UNIVERSIDADE FEDERAL DE SÃO CARLOS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA EVOLUTIVA E BIOLOGIA MOLECULAR

Caracterização de pontos de quebras de DNA e rearranjos cromossômicos no gênero *Harttia* (Siluriformes: Loricariidae)

Geize Aparecida Deon

SÃO CARLOS
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Tese de doutorado apresentada ao Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos – UFSCar, como parte dos requisitos necessários para obtenção do título de Doutora em Ciências (Ciências Biológicas), área de concentração: Genética e Evolução.

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que me guiaram até aqui.

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“Tenho a impressão de ter sido uma criança
brincando à beira-mar, divertindo-me em descobrir
uma pedrinha mais lisa ou uma concha mais bonita
que as outras, enquanto o imenso oceano da verdade
continua misterioso diante de meus olhos”.

(Isaac Newton)

DADOS CURRICULARES DA AUTORA

Geize Aparecida Deon iniciou sua graduação em Ciências Biológicas na Universidade Estadual do Paraná, *campus* União da Vitória-PR no ano de 2011 e obteve seu título no ano de 2015. Durante a graduação foi aluna do Programa Institucional de Bolsas de Iniciação à Docência (PIBID). Concomitantemente a esse projeto também atuou como monitora no Laboratório de Zoologia e como voluntária no Laboratório de Citogenética, onde ingressou nesta área com estudos de citogenética clássica sob supervisão da Prof. Dr. Carla Andreia Lorscheider. Após o contato com a área durante a graduação, ingressou no mestrado pelo Programa de Pós-Graduação em Biologia Evolutiva da Universidade Estadual de Ponta Grossa no ano de 2015, como bolsista CAPES. Durante este período aprendeu e desenvolveu habilidades na área da Citogenética Clássica e Molecular de peixes estudando diferentes populações de *Neoplecostomus* em bacias hidrográficas do estado do Paraná. Também desenvolveu habilidades na área de Genética Molecular com amplificação e análise de sequências do gene Citocromo C oxidase. Além disso, exerceu o cargo de representante discente junto ao colegiado deste programa. Obteve seu título de mestre no ano de 2017 com a dissertação intitulada: "Citogenética comparativa e diferenciação genética em populações de *Neoplecostomus* (Siluriformes: Loricariidae) em afluentes do rio Paraná" sob orientação do Prof. Dr. Marcelo Ricardo Vicari. Ingressou no doutorado em 2017 pelo Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular da Universidade Federal de São Carlos sob orientação do Prof. Dr. Orlando Moreira-Filho e coorientação do Prof. Dr. Marcelo Ricardo Vicari. Em 2019, realizou um estágio no Laboratory of Molekulare Zytogenetik da Universitätsklinikum na cidade de Jena (Alemanha) liderado pelo Dr. Thomas Liehr onde realizou experimentos de microdissecção e pintura cromossômica e aprimorou seus conhecimentos. Durante o doutorado participou de três projetos de pesquisa, ministrou cursos de curta duração e foi coorientadora de um trabalho de conclusão de curso. Além disso, participou de diversos eventos nacionais e internacionais da área com apresentação de trabalhos, incluindo um prêmio de menção honrosa. Até o momento publicou nove artigos científicos (além de quatro artigos em processo de submissão), dos quais três estão diretamente relacionados a sua tese.

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CAPÍTULO 1

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LISTA DE ABREVIAÇÕES

2n	Número diploide
NF	Número fundamental
DNAr	DNA ribossômico
ITS	Sítio telomérico intersticial
EBR	*Evolutionary breakpoint region*
mm	Milímetro
ml	Mililitro
mg/ml	Miligrama por mililitro
ng/ml	Nanograma por mililitro
µl	Microlitro
mM	Milimolar
µM	Micromolar
µm	Micrômetro
m	Cromossomo metacêntrico
sm	Cromossomo submetacêntrico
st	Cromossomo subtelocêntrico
a	Cromossomo acrocêntrico
PCR	*Polymerase chain reaction*
dNTP	*Deoxynucleotide triphosphates*
dUTP	*Deoxyuridine Triphosphate*
DOP	*Degenerate oligonucleotide-primed*
FISH	*Fluorescence in situ hybridization*
WCP	*Whole chromosome painting*
CGH	*Comparative genomic hybridization*
gDNA	*Genomic DNA*
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
$MgCl_2$	Cloreto de Magnésio
rpm	Rotação por minuto
M	Molaridade
N	Normalidade
PBS	*Phosphate-bufferid saline*

RESUMO

O gênero *Harttia* (Siluriformes: Loricariidae) compreende um grupo de peixes amplamente distribuído em bacias hidrográficas da América do Sul. Do ponto de vista citogenético, apresenta ampla variação cariotípica, incluindo uma extensa variação na posição dos sítios de DNAs ribossômicos, a presença de cromossomos supranumerários e de sistemas de cromossomos sexuais diversificados. Acredita-se que a ocorrência de sítios de instabilidade cromossômica presentes no genoma destas espécies tem levado a quebras de dupla fita de DNA, as quais desencadearam esses rearranjos cromossômicos. Assim, este trabalho teve como objetivo analisar os rearranjos e os cromossomos envolvidos na diferenciação cariotípica de espécies de *Harttia*, bem como a organização de sequências repetitivas próximas a essas regiões. Para isso, foram utilizadas dez espécies coletadas em diferentes bacias hidrográficas da região sul, sudeste e centro oeste do Brasil, incluindo duas novas espécies ainda não descritas na literatura. Na etapa inicial foram utilizadas ferramentas de citogenética clássica e molecular incluindo hibridização *in situ* de genes ribossômicos e sequência telomérica, além de ensaios de hibridização genômica comparativa. Os resultados revelaram a ocorrência de dois novos casos do sistema de cromossomos sexuais múltiplos XX/XY_1Y_2, além de uma espécie não descrita com o número diploide mais alto já encontrado para o gênero. Na investigação dos rearranjos cromossômicos foi utilizada a técnica de microdissecção e pintura cromossômica total. Foram isolados seis cromossomos diferentes: 25 (X_1) e 26 (X_2) de *H. punctata*, 9 e X (par 1) de *H. carvalhoi*, cromossomo X (par 1) de *H. intermontana* e cromossomo 1 de *H. torrenticola*. Os dados de pintura cromossômica utilizando as sondas X_1 e X_2 indicaram uma série de rearranjos cromossômicos ocorridos durante a diversificação de *Harttia* da região sul e sudeste. Com esses resultados foi possível detectar que pontos de quebras cromossômicas podem estar localizados dentro ou adjacentes aos sítios de DNAs ribossômicos nos genomas de *Harttia*, e que esses sítios desencadearam inúmeros eventos de remodelação cromossômica. Já os resultados de pintura cromossômica comparativa obtidos com as sondas do cromossomo X e do cromossomo 9 de *H. carvalhoi* mostraram que eventos de fusão cêntrica foram responsáveis pela origem desses cromossomos e pela diminuição do número diploide nessa linhagem. O cromossomo 1 de *H. torrenticola* demonstrou alta homeologia ao cromossomo X de *H. carvalhoi*. Por sua vez, embora com morfologia similar, o cromossomo X de *H. intermontana* foi oriundo de uma translocação entre X e autossomo (cromossomo 9 de *H. carvalhoi*), além da identificação de outros rearranjos cromossômicos que desencadearam o reposicionamento de blocos homeólogos no cariótipo dessa espécie. Além disso, demonstrou-se também que os dois diferentes sistemas de cromossomos sexuais múltiplos encontrados no gênero tiveram origem evolutiva independente. A respeito da investigação de sequências repetitivas associadas a essas regiões de instabilidade, foram mapeadas sequências microssatélites e analisadas quanto ao acúmulo em regiões onde ocorreram rearranjos cromossômicos. Foi observado um enriquecimento de sequências em regiões de quebra de dupla fita. Embora uma caracterização genômica em *Harttia* possa ser necessária para identificar os sítios instáveis, os resultados obtidos indicam para pontos de quebra de dupla fita no genoma de *Harttia* e reafirmam a ampla diversidade cariotípica no gênero.

Palavras-chave: instabilidade cromossômica, rearranjos cromossômicos, cromossomos sexuais.

ABSTRACT

Harttia genus comprises a fish group widely distributed in hydrographic basins of South America. In the cytogenetic point of view, present large karyotypic variation, including an extensive variation in position of ribosomal DNA, B chromosome presence and diversified sex chromosome systems. It is proposed the occurrence of chromosomal instability sites present on the genome of this species could lead the DNA double strand breaks, which trigger these chromosomal rearrangements. Thus, this work aimed to analyze rearrangements and the chromosome involved on karyotypic differentiation in *Harttia* species, as well the accumulation of repetitive sequence in these regions. Ten species were collected in different hydrographic basins of south, southeast and middle-west regions, including two new not described species. Firstly, classical, and molecular cytogenetic tools were used, including in situ hybridization of ribosomal genes and telomeric sequences, in addition to comparative genomic hybridization essays. The results reveal two new cases occurrence of multiple sex chromosome XX/XY_1Y_2, besides of new not described species with the highest diploid number founded in the genus. In the investigation of chromosomal rearrangements, was used the microdissection and whole chromosome painting techniques. Were isolated six different chromosomes: 25 (X_1) and 26 (X_2) from *H. punctata*, 9 and X (pair 1) chromosome from *H. carvalhoi*, X chromosome (pair 1) from *H. intermontana* and chromosome 1 from *H. torrenticola*. The chromosome painting results using the X_1 and X_2 probes indicated several chromosomal rearrangements occurred during the diversification of *Harttia* species from south and southeast regions. With these results, was possible detect that chromosomal breakpoint regions are located inside or surrounding the ribosomal DNA sites in *Harttia* genome, and these sites triggered several events of chromosomal remodeling. The chromosome painting results obtained with X chromosome probes and chromosome 9 of *H. carvalhoi* showed that fusion centric events were responsible for the origin of these chromosomes and for decrease in diploid number variation in this lineage. The chromosome 1 of *H. torrenticola* demonstrated high homeology to chromosome X of *H. carvalhoi.* Although with similar morphology, the X chromosome of *H. intermontana* arise from a translocation event between X and autosome (chromosome 9 of *H. carvalhoi*) besides the identification of other chromosomal rearrangements that triggered the repositioning of homeology blocks on the karyotype of this species. Furthermore, demonstrated two different types of multiple sex chromosome systems founded in the genus, had independent evolutive origin. About the investigation of repetitive sequences associated with this instability regions, were mapped microsatellite sequences and analyzed regarding to the accumulation in chromosomal rearrangements occurred regions. It was observed an enrichment of different sequences in double strand break region. Although a genomic characterization can be necessary to identify the instable sites, the results obtained point to double strand breakpoints in *Harttia* genome and reaffirm the high karyotypic plasticity in the genus.

