btconnect.com (see Section 7).

3. PREPARING YOUR SUBMISSION

Authors should consult recent issues of *JFB* for examples of content, emphasis and presentation. Authors whose first language is not English are encouraged to have their manuscript carefully checked before submission by an expert in English or a native English speaker. Wiley Editing Services (wileyeditingservices.com/en/) offer expert help in English language editing, translation, manuscript formatting and figure preparation to ensure that a manuscript is ready for submission.

As *JFB* serves an international community of fish biologists, some conventions are required (see Section 5).

3.1 Preparing an Original Research Article

Authors may submit a manuscript using either UK or North American English spelling, with the exception of exact quotations that are placed in quotation marks. Accepted papers will be converted to **UK English** (the standard is the *Concise Oxford English Dictionary*) during the production process. Latin words, e.g., a genus and species, appear in italics. A cover letter is not mandatory.

An **Original Research Article** consists of 12 essential parts:

Title page
Abstract
Significance Statement
Introduction
Materials and Methods
Results
Discussion
Acknowledgements
Contributions
References
Tables
Figures

When appropriate, submissions may include Supporting Information.

Title page

The title page must contain the following information:

1. **Title of the paper**, which should be short, informative and avoid any geographical or regional references, unless they are fundamental to the scientific thrust of the paper. If a species name is used in the title, we require a common name (if available) followed by the full scientific name. See Wiley's tips for search engine optimization: https://authorservices.wiley.com /author-resources/Journal-Authors/Prepare/writing-for-seo.html;
2. The **family (or formal) name** by which each author is known **plus the initials for their given or familiar names** (see Section 6 for criteria on author eligibility);
3. The **address in full of each author's primary affiliation** (university, research institute, etc.) as a numbered list below the Author list;
4. The **corresponding author**, their **telephone number** and their **email address**. Where necessary, a **footnote** can be added stating that certain authors '… made an equal contribution to this work'. An author's current address can be added to a footnote when different from that at the head of the page.

Abstract

The **Abstract** must be a concise and accurate summary of the **significant findings** of the paper without any introductory or contextual information. Methods can be identified only as part of a result (e.g., Respirometry revealed that exercise increased…; GWAS identified a significant number of SNPs…). If a species name is used in the Abstract, we require a common name (whenever available) followed by the full scientific name.

A list of up to 6 descriptive **Key Words** (maximum 100 characters) in alphabetical order follow the Abstract. Specific geographical (e.g., Baffin Island, Amazon Basin) or regional references (e.g., south-east Asia) can be included here.

Significance Statement

The **Significance Statement** (no more than 75 words) will be available for reviewers as part of the peer review process. It should explain the significance and relevance of the findings of the manuscript to a broad readership and will ultimately appear directly below the online title within the online table of contents. Suggested content includes: an introductory sentence and/or why a problem/unanswered question was important to address; what has been shown/what does the manuscript do to fill a gap in our knowledge; what it means to the field as a whole. A Significance Statement may undergo editorial revision.

Introduction

The **Introduction** alerts readers to literature relevant to the research discovery. The originality of the research cannot be easily assigned without a proper description of the relevant literature. Also, the Introduction must state the intent of the research in the form of a research question or hypothesis so that no confusion arises as to what advance in fish biology is being sought. Footnotes to the text are not allowed; any such material should appear in the text as parenthetical matter.

Examples of text citations of references

Text citations of references use the style "author, date" and multiple references are list in chronological order.

For example: '…as demonstrated by McKenzie (2001) and by McKenzie & Farrell (2010)'; '…as suggested previously in some works (Sloman, 2010), but not others (McKenzie & Farrell, 2010)'; '…consistent with earlier studies (Blaber, 1975, 1988; Prodöhl, 1988; Lujan, 2011a,b)'. Three or more authors are cited with the name of the first author followed by *et al*. (in italics): e.g., (Sloman *et a*l., 2002) or Sloman *et al*. (2002). Authors sharing the same surname and year of publication are distinguished by their initials: e.g., (Young, L., 2012; Young, T., 2012).

Materials and Methods

The **Materials and Methods** may contain up to two levels of sub-headings and must provide sufficient detail so that the work can be replicated by others. Established methods can be simply referenced, preferably acknowledging the original work (rather than a recent user of that method), even if minor changes have been made (which should be described). Materials and Methods must also include information on how observations were analysed to derive the quantitative

results. Statistics should be based on independent biological samples. Technical replicates should be averaged before statistical treatment and not used to calculate deviation parameters. In the case of multiple comparisons (e.g., microarray data), the probability of false positives should be considered in the analysis.

