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DOS RIOS AO MAR: ESTUDOS DE GENÉTICA E CONSERVAÇÃO DE RAIAS (BATOIDEA, ELASMOBRANCHII) NEOTROPICAIS

FORTALEZA
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sua luz guiou meu caminho até aqui.

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“Sit down before fact like a little child and be prepared to give up every preconceived notion, follow humbly wherever and to whatever abyss nature leads or you shall learn nothing” (HUXLEY, 1903).

RESUMO

Em nosso planeta, o impacto das diversas ações antrópicas no meio ambiente levou a um aumento na velocidade de declínio e extinção de diversas espécies. Dentre as espécies viventes, existem alguns grupos mais sensíveis a impactos antrópicos que outros. Os Chondrichthyes, ou peixes cartilaginosos, são um exemplo desses grupos vulneráveis. Dentre os condrictes, destaca-se o grupo Batoidea (raias) que apesar de mais especioso e com número maior de espécies em algum nível de ameaça, apresentam menos estudos. Logo, o desenvolvimento desses estudos é importante para a elaboração de estratégias de manejo e conservação eficazes. Nesse contexto, o presente estudo tem como objetivo desenvolver inferências que subsidiem o desenvolvimento de ações de conservação para batóideos neotropicais. No primeiro capítulo, foi realizada a primeira confirmação de múltipla paternidade na subfamília Potamotrygoninae. Essa confirmação expande o entendimento sobre aspectos reprodutivos do grupo e abre possibilidades para o entendimento do policromatismo observado em muitas espécies. O segundo capítulo analisa a estrutura populacional da raia-viola (*Pseudobatos horkelii*). Pelo menos dois grupos foram identificados havendo considerável diferença genética a nível populacional entre eles. Esses dados devem ser considerados nos planos de manejo visando a manutenção da diversidade genética dessa espécie. O terceiro capítulo apresenta uma revisão acerca dos dados genéticos conhecidos para o grupo das raias de água doce neotropicais. Nesse trabalho foram identificados e analisados os estudos realizados até o momento bem como, as lacunas de conhecimento presentes, assim como as possíveis alternativas para preenchê-las. No quarto capítulo, foi realizada uma análise das principais ameaças para espécies de Potamotrygoninae. A partir dos resultados, foi possível projetar perspectivas e ações de manejo para o grupo considerando variação regional. No quinto capítulo foi avaliada a diversidade policromática dorsal em *Potamotrygon leopoldi*. Nessa espécie foi descrita a variação ontogenética na coloração e identificado padrões de coloração associados à localidade. Esses dados são importantes ferramentas para a fiscalização da exportação e podem ser utilizados para auxiliar ações de fiscalização. Dessa forma, os estudos apresentados fornecem um aporte de informações importante para o manejo e a conservação de espécies de batóideos neotropicais.

Palavras-chave: elasmobrânquios; genômica; Potamotrygoninae; Rhinobatidae.

ABSTRACT

On our planet, the impact of various anthropogenic actions on the environment has led to an increase in the decline and extinction of various species. Among living species, there are some groups that are more vulnerable to anthropogenic impacts than others. Chondrichthyes, the cartilaginous fishes, are an example of these vulnerable groups. Among chondrichthyans, the Batoidea (rays) group is particularly diverse and has more species that are threatened in some way, but it has received less attention in research. Therefore, the development of such studies is important for the formulation of effective management and conservation strategies. In this context, the goal of this study is to develop research that can support the development of conservation measures for Neotropical batoids. The first chapter provided the first confirmation of multiple paternity in the subfamily Potamotrygoninae. This confirmation expands the understanding of the reproductive aspects of the group and opens up possibilities for understanding the polychromatism observed in many species. In the second chapter, the population structure of the Brazilian Guitarfish (*Pseudobatos horkelii*) is analyzed. At least two groups were identified between which significant genetic differences exist at the population level. These data should be considered in management plans aimed at maintaining the genetic diversity of this species. The third chapter reviews the known genetic data of the Neotropical freshwater ray group. This work identified and analyzed the studies conducted to date, the knowledge gaps that exist, and the possible alternatives to fill them. In the fourth chapter, an analysis of the main threats to Potamotrygoninae species was carried out. Based on the results, it was possible to design management perspectives and measures for the group, taking into account regional differences. In the fifth chapter, polychromatic dorsal diversity in *Potamotrygon leopoldi* was evaluated. Ontogenetic variation in coloration was described in this species, and coloration patterns associated with location were identified. These data are important tools for export monitoring and can be used to support enforcement actions. Thus, the studies presented provide an important contribution of information for the management and conservation of Neotropical batoid species.

Keywords: elasmobranchs; genomics; Potamotrygoninae; Rhinobatidae.

SUMÁRIO

1 INTRODUÇÃO

1.1 Elasmobrânquios

Atualmente, no nosso planeta há uma crise global na biodiversidade (RULL, 2021). O impacto das diversas ações antrópicas no meio ambiente levou a um aumento na velocidade de declínio e extinção de diversas espécies (CARDINALE et al., 2012). A magnitude dessa aceleração na perda de biodiversidade é tamanha que pode ser encarada como a sexta extinção em massa do planeta (COWIE; BOUCHET; FONTAINE, 2022). Essa crise na biodiversidade compromete a conservação de diversos ecossistemas (ALBERT et al., 2020; FANIN et al., 2017). Dessa forma, esforços para garantir a conservação de espécies são impreteríveis para a manutenção dos ecossistemas.

Na priorização dos esforços para a conservação, alguns grupos de organismos são mais sensíveis a impactos antrópicos que outros. Essa suscetibilidade pode vir de características biológicas intrínsecas, como baixa taxa de fertilidade ou maturação sexual tardia, que reduzem a resiliência dessas espécies frente a exploração direta ou alteração de hábitats (CHARVET-ALMEIDA; ARAÚJO; ALMEIDA, 2005). Os condrictes, ou peixes cartilaginosos, são um exemplo desses grupos vulneráveis (LAST et al., 2016). Os condrictes são uma linhagem antiga de vertebrados que compreende tubarões, raias e quimeras (EBERT; DANDO; FOWLER, 2021).

Atualmente, as espécies de condrictes são classificadas em dois grupos: Elasmobranchii é o que contém o maior número de espécies, compreendendo tubarões e raias (aproximadamente 1.206); e Holocephalii com aproximadamente 51 espécies de quimeras (WEIGMANN, 2016; LAST et al., 2016). A diferença entre esses grupos está principalmente na presença de escamas placoides e de 5-7 pares de brânquias sem um opérculo membranoso em Elasmobranchii (Figura 1) (EBERT; DANDO; FOWLER, 2021). Por sua vez, os elasmobrânquios são divididos em dois grandes grupos Selachii (tubarões) e Batoidea (raias), diferenciados pela posição ventral das fendas branquiais e da fusão, total ou parcial, das nadadeiras peitorais a cabeça nas raias (LAST et al., 2016).

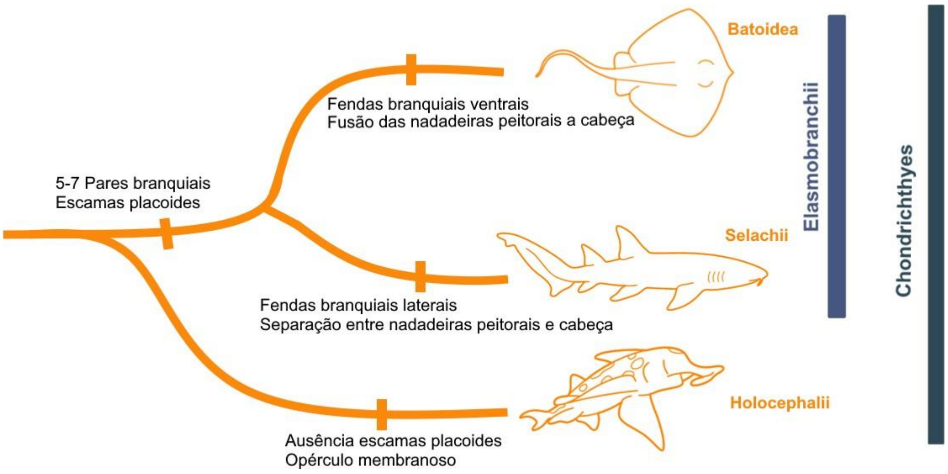

Figura 1. Relações evolutivas entre as principais linhagens de Chondrichthyes. Fonte: Elaborado pelo autor.

Elasmobrânquios são um grupo vulnerável exposto a uma miríade de ameaças. As baixas taxas de fertilidade, crescimento lento e maturidade tardia os tornam muito vulneráveis a impactos tantos de exploração direta quanto degradação de habitat (COMPAGNO; COOK, 1995; ARAÚJO et al., 2004). A pesca figura como principal ameaça à maioria das espécies, especialmente espécies marinhas. Em especial as artes pesqueiras que utilizam redes de arrasto de fundo, que acaba capturando como fauna acompanhante (bycatch) a maioria das espécies. Contudo, ameaças relacionadas à perda de habitat ou a degradação de hábitat por contaminantes são um problema para todas as espécies de elasmobrânquios (MARTIN, 2005). O considerável número de espécies ameaçadas do grupo aponta como a combinação entre vulnerabilidade e ameaças pode ser desastrosa e reforça a importância dos esforços para a conservação dos condrictes (DULVY et al., 2021).

Além do nível atual de ameaça das espécies, a conservação de condrictes é também relevante, pois se tratam de uma linhagem evolutivamente distinta e importante componente em diversos ecossistemas (STEIN et al., 2018). A linhagem dos peixes cartilaginosos é uma das mais antigas entre os vertebrados, com origem estimada em 420 milhões de anos, tendo sobrevivido a extinções em massa (DULVY et al., 2021). Logo, é um grupo interessante a ser estudado pelo ponto de vista evolutivo (DULVY et al., 2014). Ademais, diversas espécies do grupo desempenham papéis como predadores, que são significativos para a homeostase em seus ecossistemas (HUSSEY et al., 2013; SHERMAN et al., 2020). Devido a estas características biológicas, configura-se a necessidade do desenvolvimento de estratégias de conservação.

A obtenção de dados acerca das espécies é um dos obstáculos para o desenvolvimento de estratégias de conservação eficazes para os peixes cartilaginosos. Apesar da alta proporção de espécies ameaçadas e da escassez de ações de conservação faltam informações acerca da maioria das espécies, o que dificulta a elaboração de legislações e ações adequadas de conservação (DULVY et al., 2014). Entre os grupos de elasmobrânquios, as raias (Batoidea) são o grupo mais especioso (WEIGMANN, 2016). No entanto, apesar do número maior de espécies, em geral há menos estudos envolvendo espécies de batóideos (HIRSCHFELD et al., 2021), mesmo este táxon apresentando maior número de espécies ameaçadas (DULVY et al., 2021). Diferenças existem também em nível geográfico, regiões tropicais possuem em geral mais espécies ameaçadas e menos estudos (REIS et al., 2016). Diante desse cenário, é então interessante aplicar esforços para obtenção de dados acerca de espécies de batóideos, especialmente de regiões tropicais.

A costa brasileira apresenta 211 espécies de elasmobrânquios, com considerável número de representantes de água doce (31) (KOTAS et al., 2023). Das espécies marinhas (179) há 55 espécies em algum grau de ameaça segundo o Ministério do Meio Ambiente (MMA) e 51 segundo critérios da União Internacional pela Conservação da Natureza (IUCN). Considerando as espécies de água doce há uma espécie ameaçada pelo MMA e duas pela IUCN, com a maioria das espécies classificadas como dados deficientes. Esses dados ressaltam a necessidade de desenvolvimento de estudos com elasmobrânquios da costa brasileira.

1.2 Potamotrygoninae: Raias de água doce

Dentre os batóideos tropicais, as raias de água doce se destacam com um grupo evolutivamente único e ameaçado. Essa é a maior linhagem vivente dos elasmobrânquios totalmente adaptada a ambientes dulcícolas (THORSON; WOTTON; GEORGI, 1978). Atualmente, os membros da subfamília Potamotrygoninae compreendem 39 espécies de raias adaptadas a rios e estuários da América do Sul (ROBERTS, 2020; FONTENELLE et al., 2021a;). Estas espécies estão divididas atualmente em quatro gêneros: *Heliotrygon* Carvalho and Lovejoy, 2011, *Paratrygon* Duméril, 1865; *Plesiotrygon* Rosa, Castello & Thorson, 1987; *Potamotrygon* Garman, 1877 (Figura 2). O ancestral do grupo, provavelmente, invadiu o continente durante uma incursão marinha no continente havendo posteriormente a diversificação pelos ambientes dulcícolas (FONTENELLE et al., 2021b). Essa diversificação ocorreu dentro de Potamotrygon, o gênero mais especioso (32) e o único encontrado fora da região Amazônica. Em adição ao exposto, Potamotrygoninae é um grupo em que a informação é relativamente escassa, evidenciando assim a importância do desenvolvimento de estudos com espécies do grupo.

Além da intrínseca vulnerabilidade dos elasmobrânquios, as raias de água doce estão expostas as específicas ameaças do ambiente dulcícola. A presença dos potamotrigonineos nos ambientes dulcícolas os expõe ameaças como fragmentação e degradação de habitat (ARAÚJO et al., 2004; MARTIN, 2005). Essas ameaças ocorrem no ambiente marinho, mas possuem um impacto maior na água doce devido à restrição espacial que reduz a mobilidade das espécies e dispersão dos efeitos. Além disso, algumas espécies do grupo sofrem pressão por diversos tipos de pesca. A pesca ornamental é realizada em várias espécies que apresentam variações nos padrões de coloração como *Potamotrygon leopoldi* Castex & Castello, 1970. Outrossim, o impacto da pesca negativa, a qual acarreta mutilação da cauda ou morte dos indivíduos, é realizada com intuito de evitar acidentes com os ferrões, especialmente em regiões onde há turismo (COMPAGNO; COOK, 1995; ARAÚJO et al., 2004). A pesca direcionada alimentação, apesar de não ser tradicionalmente difundida, a mesma é realizada em especial, na região amazônica pelas comunidades ribeirinhas destas localidades.

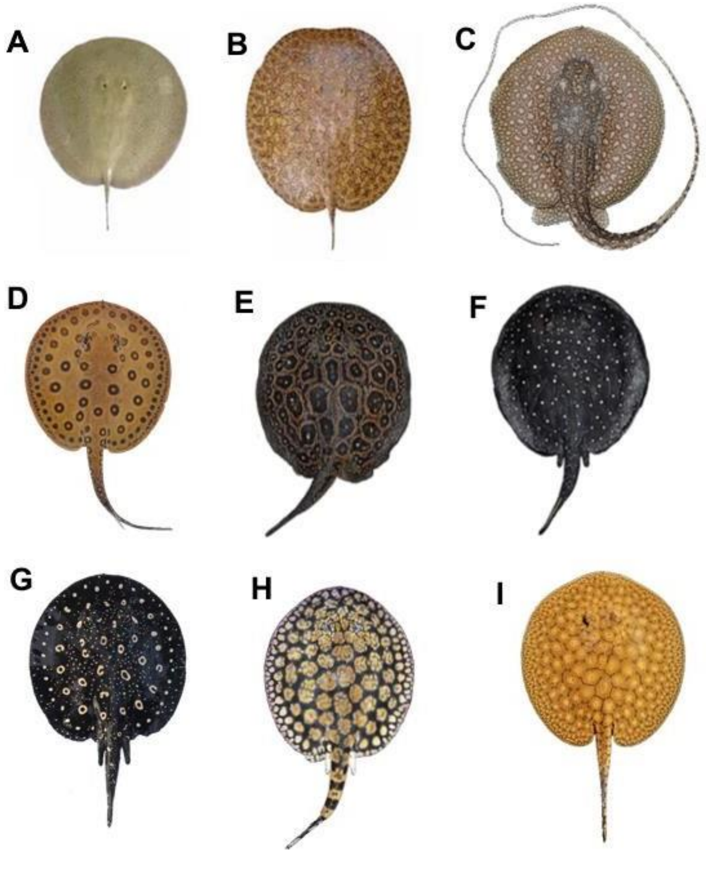

Figura 2. Espécies da subfamília Potamotrygoninae. **A,** *Heliotrygon rosai* Carvalho & Lovejoy, 2011, **B**, *Paratrygon aiereba* (Müller & Henle, 1841). **C**, *Plesiotrygon iwamae* Rosa, Castello & Thorson, 1987. **D**, *Potamotrygon motoro* Müller & Henle, 1841. **E**, *Potamotrygon jabuti* Carvalho, 2016. **F**, *Potamotrygon albimaculata* Carvalho, 2016. **H**, *Potamotrygon leopoldi* Castex & Castello, 1970**. I** *Potamotrygon orbignyi* (Castelnau, 1855). Adaptado de: A, B - FONTENELLE et al. (2021b); C - CARVALHO & RAGNO (2010); E, F - CARVALHO (2016); G – CHARVET, P. Imagem cedida pela autora; D, H, I - MARK SABAJ PEREZ, 2023.

1.3 Rhinobatidae: raias-viola

Dentre todos os batoideos que possuem espécies com status de ameaça de extinção, as raias-viola (Rhinobatidae) são o táxon com mais espécies ameaçadas dentro de Elasmobranchii (MOORE, 2017). Como exemplo de espécies da família, temos as espécies que ocorrem no Brasil: *Pseudobatos lentiginosus* (Garman, 1880), *Pseudobatos horkelii* (Müller & Henle, 1841) e *Pseudobatos percellens* (Walbaum, 1792) que ocorrem na costa sul do Atlântico ocidental (LAST et al., 2016). Devido à pressão pesqueira estas espécies sofreram fortes reduções nas últimas décadas (VASCONCELLOS et al., 2011; VOOREN et al., 2018). Em consequência das características intrínsecas dos elasmobrânquios, ações de conservação efetivas são necessárias para promover a recuperação dessas espécies (WARD-PAIGE et al., 2012). Para o desenvolvimento de estratégias desse tipo, a obtenção de dados confiáveis acerca das espécies é crucial.

As raia-viola são animais demersais que habitam zonas até 400 m de profundidade com substrato arenoso (LAST et al., 2016). Para *P. horkelii*, durante o período reprodutivo se formam agrupamentos em zonas mais rasas (<20m). A alimentação é baseada em invertebrados e pequenos peixes (LAST et al., 2016). A pesca de arrasto, tanto a direta como indireta, principalmente durante o período reprodutivo são uma ameaça para as espécies. Possuem capacidade de dispersão limitada, o que facilita a formação de estruturação genética para o grupo. Dessa forma, a presença de correntes ou ressurgências podem se estabelecer como barreiras para fluxo gênico entre populações. Daí a aplicação de ações de conservação são fulcrais para a conservação do grupo.

No desenvolvimento de ações de conservação, a implementação de ferramentas genéticas tem sido informativa na resolução de questões de conservação. Por exemplo, a partir de análises genéticas podemos obter informações sobre estrutura populacional, fluxo gênico, estimativas de redução populacional e estratégias de reprodução (DOMINGUES; HILSDORF; GADIG, 2017; HIRSCHFELD et al., 2021). Além disso, importantes resultados com filogenias e delimitação de espécies são essenciais para desenvolvimento de planos de conservação adequados (BALL et al., 2016). Como como observado em *Carcharhinus galapagensis* Snodgrass & Heller 1905, dados genéticos proveem informações acerca de estrutura populacional importante no manejo de estoques da espécie e criação de áreas prioritárias de conservação (PAZMIÑO et al., 2017, 2018). Outra importante aplicação de dados moleculares é a detecção da presença de espécies ameaçadas em mercados, evidenciando que o nível de proteção não está sendo efetivo (CAMACHO-OLIVEIRA et al., 2020). Ademais dados genéticos podem fornecer informações acerca de adaptação ecológica enquanto ainda não há variação morfológica evidente (SANDOVAL-CASTILLO; BEHEREGARAY, 2020), ou até a detecção de hibridização (CRUZ et al., 2014).

Nesse cenário, devido ao desenvolvimento de novas técnicas de sequenciamento em nível genômico e seu consequente barateamento, novas possibilidades de análise estão disponíveis (FUENTES-PARDO; RUZZANTE, 2017). Dessa forma, a aplicação de estudos genômicos deverá em breve aprofundar e ampliar nosso entendimento de diversos aspectos nas espécies de elasmobrânquios (DOMINGUES; HILSDORF; GADIG, 2017). Logo, é possível obter dados fulcrais para desenvolvimento de estratégias de conservação eficazes através de estudos genéticos.

Dentro desse contexto, é claro a existência de lacunas de conhecimento que são obstáculos para a conservação de espécies de batóideos neotropicais. Logo, a presente tese tem como objetivo principal desenvolver de estudos que subsidiem o desenvolvimento de ações de conservação para batóideos neotropicais. O primeiro objetivo específico é a aplicar análises genéticas em espécies de batóideos para coadjuvar o manejo de espécies ameaçadas. O segundo objetivo é a produzir revisões bibliográficas que auxiliem o desenvolvimento de ações e avaliações de conservação. O terceiro objetivo é avaliar o policromatismo em uma espécie ameaçada de raia de água doce.

1.4 Apresentação dos capítulos

O presente trabalho se subdivide em cinco capítulos referentes aos estudos (publicados e em submissão) desenvolvidos e alinhados aos objetivos específicos da presente tese. Os primeiros dois capítulos focam em uso de ferramentas moleculares aplicadas para a conservação de raias de água doce e raia-viola. O capítulo 1 apresenta a primeira evidência de paternidade múltipla em raias de água doce obtida a partir de dados genéticos. O capítulo 2 analisa a estrutura populacional de Pseudobatos horkelii na costa brasileira a partir de dados moleculares, com evidência de efeito gargalo para a espécie.

Em continuidade, o capítulo 3 aborda a revisão acerca dos dados genéticos para as espécies de Potamotrygoninae, identificando principais lacunas e perspectivas de estudo. Na sequência o capítulo 4 apresenta a revisão das ameaças para Potamotrygoninae considerando a variação regional por bacia hidrográfica, apresentando um amplo panorama das ameaças para raias de água-doce. O capítulo 5 apresenta o estudo de variação de coloração dorsal em *P. leopoldi*, com implicações para fiscalização e perspectivas evolutivas para a espécies

CAPÍTULO 1: EVIDENCE OF MULTIPLE PATERNITY FOR THE ENDEMIC XINGU RIVER STINGRAY

Scientific publication related to this chapter:

TORRES, Y., CHARVET, P., FARIA, V. V., de CASTRO, A. L. (2022). Evidence of multiple paternity for the endemic Xingu River stingray. *Journal of Fish Biology*, 100(5), 1315–1318. https://doi.org/10.1111/jfb.15038

Abstract

Multiple paternity (MP) is a phenomenon observed for more than 30 elasmobranch species. The Batoidea is more specious than the Selachii, however, only three studies of multiple paternity have been conducted on batoids. The occurrence of MP in freshwater stingrays was tested using microsatellite markers, which were developed for *Potamotrygon leopoldi*. Six mothers and their litters were genotyped, providing the first evidence of multiple paternity for Potamotrygonidae, with a MP frequency of 33%.

Introduction

Multiple paternity (MP) is a phenomenon when two or more males contribute genetically to a litter (Daly-Engel et al., 2010). Several species from different vertebrate taxa are known to adopt this strategy (Correia et al., 2021; Gayet et al., 2021; Zajdel et al., 2019). Hypotheses for the widespread occurrence of MP encompass the points of view of both genders. From a male perspective, copulating with more females would be desirable because it could increase its reproductive fitness (Daly-Engel et al., 2010). On other hand, mating with multiple males would be desirable for females because it could reduce genetic incompatibility and increase offspring fitness (Lyons et al., 2017). Furthermore, comprehension of the evolutionary mechanisms behind this phenomenon requires investigating species with different reproductive biology (Nehmens et al., 2020).

MP has been an important topic for research in the Elasmobranchii, which includes sharks and rays (Lyons et al., 2021). To date, MP is reported for 35 elasmobranchs (Lamarca et al., 2020a); only three of them being rays (Batoidea), two myliobatiform species (Janse et al., 2013; Lyons et al., 2017) and one rajiform species (Chevolot et al., 2007). This unbalanced number of studies on MP for sharks and rays does not reflect the fact that the Batoidea is more specious than the Selachii (Last et al., 2016). Due to the small number of studies assessing MP in batoid species, it is hard to tell how widespread MP is in rays, skates and their relatives. Batoidea is known mainly for its marine representatives; nonetheless within the Potamotrygonidae family there is an extant lineage that successfully colonized South American freshwater environments, the Potamotrygoninae (Fontenelle et al., 2021). This taxon comprises the Neotropical freshwater stingrays, which are widespread throughout the main river drainages in South America, with endemisms often associated with river basins (Rosa et al., 2010). Striking colour patterns are featured by some species, making them heavily targeted for the ornamental market, which if unregulated poses major threats for the group (Araujo et al., 2004). The Xingu River Stingray, *Potamotrygon leopoldi* (Castex & Castello, 1970), is one of the most valued species by aquarists and is endemic to the Xingu River Basin (Charvet-Almeida, 2006). It has a dark (greyish to black) dorsal colour background with white to yellowish spots. This colour pattern makes this species highly valued in the ornamental fish market. Nevertheless, it has been exploited for decades without adequate monitoring to ensure sustainable exploitation and population assessments to improve management are still needed (Charvet-Almeida, 2006). Understanding aspects of reproductive biology such as mating systems could be useful for improving assessments of genetic population structure, aiding the development of conservation actions (Portnoy, 2010). There have been no assessments of MP in this unique freshwater stingray lineage. In this context, the present study assessed the occurrence of MP in *P. leopoldi*.