Keywords: chromosomal instability, chromosomal rearrangements, sex chromosome systems.

1. INTRODUÇÃO

1.1 Peixes neotropicais

Peixes ocorrem em lagos, córregos, estuários e oceanos em todo mundo e representam um grupo de vertebrados extremamente diversificado (NELSON; GRANDE; WILSON, 2016). O número de espécies de peixes válidas é de 36.128, das quais mais de 18.260 são espécies de peixe de água doce (FRICKE; ESCHMEYER; FONG, 2022). É notável que cerca de 50% do total de espécies de peixes habita ambientes de água doce, os quais compreendem uma pequena parcela da superfície de água do planeta (cerca de 1%). Ou seja, uma parcela significativa da diversidade ictiológica está desproporcionalmente distribuída em uma área relativamente pequena quando comparada ao da superfície terrestre (LÉVÊQUE *et al*., 2008).

Três grandes bacias hidrográficas de água doce predominam no continente Sul-Americano: bacia Amazônia, bacia do Orinoco e a bacia Paraná-Paraguai (também denominada como bacia La Plata). A bacia Amazônica é a maior e mais diversa em espécies de peixe de água doce no mundo e drena rios da Colômbia, Equador, Peru, Bolívia e Brasil. A segunda bacia hidrográfica mais diversa é a do rio Orinoco. Esta bacia abrange áreas da Colômbia e Venezuela e constitui a maior área de planície alagada da América do Sul, e que compartilha muitos gêneros de peixes com a bacia Amazônica (REIS *et al*., 2016). Juntas, essas duas bacias representam o centro da diversidade para a maior parte dos peixes neotropicais, onde aproximadamente 65% das espécies que habitam a região neotropical estão distribuídas nessa região. Por fim, a bacia La-Plata que possui a segunda maior planície alagada do continente, incluindo o Pantanal, drena áreas da Argentina, Bolívia, Brasil, Paraguai e Uruguai (ALBERT; REIS, 2011; REIS *et al*., 2016; ALBERT; TAGLIACOLLO; DAGOSTA, 2020).

O maior número de famílias de peixes totalmente de água doce é encontrado na região Neotropical, com uma alta proporção de espécies endêmicas (LÉVEQUE *et al*., 2008). Os peixes de água doce da região Neotropical incluem entre 20 e 25% de toda a diversidade de peixes do mundo, com mais de 6.000 espécies, e estima-se que o número total exceda 8.000 espécies apenas na América do Sul (ALBERT; TAGLIACOLO; DAGOSTA, 2020; REIS *et al*., 2016). Nesta região, um número considerável de espécies encontra-se agrupadas em algumas ordens como: Siluriformes (37,1%), Characiformes (31,2%), Cyprinodontiformes (12,7%) (MALABARBA; MALABARBA, 2020; VAN

DER SLEEN; ALBERT, 2021). Uma importante característica dos peixes neotropicais é a abundância de espécies de pequeno porte (20 a 30 milímetros) entre as três ordens predominantes. Por outro lado, também existe a ocorrência de espécies de grande porte como por exemplo, o bagre amazônico gigante pertencente ao gênero *Brachyplatystoma* que pode alcançar 3 metros e 140 quilos e a espécie *Arapaima gigas* que pode alcançar 4,5 metros e até 200 quilos (LÉVÊQUE *et al*., 2008).

O elevado número de espécies presente nessa região reflete não apenas a grande diversidade existente nas bacias hidrográficas tropicais e subtropicais da região Neotropical, como também um constante e significativo aumento do conhecimento sobre a ictiofauna. Esse crescimento do número de espécies de peixes confirma a expectativa de que a biodiversidade da Região Neotropical é bastante elevada, existindo ainda muitas espécies desconhecidas (BUCKUP; MENEZES; GHAZZI, 2007).

1.1.2 Ordem Siluriformes com ênfase na família Loricariidae

Loricariidae corresponde a um dos principais clados da ordem Siluriformes com elevado número de representantes, atualmente com mais de 1000 espécies válidas distribuídas em 114 gêneros e que são conhecidos popularmente como “cascudos” (REIS; KULLANDER; FERRARIS Jr, 2003; FRICKE; ESCHMEYER; FONG, 2022). De modo geral, habitam ambientes lóticos de pouca profundidade e com fundo arenoso, mas podem ser encontrados também em lagos e rios com correnteza fraca a moderada, pequenos arroios e até rápidos tributários (NELSON, 2006; COVAIN; FISCH-MULLER, 2007). Membros dessa família possuem corpo achatado recoberto por placas ósseas e possuem a boca modificada em forma de disco que permite a aderência em diferentes tipos de substratos, até mesmo em lugares com rápido fluxo de água (COVAIN; FISCH-MULLER, 2007). O formato da boca e dos dentes consistem em uma importante adaptação para este grupo de peixe, permitindo a raspagem de substratos para se alimentar de algas, detritos e pequenos invertebrados (COVAIN; FISCH-MULLER, 2007). Com relação a estrutura bucal, os loricarídeos apresentam os mais variados tamanhos que variam com relação a composição tecidual, que está diretamente relacionada ao tipo de ambiente (BRESSMAN *et al*., 2020).

Durante o decorrer dos últimos anos, diversas hipóteses têm sido levantadas para explicar a relação entre os táxons agrupados em Loricariidae (ARMBRUSTER 2004; REIS; PEREIRA; ARMBRUSTER, 2006; CRAMER; BONATTO; REIS, 2011; LUJÁN

et al., 2015). Parte das espécies desta família foram historicamente agrupadas em subfamílias com pouco ou nenhum suporte filogenético, entretanto atualmente compõem seis subfamílias: Lithogeninae, Delturinae, Rhinelepinae, Hypoptopomatinae, Hypostominae e Loricariinae (ROXO *et al*., 2019). Loricariinae é a mais especiosa subfamília de Loricariidae com 30 gêneros e 257 espécies válidas, distribuídas por drenagens de toda América do Sul e América Central (FRICKE; ESCHMEYER; FONG, 2022).

Estudos citogenéticos em Loricariinae já foram realizados em pelo menos 35 espécies e os dados indicam uma notável variação cromossômica (TAKAGUI *et al*., 2020). Com relação ao número diploide (2n), a variação encontrada é de 2n=36 cromossomos em *Rineloricaria latirostris* (GIULIANO-CAETANO, 1998) até 2n=74 cromossomos em *Sturisoma nigrirostrum* (ARTONI; BERTOLLO, 2001). Além disso, nesse grupo também já foram relatados casos de cromossomos B (SCAVONE; JULIO Jr., 1994; PORTO *et al*., 2011; BLANCO *et al*., 2012), diversos tipos de sistemas de cromossomos sexuais (SCAVONE; JULIO Jr., 1995; CENTOFANTE; BERTOLLO; MOREIRA-FILHO, 2006; BLANCO *et al*., 2013 e 2014), polimorfismos populacionais com relação ao número diploide e fórmulas cariotípicas (GIULIANO-CAETANO, 1998; ROSA *et al*., 2012; PORTO *et al*., 2014a) e com relação a distribuição de DNA ribossômico (DNAr) (PORTO *et al*., 2014b).

Nas últimas décadas, o número de espécies descritas para a família Loricariidae tem crescido, especialmente na região do escudo cristalino brasileiro. A maioria dos gêneros possui mais de uma espécie válida distribuída nas bacias do rio Doce, rio Paraíba do Sul, Alto rio Paraná e rio São Francisco, e em alguns deles com ocorrência de espécies simpátricas (OLIVEIRA; OYAKAWA, 2019). Os gêneros mais especiosos desta subfamília são *Rineloricaria* Bleeker, 1862 (63 espécies), *Farlowella* Eigenmann & Eigenmann, 1889 (29 espécies), *Harttia* Steindachner, 1877 (27 espécies), *Loricariichthys* Bleeker, 1862 (18 espécies) e *Loricaria* Linnaeus, 1758 (17 espécies) (MARAJÓ *et al*., 2018; OLIVEIRA; OYAKAWA, 2019).

1.1.3 Gênero *Harttia*

O gênero *Harttia* constitui um grupo de peixes endêmicos da região Neotropical e amplamente distribuídos em vários rios e riachos da América do Sul, incluindo tributários do Orinoco, Escudo das Guianas, Amazônia e drenagens costeiras do Brasil

(OYAKAWA; FICHBERG; RAPP-PY DANIEL, 2018). Este gênero foi descrito por Steindachner em 1876 baseado no formato corporal achatado e alongado, pedúnculo caudal curto, ausência da quilha lateral do tronco e nadadeira adiposa, olhos situados na região dorso-lateral da cabeça, nadadeira caudal emarginada e grandes placas ósseas que circundam a papila anal (OYAKAWA, 1993).