Results

This section, which may contain up to two levels of sub-headings, presents a concise and accurate description of the results of the research. Figures and Tables, which are numbered consecutively in order of their mention in the text, increase the clarity and conciseness of the result presentation, but should not excessively duplicate material. All statements concerning quantitative differences between experimental conditions require quantitative data and adequate statistical treatment. The deviation parameter, the number of biological samples and the statistical procedures should be provided for each dataset either in the main text or as part of a Figure or Table.

Discussion

The Discussion, which may contain up to two levels of sub-headings, is intended to place the results into the broader context of existing literature so that the significance, quality and novelty of the work can be established. The Discussion should return to and address the original research question or hypothesis, as stated in the Introduction. Excessive repetition of results should be avoided. The potential for future work or a brief perspective on the findings can be included in the Discussion.

Acknowledgements

Contributions from anyone who does not meet the criteria for authorship should be listed here with their initial only and without titles or honorifics, e.g., A. P. Farrell, not Prof. Tony Farrell. Thanks to editors and anonymous reviewers are not appropriate. All sources of financial and material support for individual authors (identified by their initials) funding in support of the research described must be declared here when submitting the manuscript. Authors are responsible for the accuracy of their funder designation. If in doubt, please check the Open Funder Registry for the correct nomenclature: https://www.crossref.org/services/funder-registry/

Contributions

The contributions of each author (their initials only, e.g., A. P. F.) must be listed here. Contributions include ideas, data generation, data analysis, manuscript preparation and funding.

References

All published citations mentioned in the text, tables or figures must be listed in the reference list, which includes all key elements of each reference, including the names of journals in full. A manuscript title must appear exactly as in the original publication. However, manuscript submissions are not required to use JFB reference formatting, which will be corrected during the publication process if the article is accepted. Examples of reference content requirements are shown below.

Journal Article: Lacomme, C. & Santa Cruz, S. (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proceedings of the National Academy of Sciences of the USA* **96**, 7956–7961. doi.org/10.1111 /rego.12074
Online Article Not Yet Published in an Issue: Lacomme, C. & Santa Cruz, S. (1999) Bax-induced cell death in tobacco is similar to the hypersensitive response. doi.org/10.1111/rego.12074 An online article is cited by its Digital Object Identifier (DOI), which remains valid and allows article tracking even after its allocation to an issue. It has no volume, issue or page numbers.
Book: Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Chapter in a Book: Shah, J. & Klessig, D.F. (1999) Salicylic acid: signal perception and transduction. In *Biochemistry and Molecular Biology of Plant Hormones* (Hooykaas, P. P. J., Hall, M. A. & Libbenga, K. R., eds), pp. 513–541. New York, NY: Elsevier Science.
Doctoral Thesis: These must have a permanent record of where they are held (e.g. thesis has been lodged at the individual's University or Institution Library as a permanent addition to the collection there), e.g., Lockwood, S. J. (1972). An ecological survey of an 0-group plaice

population, Filey Bay, Yorkshire. Ph.D. Thesis. University of East Anglia, Norwich, U.K.
Master's Thesis: These must be readily available electronically and the URL provided, e.g., Cox, G. K. (2010). Anoxic survival and cardiovascular responses of the Pacific hagfish, Eptatretus stoutii. https://open.library.ubc.ca/ UBC cIRcle.
Electronic References: These include references not subject to peer review and formal publication and can be set out as shown given below. ICES (2016). Report of the Baltic salmon and trout assessment working group (WGBAST).
ICES CM 2016/ACOM:09. Available at:
http://ices.dk/sites/pub/Publication%20Reports/Expert%20Group%20Report/acom/2016/WGBAST/wgbast_2016.pdf
Marshall, A., Bennett, M. B., Kodja, G., Hinojosa-Alvarez, S., Galvan-Magana, F., Harding, M., Stevens, G. & Kashiwagi, T. (2011). *Manta birostris*. In *IUCN Red List of Threatened Species* Version 2013.2. Available at http://www.iucnredlist.org/details/198921/0 (last accessed 9 December 2013).