Methods

A total of 166 individuals were sampled at locations along the mid Xingu River (State of Pará, Brazil), from six points distributed in a stretch of approximately 145 km, between 2002 and 2005. Nine pregnant females were subject to lethal levels of anaesthesia and heart tissue samples were collected from them and from each pup in their litters. The handling, care and use of the specimens was in accordance with the Instituto Brasileiro do Meio Ambiente e Recursos Naturais (IBAMA; Brazilian Environmental Institute) as approved by the Brazilian national permitting system for biological sampling at the time when the samples were taken (IBAMA; permit reference number 44/2002). The samples were fixed and preserved in ethyl alcohol (95%), then stored at temperatures of -18 to -25 ºC. An enriched genomic library was produced from these samples for microsatellite isolation. Five polymorphic loci isolated were characterized using a subsample of 73 individuals from different locations (Figure S1).

Six pregnant females (disc width 466–631 mm) with litters ranging from three to eight embryos were selected for MP analyses. The mid to near-term developed embryos were genotyped using three polymorphic microsatellite loci (*Pleo*83, *Pleo*120, *Pleo*187). The loci *Pleo*83, *Pleo*120 and *Pleo*187 did not differ significantly from expectations of the Hardy–Weinberg equilibrium and presented five to 20 alleles with an expected heterozygosity of 0.95, 0.48 and 0.55, respectively (Table S1). Unfortunately, the loci *Pleo*017 and *Pleo*125 did not successfully amplify for all litters, so they were excluded from the analysis. MP was determined when, in the same litter, at least three different paternal alleles were found for more than one locus. In addition, the number of sires was estimated using softwares Gerud 2.0 and Colony ver. 2.0.6.1. The former, Gerud 2.0, estimates the minimum number of sires through an exhaustive algorithm based on the offspring genotypes array, with or without a known parent (Jones, 2005), while the latter, Colony, uses a maximum likelihood approach to cluster individuals into half sib and then full sib groups, and then estimates the most likely number of sires (Jones & Wang, 2010). The parameters for running Colony analysis were a polygamous mating system for both sexes, full-likelihood with long run model, genotyping error rate set to 0.02% and allele dropout set to 0% through 10 replicates. The allele frequencies used were inferred from the offspring array. The combination of two methods provides a reliable estimation of the number of sires, since Gerud estimates the minimum number of sires and Colony tends to overestimate it, providing a maximum number (Lyons et al., 2017). The probability of detecting MP was estimated using the software PrDM (Neff & Pitcher, 2002).

Results and Discussion

MP was confirmed in two of the six (33%) litters analysed by allele counting (Table S2). In both confirmed cases (litters B and D), three possible paternal alleles at loci *Pleo*83 and *Pleo*120 were observed (Table 1). The estimated number of sires ranged from two to four (Table 2). The probability of MP detection increased with the number of sires and litter size (Table S3). Gerud estimations were congruent with the allele counting observations, although Colony indicated MP in five of the six (83%) litters. The full-sib family probabilities from Colony analysis indicated oversplitting for some litters, which explained this discrepancy in the frequencies (Table S4). Moreover, MP was confirmed for more numerous litters (seven and eight pups), which was supported by PrDM estimates since MP is likely to be easier to detect in larger arrays of progeny (Table S3).

Table 1 – Maternal and paternal alleles identified in the multiple paternity confirmed litters of *Potamotrygon leopoldi*. Bold alleles confirm the existence of multiple paternity with> 2 identified paternal alleles.

	Loci		
	*Pleo*83	*Pleo*120	*Pleo*187
Litter B			
M	178/194	144/154	185/187
O1	194/**196**	**144**/144	185/187
O2	**192**/194	**142**/144	185/187
O3	178/**194**	**142**/144	185/187
O4	194/**194**	**154**/154	185/187
O5	194/**194**	144/154	185/187
O6	178/**194**	144/154	185/187
O7	178/**196**	144/154	185/187
O8	178/**196**	144/154	185/187
Paternal Alleles	**192/194/196**	**142/144/154**	185/187
Litter D			
Mother	192/194	142/144	187/187
O1	**192**/192	142/**154**	185/187
O2	194/**196**	142/**144**	187/187
O3	192/**196**	142/**142**	187/187
O4	194/**196**	142/**144**	187/187
O5	194/**194**	142/**144**	185/187
O6	194/**196**	142/**144**	187/187
O7	192/**196**	144/**144**	187/187
Paternal Alleles	**192/194/196**	**142/144/154**	185/187

Table 2 – Estimated number of sires according to Gerud and Colony analyses.

	Gerud	Colony
Litter A	1	3
Litter B	2	4

Litter C	1	1
Litter D	2	3
Litter E	1	3
Litter F	1	2

The present study is the first report of MP in Potamotrygonidae and the fourth in the Batoidea. MP was observed in *Urobatis helleri*, a species of Urotrygonidae (Lyons et al., 2017), which is considered a closely related clade to Potamotrygonidae (Last et al., 2016), therefore supporting the previous findings of the widespread presence of MP in elasmobranchs (Lamarca et al., 2020a). Considering this, the detection of MP in *P. leopoldi* points to the likely occurrence of MP in other neotropical freshwater stingrays. The confirmation of multiple sires in *P. leopoldi* is indisputable. Even though the small number of loci used here restrained the capacity to detect MP in smaller litters, MP was detected under similar conditions for other elasmobranch species (i.e., loci number, litter size) (Lamarca et al., 2020b; Scheepers et al., 2018). Nonetheless, if a larger number of loci were deployed, this would possibly confirm that all litters have multiple sires. Therefore, MP could not be ruled out for the other litters in this study.

The evolutive drivers behind MP evolution in elasmobranchs are still being discussed. Convenience polyandry is when multiple mating occurs with females to avoid the physical costs of resisting copulation with different males and is widely used to explain MP in elasmobranchs (Daly-Engel et al., 2010). On the other hand, some evidence points to females having an active role in the reproductive system selection rather than being passive, due to potential benefits obtained from multiple mating (Lyons et al., 2021). Despite the different perspectives in female choice role, these concepts are not mutually exclusive and can occur simultaneously during a lifespan. One of those benefits for *P. leopoldi* could be the increase in offspring fitness by the increment of genetic diversity within a litter. This advantage could be especially profitable for an endemic species restricted to a single river basin, such as *P. leopoldi*. Another potential benefit for females would be limiting fertilization by preferred males. Some reproductive mechanisms present in *P. leopoldi*, such as a relatively long copulation window sperm storage and undeveloped eggs, could possibly be indicators of some level of female ‘control’ or reproductive control mechanisms related to fertilization and litter size (Charvet-Almeida, 2006). Other neotropical freshwater stingray species present similar features, and some reproductive strategies variation (Charvet-Almeida et al., 2005). Although not evaluated in the present study, Potamotrygoninae species might be an interesting model for understating the drivers behind MP evolution in elasmobranchs.

The detection of MP could also help shed light on the source of the typical Potamotrygoninae intraspecific polychromatism. The occurrence of different colour patterns within the same species and within litters of a same female is known for *P. leopoldi* and other freshwater stingrays (Castex & Castello, 1970; Charvet-Almeida, 2006; Rosa et al., 2010). The different colour patterns in the same litter could possibly be associated with MP events. There is recent evidence that other Potamotrygon species (*P. motoro*) can identify colours (Schluessel et al., 2021). Therefore, it is speculated that *P. leopoldi* females could identify colour patterns of potential sires and might use visual identification of colour patterns to choose males in courtship and mating, possibly resulting in polychromatic offspring.

This first confirmation of MP in a Neotropical freshwater stingray species possibly implies that mating systems in this group might be more complex than expected. Until now, in other Batoidea, high MP frequencies (>90%) have been observed (Chevolot et al., 2007; Janse et al., 2013; Lyons et al., 2017). High levels of MP frequency are hypothesised to be related with philopatry, as a strategy to avoid inbreeding (Chapman et al., 2004). Considering the species ecology and distribution, a philopatric condition would be more likely for *P. leopoldi*, so a higher MP frequency might be expected (Charvet-Almeida, 2006). This way, the relatively low MP frequency found for *P. leopoldi* might be attributed to an underestimation due to the small number of loci analysed (Barker et al., 2019). Thus, new studies could focus on a profile with more loci to evaluate MP frequency estimation for this and other species of freshwater stingrays.

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3.6 Supporting Information

Table S1 – Five microsatellite loci for *Potamotrygon leopoldi*. Loci name, primer sequence, annealing temperature (Ta), sequence motif, size range, number of allelles (Na), sample size (n) and observed and expected heterozygosity (H_O / H_E).

Locus	**Primer**	**T_a (^{0}C)**	**Motif**	**Size (pb)**	**Na**	**n**	**H_O / H_E**
*Pleo*83	F: FAM GTGGACAAAGTGACCGGA R: CTCAATCCTATCAAATGATCTGC	56	$(CA)_{15}$	164 – 218	20	73	0.92 / 0.95
*Pleo*120	F: NED TCTCCTGGACCTCCTCTGGA R: CCTTCAGCATTTTGTGCGTA	56	$(CA)_{12}$	142-154	6	68	0.46 / 0.48
*Pleo*187	F: PET GGCTCACACTCAGCAATTCA R: TTCATGACATAAGCCGGTGA	60	$(CA)_9$	163-193	5	72	0.51 / 0.55
*Pleo*017	F: VIC TTTGGTCTTGGTGGTGTTGA R: CTCACAAACACGTGCACTCA	59	$(GT)_8$ $(GTAT)_3$	223-247	10	68	0.69 / 0.68
*Pleo*125	F: NED	60	$(TATC)_7$	214-266	18	73	0.89 /

AACTGTCGTGATCCAGAAAAA R: GGCATGTTCAAGGTGATCTT	0.93

Table S2 -Maternal and paternal alleles identified in 6 litters of *Potamotrygon leopoldi*. The litter size is shown in parentheses. Bold alleles confirm the existence of multiple paternity with> 2 identified paternal alleles.

	Loci		
	*Pleo*83	*Pleo*120	*Pleo*187
Litter A			
M	192/194	142/144	187/187
O1	192/194	144/154	187/187
O2	192/194	144/154	187/187
O3	192/192	142/154	187/187
O4	190/192	142/142	185/187
O5	192/194	142/144	187/187
Paternal Alleles	190/192	142 /154	185/187
Litter B			
M	178/194	144/154	185/187
O1	194/**196**	**144**/144	185/187
O2	**192**/194	**142**/144	185/187
O3	178/**194**	**142**/144	185/187
O4	194/**194**	**154**/154	185/187
O5	194/**194**	144/154	185/187
O6	178/**194**	144/154	185/187
O7	178/**196**	144/154	185/187
O8	178/**196**	144/154	185/187
Paternal Alleles	**192/194/196**	**142/144/154**	185/187
Litter C			
Mother	192/194	142/144	187/187
O1	178/194	142/150	185/187
O2	192/196	142/150	185/187
O3	178/194	142/144	187/187
Paternal Alleles	178/196	142 or 144/150	185 /187
Litter D			
Mother	192/194	142/144	187/187
O1	**192**/192	142/**154**	185/187
O2	194/**196**	142/**144**	187/187
O3	192/**196**	142/**142**	187/187
O4	194/**196**	142/**144**	187/187
O5	194/**194**	142/**144**	185/187
O6	194/**196**	142/**144**	187/187
O7	192/**196**	144/**144**	187/187
Paternal Alleles	**192/194/196**	**142/144/154**	185/187
Litter E			
Mother	194/196	142/144	187/187
O1	194/196	142/144	187/187
O2	194/196	142/142	187/191
O3	194/196	144/154	187/187
O4	194/196	142/142	187/187
Paternal Alleles	194/196	142 /154	187/191

Litter F			
Mother	194/196	144/154	187/191
O1	196/196	144/144	187/187
O2	194/196	150/154	187/187
O3	194/196	144/144	187/187
O4	194/196	144/154	187/187
Paternal Alleles	194/196	144/150	187

Table S3 – Estimation of detection probability of multiple paternity (PrDM) with different number of sires, paternity skew and litter size. Based on the allele frequency of *Potamotrygon leopoldi* population.

Sires	Skew					Litter Size					
						3	4	5	6	7	8
2	0.5	0.5				0.14	0.28	0.39	0.47	0.53	0.58
	0.67	0.33				0.12	0.24	0.35	0.42	0.49	0.54
	0.75	0.25				0.10	0.21	0.30	0.37	0.43	0.48
3	0.33	0.33	0.33			0.20	0.39	0.52	0.63	0.70	0.76
	0.57	0.29	0.15			0.17	0.34	0.46	0.56	0.63	0.69
4	0.25	0.25	0.25	0.25		0.24	0.44	0.59	0.70	0.78	0.83
	0.52	0.27	0.14	0.07		0.19	0.37	0.51	0.60	0.68	0.74
5	0.2	0.2	0.2	0.2	0.2	0.26	0.48	0.63	0.74	0.81	0.86
	0.5	0.26	0.13	0.07	0.04	0.20	0.39	0.53	0.63	0.71	0.77

Table S4 – Full-sib family inclusion and exclusion probabilities from Colony analysis for *Potamotrygon leopoldi* litters.

Full-sib Index	**Inclusion Probability**	**Exclusion Probability**	**Member 1**	**Member 2**	**Member 3**	**Member 4**	**Member 5**
1	0.62	0.41	A1	A2	A3		
2	1	0.51	A4				
3	1	0.22	A5				
4	0.53	0.22	B1	B7	B8		
5	1	0.18	B2				
6	1	0.12	B3				
7	0.43	0.18	B4	B5	B6		
8	0.29	0.29	C1	C2	C3		
9	1	0.36	D1				
10	0.42	0.42	D2	D3	D4	D6	D7
11	1	0.23	D5				
12	0.57	0.07	E1	E4			
13	1	0.09	E2				
14	1	0.33	E3				
15	0.44	0.32	F1	F3	F4		

16	1	0.29	F2

CAPÍTULO 2: POPULATION STRUCTURE OF THE CRITICALLY ENDANGERED BRAZILIAN GUITARFISH *PSEUDOBATOS HORKELII* REVEALED BY ddRADseq ANALYSIS

Scientific publication related to this chapter:

TORRES, Y., ROTUNDO, M.M., VIANNA, M., CHARVET, P., FARIA, V. V., OLIVEIRA, C., FORESTI, F., CRUZ, V.P. (submitted). Population structure of the critically endangered Brazilian Guitarfish *Pseudobatos horkelii* (Rhinobatidae) revealed by ddRADseq analysis. *Aquatic Conservation: Marine and Freshwater Ecosystems*

Journal Guidelines: Aquatic Conservation: Marine and Freshwater Ecosystems

Population structure of the critically endangered Brazilian Guitarfish *Pseudobatos horkelii* (Rhinobatidae) revealed by ddRADseq analyses

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Keywords: genomic, SNPs, threatened species, conservation, elasmobranchs

Abstract

Molecular analysis is a powerful tool able to aid in assessing and developing conservation strategies for endangered species. Elasmobranchs comprise one of the most threatened groups of vertebrates, and about one-third are subject to some degree of threat. This is the case for *Pseudobatos horkelii*, the Brazilian Guitarfish, which has suffered severe population reductions (>80%) in recent decades, mainly due to coastal overfishing. Understanding the genetic diversity of this species is paramount to apply adequate management and conservation strategies. In this context, the present study evaluated the genetic diversity of *P. horkelii* employing single nucleotide polymorphisms (SNPs) nuclear markers obtained by double digest Restriction-Site Associated DNA sequencing (ddRADseq). Samples collected in three areas along the Brazilian Coast (Rio de Janeiro, São Paulo, and Rio Grande do Sul) provided an average of 1,565,339 reads and 536 SNPs. The results indicate the presence of at least two genetically distinct populations along the Brazilian Coast, with molecular diversity differences noted among clusters. The heterozygosity excess observed in all populations is likely the result of a bottleneck due to overfishing and habitat fragmentation. Moreover, the upwelling phenomenon in Cabo Frio, in the state of Rio de Janeiro, may be a significant influence towards the observed genetic structure. The gene flow between the clusters was estimated and was noted as assymetric between the studied populations. Therefore, the observed patterns may be the result of the combination of biological characteristics and overfishing pressure in the past decades. The evidence of structured populations presenting genetic diversity differences provides insightful information for the development of stock management and conservation strategies for the Brazilian Guitarfish.

1 Introduction

The significant decline in global biodiversity in the past decades can be considered the sixth mass extinction event on Earth, with land vertebrates considered the most threatened group (Cowie et al., 2022). Despite that, some marine groups, such as elasmobranchs, face comparable threat risks (Dulvy et al., 2014). Within elasmobranchs, Batoidea (skates and rays) comprises the most threatened group, with 36% of species assigned to one of the threat International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species categories (Dulvy et al., 2021). In this regard, the combination of biological features such as long lifespan, late maturity, low fertility, slow growth, and overfishing has led to a severe depletion in several skate and ray species (Worm et al., 2013).

Rhinobatidae, or guitarfishes, comprise 37 shark-like ray species (Fricke and Van der Laan,2022) whose populations have suffered strong depletions (Moore, 2017). This family is among the most threatened elasmobranch groups, with 23 (62%) of its species included in one of the IUCN Red List of Threatened Species threat categories (Last et al., 2016; IUCN, 2021; Dulvy et al., 2021). According to The Food and Agriculture Organization (FAO, 2015), Brazil is among the world's main shark meat consumers, although many ray species, are often marketed as "sharks". This is the case of the Brazilian Guitarfish, *Pseudobatos horkelii* (Müller & Henle, 1841), which, despite being undervalued by consumers and not comprising a direct fishery target species, is vulnerable to unregulated fishing pressures throughout its entire distribution range. In fact, this species is listed as Critically Endangered (CR), mainly due to overfishing, resulting in an estimated population reduction of >80% over the last three generations (Pollom et al., 2020). Therefore, the development of adequate management strategies is crucial to ensure this species' survival, as overfishing decreases genetic richness and evolutionary potential by increasing the influence of inbreeding and genetic drift

(Domingues et al., 2017; Benestan, 2019; Benestan et al., 2021). In this regard, population genetic studies provide relevant insights towards managing impacted and threatened populations (Allendorf et al., 2008; Bernatchez, 2016; Bernos et al., 2020). For example, genetic structure and diversity, and gene flow between populations are crucial data to establish evolutionarily significant units (Hutama et al., 2017). Maintaining genetic diversity is essential for a species to persist during shifts due to anthropic actions (Ramos et al., 2018; Hoban et al., 2022). Genetic diversity can decrease due to genetic drift under limited gene flow, genetic recombination, and selection against poorly adapted genotypes (Davis and Shaw, 2001). Consecutive genetic bottlenecks, where a reduction in population size is noted, or founder effects can result in genetic drift and inbreeding depression (Hallatschek and Nelson, 2010; Signorile et al., 2014; Willi et al., 2022). Thus, genetic diversity data for overfished species is an extremely relevant parameter for conservation management.

Genetic structure analyses comprise another important information source for conservation development. Physical barriers, such as currents, depth, temperature and salinity, can influence the genetic structure of elasmobranch species (Hirschfeld et al., 2021). However, physical barriers are not the only factors able to shape the genetic structure of elasmobranchs, as reproductive behavior or habitat preferences should also be considered (Flowers et al., 2016). Likewise, human exploitation can also influence population structure (Ovenden et al., 2019; Delaval et al., 2021). Therefore, the development of population genetics studies provides crucial data for monitoring and developing species management and conservation strategies.

Genetic structure studies have, however, mainly focused on shark species, with only half of them conducted on Batoidea (Hirschfeld et al., 2021). In this context, the Brazilian Guitarfish genetic structure was previously studied based on a mitochondrial marker (D-loop control region), and the occurrence of structures between populations throughout the species distribution range was suggested (Cruz et al., 2021). Genomic tools are a rich source of information in population genetic analyses, especially in identifying single nucleotide polymorphisms (SNPs) (Johri et al., 2019). Techniques developed to obtain SNPs, such as the double digest Restriction-Site Associated DNA sequencing (ddRADseq), can provide several reliable SNPs (Peterson et al., 2012), and SNPs have revealed detailed population structure outputs in several elasmobranch population genetic studies, (Junge et al., 2019; Kraft et al., 2020). Thus, the present study aimed to analyze the genetic structure of *P. horkelii* throughout three areas of its distribution in southeast coast of Brazil for the first-time using SNPs obtained through ddRADseq.

2 Methods

2.1 Sampling and DNA extraction

Pseudobatos horkelii tissue samples were obtained at three localities along the Brazilian Coast: Rio de Janeiro (RJ, n=15), São Paulo (SP, n=14), and Rio Grande do Sul (RS, n=14), collected by local fishers and research collaborators. A total of 43 samples were sequenced and analyzed (Figure 1). The vouchers were deposited at the UNESP Laboratory of Fish Biology and Genetics collection, in Botucatu, São Paulo, Brazil. The genomic DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen) following total DNA from animal tissues protocol and quantified using a Qubit 4.0 Fluorometer (Invitrogen).

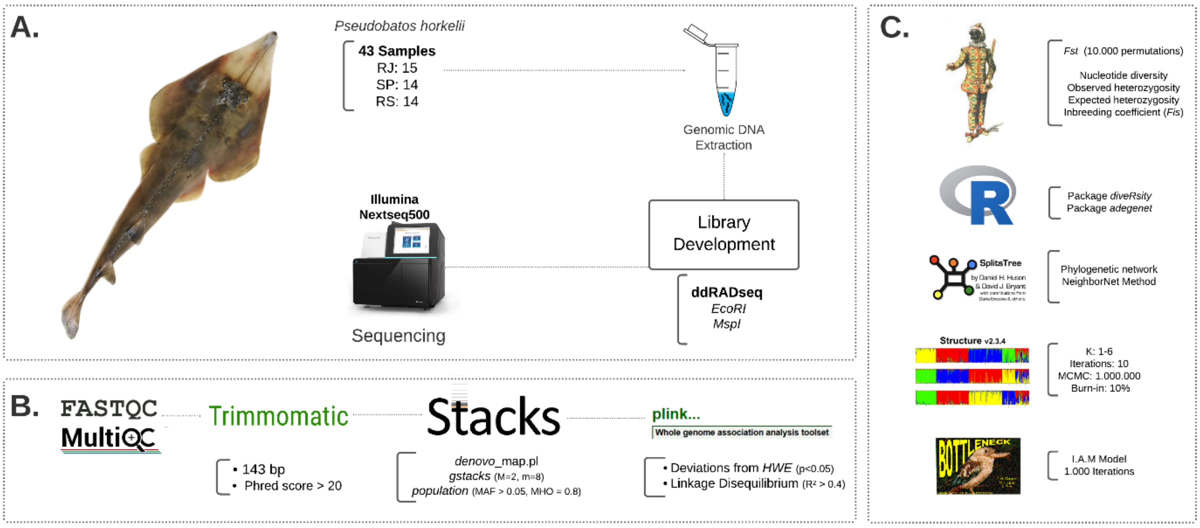

Figure 1. Method workflow: (A) Sampling data and DNA sequencing; (B) Bioinformatics cleaning and filtering; (C) Genetic analysis.

2.2 SNP Library Construction

The samples undeerwent a double digest Restriction-Site Associated DNA sequencing (ddRADseq) (Peterson et al. 2012). The *EcoRI* and *MspI* restriction enzymes were used in the digestion, according to Campos et al. (2017). Following the digestion, a pair of adaptors were attached to the fragments of each enzyme. The Nextera® Index Primers (Illumina, San Diego EUA) i5 e i7 (Nextera DNA CD Indexes – 96 indexes, 96 samples) were used to index the samples. A pool of the processed samples was prepared and submitted to 1% agarose electrophoresis. The fragments within 300-500 base pairs (bp) were removed and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, EUA). The pool was then sequenced employing a NGS Illumina Nextseq500 at the UNESP Biotechnology Institute (IBTEC), in Botucatu, São Paulo, Brazil.