Assim como os demais representantes de Loricariidae, esses peixes apresentam hábito de vida sedentário e tendem ocupar porções específicas dos corpos hídricos (PINNA, 1998). Algumas espécies habitam a calha principal do rio e regiões hídricas de grande porte, a exemplo de *H. longipinna* que ocorre no médio São Francisco. Por outro lado, a maioria das espécies de *Harttia* habita pequenos córregos e riachos. Outra característica interessante deste grupo de peixes é o fato de apresentarem desova parcial, principalmente naquelas espécies encontradas em riachos. Isso ocorre devido as condições ambientais deste tipo de ambiente, como temperatura e nível de água oscilarem constantemente. Com isso as espécies podem desovar em épocas propícias para a reprodução com maiores condições de sobrevivência.

O gênero *Harttia* é composto por 27 espécies válidas (**Tabela 1**) (OLIVEIRA; OYAKAWA, 2019) e esse número tende a aumentar visto que existem relatos de espécies não descritas para diversos tributários. Uma dessas espécies, até então denominada como *Harttia* sp. 3, tem ocorrência no rio do Peixe, município de Altamira no Pará, e está em processo de descrição taxonômica (SASSI *et al*., 2021). Além disso, nesse mesmo estudo, uma espécie anteriormente descrita apenas para a região das Guianas (*H. guianensis*), foi reportada em bacias hidrográficas da região brasileira, estendendo assim a distribuição geográfica desta espécie. Destas 27 espécies válidas, 22 delas ocorrem distribuídas dentro do território brasileiro e 13 apresentam dados de descrição citogenética clássica (RODRIGUES, 2010; BLANCO *et al*., 2017; SASSI *et al*., 2020).

Estudos de caracterização citogenética no gênero *Harttia* têm indicado uma considerável diversidade cariotípica e uma série de rearranjos visualmente detectáveis. Com relação ao número diploide, a variação encontrada é de 2n=52 cromossomos em indivíduos fêmea de *H. carvalhoi* do ribeirão Grande (Bacia do rio Paraíba do Sul) (CENTOFANTE; BERTOLLO; MOREIRA-FILHO, 2006) até 2n=62 cromossomos em *H. absaberi* do rio Passa Cinco (Bacia do alto rio Paraná) (RODRIGUES, 2010). Além disso, um caso de cromossomo B já foi descrito para *H. longipinna* com 2n=58 cromossomos e a presença de 0-2 micro cromossomos (BLANCO *et al*., 2012).

Tabela 1: Espécies do gênero *Harttia* e suas respectivas distribuições geográficas. Em negrito, espécies que possuem estudos citogenéticos.

Espécie	Ecorregião *	2n	Referência
Harttia fowleri (Pellegrin 1908)	Guiana oriental		
Harttia surinamensis Boeseman 1971	Guiana oriental		
Harttia fluminensis Covain, Fisch-Muller 2012	Guiana oriental		
Harttia tuna Covain, Fisch-Muller 2012	Guiana oriental		
Harttia merevari Provenzano, Machado-Allison, Chernoff, Willink & Petry 2005	Orinoco-Escudo das Guianas		
***Harttia guianensis* Rapp Py-Daniel & Oliveira 2001**	**Amazonas-Escudo das Guianas e Guiana oriental**	**2n=58♀♂**	**Sassi *et al*. (2021)**
Harttia depressa Rapp Py-Daniel & Oliveira 2001	Amazonas-Escudo das Guianas		
Harttia trombetensis Rapp Py-Daniel & Oliveira 2001	Amazonas-Escudo das Guianas		
Harttia uatumensis Rapp Py-Daniel & Oliveira 2001	Amazonas-Escudo das Guianas		
***Harttia dissidens* Rapp Py-Daniel & Oliveira 2001**	**Tapajós-Juruena**	**2n=54♀♂**	**Sassi *et al*. (2021)**
***Harttia rondoni* Oyakawa, Fichberg & Rapp Py-Daniel 2017**	**Alto Xingu**	**2n=54♀♂**	**Sassi *et al*. (2020)**
Harttia panara Oyakawa, Fichberg & Rapp Py-Daniel 2017	Alto Xingu		
***Harttia villasboas* Oyakawa, Fichberg & Rapp Py-Daniel 2017**	**Alto Xingu**	**2n=56♀/55♂**	**Sassi *et al*. (2020)**
***Harttia duriventris* Rapp Py-Daniel & Oliveira 2001**	**Tocantins-Araguaia**	**2n=56♀/55♂**	**Sassi *et al*. (2020)**
***Harttia punctata* Rapp Py-Daniel & Oliveira 2001**	**Tocantins-Araguaia**	**2n=58♀/57♂**	**Blanco *et al*. (2014)**
Harttia rhombocephala Miranda-Ribeiro 1939	Mata Atlântica		
Harttia garavelloi Oyakawa 1993	São Francisco		

Tabela 1: Espécies do gênero *Harttia* e suas respetivas distribuições geográficas. Em negrito, espécies que possuem estudos citogenéticos. (continuação)

Espécie	Ecorregião *	2n	Referência
Harttia leiopleura Oyakawa 1993	São Francisco		
***Harttia longipinna* Langeani, Oyakawa & Montoya-Burgos 2001**	**São Francisco**	**2n=58♀♂+0-2Bs**	**Blanco *et al*. (2012)**
Harttia novalimensis Oyakawa 1993	São Francisco		
***Harttia torrenticola* Oyakawa 1993**	**São Francisco**	**2n=56♀♂**	**Blanco *et al*. (2013)**
Harttia intermontana de Oliveira; Oyakawa 2019	Mata Atlântica		
***Harttia carvalhoi* Miranda-Ribeiro 1939**	**Paraíba do Sul**	**2n=52♀/53♂**	**Centofante; Bertollo; Moreira-Filho (2006)**
***Harttia loricariformis* Steindachner 1877**	**Paraíba do Sul, Mata Atlântica**	**2n=56♀♂**	**Kavalco *et al*. (2004)**
***Harttia absaberi* Oyakawa, Fichberg & Langeani 2013**	**Alto Paraná**	**2n=62♀♂**	**Rodrigues (2010)**
***Harttia gracilis* Oyakawa 1993**	**Alto Paraná**	**2n=58♀♂**	**Blanco *et al*. (2017)**
***Harttia kronei* Miranda-Ribeiro 1908**	**Alto Paraná, Ribeira de Iguape**	**2n=58♀♂**	**Alves; Oliveira; Foresti (2003)**

* Ecorregiões de acordo com Albert; Petry e Reis (2011).

Diversos rearranjos cromossômicos ocorridos no grupo culminaram em uma ampla variação da posição e do número de sítios de DNAr (BLANCO *et al*., 2017; SASSI *et al*., 2020 e 2021). Enquanto a maioria das espécies apresenta sítios do DNAr 5S e 18S em cromossomos diferentes, como em *H. kronei, H. gracilis, H. longipinna* e *H. torrenticola*, outras apresentam esses genes em sintenia, como em *H. loricariformis* e *H. carvalhoi* (sendo a última detentora de um sítio extra de DNAr 5S) (BLANCO *et al*., 2017). Além disso, esses genes também foram mapeados em cromossomos sexuais, como é o caso de *H. duriventris, H. punctata, H. rondoni* e *H. villasboas* (BLANCO *et al*., 2014; SASSI *et al*., 2020).

Em *Harttia*, os rearranjos robertsonianos além de atuarem na diversidade cariotípica geral de inúmeras espécies, estão frequentemente associados à diferenciação dos sistemas de cromossomos sexuais múltiplos (BLANCO *et al*., 2017). No que diz respeito aos sistemas de cromossomos sexuais, três diferentes tipos de sistemas sexuais com heterogametia masculina foram relatados no gênero: XX/XY_1Y_2 em *H. carvalhoi* (CENTOFANTE; BERTOLLO; MOREIRA-FILHO, 2006), $X_1X_1X_2X_2/X_1X_2Y$ em *H. punctata* (BLANCO *et al*., 2014), *H. villasboas* e *H. duriventris* (SASSI *et al*., 2020) além da ocorrência de um sistema proto neo-XY em *H. rondoni* (SASSI *et al*., 2020).

Até o momento, apenas *Harttia carvalhoi* foi caracterizada com a presença do sistema de cromossomos sexuais do tipo XX/XY_1Y_2, onde fêmeas possuem um grande par de cromossomos metacêntricos correspondente ao cromossomo X, e machos possuem um cromossomo X e outros dois cromossomos acrocêntricos, correspondentes aos cromossomos Y_1 e Y_2. Além desta, *H. torrenticola* também compartilha o maior par de cromossomos metacêntricos, embora nenhum sistema de cromossomos sexuais tenha sido detectado. Acredita-se que esse maior par de cromossomos metacêntricos tenha se originado através de eventos de fusão robertsoniana a partir de um ancestral com 2n=58 cromossomos e sem a presença de cromossomos sexuais diferenciados. Um posterior evento de fissão poderia ter levado a uma quebra do cromossomo X e originado os cromossomos Y_1 e Y_2 (BLANCO *et al*., 2013).