Tables

Tables are submitted as a separate text files (not pasted as images) and without vertical lines. They complement but do not duplicate information contained in the text. Tables are numbered in order of appearance, are self-contained and include t**he full scientific name(s) of the species** to which the table relates. The table caption should be concise and descriptive, and understandable without reference to the main text. Statistical measures such as S.D. or S.E. should be identified in the caption. Dimensions for the units should appear in parentheses in the column headings and not in the legend or body of the table. All abbreviations must be defined in footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values.

Figures

Preparing Figures
Figures complement information contained in the text, but without unnecessary duplication. Figures are submitted in digital format and as separate files. **Native file formats are not accepted**. They are listed consecutively in order of appearance in the text. Figures that contain data are intended to accurately, clearly and concisely represent the research results, while other figures may better orientate the reader. A wide variety of formats, sizes and resolutions of high quality figures are accepted for initial peer review. Labelling on Figures should be a sans serif font like Helvetica. Multi-panel Figures are identified by lower case letters [(a), (b), etc.].

More information is found at:
https://authorservices.wiley.com/asset/photos/electronic_artwork_guidelines.pdf

Line artwork (vector graphics) should normally be prepared in black and white with shades of grey, unless colour is essential for clarity. Error bars and the method used to derive them must be included in the caption. Line artwork must be saved as Encapsulated PostScript (EPS) file. **Photographs** should illustrate something that cannot adequately be displayed in any other manner. Electron and light microscope photographs must embed a magnification as a **scale bar**. Staining techniques should be described in the caption. Photographs must be saved as bitmap files (half-tones or photographic images) as Tagged Image Format (TIFF) file.
Maps and charts should be contained within a frame and show either a latitude and longitude or a single co-ordinate (N, S, E or W). The JFB standard for geographical names, countries, seas, rivers, etc. is The Times Concise Atlas of the World. London: Times Books.
Figure captions Figure captions are submitted as a separate text file along with the Figures. A Figure caption is a concise and self-contained description of the figure that can be understood without reference to the main text. They begin with a short title for the figure, which i**nclude the full scientific name(s) of the species** to which the illustration relates. Any lines fitted through data points in the figure must be statistically significant and be supported by the mathematical equation and statistical information (*P*-values and *R2* or *R* values). Keys to the symbols, formulae and regression values may be included in the figure itself. Otherwise, symbols and abbreviations appearing in the figure must be explained in the figure caption. The minimum reduction for a figure may be indicated. If material has previously been published, authors must obtain permission from the copyright owner (usually the publisher) to use any figure. Such usage should cite the author in the caption (or text), e.g., 'Reproduced with permission from Craig (1975). Note:

This requirement also applies to the reproduction of a previously published Table or an extended quotation from material.

Supporting Information

Two types are accepted in *JFB*: files containing videos and animations, and long datasets. Supporting Information contains information that is not essential to the article but is a valuable addition by providing greater depth and background. Supporting Information will be reviewed and will appear without typesetting. It is only hosted online and may include datasets, tables, figures, videos, datasets, etc. The availability of Supporting Information is indicated in the main text after the Acknowledgements, headed “Supporting Information”. Short captions list the titles of all supporting material. Supporting Information should be supplied as separate files, and not incorporated into the main manuscript text file. Wiley’s FAQs on Supporting Information is found at: https://authorservices.wiley.com/author-resources/Journal-Authors/Prepare/manuscript-preparation-guidelines.html/supporting-information.html

3.2 Preparing a Brief Research Communication

A Brief Research Communication should be **confined to a single point or issue of progress**, such as an unusual occurrence, an interesting observation, a particularly topical and timely finding or an important technical advance. It must have relevance beyond the species or locality under consideration. First records should adhere to best practices proposed by Bello *et al*. (2014) and should strive to aggregate and report regional historical records for the same species. (Bello, G., Causse, R., Lipej, L. & Dulcic, J. (2014). A proposed best practice approach to overcome unverified and unverifiable "first records" in ichthyology. *Cybium* 38, 9-14.) *JFB* no longer considers short technical notes describing molecular markers (e.g. microsatellites). A **Brief Research Communication** must be **no more than 5 printed pages** (c. 2500 words of text) and normally include no more than **one (multi-panel) figure and one table**. While it is written in freeform **without any headings**, it must follow the same format as Research Articles with respect to the Title, Authors and Affiliations, Abstract, Key Words, Statement of Significance, Acknowledgements and References (see Section 3.1). The Abstract is **no more than 90 words**.