2.3 Genetic Analysis

Sequencing quality was assessed using FastQC (Andrews et al., 2015) and MultiQC (Ewels et al., 2016). All reads were truncated to 143 bp using TRIMMOMATIC (Bolger et al., 2014) due to the presence of adapters in the first five bases of the reads. All retained reads presented a Phred quality score above 20. The ddRAD-seq filtered and trimmed sequences were analyzed using Stacks v2.0 (Catchen et al., 2013) according to Rochette and Catchen, 2017. As a reference genome was unavailable, the Stacks program denovo_map.pl was used to assemble the loci using M=2 and m=8 as parameters when processing the reads, followed by applying the 'ustacks' unit to build the stacks from the filtered reads. Subsequently, 'cstacks' was used to generate a reference catalog. The 'sstacks' unit aligned reads from each sample to the catalog, and the 'gstacks' unit called the variants. Finally, the 'population' unit selected SNPs present in all populations, in over 70% of the individuals ($r = 0.7$), with a maximum observed heterozygosity of 0.80 and a minor allele frequency (MAF) >0.05 (Supplementary Table 1 and Table 2, for more details). The output files from Stacks were converted into other formats using the PGDSpider v. 2.1.1.5 software (Lischer and Excoffier, 2011). The PLINK v1.9 software (Chang et al., 2015) filtered *loci* that significantly ($p<0.05$) deviated from the Hardy-Weinberg Equilibrium and presented linkage disequilibrium ($R^2 > 0.7$). The following population parameters were calculated to analyze genetic diversity: observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}). The ARLEQUIN v 3.5.2.2 software (Excoffier and Lischer, 2010) was used to perform the estimations.

2.4 Population Structure

Genetic distances among populations were estimated through pairwise *Fst* and their significance was tested by running 10,000 permutations in the ARLEQUIN v 3.5.2.2 software (Excoffier and Lischer, 2010). A Bayesian clustering analysis was performed using the STRUCTURE v 2.3.4 software to investigate *P. horkelii* genetic structure (Pritchard et al. 2000) to estimate the likely number of populations based on allele frequencies. K values ranged from 1 to 6, with 10 independent iterations, and one million Markov Chain Monte Carlo (MCMC) repetitions with a 10% burn-in. The most likely number of populations was evaluated by the Delta Evanno (ΔK) (Evanno et al., 2005) and Puechmaille (Puechmaille, 2016) methods using Structure Selector (Li and Liu, 2018). Graphs were generated by CLUMPAK (Kopelman et al., 2015).

In addition, a Discriminant Analysis of Principal Components (DAPC) was performed to identify genetic clusters using the adegenet v. 2.1.9 R package (Jombart and Ahmed. 2010). A phylogenetic network was generated by the NeighborNet Method using SplitsTree v4.18.3 (Huson and Bryant, 2006). The relative gene flow between sampling locations was estimated by the Alcala Method (2014), which combines *D* and *Gst* values to calculate migration rates, using the divMigrate function of the diveRsity v 1.9.89 R package (Sundqvist et al. 2016). The BOTTLENECK v 1.2.02 software was applied to test the occurrence of a recent bottleneck (Piry et al., 1999). The applied models were the Infinite Allele Method (IAM) and Stepwise Mutation Model (SMM) through 1,000 iterations.

3 Results

3.1 Genetic Diversity

The average number of reads sequenced per sample was 1,565,339, which, after the quality filter, was reduced to 744,860 reads (~78%) (Supplementary Table 3). The standard size fragment selected was 143 bp. After filtering, the dataset contained 542 SNPs. The obtained genetic diversity indices are summarized in Figure 2. Observed heterozygosity (Ho) values were higher than the expected heterozygosity (He) for all populations. The He/Ho values were similar between SP and RS and higher in RJ. The RJ location also presented a lower inbreeding coefficient when compared to the other populations. The BOTTLENECK analysis indicated the occurrence of a recent bottleneck in all three sampled locations considering the IAM model and in the RJ and RS populations according to the SMM model (Supplementary Table 4).

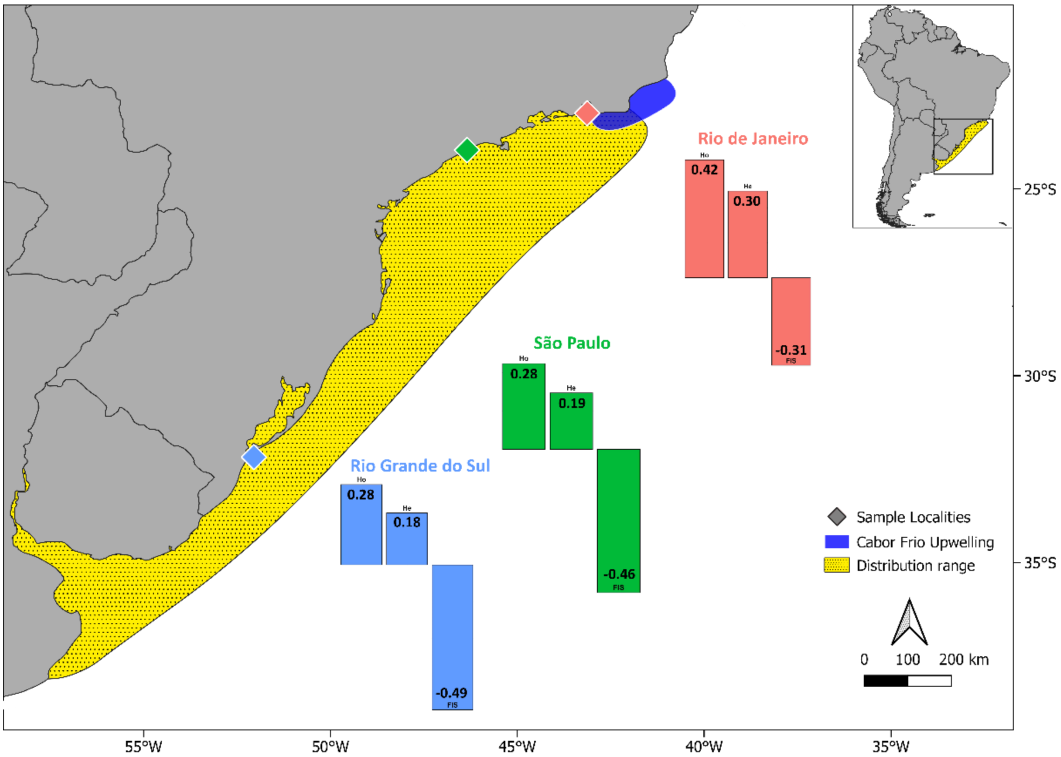

Figure 2. *Pseudobatos horkelii* diversity indices per sampling area. Ho: observed heterozygosity; He: expected heterozygosity; Fis: inbreeding coefficient. The blue area corresponds to the upwelling phenomenon off the coast of Cabo Frio.

1.1 Population Structure

Fst values were significant among all three locations, also indicating a close relationship among SP-RS individuals (*Fst* = 0.01) compared to RJ individuals (*Fst* = 0.12-0.14) (Table 1). The STRUCTURE analysis indicated two clusters (K = 2) inferred from the Delta Evanno Method (ΔK), as the most likely number of populations, with SP-RS individuals clustered in one population (Figure 3-A). Some RJ individuals clustered with the SP-RS group, while others formed a different cluster.

Table 1. Pairwise *Fst* values between *Pseudobatos horkelii* populations distributed along the Brazilian coast. Comparisons marked with * were statistically significant.

	RJ	SP	RS
RJ	-	*	*
SP	0.12	-	*

RS	0.14	0.01	-

The DAPC optimal K value was equal to 3 (BIC = 163), indicating three putative clusters. The first cluster comprised all individuals from SP-RS and some from RJ in one group (Figure 3-B; blue). This finding is similar to the results observed in the STRUCTURE analysis (Figure 3-A). The second and third DAPC clusters comprised the remaining RJ individuals (Figure 3-B; red and yellow). The observed arrangement was similar to the phylogenetic network (Figure 3-D), with individuals from RJ scattered between three different clusters. The STRUCTURE analysis, however, indicated that the remaining RJ individuals (outside of the SP-RS cluster) belonged to a single group.

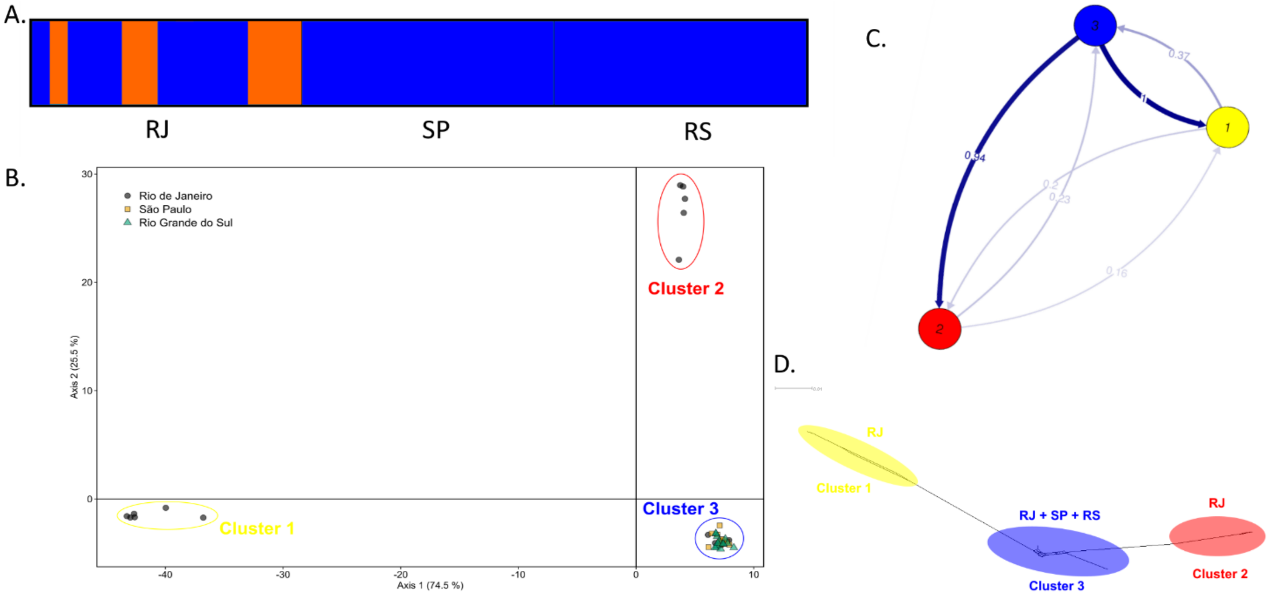

Figure 3. *Pseudobatos horkelii* population structure and connection analysis. (A) Population assignment for optimal estimation (K=2) calculated using STRUCTURE. (B) Discriminant Analysis of Principal Components for optimal estimation (K=3) calculated and plotted using the R package adegenet. (C) Relative migration network between populations calculated using the diversity R package. (D) Phylogenetic network constructed using the NeighborNet on SplitsTree software.

The estimated gene flow between the observed clusters was asymmetrical. The flow from cluster 3 was three-fold higher towards cluster 1 and cluster 2 compared to the flow to the opposite direction (Figure 3-C). These values align with the reported *Fst* values, as populations with greater gene flow exhibited lower genetic distance values, particularly between SP and RS individuals. Moreover, when considering location, the gene flow was higher from SP and RS to RJ (0.49-0.51) compared to the flow from RJ to SP and RS (0.15).

2 Discussion

The findings reported herein provide a better understanding of the genomic diversity of the threatened guitarfish *P. horkelii* from the Brazilian coast based on SNPs *loci*. Evidence of a bottleneck event was noted, with at least two genetically distinct *P. horkelii* groups along the Brazilian coast. Furthermore, genetic differentiation was evident between samples from coastal Rio de Janeiro and São Paulo-Rio Grande do Sul.

The genetic diversity values observed for *P. horkelii* were considered higher (mean Ho: 0.32) for an overexploited species when compared to a less threatened species, such as *Chiloscyllium punctatum* Müller & Henle, 1838 (mean Ho: 0.09) (Fahmi et al., 2021). This is similar to what is noted among sawfishes (Pristidae), which have been completely eradicated in many areas and have undergone severe range restrictions due to fishing (Dulvy et al., 2016). However, an unexpected high diversity pattern has been observed in *Pristis pectinata* Latham, 1794 in a study employing microsatellite markers (Chapman et al., 2011), as reported herein. Other overexploited elasmobranch species (*Carcharhinus falciformis* (Bibron, 1839) and *Sphyrna lewini* Griffith & Smith, 1834) also presented unexpectedly high genetic diversity based on mitochondrial markers (Clarke et al., 2015; Hadi et al., 2020). This may be due to longevity and large population sizes being enough to retain original diversity and counterbalance genetic drift effects (Chapman et al., 2011). Brazilian Guitarfish longevity (estimated as 28 years) is shorter than that estimated for *P. pectinata* (30 years), albeit with similar generation lengths (Lessa et al., 1986, Vooren et al., 2005, Carlson et al., 2013). Therefore, some life-history traits (*i.e.,* generation length) may help explain the relatively high genetic diversity in some species, despite population depletions, as noted for *P. pectinata* (Chapman et al., 2011) and *P. horkelii*.

Diversity levels can remain high for some generations following bottleneck events (Luikart et al., 1998). Therefore, the heterozygosity excess observed in all studied *P. horkelii* populations may have incurred as a result of a bottleneck event. The BOTTLENECK results corroborate this, especially considering the RJ and RS populations. This bottleneck likely results from *P. horkelii* population declines along the Brazilian coast in past years (Miranda and

Vooren, 2003). The unexpected high values are probably a reflection of the genetic impact of this demographic reduction. Therefore, valid evidence of a genetic bottleneck for *P. horkelii* populations on the Brazilian coast are described herein.

The structure analysis points to a genetically structured *P. horkelii* population along the Brazilian coast, corroborating previous studies based on D-loop mitochondrial markers in population analyses for the same species (Cruz et al., 2021). The presence of genetically structured populations is not new for Rhinobatidae species, as significant *Fst* values have been indicated within *Pseudobatos productus* (Ayres, 1854) (Sandoval-Castillo et al., 2004, Meyer, 2020) and *Zapteryx exasperata* (Jordan & Gilbert, 1880) populations (Castillo-Páez et al., 2017). The analyses conducted herein indicate at least two Brazilian Guitarfish clusters among the sampled localities, the first cluster combining all individuals from the São Paulo and Rio Grande do Sul populations and some individuals from Rio de Janeiro, which was consistent throughout all population structure analyses. Considering STRUCTURE data, the second cluster contained the remaining Rio de Janeiro individuals. Based on the DAPC and phylogenetic diagrams, an additional grouping in this remaining Rio de Janeiro group was obtained (Fig. 2 (C), (D); yellow group). Such differentiation is surprising, as the individuals are morphologically similar, the region range is not that large (<200 km) and no geographic barrier seems present. Strong structuration between Rhinobatidae species has been previously reported (Castillo-Páez et al., 2017), and some hypotheses have been postulated to explain the observed structure, detailed below.

Although no evident physical barriers separating the studied populations, other factors may explain the origin of their structure and their maintenance in *P. horkelii*. For example, the low levels of gene flow of the Rio de Janeiro population towards São Paulo and Rio Grande do Sul comprise a significant factor towards genetic differentiation. In this case, this will likely contribute to maintaining population structure due to the minor exchange of genetic material between populations. The observed levels of high diversity and low inbreeding coefficient observed in Rio de Janeiro would then derive from an ancient polymorphism. Intense fishing activities in the past decades have severely reduced populations and may have increased genetic drift throughout the Brazilian coast (Vooren et al., 2005). The depletion of populations along the Brazilian coast may have, in turn, lowered the genetic diversity in most of the studied regions, and some individuals from the Rio de Janeiro area could have retained this ancient genetic characteristic (Han et al., 2017).

Another hypothesis is that the observed genetic structure derived from environmental factors (Bernatchez, 2016). Local environmental heterogeneity is one of the factors responsible for the genetic structure of shark and ray species (Sandoval-Castillo and Beheregaray, 2015). The radiation of *Pseudobatos* Last, Seret & Naylor, 2016 species observed in the Gulf of California (Sandoval-Castillo and Beheregaray, 2020) may be employed as a model for the present results, as the Rio de Janeiro coastline presents different environmental conditions as those present in the Gulf of California, mainly promoted by the Cabo Frio upwelling phenomena (Coelho-Souza et al., 2012). The local northeastern winds contribute to upwelling during the summer, pumping the colder South Atlantic Central Water (SACW) from the shelf slope to the coast (see Campos et al., 2013), forming a complex of heterogeneous habitats. Additionally, philopatry behavior may have also influenced the observed genetic structure in this heterogenous environment (Flowers et al., 2016, Ogburn et al., 2018). This behavior is defined as individuals frequently returning to or staying in their areas of origin, birthplaces, or other specific localities, such as the different ecoregions generated by the Cabo Frio upwelling system (Mayr 1963, Speed et al., 2010, Chapman et al., 2015). Philopatric behavior has been recently confirmed for several ray species, including for other guitarfishes, such as *Z. exasperata* (Castilho-Paez et al. 2014) and *Pseudobatos productus* (Ayres, 1854) (Farrugia et al. 2011, Gong 2022). Therefore, environmental heterogeneity, combined with different habitat preferences and philopratry could generate and maintain the observed clusters in Rio de Janeiro. Thus, different habitats or behavior preferences could be able to maintain reproductive isolation and, consequently, the occurrence of clusters in sympatry (Cardeñosa et al., 2014).

Evidence of similar population structuration linked to the Cabo Frio upwelling is noted for other marine species. For example, Schroeder et al. (2022) recently analyzed body geometric morphometrics and otolith shape in Brazilian Sardine (*Sardinella brasiliensis* (Steindachner, 1879)) populations along Brazil's coastline, identifying two population units related to distinct oceanographic conditions mainly associated with the SACW. Moreover, population differences have also been observed for the Seabob Shrimp *Xiphopenaeus kroyerientre* (Heller, 1862) (Davanso et al., 2017) from the coast of Rio de Janeiro. Both studies suggest that the local upwelling system may be acting in specimen dispersion. Therefore, the results reported herein for guitarfish require further analysis with broad sampling in study areas, which could enlighten this local divergence in the Rio de Janeiro area resulting from ecological differentiation, as reported for other species.

Given the differences between the populations analyzes in this study, migration rates indicated an unsteady flow between populations. The estimated gene flow between individuals from Rio de Janeiro towards the São Paulo - Rio Grande do Sul areas presented the lowest values, while the estimated flow in the other direction was three-fold higher. The clusters generated by the genetic structure analysis indicated a similar pattern, with higher migration rates from the larger cluster (RJ-SP-RS) toward the other groupings. This pattern thus seems to suggest a source-sink metapopulation, with the RJ-SP-RS group acting as the source and the remaining RJ groups as the sink (Sandoval-Castillo and Beheregaray, 2015). Thus, it is feasible that this mechanism acts on the maintenance of *P. horkelii* genetic structuration.

These results comprise significant findings for developing *P. horkelii* management and conservation actions. The confirmation of a genetic bottleneck raises the alert to ensure monitoring and improve conservation actions. The *P. horkelii* population structure as a metapopulation with source-sink dynamics also imposes new management perspectives. Thus, these genetically differentiated groups should be approached as different management units for the preservation of the genetic diversity of this species, especially in the Rio de Janeiro area, due to the distinct lineages probably linked to the area's environmental heterogeneity. Further analysis based on similar markers for the southern region are paramount for a better gene flow resolution to develop broader conservation management actions. Finally, considering the findings reported herein, conservation actions are urgent for *P. horkelii* along the Brazilian coast, mainly since anthropogenic impacts, habitat change and the strong effects of overfishing pose ongoing conservation challenges for this species.

3 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4 Author Contributions

FF, CO and VPC conceived and designed the study. MR and MV collected the data. FF, CO and VPC contributed samples, laboratory infrastructure, and analysis tools. VPC and YT performed the analysis. YT wrote the first draft. VPC, FF, CO, PC, VF, MR, and MV commented on and reviewed the manuscript.

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7 Data Availability Statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA887371.

8 Ethics statements

Ethical review and approval were not required for the animal study because the samples collected were from museum specimens or bycatch from fishers.

9 Importance Statement

A strong decline for several cartilaginous fishes (sharks and rays) is noted, and the development of management strategies is crucial to ensure their conservation. The genetics tools can provide critical information for this management, such as the genetic structure and gene flow between populations. The Brazilian Guitarfish is a critically endangered shark-like ray species that has suffered severe depletion in the last decades, requiring adequate management. This study, based on nuclear markers, reports that the Brazilian Guitarfish is genetically structured along the Brazilian coast in at least two clusters for the first time. Furthermore, one of the areas presented higher genetic diversity than the others, probably due to an ancient polymorphism. These findings should be considered in the development of future management and conservation strategies.

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Supplementary Material

Supplementary Table 1. Filtering parameters and outputs (bold) applied in the analysis.

ustacks	**cstacks**	**gstacks**	**populations**	**plink**
m = 6	n = 2	Mean per-sample coverage: 42.3x	r = 0.7	HWE $p<0.05$
M = 2			min_maf = 0.05	$R^2 > 0.7$
			max_obs_het = 0.8	
Coverage:	**Final catalog:**	**Genotyped loci:**	**Variant Sites:**	**Variant**

25.86x	46396 loci	3687	777	**Sites:** 542

Supplementary Table 2. Depth of coverage and number of reads incorporated for each sample processed calculated by gstacks.

Sample ID	Depth of coverage	Percentage of reads incorporated (Number of reads incorporated)
1021RJ	20.75	8.04% (48421)
1022RJ	21.70	8.05% (50730)
1023RJ	25.65	8.78% (61382)
1024RJ	25.78	9.35% (61216)
1025RJ	19.57	8.8% (46116)
1026RJ	25.05	8.78% (59883)
1027RJ	21.42	8.77% (50737)
1028RJ	26.99	8.08% (63464)
1029RJ	22.26	8.06% (52070)
1030RJ	18.83	8.06% (43640)
138SP	54.86	8.55% (132027)
27468RJ	64.59	9.08% (157053)
28121RS	33.41	8.15% (80297)
28122RS	27.89	7.4% (66707)
28123RS	27.49	8.32% (65060)
28627RS	55.56	8.3% (133640)
28639RS	39.37	5.83% (94671)
28649RS	88.48	8.18% (215662)
28651RS	30.06	8.24% (70266)
28652RS	49.70	7.41% (119947)
28657SP	45.88	7.49% (110066)
28658SP	51.79	8.09% (125345)
28682RS	88.41	8.72% (214587)
28684RS	55.37	7.76% (133793)
28966RS	50.54	8.3% (121918)
28969RS	46.42	7.42% (111694)
28971RS	59.12	8.9% (143264)
28976RS	43.69	7.58% (105178)
35312SP	50.41	8.02% (121509)
35314SP	47.66	7.64% (115283)
35315SP	45.27	8.22% (109096)
35317SP	55.97	8.33% (135511)
35320SP	28.33	7.65% (67887)
35321SP	31.57	8.2% (75640)
35322SP	32.26	8.31% (77691)
35323SP	25.16	7.52% (60053)
78356SP	39.92	7.5% (96207)

78362SP	56.49	9.01% (135878)
78363SP	46.32	8.05% (111932)
89700RJ	46.30	8.06% (111270)
89701RJ	59.26	8.45% (142926)
89702RJ	61.21	9.32% (147603)
89705RJ	53.99	8.78% (131400)

Supplementary Table 3. Summary of the ddRAD sequencing and read filtering processing for *Pseudobatos horkelii* individuals from the states of Rio de Janeiro, São Paulo and Rio Grande do Sul, located along the Brazilian coast. ID: sample identificationn.

Sample	**Raw Data**	**Quality Filter**	**Final Reads (143 bp)**	**Use Rate (%)**
1021RJ	1,087,512	804,771	602,125	55.37%
1022RJ	947,234	798,145	629,852	66.49%
1023RJ	1,146,578	883,047	699,107	60.97%
1024RJ	922,347	790,441	654,781	70.99%
1025RJ	899,874	702,554	524,123	58.24%
1026RJ	1,144,023	889,971	682,288	59.64%
1027RJ	871,564	667,237	578,421	66.37%
1028RJ	1,224,778	910,985	785,446	64.13%
1029RJ	944,389	709,842	646,021	68.41%
1030RJ	860,728	660,417	541,201	62.88%
138SP	1,748,199	1,742,915	1,544,209	88.33%
27468RJ	1,937,213	1,928,565	1,730,269	89.32%
28121RS	1,389,442	1,184,021	985,209	70.91%
28122RS	1,421,374	1,099,203	901,311	63.41%
28123RS	1,127,889	897,541	781,987	69.33%
28627RS	1,788,654	1,784,599	1,610,391	90.03%
28639RS	1,819,304	1,814,106	1,622,772	89.20%
28649RS	3,476,022	3,468,598	2,637,889	75.89%
28651RS	980,763	976,488	852,527	86.92%
28652RS	1,792,334	1,789,044	1,618,037	90.28%
28657SP	1,739,130	1,732,826	1,470,186	84.54%
28658SP	1,793,367	1,787,498	1,549,810	86.42%
28682RS	2,863,728	2,858,368	2,461,304	85.95%
28684RS	1,915,433	1,911,587	1,723,475	89.98%
28966RS	1,785,955	1,782,228	1,468,311	82.21%
28969RS	1,792,269	1,786,091	1,505,819	84.02%
28971RS	1,868,853	1,865,126	1,609,496	86.12%
28976RS	1,769,688	1,762,307	1,387,788	78.42%
35312SP	1,860,896	1,854,513	1,514,717	81.40%
35314SP	1,847,176	1,835,387	1,508,825	81.68%
35315SP	1,540,413	1,535,229	1,327,879	86.20%
35317SP	1,802,822	1,796,223	1,627,558	90.28%
35320SP	1,287,245	1,098,219	887,211	68.92%

35321SP	1,348,742	1,147,541	922,811	68.42%
35322SP	1,432,102	1,301,985	934,515	65.25%
35323SP	1,050,987	949,541	798,541	75.98%
78356SP	1,465,224	1,457,016	1,283,111	87.57%
78362SP	2,064,497	2,052,380	1,507,320	73.01%
78363SP	1,548,112	1,535,206	1,390,088	89.79%
89700RJ	1,599,329	1,592,315	1,381,106	86.36%
89701RJ	1,857,494	1,854,503	1,691,126	91.04%
89702RJ	1,886,162	1,882,662	1,583,351	83.95%
89705RJ	1,659,720	1,654,937	1,497,269	90.21%
Average	1,565,339	1,477,586	1,247,897	77.79%

Supplementary Table 4. Bottleneck test results for *Pseudobatos horkelii* populations distributed along the Brazilian coast using the Sign Test under the IAM and SMM models. The number of *loci* in heterozygosis excess (HetExcess) is indicated by model. Bold values represent significant (p<0.05) results.