Por sua vez, as espécies detentoras do sistema de cromossomos sexuais $X_1X_1X_2X_2/X_1X_2Y$ aparentam ter seguido uma trajetória evolutiva diferente do sistema mencionado anteriormente. Nesse sistema, as fêmeas possuem dois pares de cromossomos X_1 e X_2 e que correspondem aos dois maiores pares de cromossomos acrocêntricos em *H. duriventris, H. punctata* e *H. villasboas.* Já os machos possuem uma cópia de cada um desses cromossomos, além de um cromossomo Y com morfologia

metacêntrica ou submetacêntrica (BLANCO *et al*., 2014; SASSI *et al*., 2020). Nesse sistema sítios de DNAr estão localizados nos cromossomos sexuais. No caso de *H. punctata*, o DNAr 5S ocorre na região terminal do braço longo do cromossomo 25 (X_1) e o DNAr 18S na região intersticial do braço longo do cromossomo 26 (X_2). O cromossomo Y, oriundo de um evento de translocação entre esses dois cromossomos, apresenta o9 DNAr 5S em região terminal (BLANCO *et al*., 2013). Nas espécies *H. duriventris* e *H. villasboas*, apenas o DNAr 18S está envolvido nos cromossomos responsáveis pela organização do sistema, enquanto o DNAr 5S está localizado em um par de autossomos. Adicionalmente a esses três casos, *H. rondoni* apresenta um sistema de cromossomos sexuais do tipo XX/XY com a ocorrência de sítio DNAr 18S (SASSI *et al*., 2020). Acredita-se que esses dois sistemas distintos (X_1X_2Y e XY) tenham origem a partir de um ancestral com 2n=58 cromossomos e sem cromossomos sexuais diferenciados (BLANCO *et al*., 2017; SASSI *et al*., 2020).

Características no cariótipo parecem estar correlacionadas com o estilo de vida e os hábitos ecológicos, uma vez que espécies migratórias e dispersivas parecem ter cariótipos mais estáveis quando comparados com aquelas com baixa vagilidade e organizadas em pequenas populações locais (BERTOLLO; MOREIRA-FILHO; GALETTI JR, 1986; BLANCO *et al*., 2011; OLIVEIRA *et al*., 2015).

Com relação aos dados filogenéticos do grupo, alguns estudos já foram realizados envolvendo espécies de *Harttia* e outras espécies pertencentes à Loricariidae (COVAIN *et al*., 2016; LONDOÑO-BURBANO; REIS, 2021). Os dados demonstram três ramos de diversificação das espécies deste gênero: i) espécies que se encontram distribuídas na região da Guiana e escudo das Guianas, ii) espécies distribuídas principalmente na região da bacia Amazônica, e iii) espécies distribuídas na região sul e sudeste do Brasil próximas ao escudo cristalino.

1.2 Regiões de instabilidade e quebras cromossômicas

Rearranjos cromossômicos são importantes fatores que têm contribuído para a diversificação genética e a sua ocorrência tem sido associada às quebras em locais de DNA altamente repetitivo (CAZAUX *et al*., 2011; BRUSCHI *et al*., 2014). Essa predisposição de determinada região do genoma ser instável é considerada como *hotspot* para quebras da dupla fita do DNA (do inglês, *double strand break* - DSB) e rearranjos cromossômicos (PEVZNER; TESLER, 2003). Estudos têm demonstrado que as regiões

das DSB são ricas em diferentes tipos de sequências repetitivas como, por exemplo, SINEs (*short interspaced nuclear elements*), LINEs (*long interspaced nuclear elements)* e LTRs (*long terminal repeats*) preferencialmente localizadas ao lado ou inclusas em segmentos duplicados (SD) dos genomas (CARBONE *et al*., 2009).

Segmentos duplicados são facilmente reconhecidos como arranjos *in tandem* das famílias gênicas, expansões microssatélites ou mesmo por unidades interespaçadas que flanqueiam um rearranjo cromossômico (FARRÉ *et al*., 2011). A associação aparente entre regiões repetitivas e sítios em que ocorreram divergências estruturais é confirmada pela determinação de pontos quentes de rearranjos cromossômicos enriquecidos por SD. Contudo, por conter sítios homólogos, os segmentos repetitivos podem apenas ter facilitado o rearranjo cromossômico, enquanto o ponto de quebra cromossômica pode estar localizado nas sequências que flanqueiam as regiões duplicadas (EICHLER; SANKOFF, 2003; KEHRER-SAWATZKI; COOPER, 2008).

Em alguns organismos foi determinado que as quebras cromossômicas ocorrem nas proximidades dos DNAs ribossômicos com localização subterminal ou proximal de cromossomos subtelocêntrico/ acrocêntricos. Em inúmeros casos, estas regiões de DNAr estão envolvidas em fusão Robertsoniana (Rb) via união não homóloga (do inglês *Non-Homologous End Joining* - NHEJ) durante o reparo do DNA (RICHARDS, 2001; HUANG *et al*., 2008; CAZAUX *et al*., 2011; BARROS *et al*., 2017; GLUGOSKI *et al*., 2018). Em Loricariidae, a localização cromossômica de pseudogenes DNAr 5S em regiões colocalizadas a sítios teloméricos intersticiais demonstraram o envolvimento de DNAr em rearranjos cromossômicos (BARROS *et al*., 2017; GLUGOSKI *et al*., 2018).

Estudos de organização genômica em eucariotos mostraram trocas de sequências entre cromossomos não homólogos durante a evolução cromossômica, promovendo evolução em concerto (LIAO, 1999). Assim, trocas não recíprocas de sequências e as duplicações entre regiões subteloméricas são frequentes nos eucariotos, especialmente quando expandem famílias gênicas próximas ao telômero (KELLIS *et al*., 2003). Os clusters de DNAr apresentam características comuns para regiões de quebras cromossômicas: são repetidos *in tandem*; geralmente são localizados em regiões pericentroméricas ou subteloméricas, podem se transpor de um cromossomo para outro; estão sujeitos à altas taxas de recombinação intra e inter-cromossômica e podem estar invadidos por elementos transponíveis (CAZAUX *et al*., 2011).

Telômeros são sequências de DNA repetitivo *in tandem* localizados na região terminal dos cromossomos que tem como função manter a sua estabilidade e integridade

(ZAKIAN, 1995). Entretanto, algumas vezes, podem ser encontradas em outras regiões do cromossomo, onde são chamados de sítios teloméricos intersticiais (do inglês, *interstitial telomeric sites* - ITS) (LEE; SASI; LIN, 1993; CAMATS *et al*, 2006). Essas sequências já foram localizadas em: roedores (VENTURA *et al*., 2006; MENCUCCI et al., 2012), anuros (TEIXEIRA *et al*., 2016; BRUSCHI *et al*., 2014; SCHMID; STEINLEN, 2016), répteis (ROVATSOS *et al*., 2015), primatas (DUMAS; CUTTAIA; SINEO, 2016), insetos (SCALI *et al*., 2016) e peixes (ROSA *et al*., 2012; ERRERO-PORTO *et al*., 2014; BARROS *et al*., 2017; BLANCO *et al*., 2017; PRIMO et al., 2017; GLUGOSKI *et al*., 2018).

Sítios teloméricos intersticiais surgem como resultado de rearranjos cromossômicos na evolução dos genomas e que podem causar instabilidade cromossômica (KILBURN *et al*., 2001; PERRY; SLATER; CHOO, 2004; BOLZÁN, 2012; AKSENOVA *et al*., 2013). Os ITS são pontos quentes de recombinação devido as alças teloméricas (*T-loops*) terem a possibilidade de interagir com sequências TTAGGG em regiões intersticiais via a proteína shelterina TRF2 (WOOD *et al*., 2014) formando uma série de ciclos de quebra-fusão-ponte (do inglês *breakage-fusion-bridge cycles*) (BOLZÁN, 2012; SLIJEPCEVIC, 2016). Nessa via, genomas detentores de ITS não silenciados epigeneticamente são conhecidos por formar outros rearranjos cromossômicos como inversões, translocações, transposições e fissões (DAY; LIMOLI; MORGAN, 1998; PERRY; SLATER; CHOO, 2004; BOLZÁN, 2012; AKSENOVA *et al*., 2013; WOOD *et al*., 2014; SLIJEPCEVIC, 2016).

Em um estudo realizado por Barros e colaboradores (2017), analisando uma espécie de peixe de *Ancistrus*, foram determinadas duas sequências de DNAr 5S, as quais foram denominadas 5S.1 e 5S.2. Essas duas sequências de DNAr 5S estavam colocalizadas em vários sítios subterminais de cromossomos subtelocêntricos/ acrocêntricos. Além disso, foi detectado um indicativo de fusão Rb, com a presença de um ITS no par metacêntrico 1, colocalizado à sonda DNAr 5S.2. Neste estudo, o DNAr 5S.2 foi descrito como um pseudogene por apresentar inúmeras mutações e ausência de regiões necessárias para a transcrição (TATA-like e sequência GC box e considerado um *hotspot* para DSB relacionado com parte das fusões robertsonianas em Loricariidae.

Pevzner e Tesler (2003) propuseram que a característica chave do modelo não randômico de quebras é o re-uso evolutivo das sequências que promovem as quebras cromossômicas em diferentes linhagens. Para testar a hipótese do re-uso evolutivo de sequências DNAr 5S em quebras cromossômicas na família Loricariidae, Glugoski e

colaboradores (2018) analisaram fusões Rb, em segmentos duplicados de DNAr 5S colocalizados à ITS, em *Rineloricaria latirostris*. Neste estudo também foram encontradas duas sequências do DNAr 5S, as quais foram denominadas de DNAr 5S e DNAr 5S degenerado, sendo que o último possuía um segmento do transponível (TE) *hAT* intercalar. Os autores propuseram que a invasão do TE *hAT* no DNAr 5S gerou por crossing-over desigual a região do DNAr 5S degenerado, a qual atuou como *hotspot* para quebras cromossômicas envolvidas nos sistemas de reparo por recombinação e NHEJ em, *R. latirostris.*

Evidências de localização *in situ* de sequências DNAr e TTAGGG*n* indicam um possível envolvimento de DNAr em quebras cromossômicas em vários grupos de Loricariidae (ROSA *et al*., 2012; FAVARATO *et al*., 2016; PRIMO *et al*., 2017). Esta via de investigação de sequências *hotspots* para DSBs em Loricariidae depende da avaliação da dispersão dos DNAr nos cariótipos e, se carreiam sequências capazes de gerar estagnação da duplicação do DNA e quebras de duplas fitas.