3.3 Preparing a Review Article

Prospective authors will submit a synopsis (two pages maximum) of their article to an Associate Editor or the Editor-in-Chief. The synopsis should outline why the review is topical, its main points and objectives, and how it will stimulate debate and research. When the proposal has been accepted, the authors will submit a manuscript within a mutually agreed upon time and page limit. Review papers must conform to the *JFB* Instructions for Authors in all respects, except for the heading requirements.

3.4 Preparing a Comment to the Editor

Comments must be no more than c. 750 words of text and deal with single significant finding or point for discussion concerning recent published papers in JFB that needs rapid publication. The submission should include a Title page, Main Text and References (maximum four). It contains no Abstract, Key Words, Tables or Figures. After satisfactory peer review, it will be sent to the original corresponding author for a Reply. The reply will take the same form and will be peer reviewed. Publication will end the debate.

4. ETHICAL CONSIDERATIONS

Ethical considerations for the use of animals

The use and treatment of fishes in research is a critical consideration and Ethical and/or Animal Welfare permits/approvals must be listed in the Materials and Methods. Also, contributors must complete a questionnaire when submitting their paper on the Editorial Manager (‘Attach files’ page; questionnaire found if you click here). Ahead of submission, authors will benefit greatly from reading our Editorials on animal welfare: http://onlinelibrary.wiley.com/doi/10.1111/j.0022-1112.2006.01035.x/full (2006) and http://onlinelibrary.wiley.com/doi/10.1111/j.1095-8649.2010.02900.x/full (2011).

Publication Ethics

The Fisheries Society of the British Isles (FSBI) considers that scientists should avoid research threatening the conservation status of any species of fish that is already regarded as threatened according to the IUCN Red List of Threatened Species and the associated current Red List

Categories and Criteria (http://www.iucnredlist.org/technical-documents/categories-and-criteria) or which is listed as such in a Red Data Book appropriate to the country or geographical area concerned. In accordance with this view, papers based on such research will not be accepted, unless the work had clear conservation objectives.

The acceptance criteria for all papers are research quality and originality, and their significance to our readership. Except where otherwise stated, manuscripts are single-blind peer reviewed. All submissions will be considered by the Editorial Board to determine whether they fall within the scope of the journal. Manuscripts deemed by the Editorial Team to be either an inappropriate subject area for *JFB*, or of inadequate scientific quality, or poor quality of English, will be quickly returned to authors without review. A cover letter will explain the decision. *JFB* is a member of the Committee on Publication Ethics (https://publicationethics.org/) and uses iThenticate's CrossCheck software to detect instances of overlapping and similar text in submitted manuscripts. Top 10 Publishing Ethics Tips for Authors are found at: https://authorservices.wiley.com/author-resources /Journal-Authors/Prepare/publishing-ethics.html. Wiley's Publication Ethics Guidelines are found at: https://authorservices.wiley.com/ethics-guidelines/index.html.

Submissions that are sent out for full external review of the scientific quality and the contribution to Fish Biology will be assessed typically by at least two experts; however, in extenuating circumstances (e.g., a delay caused by an overdue reviewer), the Handling Editor may make a decision based on the comments of only one reviewer, in addition to their own assessment of the manuscript. Any requested revisions to the manuscript will have a time line and must be completed to the satisfaction of the Handling Editor, who may consult with the original referees.

If a previously rejected manuscript has been invited to be resubmitted, the manuscript will typically be sent to the same reviewers who saw the original version, providing those reviewers are available. However, in some cases, the Handling Editor may decide that it is not appropriate to re-invite one or more of the original reviewers and/or may judge that a fresh reviewer is needed. While there is no time line for such resubmissions, authors must recognize that the impact of their work, and hence its suitability for *JFB*, may be lessened as knowledge advances with time. Wiley's policy on confidentiality of the review process is available at: https://authorservices.wiley.com/Reviewers/journal-reviewers/how-to-perform-a-peer-review/general-and-ethical-guidelines.html

Authorship

The list of authors should accurately illustrate who contributed to the work. Any person listed as an author, by definition, will have contributed substantially to the article's conception and design, or acquisition of data, or analysis and interpretation of data. All listed authors will be contacted by email after a manuscript is submitted to confirm their contribution. Listed authors should meet the following criteria:

1. Have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; given final approval of the version to be published and have participated sufficiently in the work to take public responsibility for appropriate portions of the content;
2. Been involved in drafting the manuscript or revising it critically for important intellectual content; and
3. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section (for example, to recognize contributions from people who provided technical help, collation of data, writing assistance, acquisition of funding, or a department chairperson who provided general support). Prior to submitting the article all authors should agree on the order in which their names will be listed in the manuscript. (https://www.crossref.org/services/funder-registry/).