Population	**Sign Test IAM**	**Sign Test SMM**	**HetExcess Loci (IAM/SMM)**
Rio de Janeiro	**0.000**	**0.000**	433/382
São Paulo	**0.000**	0.057	198/174
Rio Grande do Sul	**0.000**	**0.003**	189/74

CAPÍTULO 3: CURRENT STATUS AND FUTURE PERSPECTIVES OF NEOTROPICAL FRESHWATER STINGRAYS (POTAMOTRYGONINAE, MYLIOBATIFORMES) GENETICS.

Scientific publication related to this chapter:

TORRES, Y., FARIA, V. V., CHARVET, P. (2022). Current status and future perspectives of Neotropical freshwater stingrays (Potamotrygoninae, Myliobatiformes) genetics. *Environmental Biology of Fishes*,105, 1111 – 1127. https://doi.org/10.1007/s10641-022-01320-x

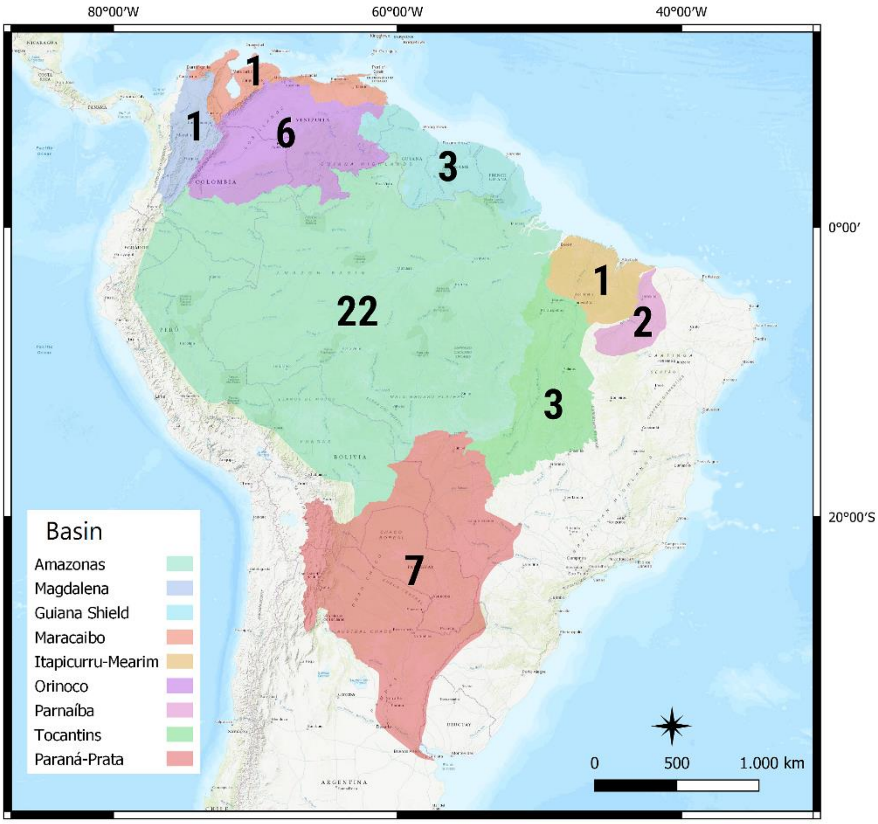
Abstract

Potamotrygoninae is a group of stingrays fully adapted to South American freshwater ecosystems. Recently, there has been an increase in the number of publications applying genetic tools. The present review compiles and discusses the recent advances of these studies and the future perspectives for the genetic studies of this group. Mitochondrial markers are the main molecular marker applied, especially cytochrome *c* oxidase I and cytochrome *b*. The genetic studies were grouped in thematic areas, and the advances and challenges of each topic comprising Evolution, Taxonomy, Phylogeography, Hybridization, Cytogenetics and Transcriptomes were discussed. Despite the recent efforts and increase in the number of studies in recent years, several questions for the group remain unanswered, mainly for *Potamotrygon* species, which present high genetic similarity despite their phenotypic plasticity. The application of genomics techniques might aid to elucidate the complex questions still open for freshwater stingrays.

Introduction

Potamotrygoninae is a group of Neotropical stingrays that comprises the only group of elasmobranchs fully adapted to freshwater ecosystems (Thorson et al. 1978; Fontenelle et al. 2021a). The colonization of South American rivers and estuaries by them is considered a unique evolutionary event for the cartilaginous fishes (Last et al. 2016). Nowadays, the 39 described species of the group are distributed mostly in the Amazon River Basin, followed by the Paraná-Prata and Orinoco basins (Figure 1). Most potamotrygonin species are endemic to a single basin, sometimes even to a single river, which contrasts to others that are widely distributed in multiple watersheds (Lasso et al., 2013). In the last decade, the group has been through a great increase of studies; for instance, it was in this period that more than 40% of the known species in the group were described (Carvalho and Lovejoy 2011; Carvalho and Ragno 2011; Fontenelle et al. 2014; Carvalho et al. 2016; Fontenelle and Carvalho 2017; da Silva and Loboda 2019; Roberts 2020; Loboda et al. 2021).

Figure 1 – Main South American river basins with records of occurrence of potamotrygonin freshwater stingrays. Bold numbers represent the number of species in each basin.



The Potamotrygoninae comprises four genera: *Paratrygon* Duméril, 1865; *Potamotrygon* Garman, 1877; *Plesiotrygon* Rosa, Castello and Thorson, 1987 and *Heliotrygon* Carvalho and Lovejoy, 2011. Most species belong to the *Potamotrygon* genus, which currently comprises 32 described species distributed in several river basins throughout South America. *Paratrygon* was a monotypic genus until a recent subdivision (Loboda et al. 2021), now it comprehends at least three species distributed in Orinoco and Amazon River basins. *Heliotrygon* and *Plesiotrygon* genera present two species each and occur throughout the

Amazon River Basin.

Like many other elasmobranchs, freshwater stingrays have low fecundity, late maturity, and long lifespan, which makes them vulnerable to overexploitation and environmental changes (Charvet-Almeida et al. 2005). Attractive and striking color patterns are a feature for some species, which makes them a target for ornamental market trade; their unsustainable exploitation is one of the main threats for the group (Pereira et al. 2004; Rosa et al. 2010). Other types of fisheries threaten the freshwater stingrays in South America, including fisheries for consumption as food and mutilation or local extirpation ('negative fishery' *sensu* Compagno and Cook 1995) (Araújo et al. 2004). Since they inhabit freshwater ecosystems, they are exposed to other impacts such habitat fragmentation (dams) and degradation (agricultural chemicals runoff, silting, and mining) (Charvet-Almeida et al. 2002). Therefore, Neotropical freshwater stingrays present limiting life history characteristics and may be subject to a combination of the threats mentioned above. Thus, population monitoring and conservation strategies should be applied to these stingrays.

Despite the need for management and conservation actions for Neotropical freshwater stingrays, the difficulty in assessing these animals in areas as remote as the Amazon Basin, coupled with the lack of knowledge about several aspects of their natural and evolutionary history challenges the development of appropriate conservation strategies (Toffoli et al. 2008; Carvalho and Lovejoy 2011; Cruz et al. 2014). It is within this context that genetic techniques, vastly applied to other elasmobranchs (Dudgeon et al. 2012; Marino et al. 2018; Gonzalez et al. 2019; Camacho-Oliveira et al. 2020) may provide useful information about freshwater stingrays (e.g. taxonomy, population structure, gene expression, reproductive biology) and help on their conservation. For instance, DNA based markers (mitochondrial and nuclear) are useful tools to evaluate species complexes and hybridization (Fields et al. 2016; Easton et al. 2020). Cytogenetic markers may provide important insights about evolutionary processes (Valentim et al. 2019). Transcriptomic analysis can address different fields that are not fully understood, as for example, venom composition and color determination, and physiological adaptations (Connon et al. 2018; Schluessel et al. 2021). Thus, genetic tools can be considered an effective way to obtain information to solve different questions for Potamotrygoninae and assist in conservation. The present study aimed to assess the state-of-art application of genetic tools to South American freshwater stingrays (Potamotrygoninae).

Literature Search

The bibliographic search was made in four online database portals (Google Scholar, Science Direct, Web of Knowledge and Shark References) using the following keyword combinations: "potamotrygonidae + genetics"; "freshwater + stingray + genetics"; "potamotrygonidae + molecular"; "potamotrygoninae + genetics"; "potamotrygon + genetics". The published articles, book sections and thesis/dissertations were compiled until December, 2021.

Where we are

Studies, species, and localities

From 1998 to 2021, a total of 46 studies using genetic data from potamotrygonin species were produced (Figure 2). Most of these studies (n = 40; 87%) were produced after 2011 and, since then, at least two studies per year have been concluded or published. Most of the Neotropical freshwater stingrays species (36 of 39) have been included in these studies. Notwithstanding, the genetic information from most of these species (n = 24, 52%) are available due to only two recent studies (Fontenelle et al. 2021a,b). Only three species have no genetic published data until this moment: *Potamotrygon ocellata* (Engelhardt, 1912); *Potamotrygon schuhmacheri* Castex, 1964; *Potamotrygon roulini* Roberts, 2020. *Potamotrygon humboldtii* (Roulin 1829) was indicated as *species inquirenda* and confirmation of its taxonomic status remains pending (Roberts 2020), therefore it was not counted as a valid species in the present review. Genetic information derived from undescribed *Potamotrygon* sp. samples were present in 16% (n = 7) of the genetic studies conducted for the group, considering that identifying some of these stingrays to species level is not always easy mainly due to polychromatism (Rosa et al. 2010). These aspects reveal a knowledge gap for genetic information about most of potamotrygonins and therefore only recently their genetics has begun to be unveiled (Figure 3).

Figure 2 – Distribution of potamotrygonin genetic studies over the years, note the increase in the past decade with at least two studies per year.

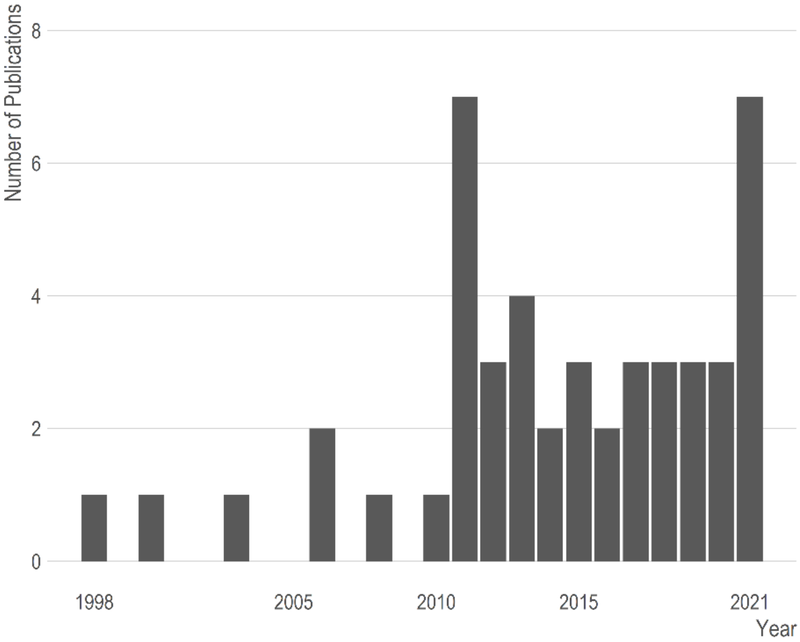
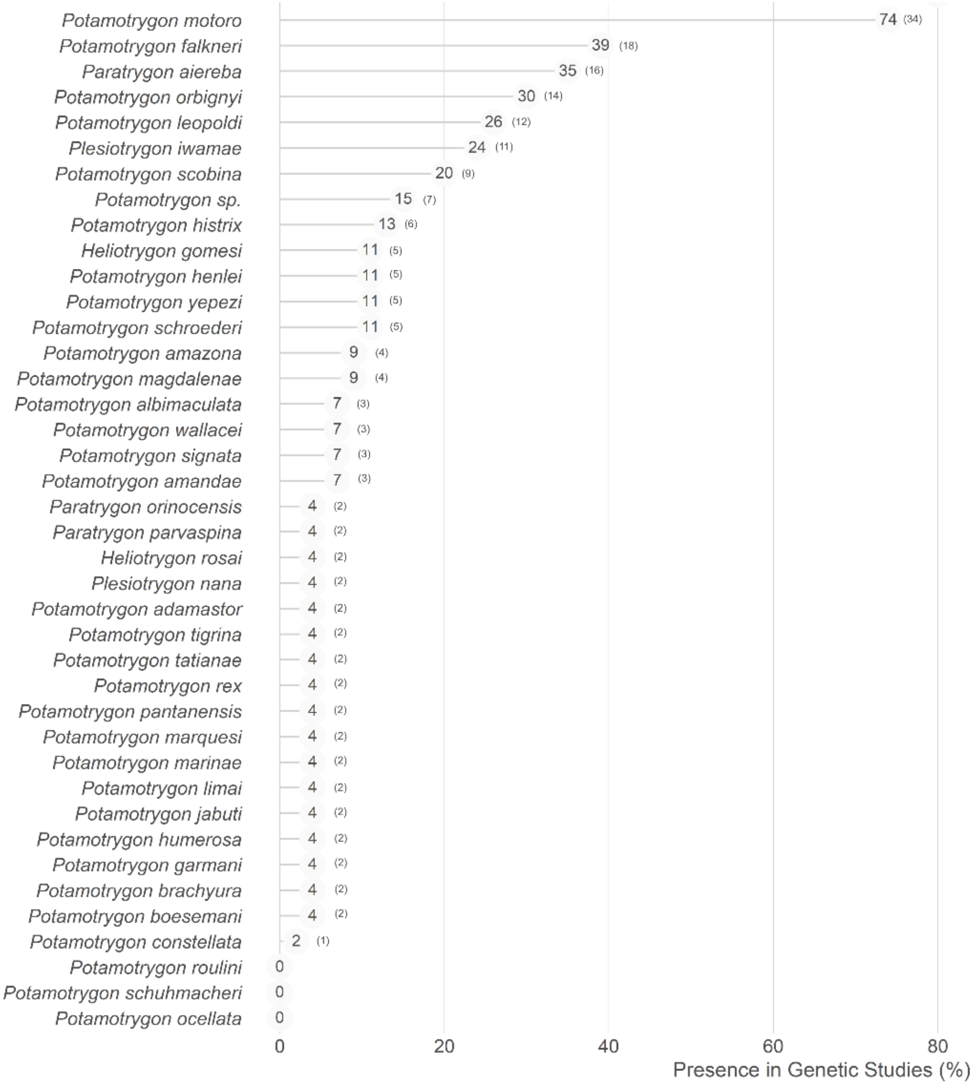


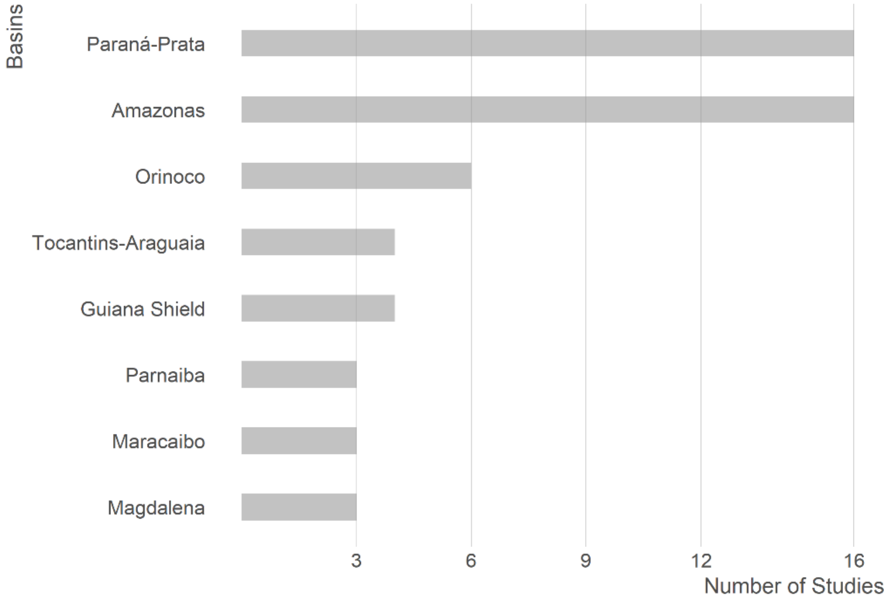
Figure 3 – Presence in genetic studies by species.



Potamotrygon motoro (Müller & Henle, 1841) was the most studied species, present in 73% (n = 33) of the studies. This species, firstly known from the Paraná-Prata Basin, was one of the earliest described species for the group and has been reported for several river basins (Rosa 1985; Loboda and Carvalho 2013). However, this species is recognized as a species complex with high phenotypic plasticity (Rosa et al. 2010). The *Potamotrygon motoro* complex shows a remarkable and typical colorful ocellated dorsal pattern and has been considered a relatively common species where it occurs, making it common in the aquarium trade (Charvet-Almeida and Almeida 2008). This may help explain why *P. motoro* was the most genetically studied species in the group.

As for sampling locality, the genetic studies are also uneven. The Amazon River Basin is one of the two most studied areas (n= 16) (Figure 4). This Basin is the richest for Potamotrygoninae species with ~60% of the described species occurring in the area. The Amazon River Basin is the largest in the world with a complex river system that can be divided into several subbasins (Abell et al. 2008). Subdivisions for this Basin were proposed by Dagosta and de Pinna (2017) and were followed here. The subbasins with higher number of studies on Neotropical stingray genetics were Negro River (n = 8), Xingu River (n = 6), and Amazonas River main channel (n = 6). Other three subbasins that were noteworthy due to their potential role as basin connectors and endemicity were: (a) the tributaries of the Tapajós and Xingu rivers (Teles-Pires, Juruena and Jamanxim rivers) with two endemic species: *Potamotrygon albimaculata* Carvalho, 2016 and *Potamotrygon leopoldi* Castex and Castello, 1970 and no study in these specific areas; (b) upper-Amazonas tributaries (Marañon-Nanay, Napo-Ambyiacu and Putumayo) with two endemic species: *Potamotrygon tigrina* Carvalho, Sabaj Pérez and Lovejoy, 2011 and *Plesiotrygon nana* Carvalho and Ragno, 2011 and five studies in these areas; and (c) upper-Madeira Basin (Mamoré, Guaporé and Beni Madre de Dios rivers) with two endemic species: *Potamotrygon tatianae* Silva and Carvalho, 2011, and *Potamotrygon limai* Fontenelle, da Silva and Carvalho, 2014 and four studies there.

Figure 4 – Number of genetic studies by river basin.



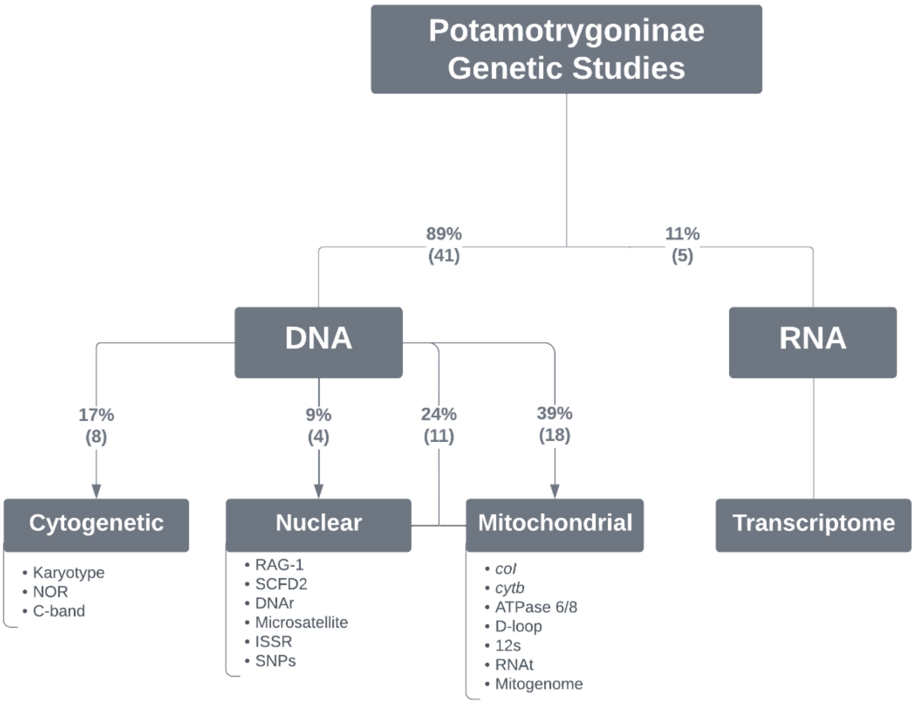
The other most studied area was the Paraná-Prata Basin (n = 16 studies). This is quite surprising since this area is relatively poor in Neotropical stingray richness; it holds only ~20% of the described species. Several pioneering studies (Castex et al. 1963; Castex 1964; Achembach 1971) with freshwater stingrays were developed in this area, which contributed to the development of genetic studies. Furthermore, this relatively high number of studies might be due to this area having most of the research centers and funding in Brazil (Sidone et al. 2016). In addition, potential sampling areas can be considered more accessible and demand less logistic efforts, in comparison to areas in the Amazon Region. This may also explain the fact that the endemic Paraná-Prata Basin *Potamotrygon falkneri* Castex and Maciel, 1963 is the second most genetically studied Neotropical stingray species. It is also worth mentioning that the distribution of the studies in the Paraná-Prata Basin are concentrated in few areas, leaving, for example, Bolivia and Paraguay with no genetic information on freshwater stingrays.

The remaining river basins are in a precarious situation regarding genetic studies. The Orinoco River Basin (n = 6) is an area where a recent increase in the number of studies has been observed in the past years. The recent description of new species (Roberts 2020; Loboda et al. 2021) for that Basin might increase this number in the near future. Other six river basins have been sampled for only a limited number of studies: (a) the Guiana Shield (n = 4), with the endemic species: *Potamotrygon marinae* Deynat, 2006 (n = 2) and *Potamotrygon boesemani* Rosa, Carvalho and Almeida, 2008 (n = 2); (b) Tocantins-Araguaia (n = 4), with the endemic species: *Potamotrygon henlei* (Castelnau, 1855) (n = 4), *Potamotrygon rex* Carvalho, 2016 (n = 2) and *Potamotrygon garmani* Fontenelle and Carvalho, 2017 (n = 2); (c) Parnaiba (n = 3), with the endemic species: *Potamotrygon signata* Garman, 1913 (n = 3); (d) Magdalenae (n = 4), with with the endemic species: *Potamotrygon magdalenae* (Duméril, 1865) (n = 4); (e) Maracaibo (n = 3), with the endemic species: *Potamotrygon yepezi* Castex & Castello, 1970 (n = 3). This was considered a very low number of genetic studies taking into account that eight endemic species are known for these regions and most information is available from two recent studies (Fontenelle et al. 2021a,b). Furthermore, for the Itapicuru-Mearim River Basin no genetic study has been conducted. These basins might comprise few species, however, despite the possible lower richness it would be important sampling on them to obtain a better profile of gene diversity in the group.

Genetic Markers

The first genetic study of potamotrygonins applied the use of mitochondrial DNA (thereinafter mtDNA) sequencing to construct a hypothesis for the origin of the group (Lovejoy et al. 1998). These authors using cytochrome *b* (*cytb*) proposed that Neotropical freshwater stingrays are monophyletic, and the ancestor invaded freshwater ecosystems during a marine invasion in the Early Miocene. Since this first study, the molecular markers used were mostly based on DNA (90%; n = 41 studies), particularly mtDNA (45%; n = 18) (Figure 5). The most used mitochondrial DNA markers were cytochrome *c* oxidase I (*coI*) and cytochrome *b* (*cytb*) (Lovejoy et al. 1998; Marques 2000; Toffoli et al. 2008; Carvalho and Lovejoy 2011; Pereira et al. 2013; Ramírez-Hernández 2018; Pereira 2018; Renza-Millán et al. 2019; Camacho-Oliveira et al. 2020; Sanches et al. 2021), other less commonly applied were: ATPase 6-8, D-loop, ND1-2, 12s, and RNAt (Dunn et al. 2003; Frederico et al. 2012; Cruz 2013; Garcia et al. 2016). Those markers were mainly applied to species delimitation/identification (n = 6) and phylogeographic (n = 6) studies. There are only mitogenome descriptions for: *Potamotrygon motoro* (Song et al. 2015) and *Potamotrygon orbignyi* (Ory et al. 2019), with no further analysis.