1.3 Cromossomos sexuais

Os vertebrados exibem duas grandes categorias de sistemas de determinação sexual. A primeira delas, onde o sexo é determinado devido a influências ambientais, do inglês *environmental sex determination* (ESD), onde fatores externos como temperatura, por exemplo, irão determinar o sexo do indivíduo. Além disso, a determinação sexual também pode ser geneticamente controlada por um ou mais loci localizados em cromossomos sexuais ou cromossomos autossomos, os quais são responsáveis pelo desenvolvimento das gônadas, conhecida como determinação sexual genotípica do inglês *genotipic sex determination* (GSD) (BULL, 1981; CHARLESWORTH, 2002). O grupo dos peixes exibe o mais amplo espectro de sistema de determinação sexual dos vertebrados, variando de diferentes tipos de determinação ambiental até uma variedade de sistemas de cromossomos sexuais (VOLFF *et al*., 2007; MARTÍNEZ *et al*., 2014; SHEN; WANG, 2019).

Cromossomos sexuais representam uma das partes dinâmicas do genoma (FARRÉ; RUIZ-HERRERA, 2020). De acordo com o modelo mais aceito, heterocromossomos ligados ao sexo evoluíram de um par de cromossomos originalmente homólogos, devido ao surgimento de um lócus sexo-específico em um dos pares. Os passos seguintes envolvem principalmente a supressão da recombinação da região contendo esse lócus e a

diferenciação via acúmulo de sequências repetitivas, elementos transponíveis e pseudogenes (CHARLESWORTH; CHARLESWORTH; MARAIS, 2005). Vários mecanismos agem de uma maneira bastante dinâmica para diminuir a recombinação entre os cromossomos sexuais, dentre eles: inversões, acúmulo e deleção de heterocromatina, fusão entre cromossomos sexuais e autossomos, entre outros (SCHARTL; SCHMID; NANDA, 2015). O acúmulo de genes sexualmente antagonistas (benéficos para apenas um dos sexos), ligados a genes sexo-determinantes, também podem, por sua vez, favorecer a supressão da recombinação (BACHTROG, 2006).

Existe uma alta diversidade de cromossomos sexuais entre os mais diversos táxons (FARRÉ; RUIZ-HERRERA, 2020). Assim como outros organismos, cromossomos sexuais em peixes possuem uma variedade de formas cariotípicas, desde mudanças sutis até grandes rearranjos cromossômicos (DEVLIN; NAGAHAMA, 2002).

Os sistemas de cromossomos sexuais apresentam heterogametia masculina (fêmeas XX e machos XY) ou feminina (fêmeas ZW e machos ZZ), e sistemas múltiplos derivados de sistemas simples (DEVLIN; NAGAHAMA, 2002). Os sistemas mais bem estudados entre os vertebrados são o sistema XY, em mamíferos, e o sistema ZW, em cobras e aves. Esses sistemas apresentam algumas características gerais semelhantes, com os cromossomos X e Z relativamente grandes e portadores de vários genes, enquanto os cromossomos Y e W são geralmente menores, com grande quantidade de heterocromatina e poucos genes ativos (EZAZ *et al*., 2006). Entretanto, algumas espécies apresentam determinação genética do sexo sem a presença de cromossomos sexuais diferenciados, o que se verifica quando a recombinação genética ainda não foi suprimida e o par sexual ainda não divergiu morfologicamente entre si (MANK; AVISE, 2009).

Sistemas simples como XX/XY e ZZ/ZW foram identificados em peixes, assim como sistemas múltiplos do tipo $X_1X_1X_2X_2/X_1X_2Y$; $X_1X_1X_2X_2/X_1Y_1X_2Y_2$; XX/XY_1Y_2; ZZ/ZW_1W_2 e $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ (MANK; AVISE, 2009). Também foram relatados casos em que ocorreu a perda do cromossomo Y ou W, provavelmente em sistemas onde o sexo é determinado ultimamente por gene e/ou genes nos cromossomos X ou Y, conhecidos como XX/X0 e ZZ/Z0. Entre as espécies com heterogametia masculina que possuem sistemas XY, esses cromossomos são citogeneticamente distinguíveis de algum modo, mas o número diploide entre os sexos é o mesmo. Em sistemas sexuais múltiplos do tipo $X_1X_1X_2X_2/X_1X_2Y$, o cromossomo Y geralmente se origina através de eventos de translocação ou fusão robertsoniana entre os cromossomos X_1 e X_2, levando a diferença

de 2n entre machos e fêmeas. Características similares ocorrem em espécies com heterogametia feminina (MANK; AVISE, 2009; DEVLIN; NAGAHAMA, 2002).

Dentre os peixes, acreditava-se que cerca de 5% das espécies analisadas possuíam cromossomos sexuais heteromórficos, incluindo cerca de 47 casos de cromossomos sexuais múltiplos (ARAI, 2011; KITANO; PEICHEL, 2012; PENNELL *et al*., 2015). Entretanto, recentemente uma revisão sobre sistemas de cromossomos sexuais múltiplos em peixes teleósteos, demonstrou 75 casos (SEMBER *et al*., 2021), e novos estudos apontam para um crescente aumento deste número.

Os sistemas de cromossomos sexuais múltiplos resultam de rearranjos entre cromossomos sexuais (XY ou ZW) e autossomos ou de fissões no par sexual ancestral, sem o envolvimento de novo material autossômico (KITANO; PEICHEL, 2012; SEMBER *et al*., 2021). Tais sistemas podem, ser facilmente detectados, pois geralmente acarretam diferentes números cromossômicos entre os sexos, assim como a presença de um cromossomo notavelmente distinto em tamanho e/ou morfologia (WHITE, 1973). Entretanto, os neo-cromossomos sexuais resultantes apresentam pouca diferenciação morfológica, sendo tipicamente eucromáticos e, aparentemente, sem grandes acúmulos de sequências repetitivas (revisado em CIOFFI *et al*., 2012).

Estudos envolvendo a origem de sistemas de cromossomos sexuais têm sido realizados nos mais diferentes gêneros de peixes utilizando técnicas citogenéticas baseadas em hibridização *in situ* fluorescente, incluindo pintura cromossômica total (WCP) e hibridização genômica comparativa (CGH). Com relação aos dados de pintura cromossômica, o uso de sondas isoladas a partir de cromossomos sexuais e o mapeamento destas sondas em espécies do mesmo gênero ou gêneros próximos tem auxiliado na identificação dos cromossomos envolvidos na origem dos sistemas de cromossomos sexuais incluindo sistemas simples e múltiplos (CIOFFI *et* al., 2011; BLANCO *et* al., 2013, 2014; YANO *et al*., 2017, 2021; de OLIVEIRA *et* al., 2018; MORAES *et al*., 2019, 2021). Enquanto o emprego de técnicas utilizando o DNA genômico como sonda em experimentos de hibridização genômica comparativa, tem revelado sequências acumuladas diferencialmente entre sexos (YANO *et al*., 2017; SEMBER *et al*., 2018), o uso de metodologias de citogenética molecular revela e elucida as homologias cromossômicas e a origem dos cromossomos sexuais em espécies de peixes da ictiofauna neotropical.

2. JUSTIFICATIVA E OBJETIVOS

Conforme destacado anteriormente, a diversidade na macroestrutura cromossômica de *Harttia* é representada por diferentes números diploides, sistemas de cromossomos sexuais, cromossomos supranumerários, distintas fórmulas cariotípicas, diferentes números fundamentais, variação na distribuição da heterocromatina, além do número e posição dos genes ribossômicos 18S e 5S. A diversidade cromossômica associada ao crescente número de espécies descritas torna esse gênero um grupo interessante para estudos sobre a diversidade das espécies, bem como para estudos genéticos e evolutivos.

Além disso, devido a indicativos de marcadores citogenéticos que já demonstraram a ocorrência de fusões robertsonianas em prováveis cromossomos homeólogos e redução do 2n em espécies de *Harttia*, este estudo teve por objetivo geral analisar os rearranjos e os cromossomos envolvidos na diferenciação cariotípica de espécies do gênero *Harttia* bem como o acúmulo de sequências repetitivas próximas a essas regiões. Assim, os objetivos específicos deste trabalho foram:

- Fornecer um conjunto de abordagens citogenéticas convencionais e moleculares (coloração por Giemsa, Bandamento C, mapeamento de DNAs repetitivos e ensaios de Hibridização Genômica Comparativa) para avançar no conhecimento dos processos que moldaram a evolução cromossômica no gênero;
- Identificar blocos cromossômicos homeólogos e prováveis regiões de pontos de quebras evolutivos responsáveis pela diferenciação cariotípica do gênero;
- Investigar os processos de diferenciação que levaram ao estabelecimento do raro sistema de cromossomos sexuais múltiplo XX/XY_1Y_2 e rastrear sua história evolutiva entre outras espécies de *Harttia*;
- Avaliar o acúmulo de sequências microssatélites $(A)_{30}$, $(CA)_{15}$, $(GA)_{15}$ e $(CGG)_{10}$ na composição da heterocromatina de regiões de pontos de quebras evolutivos e em regiões cromossômicas altamente rearranjadas.