How individual authors specifically contributed to the work is listed in the **Contributions** statement.

Additional authorship options

Joint first and/or senior authorship: In the case of a joint first authorship a footnote should be

added to the author listing, e.g., 'X and Y should be considered joint first author' and/or 'X and Y should be considered joint senior author.'

5. EDITORIAL POLICIES AND JOURNAL STYLE

Abbreviations and acronyms: All abbreviations and acronyms must be given in the fully expanded form on first mention in the text and in all figure and table captions except for the small number of abbreviations and acronyms that are scientifically accepted, e.g., DNA. Authors will find the following two publications helpful:

BSI (1967). *Recommendations for Letter Symbols, Signs and Abbreviations:* BS 1991, Part I. London: British Standards Institute.
Baron, D. N. (Ed.) (1977) *Units, Symbols and Abbreviations. A Guide for Biological and Medical Editors and Authors*, 3rd edn. London: The Royal Society of Medicine.

Units: Physical measurements only use metric units in accordance with the Systeme International d'Unites (SI), e.g., m, mm3, s (h and day are acceptable), g, m s−1, g l−1, mg l−1 (not ppm), J (not calories).
The 24-h clock is used for time of day, e.g., 1435 hours, not 2.35 p.m. Calendar dates use day month year, e.g., 15 June 1998.
Salinity has no units; do not use psu, ‰ or similar.
Ship's speed is given in km h−1; knots (nautical miles h−1) can follow in parentheses.
Latitude and longitude can be given either as degrees minute seconds, or decimal degrees, at a level of precision proportionate to the accuracy of the fix. (0.1 second of latitude is equivalent to 185 m, but this decreases for longitude by the cosine latitude).

Statistics, equations & mathematical expressions: Authors will find the following editorials useful.
Equations and mathematical expressions: *Journal of Fish Biology* **8**2, 1771–1772 DOI: 10.1111/jfb.12146 (2013); Reporting statistics: *Journal of Fish Biology* **78**, 697–699 DOI: 10.1111/j.1095-8649.2011.02914.x (2011)

Where decimal values are given, the number of decimal places should be proportional to the accuracy of the work. Thus, means and error (S.D., S.E., 95% C.L., etc.), should be to the same number of decimal places, e.g., 15.1 + 0.2 and not 15.1 + 0.19. In mathematical expressions, single letters (italic) are used for dimensions, qualified by subscripts (roman) as required, e.g., mass (not weight) M, wet mass (M_w), length L, fork length L_F (not FL), standard length L_S, index I, gonadosomatic index I_G, hepatosomatic index I_H, etc.

Statistics are presented as follows: name of test, test statistic with associated degrees of freedom (d.f.; *N.B*. an *F* distribution has two d.f. values) and probability level (*P*). Although ANOVA and regression are robust, the real *P*-values are likely to be different from the precise values provided by the statistics program, because of violations of the assumptions. If the manuscript clearly states that data conform fully to all the assumptions of the statistical method used, then precise *P*-values can be cited with three decimal places. Otherwise, *P*-values are normally limited to: > 0.05, 0.05, 0.01 and 0.001. Confidence intervals (95% C.I.) can be provided for parameters estimated by ANOVA and regression analysis. Where numerical resampling (e.g. bootstrapping) is used to assess the statistical significance of a given parameter (e.g. F_{ST}), in addition to resulting confidence intervals, the number of replicates should be also provided (e.g. 1000 bootstrap replicates).