Figure 5 – Potamotrygoninae studies according to genetic markers.



Other DNA based markers have also been applied to potamotrygonins in several areas. Cytogenetics markers (18%; n = 8) were applied to describe karyotypes and sexual determination systems and provide evolutionary evidences for the group (Valentim et al. 2006; Cruz et al. 2011; Valentim 2011; Aichino et al. 2013; Valentim et al. 2013; Cruz and Foresti 2015; Valentim et al. 2019; Cruz et al. 2021). A considerable number of studies combined nuclear DNA (nDNA) and mtDNA information (24%; n=11). This combination was employed mainly in evolutionary studies, also in hybridization and phylogenetic ones (Maniglia 2010; Aschliman 2011; Aschliman et al. 2012; Cruz et al. 2015; Batista Morales 2017; Bloom and Lovejoy 2017; Kirchhoff et al. 2017; Araújo 2020; Fontenelle et al. 2021a,b; Rizo-Fuentes et al. 2021). Few studies solely applied nDNA (9%; n=4) and were used in evolutionary and marker development [Single Nucleotide Polymorphisms (thereinafter SNPs), microsatellites] studies (Pinhal et al. 2011; Arranz et al. 2013; Cruz et al. 2015; Cruz et al. 2017). Up to date, no whole genome sequencing has been conducted for potamotrygonins.

Studies using RNA as a genetic tool are scarce (10%; n = 5 studies) and applied transcriptomes to study venom composition and visual physiology (Júnior 2014; Júnior et al. 2016; Silva et al. 2018; Kirchhoff et al. 2021; Schluessel et al. 2021). No study applied biochemical markers. Therefore, based on those different topics assessed by genetic tools in Neotropical freshwater stingrays, this review was structured in the following topics: Evolution, Taxonomy, Phylogeography, Hybridization, Cytogenetics and Transcriptomes.

Evolution

One of the evolutionary questions in which molecular markers were applied for Potamotrygoninae is ribosomal DNA evolution (Pinhal et al. 2011; Cruz et al. 2015). Elasmobranchs possess a unique class of 5s rRNA gene which makes them suitable for studying this gene evolution process in vertebrates (Pinhal et al. 2011). In three potamotrygonin species (*Paratrygon aiereba, Potamotrygon motoro, P. falkneri*) two classes of 5s rRna were identified: class I and II. Class II is more polymorphic among the species than class I and evolutionary analysis suggested that this pattern was due to a birth-and-death effect related to evolutionary processes (Pinhal et al. 2011). This variation in the region of 5srRNA class opened a possibility for a nuclear marker development, since the differences between genus and species studied were considered enough to separate them (Pinhal et al. 2011; Cruz et al. 2015).

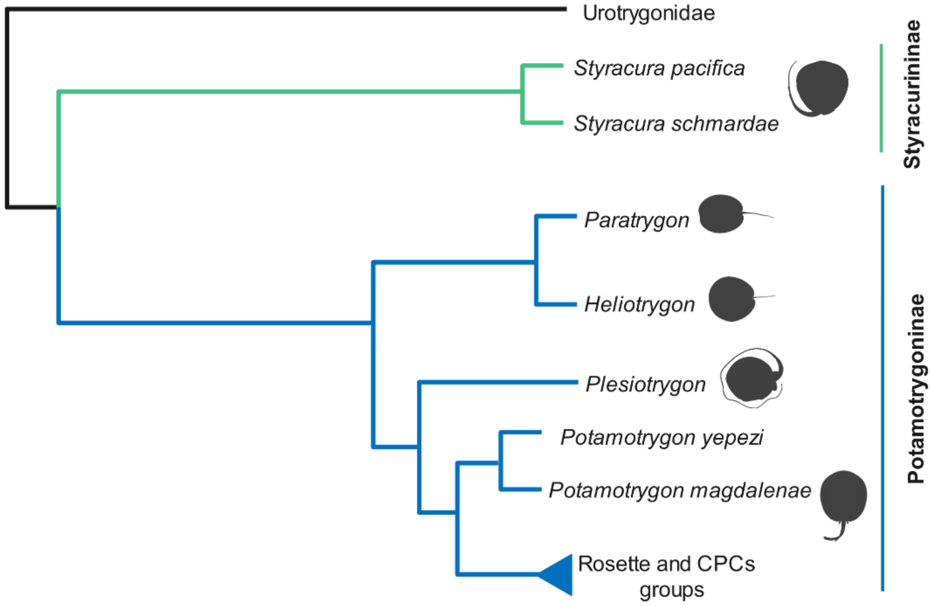
There has been a long debate on how the Neotropical elasmobranch freshwater lineage emerged. Despite the different time estimations, the consensus points to a single invasion derived from a marine incursion event in northern South America (Bloom and Lovejoy 2017; Kirchhoff et al. 2017; Fontenelle et al. 2021b). The marine genus *Styracura* has been considered the sister group to Potamotrygoninae (Dunn et al. 2003; Last et al. 2016). Now the debate focuses on the invasion's estimation time. One hypothesis, based on molecular models, points to a recent event (26.4 m.y.a) during the Oligocene-Miocene transition associated with the Pebas System Formation (Rosa et al. 2010; Fontenelle et al. 2021b). Nonetheless, other models based on molecular evidence calibrated by the fossil record indicated an older invasion (72 m.y.a) in the transition of Late Cretaceous/Early Eocene (Kirchhoff et al. 2017; based on fossil record from Adnet et al. 2014).

There have been limited studies regarding the diversification of Potamotrygoninae lineages to different regions in South America but new discoveries shed light on this event. Despite the different invasion time estimates, studies agreed on a single invasion followed by a diversification event (Bloom and Lovejoy 2011, Kirchhoff et al. 2017). Recent discoveries suggested that this diversification in the continent occurred from the Upper Amazon Region acting like a 'species pump' to other areas (Fontenelle et al. 2021b). This hypothesis contributed to explain the high number of species in the Amazon River Basin, since it was the first colonized area and from there the lineages spread to other basins. This complex process was estimated to have started 15 m.y.a and, since then, changes in landscapes connected and separated different lineages. The exchange between lineages could help explain the high genetic similarity degree found in some potamotrygonins (Fontenelle et al. 2021b).

Taxonomy

The Potamotrygoninae phylogeny is an aspect that has been studied since the late 1990s until nowadays (Lovejoy et al. 1998; Lovejoy et al. 2006; Aschliman et al. 2012; Bloom and Lovejoy 2017; Fontenelle et al. 2021a). For phylogenetic investigations *cytb* was the main marker applied (Marques 2000; Dunn et al. 2003; Carvalho and Lovejoy 2011). Recently, the first phylogeny based on wide taxon sampling applied other markers such as ATPsintase 6/8, *coI* and the first nuclear marker: nuclear ribosomal internal transcribed spacer 1 (*its1*) (Fontenelle et al. 2021a). Previously, the largest phylogenetic analysis regarding taxon sampling size included 11 species (Marques 2000; no species from the Paraná-Prata Basin was included). The monophyly of the family has been a consensus and most of the studies indicated an arrangement of the genus in two clades (Figure 6): (*Paratrygon* + *Heliotrygon*) + (*Potamotrygon* + *Plesiotrygon*) (Marques 2000; Carvalho and Lovejoy 2011; Fontenelle et al. 2021a). Other recurrent topic was the suggested *Potamotrygon* paraphyly, with the genus *Plesiotrygon* nested inside the *Potamotrygon* clade (Marques 2000; Toffoli et al. 2008; Kirchhoff et al. 2017; Araújo 2020). Recent evidence pointed to a *Plesiotrygon* monophyly, with *Potamotrygon brachyura* as a sister group (Fontenelle et al. 2021a).

Figure 4 – Phylogenetic tree of the Potamotrygonidae family adapted from Fontenelle et al. (2021a) and Toffoli et al. (2008). The Core Potamotrygon Clade (CPC) corresponds to the group of species of the genus Potamotrygon which failed to form monophyletic clades (Fontenelle et al. 2021b) and comprises the “rosette-group” species that presented similar issues (Toffoli et al. 2008).



Genetic tools have aided to solve taxonomic problems in different elasmobranch taxa (Dudgeon et al. 2012; Faria et al. 2013; Ball et al. 2016; Marino et al. 2018; Fahmi et al. 2021). Some attempts to solve taxonomic issues were successful to identify species using mitochondrial markers (Garcia et al. 2016; Camacho-Oliveira et al. 2020). Nonetheless, genetic tools did not seem so useful for some potamotrygonins. The *coI* barcode analysis for some species (Toffoli et al. 2008; Pereira 2018) showed low sequence divergence levels (0.5%-2.5%) between species when compared to other batoid species within: families [Dasyatidae (7%-10%) and Rajidae (3%-32%); Ramirez-Amaro et al. 2018]; genus [*Myliobatis* sp. (6.1%), Ruocco et al. 2012; *Himantura* (7.84%), Bineesh et al. 2016] or putative cryptic species [*Rhinoptera steindachneri* (7%-10%), Sandoval-Castillo and Rocha-Olivares 2011]. In light of this, the effectiveness of barcoding to identify potamotrygoninae species has been discussed (Toffoli et al. 2008; Pereira 2011; Pinhal et al. 2011; Pereira et al. 2013; Garcia et al. 2016; Pereira 2018; Camacho-Oliveira et al. 2020; Sanches et al. 2021). Results using *cytb* presented better resolution between species, thus this marker might be a more appropriate barcode for Neotropical freshwater stingrays (Garcia et al. 2016).

The reasons explaining the lack of resolution in molecular species delimitation for potamotrygonin species have been debated. When the monophyly of a species is considered *a priori* without properly testing it, the molecular species delimitation might be unclear (Toffoli et al. 2008). This is a valid concern since the use of molecular methods without considering other taxonomic features could lead to biased results (Schlick-Steiner et al. 2010). In a more complete genetic analysis for the group based on mitochondrial (*coI*, *cytb*, ATPase 6-8) and nuclear (*its1*) genes, a group of *Potamotrygon* species called "Core *Potamotrygon* Clade" presented few genetic differentiation between species and most of the species failed to form monophyletic clades (Fontenelle et al. 2021a). Within this group there are species of the "rosette-group" (*Potamotrygon falkneri, P. henlei, P. leopoldi, P. motoro* and *P. orbignyi*) proposed by Toffoli et al. (2008), which presented similar issues in obtaining monophyletic clades. Although both studies did not form monophyletic clades regarding those species, they were grouped when considering morphological identification and formed clades based on the sample location. This observed pattern could be resulting from a rapid lineage expansion, hybridization and ecological diversification (Fontenelle et al. 2021a). Therefore, to deal with this species group an integrative taxonomy coupled with appropriate geographic sampling could generate clearer results (Cicero et al. 2021).

Another important point is that most of the studies applied mainly mitochondrial genes to deal with taxonomic problems. Since confirmed cases of hybridization are known for this group (Maniglia 2010; Cruz et al. 2014), the solely use of mitochondrial markers can lead to unclear results. Furthermore, recent speciation processes and elasmobranch low mitochondrial mutation rate might explain the low level of divergence found when using mitochondrial markers for taxonomic issues (Martin et al. 1992). Therefore, the use of mitochondrial markers applied to Potamotrygoninae taxonomy should be carefully considered.

Phylogeography

Phylogeographic studies have been conducted for five potamotrygonin species; *Paratrygon aiereba* (Frederico et al. 2012; Rizo-Fuentes et al. 2021), *Potamotrygon motoro* (Maniglia 2010; Cruz 2013; Renza-Millán et al. 2019), *Potamotrygon falkneri* (Maniglia 2010; Cruz 2013), *Potamotrygon magdalenae* (Ramírez-Hernández 2018), and *Potamotrygon orbignyi* (Batista-Morales 2017). The mtDNA (*coI*, *cytb*, ATPase 6-8) was the primary source of information in those studies, while only two other studies applied nuclear markers (microsatellite) (Cruz 2013; Batista-Morales 2017). Four of these species (*Paratrygon aiereba*, *Potamotrygon motoro*, *P. magdalenae* and *P. orbignyi*) showed evidence of structured populations, with only the Paraná-Prata species (*P. falkneri*) showing genetic homogeneity between populations (Maniglia 2010; Cruz 2013). *Paratrygon aiereba* was the species that exhibited the highest values for within-species genetic divergence (Table 1), with populations of the Amazon and Orinoco basins divergent at a species-level (Frederico et al. 2012, Rizo-Fuentes et al. 2021). Indeed, analysis based on nuclear and mitochondrial data indicated that each *Paratrygon aiereba* river basin population was clearly separated from each other, suggesting a more complex subdivision for this species (Fontenelle et al. 2021a). The description of two new *Paratrygon* species from the Orinoco River basin (Loboda et al. 2021) corroborated the observed divergence between river basin populations (Loboda 2016).

Table 1 – Intraspecific genetic distance among Potamotrygoninae and other batoids species.

Species	**Marker**	**Intraspecific genetic distance (%)**	**Source**
Potamotrygonidae			
Paratrygon aiereba	*coI*	0.4-4.9	Frederico et al. (2012)
Potamotrygon falkneri	*coI*	0.15	Maniglia (2011)
Potamotrygon falkneri	*coI*	0.2	Cruz (2014)
Potamotrygon motoro	*coI*	0.15	Maniglia (2010)
Potamotrygon motoro	*coI*	0.3	Cruz (2013)
Potamotrygon orbignyi	*coI + cytb*	3.0	Batista-Morales (2017)
Myliobatidae			
Aetobatus narinari	*coI*	2.6	Richards et al. (2009)
Dasyatidae			
Himantura sp. (average)	*coI*	0.84	Bineesh et al. (2016)
Urotrygonidae			
Urotrygon rogersi	nd2	0.04	Ehemann et al. (2022)

The genetic evidence obtained so far indicated that freshwater stingray species tend to have structured populations between river basins (Maniglia 2010; Frederico et al. 2012; Rizo-Fuentes et al. 2021) and sometimes within them (Cruz 2013; Ramírez-Hernández 2018; Renza-Millán et al. 2019; Fontenelle et al. 2021a; Rizo-Fuentes et al. 2021). The lower values of differentiation for the Upper Paraná-Prata species could be due to the history of the site. The upper part of Paraná-Prata Basin was recently colonized by *Potamotrygon* species due to damming in that area (Garrone-Neto et al. 2014). It could be hypothesized that the populations established in the new area might not have had enough time to accumulate detectable differences on their mitochondrial genes. For example, a difference between *P. falkneri* populations could only be detected using nuclear markers (microsatellites) instead of mitochondrial markers (Cruz 2013). This similarity could also be a hybridization event artifact, considering species hybridization was confirmed between species in this area (Cruz et al. 2015). It is important to mention that the observed pattern of clear population structures associated with river basins might not reflect a rule for potamotrygonins since the studied species (n= 5) represented only 11% of this subfamily species.

Hybridization

Hybridization is a rare phenomenon in elasmobranchs with only few cases reported (Morgan et al. 2012; Marino et al. 2015). Among freshwater stingrays, it was reported between *P. falkneri and P. motoro* in the Paraná-Paraguay Basin (Maniglia 2010; Cruz et al. 2014). In both studies, some individuals morphologically identified as *P. motoro* had exclusive *P. falkneri* mitochondrial haplotypes. Thus, this suggested breeding of *P. falkneri* females with *P. motoro* males (Maniglia 2010; Cruz et al. 2014). Moreover, both studies bring genetic (mitochondrial and nuclear markers) evidence that those are not first-generation hybrids, meaning this hybridization has been going on for generations. Although this is the only confirmed case, it has been suggested that hybridization might occur for other *Potamotrygon* species in different watersheds (Toffoli et al. 2008; Rosa et al. 2010; Sanches et al. 2021).

Conditions favoring hybridization can be similar chromosome number and recent speciation events (Abbott et al. 2013). Some *Potamotrygon* species seem to fulfill those conditions. A group of *Potamotrygon* species that shares similar dorsal color pattern, the “rosette-group” (Toffoli et al. 2008) or “Core *Potamotrygon* Clade” (Fontenelle et al. 2021a), also presents similar chromosomal numbers (2n=64-66) (Cruz et al. 2011; Valentim et al. 2019). This group of species presented high mitochondrial genetic similarity and the hypothesis for this due to recent speciation events but might be combined or a result of hybridization events as well (Toffoli et al. 2008; Fontenelle et al. 2021a). These cases should be further investigated to clarify if this genetic high similarity could be due to hybridization events, ancient polymorphism or speciation ongoing process (Han et al. 2017).

Cytogenetics

Neotropical freshwater stingray cytogenetic studies have recently increased a lot (Valentim et al. 2006; Cruz et al. 2011; Valentim 2011; Valentim et al. 2013; Aichino et al. 2013; Valentim et al. 2019; Cruz et al. 2021). Karyotype data was available for 15 species, covering three of the four genera: *Paratrygon, Plesiotrygon* and *Potamotrygon* (Valentim et al. 2019). Two cytogenetics patterns were observed in Potamotrygoninae. First, the constitutive heterochromatin region is at the centromeric region, with exception only to *P. motoro* individuals from Jauaperi River, that also presented terminal heterochromatin regions on the long arms (Valentim et al. 2019). Second, the nucleolar organizing regions (NOR) were situated on the terminal portion of long arms, with exception for *Potamotrygon constellata* and *Potamotrygon* aff. *wallacei* (Valentim 2011). Based on the recent data, a karyoevolutionary hypothesis of reduction in diploid numbers with a decrease in acrocentric chromosomes in the group by chromosomal rearrangements (Robertsonian and non-Robertsonian) was proposed (Valentim et al. 2019).

The sexual chromosome systems are a remarkable feature for the genus *Potamotrygon*. The presence of sex chromosomes seemed to be an apomorphy in *Potamotrygon*, with the male being the heterochromatic sex (Valentim et al. 2014). Three sexual chromosome systems have been reported: XX/XY, XX/X0 and $X_1X_1X_2X_2/X_1X_2Y$ (Valentim et al. 2014). Multiple sex determination ($X_1X_1X_2X_2/X_1X_2Y$) systems were a trait found only in the Paraná-Prata Basin species: *P. falkneri* and *P. motoro* (Cruz et al. 2011; Valentim et al. 2019).

A variation in chromosome number and formula was reported for two species: *P. wallacei* and *P. motoro* (Valentim et al. 2019). For *P.* wallacei, one individual (indicated as *Potamotrygon* aff. *wallacei*) presented a karyotype formula differing in the number of metacentric and submetacentric chromosomes and NOR location. Considering *P. motoro*, four different chromosome formulae and two chromosome numbers were reported. There were differences between specimens of *P. motoro* collected in the Amazon, with both sexes having the same chromosome number (66♂/♀) (Valentim et al. 2006), while others from the Amazon and Paraná basins presented different number for females and males (65♂/66♀) (Cruz et al. 2011; Aichino et al. 2013; Cruz et al. 2021). Furthermore, individuals from Amazonian karyotypes presented more acrocentric chromosomes than the ones from the Paraná Basin. Also, there was some karyotype variation among individuals from the Paraná Basin. Cruz et al. (2011) found different formula numbers for *P. motoro* individuals from the Upper-Paraná River. These cytogenetic differences might reinforce the status of *P. motoro* as a species complex. Nevertheless, this array of karyotype variation could be an artifact due to different cytogenetic results interpretation or species divergence (Aichino et al. 2013).

Transcriptomic studies

Neotropical freshwater stingray injuries can cause serious envenomation and there is no specific venom antidote (Pedroso et al. 2007; Junior et al. 2013; Lameiras et al. 2019). In this context, transcriptomic analysis of glandular epithelium with venom-producing cells was applied to better characterize the venom composition and understand its physiological effects. Only four transcriptomic venom studies were available for stingray species, three of them comprising Potamotrygoninae species (*Potamotrygon amandae*, *P. falkneri*, *P. leopoldi* and *P. motoro*) (Júnior et al. 2016; Silva et al. 2018, Kirchhoff et al. 2021). The presence of some venom known transcripts (e.g. phospholipase, metalloproteinase, hyaluronidase) corroborates that Neotropical freshwater stingrays venom have provoked more severe envenomation accidents than their marine counterparts (Pedroso et al. 2007; Júnior et al. 2016; Lameiras et al. 2019). The description of the envenomation symptom mechanisms (i.e. disruption of tissue matrix and hemostasis; induction of pain, pro-inflammatory and cardiotoxic activity) are important tools for developing treatments to human accidents (Kirchhoff et al. 2021). The venom of *P. motoro* differed from the *P. amandae* and *P. falkneri*, mainly by the absence of phospholipase (Júnior et al. 2016). This suggested a diversity of venom composition among Neotropical freshwater stingray species. Nonetheless, since *P. motoro* has been considered a species complex with records of hybridization with other species (Cruz et al. 2014), the origin of samples should be clearly specified. The *P. motoro* specimens with RNA sequenced were not captured in nature and did not have a specification of its lineage origin (Silva et al. 2018;

Kirchhoff et al. 2021). Thus, the venom diversity hypothesis between species must be further investigated.

The potamotrygonin venom studied contained transcripts related to antibiotic and cancer pathways indicating bioprospecting potential (Júnior et al. 2016; Silva et al. 2018; Kirchhoff et al. 2021). This highlights how informative transcriptomic analysis can be, indicating that it could also be applied to understanding species' physiological and ecological aspects (Connon et al. 2018). For instance, transcriptomics profiles were also applied to identify visual pigments in *P. motoro* and predict their spectral range sight (Schluessel et al. 2021). Future applications of transcriptomic should be encouraged since it has the potential to solve many questions for this group.

Where to go: The frontiers in Potamotrygoninae genetics

The relatively high number of Neotropical freshwater stingray species (~50%) for which genetic information is scanty is a gap of knowledge waiting to be filled. One of the challenges to fulfill this gap is to sample some of those species, which are sometimes in isolated areas (Rosa et al. 2010; Lasso et al. 2016). Secure tissue samples from those little-known species are crucial to a better understanding of the group. Especially, from species with no genetic information available (*P. schuhmacheri*, *P. ocellata* and *P. roulini*). Moreover, for species complex cases such as *P. motoro* and *P. orbynyi*, geographically widespread sampling from diverse watersheds is strongly recommended to obtain better genetic profiles. The Paraná-Prata River basin concentrated almost the same number of studies as the Amazon River basin, despite the majority of species being located in the later. An effort to increase sampling in the Amazon Region, mainly in Amazonian regional gaps pointed out here, is encouraged for a better genetic diversity portrait of this group. The other regions with lower species richness should receive attention as well, since many of them harbor endemic species. Fulfilling those gaps and establishing more comparisons between basins is an important step for solving species complexes and for a better profile of gene diversity.

The mitochondrial DNA analysis was the main source of genetic information but mostly failed in providing species identification (Toffoli et al. 2008; Pereira 2018). Therefore, the identification of alternative mitochondrial barcoding genes is necessary to improve the genetic analyzes for this group. In this context, the use of mitogenomes can be insightful, since is a reliable approach to find alternative barcoding markers when traditional markers are not so resolutive (Chagas et al. 2020). Furthermore, it is also recommended that future efforts should be employed on nuclear markers, since nuclear genes might ravel Potamotrygoninae species complexes. In fact, there have been some attempts to develop and apply microsatellites and SNPs, but they are still limited (Arranz et al. 2013; Cruz et al. 2014; Cruz et al. 2017). New generation sequencing (NGS) is a promising technique that could be more explored with its cost decreasing and increasing widespread use to assess nuclear and mitochondrial genome information. NGS has been considered potentially useful for addressing issues on hybridization (Twyford and Ennos 2012), species complexes (Dupuis et al. 2017), population structure (Moura et al. 2020) and taxonomic issues (Liu et al. 2017) with a genomic approach. In fact, NGS has been applied to transcriptomic studies (Júnior 2014) but remains underexploited in other fields (e.g. taxonomy, phylogeography). Nonetheless, the lack of a reference genome and large genomes of the group are challenges for NGS application (Fuentes-Pardo and Ruzzante 2017). Despite these challenges, this technique might be the key to potamotrygonin genetic research.

Other tools that are unexplored for the group are the use of environmental DNA (eDNA) and metabarcoding (Bakker et al. 2017). The eDNA is a non-invasive method that could possibly easily indicate the presence of species in an area from environmental samples (Bohmann et al. 2014). Metabarcoding is sequencing multiple species simultaneously (Thomsen and Willerslev 2015). These techniques combined would make it possible to detect species from water samples, or detect the diet from stomach content or feces. Although there are still improvements to be made with eDNA application (Barnes and Turner 2016), a similar efficiency with species' traditional detection methods has already been documented (Hänfling et al. 2016; Valentini et al. 2016).