3. MATERIAL E MÉTODOS

3.1 Amostragem do material biológico

Para a realização deste trabalho, foram amostradas dez espécies de peixes pertencentes ao gênero *Harttia* em diversas bacias hidrográficas das regiões sul, sudeste e centro-oeste (**Figura 1, Tabela 2**). Foram coletados exemplares machos e fêmeas das espécies: *H. carvalhoi*, *H. gracilis*, *H. intermontana*, *H. kronei*, *H. longipinna*, *H. loricariformis*, *H. punctata*, *H. torrenticola*, *Harttia* sp. 1 e *Harttia* sp. 2 (**Figuras 2, 3, 4**). Os exemplares foram coletados com autorização do Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO), Sistema de Autorização e Informação em Biodiversidade (SISBIO) com as licenças n˚ 10538-3 e 15117-1 e o Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN - A96FF09). Os animais foram coletados com o uso de peneiras e tarrafas e transportados vivos para o Laboratório de Citogenética de Peixes (Universidade Federal de São Carlos, São Carlos-SP) ou para o Laboratório de Biologia Cromossômica: Estrutura e Função (Universidade Estadual de Ponta Grossa, Ponta Grossa-PR) para a obtenção de suspensões celulares e coleta de tecido. Os procedimentos realizados neste trabalho estão de acordo com os procedimentos éticos aprovados pelo Comitê de Experimentação animal da Universidade Federal de São Carlos (Número do processo CEUA 1853260315).

Os peixes foram numerados seguindo o registro de cada laboratório, fixados em formol 10 % por 48 horas e armazenados em álcool 70 %. Posteriormente foram enviados para tombo no museu de Zoologia da Universidade de São Paulo e propriamente identificados pelo Dr. Oswaldo Oyakawa, especialista em taxonomia e Sistemática de Peixes. As espécies nominadas como *Harttia* sp. 1 e *Harttia* sp. 2 correspondem a duas novas espécies não descritas na literatura e que estão em processo de descrição taxonômica.

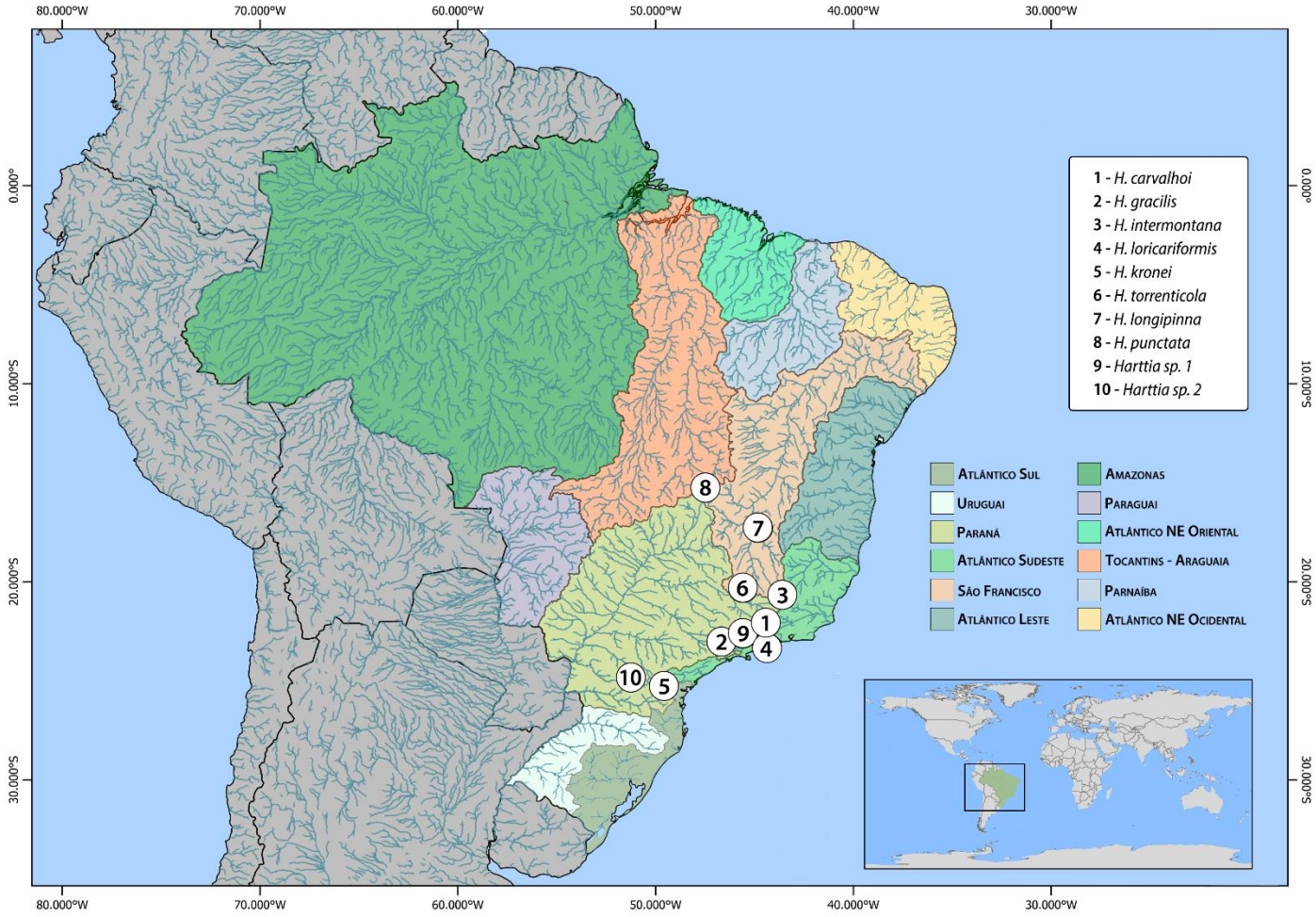

Figura 1: Mapa representando os pontos de coleta das espécies de peixes do gênero *Harttia* analisadas no presente estudo e suas respectivas distribuições nas bacias hidrográficas.

Tabela 2: Espécies de *Harttia* utilizadas no presente estudo.

Espécie	Rio	Município/Estado	GPS	Número do Voucher	Espécimes coletados
H. carvalhoi	Ribeirão Grande	Pindamonhangaba-SP	22º47’8” S; 45º27’19” O	MZUSP 109782	17♀, 12♂
H. gracilis	Córrego Machadinho	Santo Antônio do Pinhal-SP	22º48’31” S; 45º41’21” O	MZUSP 111384	18♀,15♂
H. intermontana	Rio Piranga	Carandaí-MG	20°59’34.0” S; 43°43’30.0” O	MZUSP 126520	20♀/ 13♂
H. kronei	Rio Açungui	Campo Largo-PR	25º22’44” S; 49º39’08” O	MZUSP 109783	10♀, 5♂
H. longipinna	Rio São Francisco	Pirapora-MG	17º21’22,8” S, 44º51’0,2” O	MZUSP 106767	13♀,16♂
H. loricariformis	Rio Paraitinga	Cunha-SP	22º52’22” S, 44º51’0.2” O	MZUSP 111386	7♀, 3♂
H. punctata	Ribeirão Bandeirinha	Formosa-GO	15º19’25” S, 47º25’26” O	MZUSP 111385	18♀,25♂
H. torrenticola	Ribeirão das Araras	Piumhi-MG	20º16’15” S; 45º55’39” O	MZUSP 109704	8♀, 6♂
Harttia sp. 1	Ribeirão dos Macacos	Silveiras-SP	22°40’43.0” S, 44°51’25.0” O	-	10♀/ 7♂
Harttia sp. 2	Rio Barra Grande	Prudentópolis-PR	24°58’40.72” S 51°7’34.25” O	MZUSP 126000	17♀/ 11♂