Species nomenclature, authority and nomenclature: The plural of more than one individual of a single species is 'fish', but it is 'fishes' if there is more than one species. First use of species names in the Title and Abstract must include common (if available) and scientific names without describing the authority and date of authorship. **First mention of a fish species in the main text must include the common name (if available) the binomial scientific name (in italics) and the describing authority and date of authorship**, e.g., rainbow trout *Oncorhynchus mykiss* (Walbaum 1792), not (Walbaum, 1792). Naming authorities must appear in full except Linnaeus, 1758 e.g., Atlantic salmon *Salmo salar* L. No commas are necessary to separate either the common name from the species, or the authority from the date. **After this first mention, the species should then ONLY be referred to by its scientific name**. There should then be no further reference to the common name, describing author or date. The genus name can be abbreviated to a single letter (e.g., *C. carpio* and *O. mykiss*), except either at the start of a

sentence, or where confusion arises from multiple genera with the same first letter, when genus is given in full or the first three letters of the genus provides a clear distinction.
The use or absence of parentheses around the naming authority's name and date is covered by strict scientific rules. If the current accepted genus and species name is the same as that given by the original naming author, the name appears without parentheses, e.g., *Pleuronectes platessa* L. 1758, but if the current accepted scientific name differs from that given by the original naming author, the original author's name appears within parentheses, e.g., *Platichthys flesus* (L. 1758).
Please see for **correct scientific names and formatting of naming author**: Eschmeyer, W. N. (Ed.) *Catalog of Fishes* electronic version (15 November 2013). http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp
Please see for **accepted common names of fishes**:
Wheeler, A. (1992). A list of the common and scientific names of fishes of the British Isles. *Journal of Fish Biology* **41**(Suppl. A), 17–26. doi: 10.1111/j.1095-8649.1992.tb05644.
Wheeler, A. C., Merrett, N. R. & Quigley, D. T. G. (2004). Additional records and notes for Wheeler's (1992) List of the Common and Scientific Names of Fishes of the British Isles. *Journal of Fish Biology***65**(Suppl. B), 1–40. doi: 10.1111/j.0022-1112.2004.00583.x
Nelson, J. S., Crossman, E. J., Espinosa-Perez, H., Findley, L. T., Gilbert, C. R., Lea, R. N. & Williams, J. D. (2004). Common and scientific names of fishes from the United States, Canada, and Mexico, 6th edn. Special Publication 29. Bethesda, MD: American Fisheries Society.
Froese, R. & Pauly, D. (Eds) (2013). *FishBase*. World Wide Web electronic publication. Available at http://www.fishbase.org/Search.php
FAO (2013). A*SFIS List of Species for Fishery Statistics Purposes*. Rome: Fisheries & Aquaculture Department, FAO. Available at http://www.fao.org/fishery/collection/asfis/en

Synonyms for a species require the following style: *Eptatretus cirrhatus* (Forster 1801) *Homea banksii*Fleming 1822: 375 (original description; type locality: South Seas; holotype: unknown); *Bdellostoma heptatrema* Muller 1836: 79 (original description; type locality: South seas; holotype: unknown); *Bdellostoma forsteri* Muller 1836: 80 (original description; type locality: Queen Charlotte Sound, New Zealand; holotype: unknown). Conel, 1931: 76 *Bdellostoma forsteri* var. *heptatrema*. Muller, 1838: 174 (new combination); *Bdellostoma cirrhatum*. G¨unther, 1870: 511 (in part). Hutton, 1872: 87 (in part). Putnam, 1874: 160 (in part); Gunther, 1880: 27. (Note that species names that are modifications of an existing binomial, rather than an original description, are separated from the author name by a full stop, *Bdellostoma cirrhatum*. Gunther, 1870: 511 (in part). [based in part on: Mincarone, M. M. & Fernholm, B. (2010). Review of the Australian hagfishes with description of two new species of *Eptatretus* (Myxinidae), *Journal of Fish Biology* **77**, 779–801. doi: 10.1111/j.1095-8649.2010. 02661.x]

New species: The International Code of Zoological Nomenclature (Article 8.5, amendment) requires that a work bearing a new taxonomic name, issued and distributed electronically must be registered in the Official Register of Zoological Nomenclature (ZooBank) and contain evidence in the work itself of such registration. Any manuscript dealing with the description of new species, genera, or family submitted to JFB must be registered in ZooBank and the name of each new taxonomic name (e.g., new family, genus or species) should be added to ZooBank. Read http://zoobank.org/ and associated video tutorials (http://zoobank.org/VideoGuide) and the Editorial on this subject in *JFB* 90, 1167–1169.

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