Finally, it is worth mentioning that the use of genetic tools solved and clarified many questions related to Neotropical freshwater stingrays, such as evolutive origins or phylogeographic structures. Nevertheless, there are still knowledge gaps regarding species and sampling locations that need to be fulfilled. Taxonomy is the area where genetic information provided few advances until now and genomic level studies are likely to provide interesting species delimitation data. In addition, new approaches (e.g. NGS, eDNA) will provide important tools to elucidate further genetic information and therefore contribute to

Potamotrygoninae species management and conservation.

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CAPÍTULO 4: TROUBLED WATERS FOR THE POTAMOTRYGONINAE: A REVIEW OF THREATS TO THE LARGEST FRESHWATER ELASMOBRANCH LINEAGE

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Title: Troubled waters for the Potamotryginae: a review of threats to the largest freshwater elasmobranch lineage

Running title: A review of Potamotrgoninae threats

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Abstract (150-250 words):

Freshwater ecosystems are often less adressed than terrestrial ones from a conservation perspective. Tropical freshwater environments, such as those in South America, are particularly of high concern since they comprise most freshwater on Earth and have high fish diversity. One of these unique biodiversity components subject to a number of impacts is the Potamotrygoninae, the largest living lineage of elasmobranchs fully adapted to freshwater environments. This group is of concern because it is exposed to general threats to freshwater ecosystems while exhibiting the life-history vulnerability inherent to elasmobranchs. In the present study, reported threats to Potamotrygoninae species were assessed in seven main river basins of South America considering regional differences. Freshwater stingrays face several threats, including habitat destruction (mining, urban and agribusiness expansion), fisheries (ornamental, consumption, and negative) and climate change. Habitat fragmentation caused by dam expansion is also a major issue. Management and conservation measures such as law enforcement, regulation, and the establishment of protected areas are needed to effectively protect these species.

Keywords (4-6): Stingrays, Potamotrygonin, Conservation, Impacts, Risk assessment

Declarations

Author's Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yan Torres. The first draft of the manuscript was written by Yan Torres and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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1. Introduction

The global biodiversity crisis is a conservation challenge, particularly in freshwater ecosystems (Harrison et al. 2018). Efforts are primarily focused on terrestrial ecosystems, so that impacts on freshwater environments are usually less discussed (Dudgeon et al. 2006; Albert et al. 2021). In this scenario, tropical freshwater environments are an example of an impacted environment, considering that about one-third of tropical fish species are threatened (Arthington et al. 2016). There are several challenges in addressing this situation, such as the lack of risk profiles for freshwater species (Arthington et al. 2016). Thus, knowledge gaps need to be filled, especially in less studied areas, to develop strategies to overcome this problem (Fonseca et al. 2021). Therefore, identifying and assessing threats is an important step in the necessary conservation efforts for these habitats.

South America is the continent with the most freshwater on Earth, and its ecosystems are also leaders in continental fish biodiversity (Reis et al. 2016). Neotropical fish species diversity is composed of several distinct evolutionary groups (Reis 2013). This diversity is due to several evolutionary processes, such as the various marine invasions that have occurred in the continent (Bloom and Lovejoy 2017). During one of these invasions, the lineage of Potamotrygoninae stingrays invaded the continent and diversified (Fontenelle et al. 2021). The Neotropical freshwater stingray group (hereafter referred to as freshwater stingrays) comprises the largest living lineage of elasmobranchs fully adapted to freshwater environments (Rosa 1985). The Potamotrygoninae species occur in diverse habitats throughout South America, with

39 recognized species divided in four genera: *Paratrygon* Duméril, 1865; *Potamotrygon* Garman, 1877; *Plesiotrygon* Rosa, Castello and Thorson, 1987; and *Heliotrygon* Carvalho and Lovejoy, 2011. These species occur in several South American ecosystems, most of them in the Amazon River basin and its sub-basins. From this river basin, the lineage spread from other basins throughout the continent (Fontenelle et al. 2021). The second most species-rich basin is the Orinoco River basin, which shares several species with the Amazon due to its proximity and occasional connectivity (Winemiller et al. 2008). The third area in species richness is the Paraná-Paraguay River basin, located in the southern part of the continent. This area has high endemism and is one of the best studied areas (Torres et al. 2022). The other river basins (Tocantins-Araguaia, Guyana rivers, Parnaíba, Magdalena, Maracaibo, Itapicuru-Mearim) have fewer and mostly endemic species. The occurrence in these different environments reflects not only the heterogeneity of the environments, but also the threats.

The biology of Potamotrygoninae is similar to that of their marine relatives, however, there are some considerations when evaluating threats due to the specifics of the freshwater environment. Elasmobranchs are known for their susceptibility to overfishing. One-third of elasmobranch species are threatened to some degree (Dulvy et al. 2021). This vulnerability is due to their slow growth, late maturation, and low fecundity (Last et al. 2016). As elasmobranchs, freshwater stingrays share the same characteristics and overfishing is a concern for them. However, there are some differences in threat assessment for Potamotrygoninae (Compagno and Cook 1995; Charvet-Almeida et al. 2002; Martin 2005). Approaches to threat assessment in the ocean cannot be applied to river and lake ecosystems because they are subject to flooding pulses, limited connectivity, smaller population sizes, and sources of degradation (e.g., deforestation, mining, damming) that are not present in the ocean (Barletta et al. 2010). These characteristics bring potamotrygonins closer to freshwater lineages in terms of threat, along with their typical elasmobranch biological characteristics. Therefore, Potamotrygoninae species must be evaluated considering this complex mix of aspects.

The combination of threats to Potamotrygoninae and characteristics of freshwater ecosystems is alarming. South American freshwater environments have faced an increase in many threats in past decades (Reis 2013; Barletta et al. 2016; Torremorell et al. 2021). On the other hand, there is no extinction risk assessment for most freshwater stingrays, and one challenge in filling this gap is the general lack of information on this group. In terms of conservation, this combination of increasing pressure and lack of knowledge can be devastating (Darwall and Freyhof 2015). In analyzing threats to this group, previous studies have examined threats specific to freshwater stingrays (Compagno and Cook 1995; Charvet-Almeida et al. 2002; Martin 2005). However, a more comprehensive analysis that considers the complex spatial heterogeneity in South America is lacking, as threats are not evenly distributed across river basins. Recognizing the need to identify and discuss risks in light of regional differences, this paper provides an overview of key threats to Neotropical freshwater stingrays in seven main river basins of South America.

2. Environment impacts and habitat loss

2.1 Fishing

Fishing is a common threat to elasmobranchs, but special considerations in this regard apply to freshwater stingrays. Fishing is generally considered to be one of the primary conservation concerns for marine elasmobranch species (Dulvy et al. 2017), which are primarily caught to supply meat and fin markets. For Neotropical freshwater stingrays, fishing for consumption is also a concern, but in different ways for most species than for most of their marine counterparts. In this context, three main types of fisheries are distinguished: ornamental fisheries, negative fisheries, and consumption fisheries. Therefore, to properly assess them and develop conservation strategies for the group, it is necessary to understand the differences between these fisheries.

Some of the Neotropical freshwater stingrays, particularly species from the genus *Potamotrygon*, are highly sought after by the ornamental fish market (Araújo et al. 2004). Data on exports are scarce, but available data between 2002-2003 report 13,924 potamotrygonins exported from Amazonas State, Brazil. International trade was unregulated for decades, resulting in the uncontrolled extirpation of several populations from their natural habitats. Specimens demanded for the market vary in species, sex, and age over time (Sánchez-Duarte et al. 2013). Yet, larger females are usually preferred to reproduce desirable patterns and sometimes even hybrids in captivity (Sánchez-Duarte et al. 2013). In this case, such directed fishing may accelerate the decline of populations of exploited species. Setting catch quotas (Colombia, Surinam), especially based on disk width (Brazil), is an important mechanism introduced by some South American countries (Baptiste and Sánchez-Duarte 2016). The recent approval of the inclusion of some *Potamotrygon* species and their look-alikes in the Convention on International Trade in Endangered Species (CITES) Appendix II added another layer of control on international trade (CITES 2022). On the other hand, the illegal market should not be considered as terminated because market control is weak in most countries. Therefore, for these strategies to be effective, increased monitoring by national environmental authorities is needed. A challenge for this control mechanism is its dependence on the correct identification of exported species. Due to taxonomic uncertainties, identification of some species is difficult. In addition, the possibility of hybridism poses another difficulty for species identification. Therefore, ornamental fishing for Potamotrygoninae species is likely to remain a problem despite the great progress that has been made in CITES listing.

The occasional stinging accidents with humans generate a hostility towards freshwater stingrays (Lameiras et al. 2019; Santos et al. 2019). To avoid these accidents, freshwater stingrays are targeted for capture, with the tail being mutilated or the animals killed and disposed (Araújo et al. 2004). This type of fishing was first described as ‘negative fishing’ by Compagno and Cook (1995). For this reason, it is of concern any urban expansion and increased tourism near river and lake margins, which are preferred habitats for Potamotrygoninae species (Abati et al. 2017; Moreira and Haddad-Junior 2022). Furthermore, the invasion of Potamotrygoninae species in the upper Paraná River have led to an increase in stinging accidents and cases of negative fishing have been already reported for the area (Garrone-Neto et al. 2014; Santos et al. 2019). Therefore, negative fishing is an important aspect to consider, especially as an additional impact for species already affected by other threats.

Traditionally, freshwater stingray products (meat, sting, or liver oil) have been used only occasionally. Some indigenous communities used the stings in religious ceremonies and a few riverine communities use the liver oil as a panacea (Rosa et al. 2010). However, this scenario has recently changed. In some species, especially the larger ones, the meat is used as food. This preference is evident in the directed fisheries on some species such as *Paratrygon aiereba* (Müller & Henle, 1841) and *Potamotrygon motoro* Müller & Henle, 1841. Between 2015-2020, approximately 76.9 tons of freshwater stingrays were caught in the Brazilian state of Amazonas (Santos 2022). The caught meat is not only consumed locally, in the southern regions of Brazil the trade of these species in Brazilian markets under the common name 'cação' is already documented (Camacho-Oliveira et al. 2020). The capture of freshwater stingrays for consumption is not so far common in most areas where potamotrygonins occur, but the increasing market demand for its products should not be neglected.

2.2 Habitat loss

Habitat alteration, either through degradation or fragmentation, is one of the major threats to Potamotrygoninae. Human activities can have brisk and severe impacts on freshwater ecosystems (Barletta et al. 2015). This habitat degradation can affect the survival and reproduction of several freshwater stingrays. These impacts can result from direct pollution of rivers and lakes (e.g., mining, agricultural runoff) or indirect habitat alterations (e.g., deforestation and fires). In addition, habitat fragmentation, mainly caused by dams, is a common problem in South American freshwater areas (Reis 2013; Barletta et al. 2015). Therefore, to profile how habitat changes affect potamotrygonin species, it is necessary to understand the sources and mechanisms of these impacts.

Illegal mining is a harmful threat to freshwater stingrays. Recently, illegal mining has increased throughout the Amazon (Torremorell et al. 2021). The main impact of this mining (mainly alluvial gold) is mercury contamination of freshwater bodies (Lino et al. 2019; Pestana et al. 2022). Mercury intoxication can lead to physiological changes and death (Pereira et al. 2019). The presence of mercury at different trophic levels is confirmed in several regions (Castello and Macedo 2016). Since freshwater stingrays are predators, this is even more alarming due to the known biomagnification effects. This contamination is of particular concern for endemic species due to their spatially restricted habitats, which, once altered, can lead to abrupt population declines. Consequently, the presence of illegal mining is an extremely concerning threat to Potamotrygoninae species.

There are other important sources of chemical pollution of freshwater bodies in South America (Furley et al. 2018). The presence of agricultural runoff containing inorganic and organic pollutants in freshwater ecosystems is confirmed in several habitats across the continent (Barletta et al. 2019). Oil extraction is also known to be associated with pollution of freshwater systems (Rodríguez-Jorquera et al. 2017). Another source of chemical pollution is urban wastewater, which contains several emerging pollutants (Peña-Guzmán et al. 2019). The effects of these pollutants in food webs are not fully understood, although they are commonly associated with effects on ecosystems (hypoxia, eutrophication) and organisms (susceptibility to disease, cancer, nerve damage, and reproductive disorders) (Barletta et al. 2019; Morin-Crini et al. 2022). Animals exposed to these pollutants sometimes have a restricted range of movement due to the spatial limitations of freshwater ecosystems and permanent (e.g., waterfalls) or temporal barriers (e.g., connectivity between rivers only during the flood season), so they may be permanently exposed to those contaminants. Although the effects of these pollutants to the freshwater biodiversity are not as well understood as others, it can be expected that they pose a serious threat to freshwater stingrays.

Loss of forest cover can have serious impacts on freshwater ecosystems. Once riparian vegetation is removed, siltation increases, resulting in higher inputs of sediment to the water (Reis 2013; Foucher et al. 2023). Siltation can alter visibility as well as other chemical properties of the water, potentially mimicking the effects of a collapse in fish diversity caused by overfishing (Barletta et al. 2015). This process may be of particular concern in blackwater rivers that rely on nutrient inputs from flooded forests to maintain their physicochemical properties (Sioli 1984). Loss of vegetation cover is known to reduce fishery yields (Castello et al. 2018) and likely affects the abundance of potamotrygonins. Vegetation loss has also been linked to changes in regional climate, affecting temperature and precipitation (Foley et al. 2005; Castello and Macedo 2016). Thus, although deforestation is not a direct intervention in water, it can directly alter environmental conditions and lead to significant impacts on freshwater ecosystems.

Freshwater and marine ecosystems differ in several ways, including connectivity between areas (Barletta et al. 2010). Freshwater ecosystems are much more easily fragmented, or have their connectivity impaired, than marine ecosystems (Castello and Macedo 2016). Currently, fragmentation is mainly due to the construction of dams that isolate subpopulations and impede gene flow (Barletta et al. 2015). Dams can also alter the dynamics of nutrient distribution in rivers (Dethier et al. 2022) by acting like a trap for sediments that affect the entire food web (Pestana et al. 2019). Other changes such as dredging or the water use for irrigation can also create barriers and fragment freshwater environments (Reis 2013; Castello and Macedo 2016). Even though migration movements are not fully studied in Potamotrygoninae the consequences of habitat structuration could compromise species survival by altering ecological parameters and limiting gene flow. Therefore, habitat fragmentation is a serious problem for

Potamotrygoninae, considering its impact and the fact that it is much more common in freshwater environments than in marine environments.

2.3 Climate Change

Climate change poses an increasing threat to freshwater ecosystems. Due to the complexity of cofactors, the effects of climate change on freshwater ecosystems are not fully clear, but it is known that temperature increases alter water chemical properties and precipitation regimes (Farinosi et al. 2019; Lucas et al. 2021; Costa et al. 2021; Reboita et al. 2021). The increase in water temperature may affect some thermosensitive species (Rose et al. 2023). In addition, temperature rise can reduce dissolved oxygen levels, which is of particular concern for tropical ecosystems because hypoxia is an important ecological feature in these environments (Barletta et al. 2010). These changes in environmental conditions may directly affect potamotrygonin species and also the abundance of its prey. Therefore, these impacts to freshwater ecosystems can threaten the survival of multiple species and should not be ignored in conservation planning.

In addition, other effects of climate change can be enumerated. For example, the reproductive cycles of many freshwater stingrays depend on the dynamics of floods and droughts (Charvet-Almeida et al. 2005). These dynamics are maintained by the annual cycle of rainy periods; however, shifts in this cycle have been noted in some potamotrygonin habitats (Farinosi et al. 2019; Lucas et al. 2021; Costa et al. 2021). These shifts may cause reproductive capacity to increase during prolonged floods and decrease during long drought events. An example of these shifts is thought to occur in impounded areas where constant flood conditions are simulated. It is suspected that under these conditions, the reproductive capacity of a freshwater stingray species has increased (Rincon 2006). Another alarming problem is the increasing desertification of semiarid areas. These areas are habitat for some potamotrygonin species that already struggle with harsh conditions such as high temperatures and low rainfall (Rosa et al. 2009; Moro and Rosa 2016). Increasing desertification threatens the survival of these species by pushing these conditions to extremes, as rising temperatures and lower water levels lead to a drastic decrease in oxygen levels. These impacts of climate change exacerbate the vulnerability of Potamotryogoninae to changes in ecosystem dynamics.

3. Main threats in main river basins

3.1 Amazon River basin

The Amazon River basin is the largest river basin in the world and consists of a complex network of ecosystems and social features (Barlow et al. 2011). Most potamotrygonin species occur in this river basin. In terms of ecological characteristics, Amazonian rivers can be divided into three main types of water: black, white, and clear (Sioli 1984). These types are distinguished by chemical characteristics that, together with other environmental features, create a complex of habitats used by different species (Duncan and Fernandes 2010; Dagosta and de Pinna 2017). Therefore, the mosaic of different environments and socioeconomic conditions must be considered when assessing species risk. Given this environmental and social heterogeneity, the Amazon River Basin should not be considered as a single entity. It is therefore necessary to consider the basin in terms of regional differences. Therefore, threats are analyzed considering the main sub-basins where Potamotrygoninae occur.

3.1.1 Amazon Main Channel

The Amazon basin includes many tributaries with different characteristics, but its main channel has its own characteristics, species, and threats. The main channel of the Amazon River is a long-extended white-water area heavily used for several activities. Some potamotrygonins have a preference and are distributed only in the main channel, such as *Plesiotrygon iwamae* Rosa, Castello & Thorson, 1987 and *Heliotrygon rosai* Carvalho & Lovejoy, 2011. In addition, other species such as *P. aiereba*, *P. motoro*, *Potamotrygon constellata* (Vaillant, 1880), and *Potamotrygon scobina* Garman, 1913 are also reported. The loss of forest cover in the region is known to affect fish diversity (Arantes et al. 2018), which makes an impact on potamotrygonins likely, especially for the piscivore species. In addition, the presence of industrial fishing fleets that occasionally target the largest species such as *P. iwamae*, *H. rosai* and *P. aiereba* poses a significant threat to them in the area (Charvet-Almeida and Almeida 2008; Rosa et al. 2010). Ornamental fishing occurs in the area, especially towards *P. iwamae* and *H. rosai*, which are occasionally offered in the ornamental fish market (Sánchez-Duarte et al. 2013). Negative fishing has also been reported in the area (Charvet-Almeida and Almeida 2008), and its impact could be significant given the increasing urbanization in the area. The effects of climate change are not yet fully known, but projections indicate a reduction in habitat suitability for Potamotrygoninae (Dubos et al. 2022).

3.1.2 Upper Amazon

The upper Amazon region where freshwater stingrays occur (up to 400 m) corresponds to the western Amazon region, which includes parts of Brazil, Ecuador, Colombia, and Peru. There are few data for this area and based on information from Peru, where nine potamotrygonin species are reported to occur mainly in the Itaya, Nanay, and Amazon rivers (Ortega et al. 2016). There was a high demand for ornamental fish in this area, with thousands of specimens exported annually between 2004 and 2014. According to reports from local fishers, there are signs of stock depletion as catches decline and longer trips are required to maintain catch levels (Ortega et al. 2016). This is of concern because *Potamotrygon tigrina* Carvalho, Sabaj Pérez & Lovejoy, 2011, an endemic and threatened species, is listed as Endangered on the International Union for Conservation of Nature (IUCN) of Threatened Species and a single specimen can fetch up to US$ 200,00 locally, which is of great concern given that this is a species with limited distribution range (Ortega et al. 2016; Vásquez et al. 2016). Another endemic species in the region is *Plesiotrygon nanai*, but it is not clear under how much fishing pressure this species is currently. There are reports of freshwater stingray consumption in the region, but its status is unknown (Vásquez et al. 2016). Metal pollution contributes to the general concern about habitat degradation in the Ecuador (Capparelli et al. 2020). Shifts in pluviometry are predicted for the region (Costa et al. 2021), which could affect the reproductive cycles of Potamotrygoninae species.

3.1.3 Negro River

The Negro River is a unique habitat with endemic species that are threatened by various factors. The Negro River is a blackwater river characterized by its acidity, low sediment load and primary productivity derived mainly from the seasonally flooded forest, the Igapó (Sioli 1984; Bogotá-Gregory et al. 2020). Therefore, losses in the Igapó forest can have profound effects on the entire ecosystem. Six potamotrygonin species occur in the Negro River basin: *P. aiereba*, *Potamotrygon adamastor* Fontenelle & Carvalho, 2017, *P. motoro*, *Potamotrygon orbignyi* (Castelnau, 1855), *Potamotrygon schroederi* Fernández-Yépez, 1958, and *Potamotrygon wallacei* Carvalho, Rosa & Araújo, 2016 (Duncan et al. 2016). The Cururu Ray or Wallace's Freshwater Stingray (*P. wallacei*) is an endemic species that occurs in Igapó forests (Carvalho et al. 2016). Therefore, protection of Igapó habitats is important to ensure the conservation of this species. Changes in forest cover due to fire, whether natural (favored by climate change) but mainly human-induced (Flores et al. 2016), may reduce suitable habitat for

P. wallacei and threaten its survival. Therefore, habitat degradation in this area is one of the main problems for potamotrygonin diversity in the Negro River. In addition, other threats in this region should also be considered. First, the ornamental fisheries pressure, which is mainly directed to *P. wallacei*. This species is the smallest potamotrygonin species and was highly valued in the ornamental fish trade for many years (Araújo et al. 2004; Duncan et al. 2016). Despite a decline in market demand, fishing pressure should not be neglected. Second, the increase in tourism and recreational fishing in the Negro River (Thomé-Souza et al. 2014) could lead to higher negative fishing pressure. Climate trends indicate a decrease in precipitation and an increase in temperature (Vasconcelos et al. 2022). Projections also show similar trends for the basin (Sorribas et al. 2016).

The Branco River, a tributary of the Negro River, has the endemic *P. adamastor* (Fontenelle and de Carvalho 2017). The range of this species is within the Yanomami indigenous protected area, but despite legal protection, illegal mining has increased in recent years, polluting rivers and posing a health risk to the community (Watts 2023). In addition, habitat alteration from increasing fires could further threaten the viability of the species (Peters 2022). Therefore, given these recent impacts and the small range of *P. adamastor*, special attention to this area is needed.

3.1.4 Xingu River

The Xingu River is characterized by a rocky bottom and clear water and is currently subject to various threats. Four Potamotrygoninae species occur in this area: *P. aiereba*, *P. motoro*, *P. orbignyi*, and *Potamotrygon leopoldi* Castex & Castello, 1970 (Charvet-Almeida 2006). Habitat degradation is a major problem in this area because it is multifaceted. Illegal mining, which has increased in recent years, and the consequent pollution and its effects can already be demonstrated by mercury being detected in different regions (Ribeiro et al. 2017). This pollution is critical in the Branco River, a tributary of the Xingu River whose water conditions have changed (Silva 2019). Along the Xingu River, the expansion of agriculture and livestock has also led to agricultural pollution (Pignati et al. 2018). Deforestation is another source of habitat alteration as it promotes local climate disturbances (Panday et al. 2015). Thus, there is evidence that multiple sources are affecting habitat quality in the Xingu River. This reduction may threaten the survival of freshwater stingrays.

In addition, there are other impacts throughout the Xingu River basin. The dam of Belo Monte Hydroelectric Power Plant is one of them. The construction of this dam drastically altered the characteristic habitat around the town of Altamira (Perez 2015) and created a barrier to the gene flow of freshwater stingrays. As there are proposals for additional dams upstream of the current barrier, they should be carefully considered because they could reduce continuity and alter flood dynamics, posing a further threat to potamotrygonins in the region (Zuanon et al. 2021). Another important issue is the impact of climate change. Changes in pluviometry and the length of flood and drought periods are observed (Lucas et al. 2021) and reinforced in climate projections (Michels-Brito et al. 2021) for the area. As mentioned earlier, these changes could affect prey availability and alter reproductive cycles.

The fishery in the Xingu River is mainly focused on the ornamental fish market. The main target is *P. leopoldi*, a species valued in the international ornamental fish trade (Charvet-Almeida 2006). Fishing pressure was very high in the Altamira region, but the construction of the Belo Monte dam flooded an important fishing ground in the area, and completely changed its habitat. Afterwards, the fishing area shifted towards São Félix do Xingu and today this region is the main source of *P. leopoldi* catches (Charvet et al. 2022). Although the demand for this species has decreased in recent years, *P. leopoldi* is still caught seasonally. Fishing for consumption of potamotrygonin has become more common in the area in recent years, but further analysis is needed to properly assess its extent (Charvet et al. 2022). Negative fishing is also a non-negligible practice in the area. Therefore, the fluctuation of fishing patterns should be monitored to properly assess the risk of potamotrygonin in the Xingu River basin.