SP – São Paulo, MG – Minas Gerais, PR – Paraná, GO – Goiás

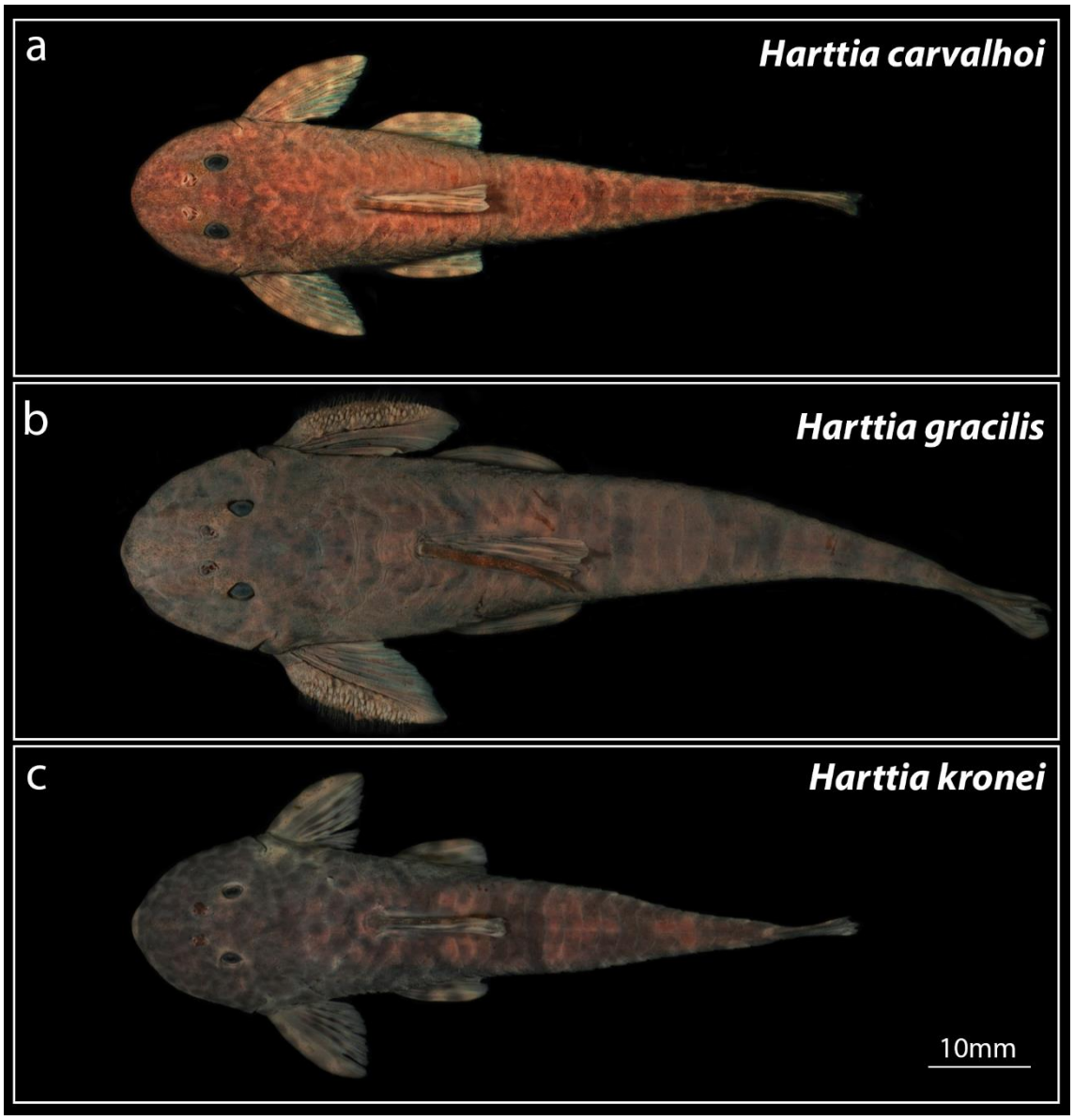

Figura 2: Vista dorsal dos exemplares de *Harttia* analisados no presente estudo: (a) *H. carvalhoi*, (b) *H. gracilis* e (c) *H. kronei.* Fonte: Blanco (2012) com modificações.

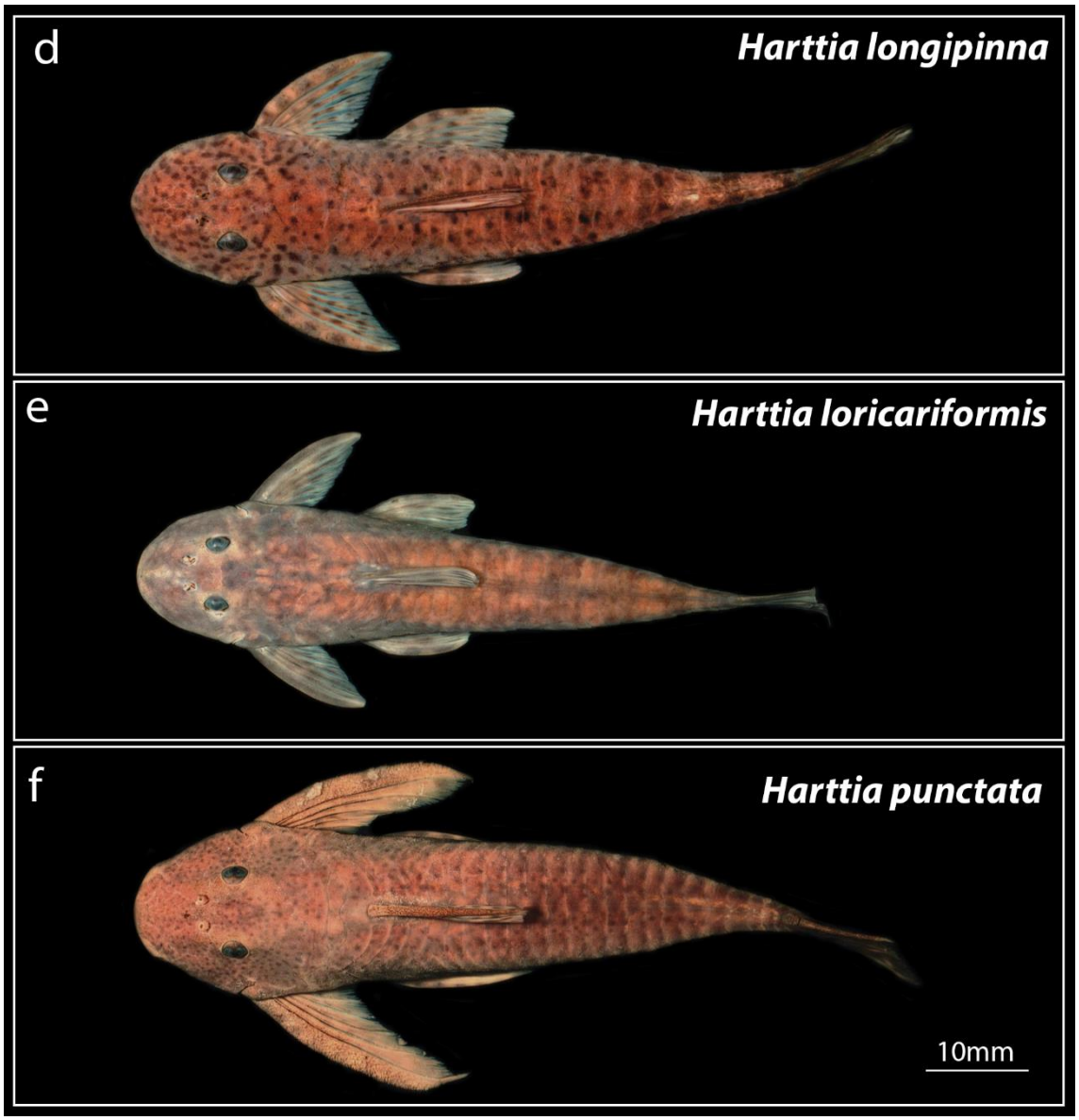

Figura 3: Vista dorsal dos exemplares de *Harttia* analisados no presente estudo: (d) *H. longipinna*, (e) *H. loricariformis* e (f) *H. punctata*. Fonte: Blanco (2012) com modificações.

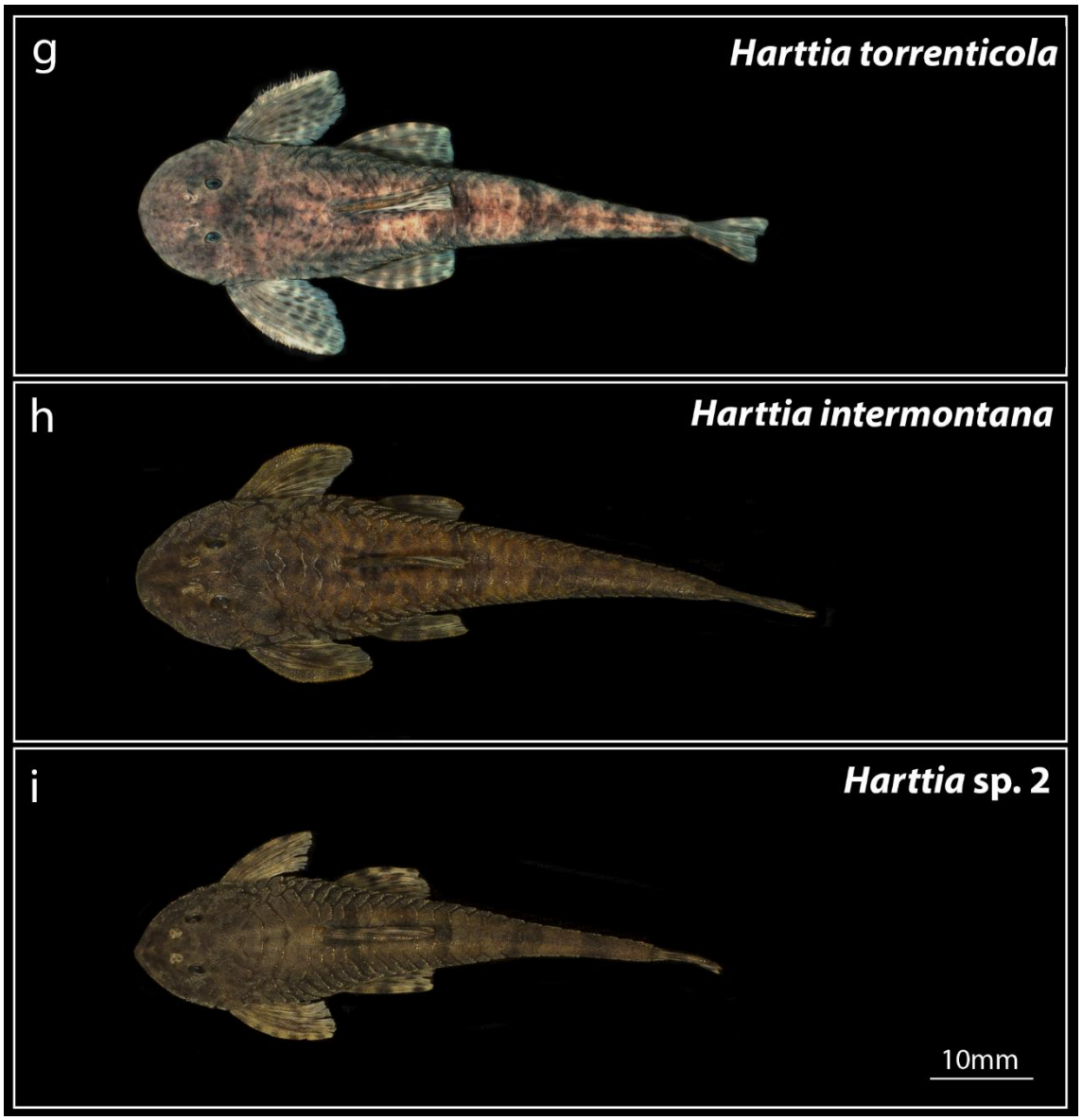

Figura 4: Vista dorsal dos exemplares de *Harttia* analisados no presente estudo: (g) *H. torrenticola* (h) *H. intermontana* e (i) *Harttia* sp. 2. Fonte: Blanco (2012) com modificações.