3.1.4 Tapajós River

The Tapajós is a clear water river with endemic species that are traded in the international market. There are six reported species in this watershed: *P. aiereba*, *Potamotrygon humerosa* Garman, 1913, *P. orbignyi*, *P. motoro* and two endemic species: *Potamotrygon albimaculata* Carvalho, 2016 and *Potamotrygon jabuti* Carvalho, 2016 (Carvalho 2016a). The Tapajós River has experienced severe habitat degradation due to agricultural expansion (Capitani et al. 2021). In addition to this impact, increased mining in the region has contaminated the main river and some tributaries (Lino et al. 2019; da Silva Montes et al. 2022). A future dam project will likely exacerbate these impacts by altering river flow and sediment dynamics (Runde et al. 2020). There is pressure from ornamental fisheries due to the valued endemic species in the area (Carvalho 2016a). Increased tourism (Ros-Tonen and Werneck 2009) is also a threat as most of Potamotrygoninae preferred habitats are visited by tourists, which may lead to an increase in negative fisheries. Changes in hydrologic cycles due to climate change are predicted (Farinosi et al. 2019), which will likely affect the reproductive cycles of Potamotrygoninae.

3.1.5 Madeira River

In the upper regions of the Madeira River basin, namely in the Beni, Mamoré, and Madre de Dios Rivers, there are four species reported: *P. aiereba*, *P. orbignyi*, *P. motoro*, and *Potamotrygon tatianae* Silva & Carvalho, 2011 (Sarmiento et al. 2016). In Bolivia, the main threat appears to be negative fishing, mainly caused by bycatch or due to the growth of tourism activities (Sarmiento et al. 2016). In addition, there are reports of fishing for consumption of *P. aiereba*, and ornamental fisheries is also mentioned, although its extent is uncertain. Few data are available, but estimates suggest that two-thirds of the upper Madeira River basin is still in good condition (Thieme et al. 2007), but increasing habitat degradation in this area is a concern (Dagosta et al. 2020).

In the middle and lower Madeira River, increasing mining, deforestation, and impoundment in recent decades are of great concern. Mercury contamination from alluvial gold mining is already recorded (Pestana et al. 2022). In addition, there is the loss of forest cover due to the expansion of agricultural activities (Trancoso et al. 2009). Increased siltation caused by deforestation is known to negatively affect fish populations (Reis 2013). Moreover, the various dams that have been built have reduced fisheries production in the region (Santos et al. 2018) and likely affected freshwater stingrays in the area. This scenario is of concern because the endemic *Potamotrygon limai* Fontenelle, Da Silva & Carvalho, 2014 occurs in this increasingly impacted area, although there are no reports of directed fisheries on this species (Fontenelle and Sayer 2021). Climate change projections indicate a change in hydrologic cycles in the Madeira River basin with a reduction in flow, so potamotrygonin reproductive cycles could change as in the watersheds previously mentioned (Júnior et al. 2015).

3.2 Orinoco River basin

The Orinoco River basin ranks second in species richness with eight species, largely due to recent descriptions (Roberts 2020; Loboda et al. 2021) that may suggest an underestimated stingray richness in the region. In this river basin, environmental degradation is due to multiple causes (Barletta et al. 2010). Habitat loss is caused by increasing urbanization and deforestation (Rodríguez et al. 2007). Agricultural development degrades habitats by altering waterways for irrigation and polluting runoff (Pacheco et al. 2014; Lasso et al. 2016; Rinaldi et al. 2021). Oil extraction (Torremorell et al. 2021) and the expansion of the Orinoco mining arc (Ebus and Martinelli 2022) are also harmful sources of pollution. Fisheries in the region are mainly focused on the ornamental international fish market. Ornamental fisheries in the region target mainly *P. motoro* and *P. schroederi*, as shown by export data (Sánchez-Duarte et al. 2013). In addition, fish are also caught for liver oil, which is used as a medicine regionally (Sánchez-Duarte et al. 2013). As in other areas, the expansion of urbanization (Best 2018) could be accompanied by an increase of negative fisheries.

3.3 Paraná-Paraguay River Basin

The Paraná-Paraguay (also called Paraná-Prata or La Plata) River basin is the second largest river basin in South America (Reis et al. 2016). This river basin ranks third in terms of potamotrygonin species richness as seven species have been recorded for its waters (Lucifora et al. 2016): *Potamotrygon amandae* Loboda and Carvalho 2013; *Potamotrygon brachyura* (Günther 1880); *Potamotrygon falkneri* Castex and Maciel 1963; *Potamotrygon histrix* (Müller and Henle 1834); *P. motoro*; *Potamotrygon schuemacheri* Castex 1964, and *Potamotrygon pantanensis* Loboda and Carvalho 2013. Endemism (71%) in this river basin is relatively high compared to other areas. Fisheries appear to pose the greatest threat to potamotrygonin in the Paraná-Paraguay River Basin (Lucifora et al. 2017). A general decline is reported from the lower Paraná River region for *Potamotrygon* species, except for *P. motoro* (Lucifora et al. 2017). The increase in commercial and recreational fishing, especially of *P. brachyura* (Lucifora et al. 2015), is of concern because elasmobranchs are vulnerable to overfishing (Frisk et al. 2005). Demand for ornamental fish is not as high as in the Amazon Basin, but some species from the basin are internationally traded (Lucifora et al. 2016). Negative fishery observations have been reported throughout the basin, including in new areas colonized by *Potamotrygon* species (Garrone-Neto 2010; Lucifora et al. 2016).

In addition, there are other threats in Paraná-Paraguay related to habitat modifications such as dams, land use, and mining (Barletta et al. 2015). The dam for the construction of the Itaipú Hydroelectric Power Plant destroyed the Sete Quedas waterfalls (Júnior et al. 2009). These falls were a barrier to dispersal in the basin, and with their removal, some potamotrygonins invaded the upper Paraná (Garrone-Neto et al. 2014). This invasion causes several problems related to human stinging (Santos et al. 2019) and could lead to an increase in negative fisheries. In addition, impoundment has altered flood pulses in the river, and future dam projects in the area could exacerbate these effects (Quirós et al. 2007). Habitat loss due to land use change is evident in parts of the Paraná-Paraguay Basin (Lee et al. 2018). In contrast, the upper Paraguay River basin appears to be the better-preserved area in the river basin (Lucifora et al. 2015). Climate change projections indicate an increase in flooding in the basin (Camilloni et al. 2013; Zandonadi et al. 2016). Although it is uncertain how these floods would alter habitats, they could affect potamotrygonin reproduction in the region.

3.4 Tocantins-Araguaia River

There are six species in the Tocantins-Araguaia River basin: *P. aiereba*, *P. motoro*, *P. orbignyi,* and the three endemic species: *Potamotrygon garmani* Fontenelle & Carvalho, 2017, *Potamotrygon henlei* (Castelnau, 1855), and *Potamotrygon rex* Carvalho, 2016 (Rincon 2006; Carvalho 2016b). Dams in this river basin are a major cause of change in hydrologic dynamics (Swanson et al. 2021). Aside from the habitat disturbance caused by the dams, there are anecdotal reports of increases in *Potamotrygon* populations (Rincon 2006). This is likely due to the constant inundation caused by the dam, which triggers continuous reproduction in the freshwater stingray species. In addition, habitat degradation has increased significantly in recent years (Swanson and Bohlman 2021). There are reports of fishing in the area, primarily for the ornamental fish market, but also of negative fisheries, especially near urban areas (Rincon 2006). Climate change impacts are likely to alter the dynamics of flood pulses (von Randow et al. 2019).

3.5 Guyana rivers

The Guyana rivers referred to in this study are a series of rivers in the northern part of South America that drain into the Atlantic Ocean, such as the Coppename-Surinam, Maroni, and Corantjn rivers (Lujan et al. 2011). For this area, there are reports of the widespread *P. orbignyi* and two endemic species, *Potamotrygon boesemani* Rosa, Carvalho & Almeida Wanderley, 2008 and *Potamotrygon marinae* Deynat, 2006. Increased mining activities in the Corantjn and Maroni areas are an alarm signal of habitat degradation (Rahm et al. 2014; Gallay et al. 2018; Willink et al. 2022). Declines in ichthyofauna in the region have been reported in altered habitats (Mol 2012; Allard et al. 2016), likely affecting freshwater stingrays. Unregulated fishing in the area has continued for a decade, although Suriname has recently established a quota system for ornamental fisheries (Verheij 2019). The main activity appears to be ornamental fishing, but occasional consumption is also likely (Mol 2012). There are no reports of negative fisheries, but this widespread practice could also occur. Although climate change predictions are scarce, the combination of uncontrolled fishing and pollution with the general lack of information about the area is troubling for the conservation of endemic species.

3.6 Parnaíba River Basin

The Parnaíba River basin is the most peculiar habitat where Potamotrygoninae species occur. This river basin is located in a semi-arid region of northeastern Brazil with a system of intermittent flows and lower annual rainfall (Moro and Rosa 2016). The species *P. orbignyi* and *Potamotrygon signata* Garman, 1913, an endemic one, occur in this watershed. In this region, the decline in river flow (Motta and Gonçalves 2016; CPRM 2017) is alarming due to limited pluviometry in the region. This decline is due to the expansion of agriculture, which changes river courses by damming and irrigation (Rosa et al. 2009). This is compounded by habitat degradation due to urban and agricultural development (Silva et al. 2014). The Parnaíba freshwater stingray, *P. signata*, is traded in the ornamental fish market (Araújo et al. 2004), but its demand has not been estimated so far. There are reports of liver oil obtained from this species being used as medicine in the region (Moro et al. 2016). Moreover, *P. signata* is known to form aggregations during dry seasons (Rosa et al. 2009), this behavior probably makes them more vulnerable to persecution by negative fishing (Moro et al. 2016). This type of fishing is widespread, and there are reports of mutilated and killed animals found on riverbanks (Moro and Rosa 2016). In addition, climate change is a cause for concern, as it could exacerbate already difficult conditions due to the expansion of desertification areas (Seyffarth and Rodrigues 2017) and the occurrence of extreme events (Rudorff et al. 2022).

3.7 Atrato-Magdalena and Maracaibo basins

The Magdalena River Basin is the fifth largest river basin in South America (Reis et al. 2016). Only the endemic *Potamotrygon magdalenae* (Duméril, 1865) occurs in this river basin. This river basin is severely degraded, as evidenced by the decline in fisheries productivity, mainly due to habitat loss (Barletta et al. 2010; Salgado et al. 2022). This reiver basin is impacted by pollution from oil exploration, mining, agriculture, and domestic sources (Tejeda-Benítez et al. 2018; Salgado et al. 2022). In fisheries, *P. magdalenae* is used for consumption and as an ornamental fish. The Magdalena freshwater stingray is one of the main exported fish from Colombia (Sánchez-Duarte et al. 2013). Occasionally, *P. magdalenae* is caught in artisanal fisheries and its liver oil is extracted as medicine (Mejía-Falla et al. 2016). In addition, there are also reports of negative fisheries in the area (Lasso et al. 2013b). Available climate change projections for the Magdalena River predict intense rainfall followed by an increase in sediment load, leading to an increase in anthropogenic pollution (Salgado et al. 2022).

In the Maracaibo Basin there is the endemic *Potamotrygon yepezi* Castex & Castello, 1970. Pollution and habitat loss are the environmental impacts reported in this area (Portillo-Quintero et al. 2012; Reis et al. 2016). The Maracaibo freshwater stingray is caught for ornamental purposes, supplying only the domestic market of Venezuela, with occasional local consumption (Lasso et al. 2013a). In addition, negative fishery has also been reported for this area (Lasso et al. 2013a).

4. Challenges and perspectives for management and conservation

The observed threats for Potamotrygoninae (Figure 1) were consistent with those general to fishes in South America, with only one exception (Reis et al. 2016; Barletta et al. 2016; Torremorell et al. 2021). This exception was the impact of invasive species, which does not appear to be the case for freshwater stingrays to date. Although Potamotrygoninae face the same threats as other fish species, their resilience to recover from these impacts is lower. The number of described Potamotrygoninae species has increased substantially in recent years, suggesting that the diversity of the group has not yet been fully assessed. This highlights the possibility that even undescribed species may be lost without discovery (Darwall and Freyhof 2015). Therefore, actions focused on critical areas and species of concern are needed to ensure the conservation of this unique lineage.

Figure 1. Distribution of threats in the main river basins.

Habitat degradation was the major threat in all river basins, although the causes varied locally. One of the most widespread activities was mining, especially in the Amazon. This damaging activity is of concern because of its abrupt and persistent impacts, so legal enforcement and regulation of this activity is urgently needed. In addition, population growth across the continent has increased pressure on freshwater environments and exacerbated pollution (Reis 2013; Barletta et al. 2016). In this context, protected areas can serve as refugia for species. However, for fish diversity in Amazonia, current protected areas do not specifically cover priority areas, and new priority regions have been identified (Dagosta et al. 2020). Some of these areas are also important for freshwater stingrays, especially when endemic species are considered. Little information is available on protected areas for the other watersheds, but given the current degradation of habitat, their establishment is recommended. Therefore, the adequacy of these areas would significantly benefit the species in the group.

Increasing habitat fragmentation was a general problem and needs to be discussed with regard to future projects throughout South America. This is a worrisome scenario in Amazonia because if all the planned dams were put into operation, only three tributaries (Juruá, Trombetas, and Içá-Putumayo) would remain as free-flowing rivers (Castello and Macedo 2016). These intense impoundments throughout the Amazon would affect all hydrological dynamics and impact most freshwater stingray species. Moreover, this grim outlook is reinforced by the proliferation of smaller dams that do not require risk assessment and have smaller impacts, but in combination could represent severe changes (Castello and Macedo 2016). This scenario is also observed to a lesser extent in the other river basins. Therefore, despite their needs (energy, water supply), the construction of dams must be carefully considered based on an adequate environmental impact assessments.

Ornamental fishing has been observed throughout the continent, but primarily in the Amazon biome (Amazon, Orinoco, Tocantins-Araguaia, and Guyana basins). There is little information on the number of exports by country or species. Even where data are available, it is important to remember that the number of specimens exported does not directly reflect the number of specimens captured. Because the process of acclimation and transport involves significant mortality (Sánchez-Duarte et al. 2013), the number of specimens caught should be higher. However, the ornamental fishery can be sustainable if the catch is regulated, especially if the specimen size is restricted (Rosa et al. 2010). Transboundary regulations and measures are important because some species are caught illegally in one country and transported to a neighboring country that allows their export (Rosa et al. 2010). Another challenge in regulation is species identification because there are similar polychromatic species that can be confused. Therefore, the development of an identification guide could assist national authorities in regulating the ornamental fish trade.

Traditionally, fishing for consumption was not considered a major threat to Potamotrygoninae, but this scenario appears to have changed. The reports summarized in this study suggest that the general reluctance to consume stingray meat has changed. This may be related to the decline of other traditionally preferred fish species (Barletta et al. 2015). In addition, the meat is exported to other regions under the non-specific name 'cação' (Camacho-

Oliveira et al. 2020), which could reduce the aversion. The increase of this fishery, combined with the overfishing vulnerability of elasmobranchs and other threats, may lead to a drastic population decline. Therefore, there is a need to closely monitor and regulate the consumer fishery for Potamotrygoninae.

A widespread practice of concern is negative fishing. Although this is not the primary cause of population declines in most regions, it may exacerbate other threats. Environmental education activities could help reduce aversion to this group, which could lead to a decline in negative fishing. Since negative fishing is associated with bycatch, measures to avoid it could be helpful (Lucifora et al. 2017). In the Paraná-Paraguay River basin, this reduction has been achieved by adjusting fishing gear (Lucifora et al. 2017). Similar initiatives in other river basins, combined with education efforts, could reduce this habit and avoid the unnecessary mutilation and killing of potamotrygonins.

The combination of climate change impacts on severely affected river basins could lead to serious consequences. There is already evidence of localized climate change in South American freshwater basins, and although no study links climate change to trends in freshwater stingray populations, its effects should not be ignored. Observed and predicted changes are likely to disrupt ecological conditions, and several potamotrygonin species have habitat preferences and may be less tolerant of change. In addition, the dynamics of the reproductive cycle depend on the dynamics of flood pulses, and the latter is one of the most altered factors in predictions. Thus, climate change would lead to a reduction in habitat suitability and changes in reproduction. Combined with other threats, this is likely to reduce the resilience of Potamotrygoninae species.

In summary, although freshwater stingrays face a variety of threats that vary regionally, general guidelines for conservation and management can be established. Habitat degradation has increased across the continent and is necessary to ensure protection of current diversity. Establishing protected areas and tightening regulations could help in this matter. In addition, curbing illegal mining activities is critical to ensure habitat suitability for potamotrygonins. Ornamental fisheries need to be more regulated, especially through cooperation between countries in the region. Consumption and negative fishing are other activities that also need to be addressed. In addition, climate change, even if it is just emerging, must be taken into account, as it could have serious consequences in synergy with other threats. All of these actions are necessary to ensure the conservation of the unique Potamotrygoninae diversity, which is not only evolutionarily unique, but also has its own unique threats.

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CAPÍTULO 5: DOTS IN THE DARK: DORSAL POLYCHROMATISM IN THE ENDEMIC XINGU FRESHWATER STINGRAY

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Journal Guidelines: Endangered Species Research | Submission guidelines

Dots in the dark: dorsal polychromatism in the endemic Xingu Freshwater Stingray

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Running title: Polychromatism in *Potamotrygon leopoldi*

Abstract:

Polychromatism refers to the presence of two or more color patterns within a species. Several species exhibit polychromatic patterns, including some elasmobranchs such as the Xingu Freshwater Stingray (*Potamotrygon leopoldi*), a threatened, endemic freshwater stingray species that is exploited in the international aquarium trade. Analysis of polychromatic patterns can provide insight into evolutionary mechanisms and be a useful tool for monitoring international trade. In this context, the present study analyzed intraspecific color variation in *P. leopoldi*. A total of 241 individuals collected in two areas along the Xingu River in Brazil were used for the study. Four dorsal color patterns of *P. leopoldi* were described. Size differences between color classes were statistically significant, suggesting that these color variations are associated with ontogenetic color changes. In addition, two color morphs specific to each locality were identified and described. Moreover, the occurrence of polychromatic forms in a Potamotrygoninae species may contribute to the understanding of diversification in this group, since some mechanisms of speciation are associated with color polymorphisms. Analysis of color variation in *P. leopoldi* is expected to help improve trade monitoring, especially given the existence of similar-looking species.

Keywords: Conservation, *Potamotrygon leopoldi*, Color Polymorphism, Elasmobranch

1. INTRODUCTION

Polychromatism or color polymorphism can be defined as the presence of two or more distinct color patterns that are genetically determined within a species (Huxley 1955, Gray & McKinnon 2007, Roulin & Bize 2007). Polychromatism is distinct from ontogenetic color change, in which different color patterns are associated with specific life stages (Booth 1990, Wilson et al. 2006). Several evolutionary factors could explain the emergence and persistence of polychromatic morphs (Huyghe et al. 2007). They could be evolutionarily neutral, arising randomly and providing no specific advantage, until some of them are stochastically maintained by drift in concert with other evolutionary mechanisms (Hoffman et al. 2006). Alternatively, these patterns may be related to habitat heterogeneity and kept by positive selection in different habitats (Chunco et al. 2007, Cupello et al. 2022). Sexual selection could also be the evolutionary pressure for the persistence of different color patterns (Roulin & Bize 2007, Hurtado-Gonzales et al. 2014). Therefore, the analysis of polychromatism may provide insights into different evolutionary and life-history mechanisms.

Polychromatic forms in cartilaginous fishes, mainly Batoidea, are not a new issue. Several studies use color polymorphism for the photo-identification of specific individuals, and as a non-invasive alternative to mark-recapture studies (Bassos-Hull et al., 2014; Benjamins et al., 2018; Cerutti-Pereyra et al., 2018; Couturier et al., 2011; Davies et al., 2012; Dudgeon et al., 2008; González-Ramos et al., 2017; Marcoux et al., 2023; Marshall et al., 2011; Navarro et al., 2018). In most of these studies, however, it is not clear whether color diversity is related to other factors such as sex, reproductive stage, or size. This is important because polymorphism may be caused by species complexes, ontogenetic color variation, or polychromatism. For example, the previously identified polychromatic forms of *Breviraja spinosa* Bigelow & Schroeder, 1950, corresponded to a species complex and therefore not a polychromatic species (McEachran & Matheson 1985). On the other hand, the seven polychromatic morphs identified in *Raja clavata* Linnaeus, 1758 are consistent between juveniles and adults that do not differ in meristic features, characterizing a case of polychromatism (Mnasri et al. 2009). Color variation is mentioned for several species from other batoid lineages, such as the Neotropical freshwater stingrays (Potamotrygoninae) (Araújo et al. 2004; Rosa et al. 2010). However, despite these efforts, no study so far has tested for an association between intraspecific color variation and other factors such as size, sexual maturation, and geographic variability, for example.

The Xingu Freshwater Stingray, *Potamotrygon leopoldi* Castex & Castello, 1970, is a species of Potamotrygoninae endemic to the Xingu River drainage (Brazil) (Rosa 1985) and known for its elaborate dorsal color patterns. This species has a dark dorsal background with white to pale yellowish spots that vary in number, size, and shape, which is why this species is referred to as polychromatic (Charvet-Almeida 2006). For decades, this species has been highly sought after by the international aquarium trade because of these color variants (Araújo et al. 2004, Charvet et al. 2022), and it is known that there is market demand for certain color patterns in different sizes (Charvet 2022). In addition, Neotropical freshwater stingrays are known to hybridize in nature (Rosa et al. 2010, Cruz et al. 2014), and many artificially bred hybrids in breeding facilities display color morphs quite different from those found in wild populations, since fertile hybrids and wild-captured specimens are bred and cross-bred to fulfill market demands for color patterns (FAO 2022). Captive bred hybridized specimens display a completely different array of color variation that are well-known by aquarists and breeding facility staff and can reach an infinite number of pattern combinations. Consequently, fishing pressure for specific patterns of *P. leopoldi* to serve in the ornamental trade as matrices for breeding could influence the color morphs observed in nature.

In addition to ornamental fishing, there are other factors that threaten the survival of the Xingu Freshwater Stingray. Habitat degradation from mining, deforestation, and water pollution reduce suitable areas for *P. leopoldi* (Neill et al. 2020; Ribeiro et al. 2017; Pignati et al. 2018; Charvet et al., 2022). Climate change is also altering flood-drought dynamics in the Xingu River (Lucas et al. 2021), which may affect *P. leopoldi* reproductive cycles which are closely linked to these dynamics (Charvet-Almeida et al. 2005). Moreover, habitat fragmentation from dams along the Xingu River basin could affect genetic connectivity among these populations (Perez 2015).

The combination of the previously mentioned threats with biological factors (i.e., low fecundity and slow growth) has led to a *P. leopoldi* population decline of over 30%, and the species is now listed as Vulnerable in the IUCN Red List of Threatened Species (Charvet et al. 2022). In addition, *P. leopoldi* was added to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (CITES 2022) to improve monitoring of its trade. Nonetheless, there are no further conservation measures in place for this species to date, which adds to the concern about its threatened status. In this context, understanding intraspecific color variation and plasticity could provide insights into evolutionary mechanisms, be a resource to improve monitoring in the international ornamental trade, and therefore contribute to this species' conservation. The present study characterized natural color patterns and variations of *P. leopoldi* from the wild.

2. MATERIAL & METHODS

Potamotrygon leopoldi individuals were sampled from two areas along the Xingu River: São Félix do Xingu (SFX) and Altamira (ATM). Fieldwork sampling campaigns were conducted in 2002-2005 (ATM) and in 2021 (SFX and ATM). Individuals were lethally anesthetized (Eugenol solution) and photographed immediately after euthanasia. Each specimen had at least three photographs taken, including an overall view and detailed view of the dorsal coloration pattern. Morphometric measurements (i.e., disc width - DW - in mm) were taken for each specimen, following Rosa (1985). The reproductive stage of each specimen was determined, following Charvet-Almeida (2006). The handling, care, and use of specimens were in accordance with the Instituto Brasileiro do Meio Ambiente e Recursos Naturais (IBAMA; Brazilian Environmental Institute) guidelines and approved by the Brazilian National Licensing System for Biological Sampling (licenses IBAMA 036/2002 and ICMBio 80415-1).

Kruskal-Wallis and Dunn post-hoc tests were applied to evaluate the relationship between color patterns and disc width (DW), while a Pearson's chi-square test was used to compare the number of reproductive stages between color classes. Plots and statistical analyzes were performed using R software (R Core Team, 2022).

3. RESULTS

A total of 241 individuals were examined: 36 from São Félix do Xingu (SFX) and 205 from Altamira (ATM). Of this total, 127 (52.7%) were females, with disc width (DW) ranging from 90-700 mm. The remaining 114 (47.3%) specimens were males, with DW ranging from 62-500 mm.

3.1 Dorsal Color Patterns

Four main patterns of dorsal coloration were identified. Pattern 1 (P1) is characterized by the presence of exclusively solid dots or circles of variable sizes (Figure 1 - A). Pattern 2

(P2) is characterized by the predominance of solid dots or circles in combination with dots or circles with fainted-centers, due to darker spots surfacing at their center and forming a darker center, and also a few hollow dots ("donut-shaped" dots or dots with a hole) with wide margins (Figure 1 - B). Pattern 3 (P3) is characterized by a predominance of the hollow donut dots, with narrower margins, over the center-faded dots (Figure 1 - C). Pattern 4 (P4) is characterized by the far predominance of hollow donut-shaped dots, with thinner margins than in the previous patterns, and vermicular or kidney-shaped spots mainly in the central area of the disc, occasionally with the presence of some smaller solid dots (Figure 1 - D). When considering these four patterns, no clear difference in color patterns was found between the sexes (Figure 2).

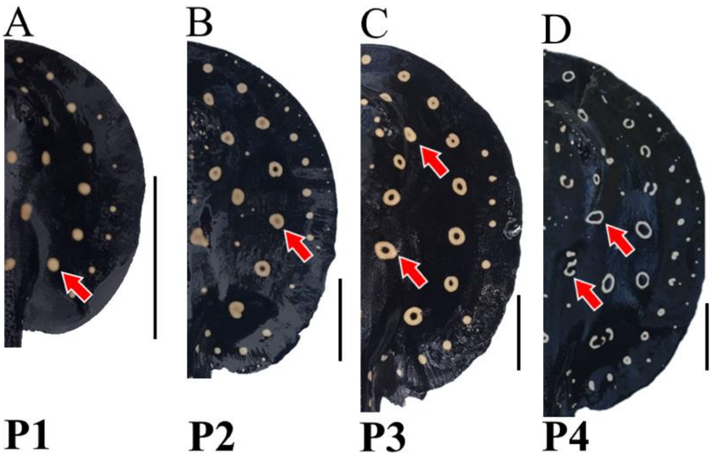

Figure 1. Dorsal right side of disc with the four main color patterns identified for *Potamotrygon leopoldi*. Scale bars: 100 mm.

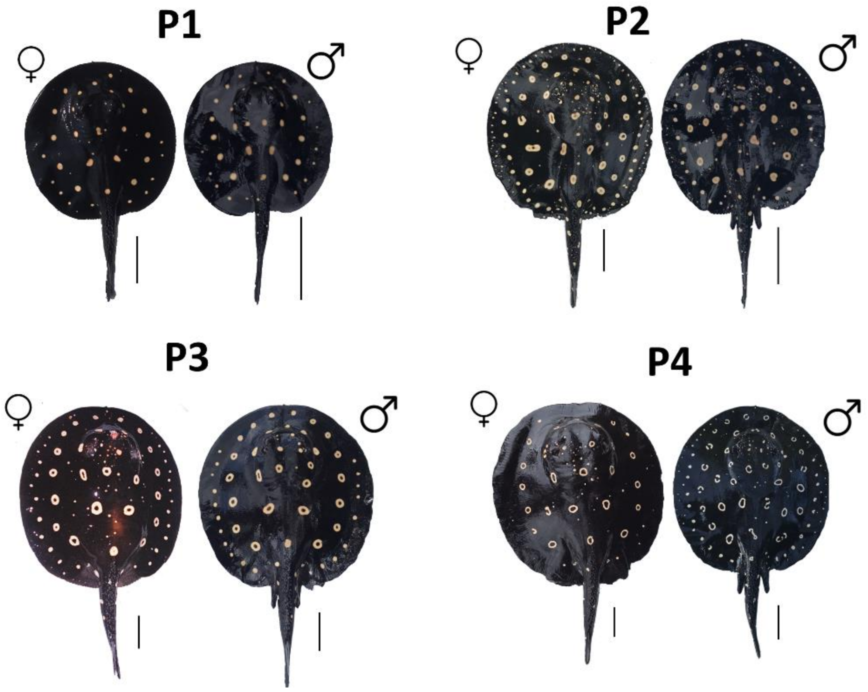

Figure 2. Disc dorsal color patterns of *Potamotrygon leopoldi* color by sex. Scale bars: 100 mm.

3.2 Patterns, Sex, Size and Sexual Maturity

The data was distributed according to color pattern, sex, reproductive stage, and size (DW) (Table 1). Individuals from SFX were observed to be sexually mature at smaller sizes compared to ATM, especially females. Another difference was the higher proportion of subadults observed in SFX.

Table 1 - Number of individuals (n) analyzed by color pattern, sex, and reproductive stage and disc width (DW) interval recorded. The proportion of individuals (%) within each color pattern by sex is indicated. Individuals are separated by sampling sites: Altamira (ATM) and São Félix do Xingu (SFX).

			Sampling locality					
			ATM			**SFX**		
Color Pattern	**Sex**	**Reproductive stage**	**n**	**%**	**DW interval (mm)**	**n**	**%**	**DW interval (mm)**
P1	♂	Juvenile	24	83	109–310	3	75	62–234
		Subadult	3	10	310–391	1	25	278
		Adult	2	7	361–415	0	-	-
	♀	Juvenile	27	87	124–400	1	100	260
		Subadult	4	13	324–418	0	-	-
		Adult	0	-	-	0	-	-
P2	♂	Juvenile	3	13	297–351	0	-	-
		Subadult	6	26	315–395	4	50	292–314
		Adult	14	61	342–416	4	50	343–422

	♀	Juvenile	2	10	294–342	2	20	90–295
		Subadult	5	25	339–458	5	50	314–369
		Adult	13	65	417–568	3	30	349–420
P3	♂	Juvenile	0	-	-	0	-	-
		Subadult	1	5	336	0	-	-
		Adult	21	95	350–500	1	100	343
	♀	Juvenile	0	-	-	0	-	-
		Subadult	1	4	423	5	55	306–403
		Adult	24	96	460–555	4	45	380–465
P4	♂	Juvenile	0	-	-	0	-	-
		Subadult	0	-	-	0	-	-
		Adult	24	100	390–493	3	100	345–352
	♀	Juvenile	0	-	-	0	-	-
		Subadult	0	-	-	0	-	-
		Adult	31	100	496–700	0	-	-

A correlation was found between dorsal color patterns, disc width size, and sexual maturity (Figure 4). The difference in the number of juvenile, subadult, and adult individuals for each color pattern was statistically significant ($\chi 2 = 203.17$, d.f. = 6, p-value < 0.001).

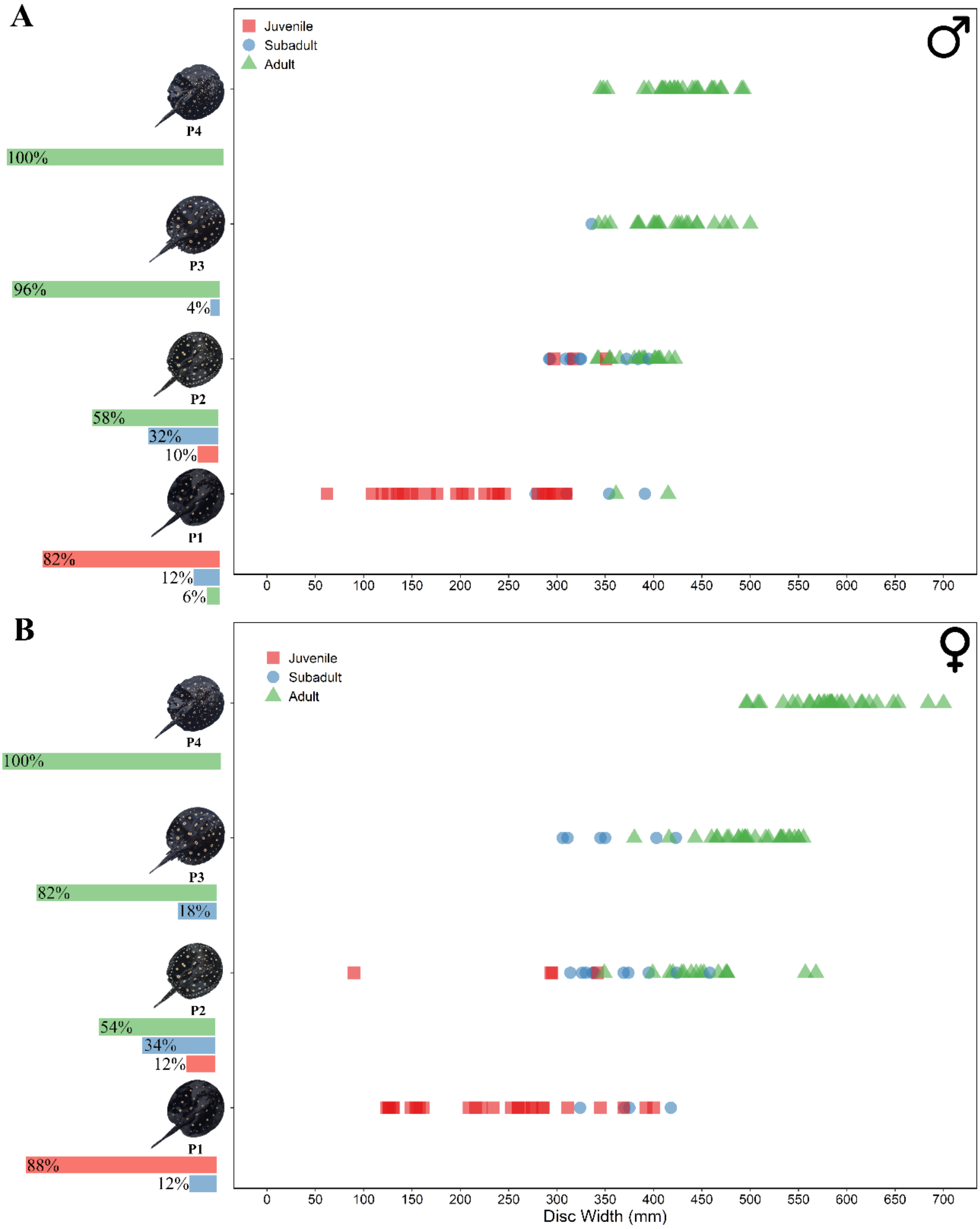

Figure 4. Distribution of color patterns (P1-P4) by size (disc width) and reproductive maturity stage in *Potamotrygon leopoldi*.

Dorsal color pattern P1 was predominant among smalland juvenile individuals (♂ 82%, ♀ 88%). Pattern P2 was predominant among small adults (♂ 58%, ♀ 54%), followed by subadults (♂ 32%, ♀ 34 %) and few juveniles (♂ 10%, ♀ 12%). Pattern 3 (P3) was predominant among adults and larger individuals, especially males (♂ 96%, ♀ 82%). The fourth pattern (P4) was found only on larger adults (100%), both males and females. In females, the predominance of P4 became more evident as disc width and overall size increased, while in males there was an overlap of individuals with P3 and P4 patterns.

The differences in disc width between color patterns were statistically significant (Kruskal-Wallis $\chi 2 = 150.70$, d. f. = 3, p-value < 0.001). The only variation that was not significant was between patterns P3 and P4, which seemed to overlap mainly amongst males (Dunn's Test = -2.31, p-value = 0.0620). A second analysis was then performed separately for each sex. For females, the difference between all classes was significant (Kruskal-Wallis $\chi 2 = 96.02$, d. f. = 3, p-value < 0.001), but again a significant difference remained lacking only between male P3 and P4 (Dunn's Test = -0.88, p-value = 1). Other male sex-related measures (e.g., testis size and weight, lobulation level, epididymis length) were tested, but none provided a significant difference between the P3 and P4 classes.

Variations in color morphs were also observed, and certain characteristics prevailed in the two sampling areas studied (Figure 3). In the SFX individuals, the predominant color pattern variation (97%; n = 35) was characterized by the presence of many very small solid dots (“snowy dots”) located mainly around or near the eyes, spiracle, and disc center (Figure 4). In the SFX region, a line, or lines, of small solid dots along the dorsal margin of the disc (“pearl string”) was observed in most specimens (80%, n = 194). In the younger or smaller animals, only this disc lining of small solid dots was noted (100%, n = 6). As disc width increased (starting at 260 mm DW), the snowy dots at the center of the disc became more frequent and were noted in 75% (n = 27) of the sampled specimens.

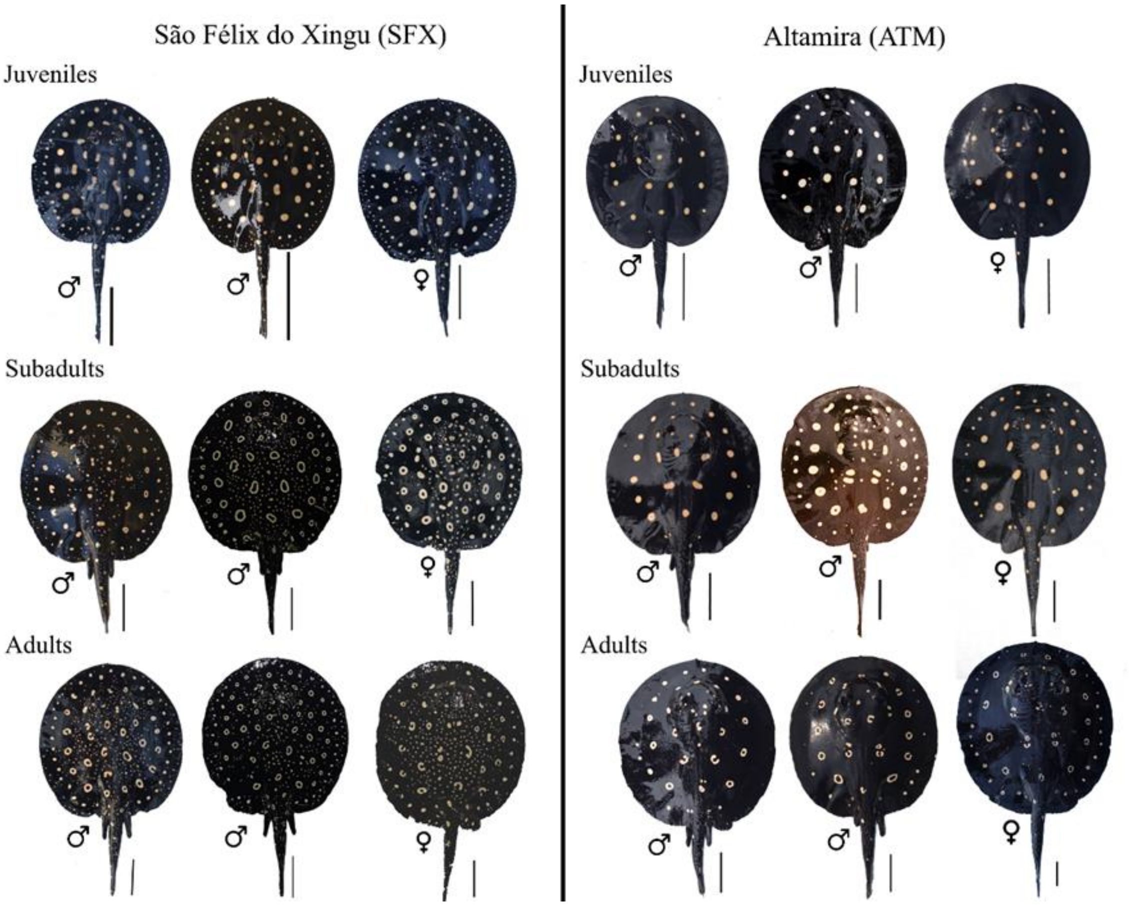

Figure 5. Color pattern variation in the *Potamotrygon leopoldi* locations of São Félix do Xingu and Altamira. Scale bars: 100 mm.

The ATM dominant color morph (94%, n = 193) displayed the presence of dots (solid or hollow), kidney-shaped forms, and vermicular forms without having the smaller dots lining the disc dorsal margin and the snowy dots were absent (Figure 4). The difference in the predominance of color morphs was significant for both SFX and ATM localities ($\chi 2$ =157.07, d.f. = 1, p-value < 0.001), suggesting that each site has its own typical color morph.

4. DISCUSSION

In the description of the Xingu Freshwater Stingray, special attention was paid to the dorsal color pattern as one of the characteristic features of the species (Castex and Castelo 1970). *Potamotrygon leopoldi* was described on the basis of a single adult male from the Upper Xingu Region. The dorsal coloration of the holotype, which was a fixed specimen, was described as dark black with yellow kidney or half-moon shaped spots that were more numerous on the margin and became irregular towards the middle of the disc (Castex and Castelo 1970). The description of this adult male is consistent with the P4 pattern in the present study, and the presence of small marginal spots on the disc resembles the São Félix do Xingu (SFX) morphotype, which is closer to the type locality than Altamira (ATM). Therefore, the intricate dorsal color pattern has been a notable feature of *P. leopoldi* since its description, and as the present results indicate, the color plasticity of this species may be associated with other biological traits.

The observed color patterns suggest that some of the polychromatic forms of dorsal coloration in *P. leopoldi* are due to an ontogenetic color change. This color transition is a phenomenon known in other species, such as cichlids (Beeching and Pike 2010), hermit crabs (Hamasaki et al. 2017) and pythons (Wilson et al. 2006). Individuals gradually transition from pattern P1 to pattern P4 as they grow and become sexually mature. This development is evident by the predominance of juveniles in P1 and adults in P4. The fact that no statistical difference was observed in males between classes P3 and P4 may be elucidated with the incorporation of physiological features (i.e., hormone detection in blood samples) in future studies. Such analyses could contribute to the understanding of these gradual changes, as hormonal changes are known to influence color patterns (Border et al. 2019). Moreover, since other potamotrygonin species exhibit size-dependent habitat segregation (Garrone-Neto & Uieda 2012), differential habitat use among individuals of *P. leopoldi* could also influence the maintenance of observed patterns (Booth 1990).

Despite ontogenetic color development, two distinct color morphs were observed for the two sampling localities. Individuals differed among themselves at each site, but a general color morphotype could be drawn for each area. The existence of some individuals that differed from the most common morphotype for each area may be suggestive of some degree of gene flow or movement between individuals from both sampling areas, as observed in other polychromatic species (Cupello et al. 2022). Since there is evidence of genetic differentiation between color patterns in other species (Gaither et al. 2020), it would be important to consider this aspect in the present case. Regarding other differences between sampling areas, sexually mature individuals tended to have a smaller disc width in the SFX area than in the ATM area. The SFX area is subject to high fishing pressure due to the presence of color morphs that are highly valued for the international ornamental trade (Charvet et al. 2022). This high demand for captures may have led to an overall reduction in size and earlier sexual maturation, a compensatory strategy that has been observed in other species subject to high fishing pressure

(Trippel 1995; Schilling et al. 2019; D'iglio et al. 2021) and could help explain the presence of smaller mature individuals in SFX.

The observed polychromatic patterns may aid in the understanding of some evolutionary events in Potamotrygoninae. The habitat of *P. leopoldi*, a clear water river, may favor the emergence of this type of pattern as it has been confirmed that Potamotrygon species can recognize colors (Schluessel et al. 2021). Since the visual signals can be perceived and polyandry has been confirmed for this species (Torres et al. 2022), the color patterns could serve as visual cues in courtship and mate choice. Thus, the prevalence of different color patterns in the SFX and ATM areas could suggest a differential mate choice for each area (Price et al. 2009, Steinwender et al. 2012). Alternatively, divergent selection, in which each color morph would have adaptative advantages in each microhabitat, could maintain polychromatic morphs being balanced by gene flow (Chunco et al. 2007, Gray & McKinnon 2007). Further analysis should consider genetic diversity to confirm whether color variation is related to genetic structure. Understanding this relationship may contribute to elucidating the complex speciation events within Potamotrygon species as these mechanisms have explained speciation events in other polychromatic groups (Elmer et al. 2010). The occurrence of such patterns in *P. leopoldi* opens the possibility to search for other polychromatic Potamotrygon species to clarify if and how this pattern diversity might explain speciation processes within the group.

The color patterns observed for *P. leopoldi* are very important for management and conservation measures of this endemic and vulnerable species. In ornamental fish catalogs *P. leopoldi* has been included in the "Large Eyed Black Rays" group, along with *P. henlei*, and both species are considered sturdy and durable aquarium occupants (Ross, 1999). One year later, these species were referred to as belonging to the "Black Stingrays" group and were characterized as having a brown or black back (meaning dorsal background color) covered with light-colored spots ringed by a dark circle, forming what was named "ocelli", with these extending distally on the tail (Ross & Schäfer, 2000). In terms of vernacular names and color codes used then, P. leopoldi was often referred to as "Black Ray" or "Eclipse-Ray" (P13) and *P. henlei* predominantly as "Black Ray" (P12), but images depicted a few other species that were considered color morphs of these two species. Another common name attributed to *P. leopoldi* was "Black Diamond" or "Black Diamond Ray", referring mainly to the SFX morphotype color which was highly valued by aquarists and potamotrygonins breeding facilities (Charvet, 2022). Moreover, the Xingu Freshwater Stingray is a threatened species subject to environmental degradation, climate change, and exploitation by the international ornamental fish trade (Charvet et al. 2022). This scenario contributed to the listing of the species under CITES Appendix II (CITES 2022). Therefore, it is expected that trade monitoring will be strengthened. One challenge associated with this is misidentifications with related species or look-alike species, such as *Potamotrygon henlei* (Castelnau, 1855) and *Potamotrygon albimaculata* Carvalho 2016, especially in the case of juveniles of *P. leopoldi* (Araújo et al. 2004, Charvet-Almeida 2006, Charvet et al. 2022). Thus, the reported color patterns can be a useful tool for the correct identification of traded individuals, since the sampling areas cover a considerable part of the species' range and the main known ornamental fishery sites for this species (Charvet-Almeida 2006, Charvet et al. 2022). In addition, evidence of movement between sampling areas highlights the importance of maintaining connectivity for the species, especially considering current and planned future dam projects for the Xingu River (Perez 2015).

The present analysis is the first to examine polychromatism in relation to other biological parameters in elasmobranchs. Part of the observed color diversity in *P. leopoldi* is due to ontogenetic development. However, another part of this diversity is associated with the two predominant color morphs found in each sampled area. The driving evolutionary mechanisms leading to color variations observed are still unclear. Further studies correlating the color patterns with population genetics and physiology are needed to help elucidate the evident color variability. The identified color patterns should be treated as an important factor for the conservation and management of *P. leopoldi*, especially since monitoring tools are needed to improve trade monitoring due to the recent listing of this species under CITES Appendix II. Analysis of other Potamotrygoninae species could also be fruitful to helping understand diversification within this group.

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6 CONCLUSÃO

Nos estudos genéticos, foram produzidas informações relevantes para a conservação das espécies. A confirmação de paternidade múltipla em raias de água doce aporta informação para o entendimento da dinâmica reprodutiva das raias de água doce. Esta informação pode auxiliar no manejo de espécies e ser uma alternativa para explicar a existência do policromatismo observado em muitas espécies. Os resultados obtidos com a análise da estrutura populacional de *P. horkelii* confirmam o efeito gargalo devido à considerável redução populacional. Esse dado reforça a necessidade de ações de manejo, já que as os efeitos da redução populacional na diversidade genética foram confirmados. A estrutura genética observada é útil no desenvolvimento de estratégias de manejo e conservação para essa espécie criticamente ameaçada, com especial atenção no manejo no Rio de Janeiro que apresenta grupo geneticamente diferenciados. Dessa forma, a região do Rio de Janeiro precisa de uma amostragem mais ampla e monitoramento para garantir a manutenção da diversidade genética para o grupo.

As revisões bibliográficas produzidas identificaram importantes lacunas e questões relevantes em Potamotrygoninae. As lacunas referentes ao conhecimento genético de raias de água doce neotropicais foram identificadas e analisadas. Através dessa análise possível identificar que a área de estudo que ainda necessita mais resolução é a taxonomia. Possivelmente, com a aplicação de análises genômicas as relações entre as espécies de raias de água doce poderão ser melhor compreendidas. A análise das ameaças para espécies de potamotrigonineos, identificou as principais dentro das principais bacias hidrográficas. A degradação de habitat foi uma ameaça comum a todas regiões, e isso demonstra a necessidade de ampliação de unidades de conservação. Ademais, o combate a mineração ilegal é crucial, considerando que é uma das atividades com maior impacto e presença pelo continente. O reforço e estabelecimento de ações de monitoramento do comércio é uma ação necessária principalmente na região amazônica devido a alta pressão por espécies na região. Outro importante ponto é o desenvolvimento de ações de educação ambiental contra a pesca negativa, que foi uma prática presente em todo o continente. A crescente pressão por pesca direcionada a consumo é uma atividade que também precisa ser regulada para manejo adequado para o grupo. Além disso, a cooperação dos países sul-americanos nessas ações é uma prática que precisa ser buscada para garantir a eficácia de aplicação das ações de conservação. Esses dados são relevantes já que incorporam a heterogeneidade de ambientes que elas ocorrem e serão importantes subsídios para desenvolvimento de ações de conservação.

A análise de policromatismo permitiu um maior entendimento da variação policromática em *P. leopoldi*. Uma parcela da variação de padrões corresponde a uma mudança ontogenética que ocorre na coloração dorsal, enquanto a outra parcela está associada a localidade de coleta. Estas informações podem auxiliar o monitoramento, especialmente importante agora que a espécies está listada no Apêndice II da CITES.

Dessa forma, todos os estudos apresentados proveram dados importantes para espécies de batóideos neotropicais. Apesar de abordarem diferentes campos, todas informações geradas são relevantes para o desenvolvimento de ações de conservação.

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