3.2 Obtenção de cromossomos mitóticos

Foi adotada a metodologia descrita por Bertollo; Cioffi e Moreira-Filho (2015) para preparações cromossômicas *in vivo*. Os animais foram previamente tratados com uma solução de colchicina 0,025 % na proporção 1 ml para cada 100 gramas de peso do animal na região intra-abdominal e mantidos em aquário aerado durante 40 minutos. A seguir foram anestesiados com solução de benzocaína e sacrificados. Fragmentos do rim foram retirados e transferidos para uma cubeta contendo 10 ml de solução hipotônica de Cloreto de Potássio (KCl) a 0,075 M e com auxílio de uma seringa hipodérmica desprovida de agulha foram dissociados com leves movimentos até se obter uma suspensão celular homogênea. A suspensão foi mantida em estufa à 37 ^{o}C durante 20 minutos e posteriormente pré-fixada com dez gotas de fixador na proporção 3:1 de álcool metílico e ácido acético por 30 minutos em temperatura ambiente. Em seguida, a solução foi transferida para um tubo de centrífuga com auxílio de uma pipeta Pasteur e centrifugada a 1000 rpm por 10 minutos. O sobrenadante foi descartado e o conteúdo celular foi ressuspendido em 10 ml de fixador e centrifugado novamente por mais duas vezes. Na última centrifugação, o sobrenadante foi descartado e cerca de 1,5 ml de fixador foi adicionado. A solução final foi transferida para microtubos os quais foram armazenados à -20 ^{o}C.

3.2.1 Preparação das lâminas

Entre 20-40 µl da suspensão celular foram pingados sobre uma lâmina limpa e aquecida em torno de 55 ^{o}C. As lâminas foram secas ao ar e posteriormente coradas com uma solução de Giemsa 5 % diluída em tampão fosfato (pH 6,8) durante 10 minutos. O excesso de corante foi retirado com água destilada. Após a análise em microscópio de campo claro com a análise e marcação em lâmina branca das melhores metáfases, o excesso de óleo de imersão foi retirado com álcool 70 % e descoradas em solução de fixador na proporção 3:1 de álcool metílico e ácido acético por 20 minutos em temperatura ambiente.

3.3 Detecção da heterocromatina constitutiva

Para a detecção da heterocromatina C-positiva, foi utilizado o protocolo descrito por Sumner (1972). Lâminas contendo as preparações cromossômicas foram tratadas com

uma solução de ácido clorídrico (0,2 N) à 37 ^{o}C por 10 minutos, lavadas com água destilada e secadas ao ar. A seguir, a lâmina foi incubada em solução recém preparada e filtrada de Hidróxido de Bário $Ba(OH)_2$ a 5 % à 42 ^{o}C por 1 minuto e 45 segundos e lavada em solução de HCl 0,2 N e em água destilada. Após secarem, as lâminas foram incubadas em solução salina (2x SSC) durante 45 minutos em estufa à 60 ^{o}C e após esse período lavadas com água destilada e secas ao ar. Os cromossomos foram corados com 20 µl da solução antifading e iodeto de propídeo (50 mg/ml) de acordo com Lui *et al*. (2012) e analisadas em microscópio de epifluorescência.

3.4 Amplificação e marcação de sondas

Porções de fígado de *H. intermontana* foram utilizadas para extração do DNA genômico total pelo método fenol-clorofórmio (SAMBROOK; RUSSEL, 2001). As amostras foram verificadas quanto a integridade em gel de agarose 1 % e posteriormente quantificadas no equipamento espectrofotômetro NanoDrop Lite (Thermo Scientific). Amostras de DNA diluídas para 50 ng/µL foram usadas de molde em reações de PCR (*Polimerase Chain Reaction)* para amplificação de segmentos do DNAr 5S usando os primers: 5Sa (5'- TACGCCCGATCTCGTCCGATC -3') and 5Sb (5'- CAGGCTGGTATGGCCGTAAGC -3') de acordo com Pendás *et al*. (1994).

Segmentos de DNAr 18S foram obtidos a partir da clonagem de vetores plasmidiais e propagação em células competentes DH5α (Invitrogen, San Diego, CA, EUA). A sonda utilizada corresponde a um segmento de 1400 pares de bases (pb) do gene RNAr 18S, obtida por PCR com a utilização dos primers 18Sf (5'- CCGAGGACCTCACTAAACCA -3') e 18Sr (5'- CC-GCTTTGGTGACTCTTGAT -3') do DNA nuclear de *Hoplias malabaricus*, de acordo com Cioffi *et al*. (2009). Após a conferência do padrão de bandas em gel de agarose 1 %, o produto da amplificação foi quantificado e utilizado em reações de marcação em Nick Translation (Jena Bioscience) com Atto550-dUTP para o DNAr 5S e AF488-dUTP para o DNAr 18S conforme as recomendações do fabricante.

Os seguintes oligonucleotídeos foram utilizados como sondas para sequências microssatélites: $d(CA)_{15}$, $d(GA)_{15}$, $d(CGG)_{10}$ e $d(A)_{30}$. Essas sequências foram marcadas diretamente com Cy3 na extremidade 5' durante a síntese, por VBC-Biotech (Viena, Áustria), segundo Kubat *et al*. (2008), com modificações.

3.5 Hibridização genômica comparativa: obtenção das sondas e marcação

Experimentos de Hibridização Genômica Comparativa (CGH) foram realizados com foco em comparações intraespecíficas e interespecíficas utilizando o DNA genômico total extraído a partir de fragmentos do fígado. Com relação aos experimentos de comparação intraespecíficas, genomas de indivíduos machos de *H. intermontana* e *Harttia* sp. 1 foram marcados por Nick Translation (Jena Bioscience) com Atto550-dUTP enquanto fêmeas foram marcadas com AF488-dUTP. Nos experimentos de comparação interespecífica com ênfase no sistema de cromossomos sexuais XY_1Y_2, o genoma de indivíduos machos foi marcado por Nick Translation (Jena Bioscience) com Atto425-dUTP para *H. carvalhoi*, Atto550-dUTP para *H. intermontana* e Atto488-dUTP para *Harttia* sp. 1.

3.6 Microdissecção cromossômica total

Os cromossomos X e 9, de *H. carvalhoi*, X, de *H. intermontana*, cromossomo 1, de *H. torrenticola* e cromossomos X_1 e X_2 de *H. punctata* nomeados respectivamente como HCA-X, HCA-9, HIN-X e HTO-1, HPU-X1 e HPU-X2, foram microdissectados e utilizados como sondas em análises de pintura cromossômica total (WCP). A escolha destes cromossomos deve-se inicialmente à fácil identificação e da suposição do envolvimento em rearranjos cromossômicos: os cromossomos X, de *H. carvalhoi*, X, de *H. intermontana*, e cromossomo 1, de *H. torrenticola*, correspondem ao maior cromossomo metacêntrico do cariótipo, o cromossomo 9 corresponde ao maior par de cromossomos submetacêntricos de *H. carvalhoi* e que os cromossomos X_1 e X_2 são os dois maiores pares de cromossomos acrocêntricos do cariótipo de *H. punctata* diferenciados entre si devido a presença de uma constrição secundária correspondente a região organizadora de nucléolo. Deste modo, 15 cópias de cada cromossomo foram microdissectadas a partir de preparações de indivíduos fêmeas, seguindo a metodologia descrita por Yang *et al*. (2009), com pequenas modificações. A obtenção das sondas foi realizada no laboratório Molekulare Zytogenetik (Universitätsklinikum Jena – Alemanha) chefiado pelo Dr. Thomas Liehr.

3.6.1 Preparação das agulhas das micropipetas

As microagulhas para a raspagem cromossômica foram preparadas em um *puller* modelo PB-7 (Narishige, Japão), utilizando bastões de vidro de 2 mm (Schott, Mainz, Alemanha), sendo expostas à luz ultravioleta por 30 minutos antes da sua utilização. As micropipetas também foram preparadas no mesmo aparelho, utilizando pipetas Pasteur de 250 mm (Assistent, Sondheim, Alemanha), as quais foram siliconizadas utilizando dimetildiclorosilano 1 % em tetracloreto de carbono e incubadas a 100 ^{o}C por 30 minutos.

3.6.2 Preparação das lamínulas e processo de microdissecção

Três a quatro gotas das suspensões cromossômicas de cada espécie foram adicionadas sobre diferentes regiões da lamínula previamente tratada em solução 10 x SDS e coradas com Giemsa 5% por 3 minutos. Posteriormente, a microdissecção dos cromossomos foi realizada utilizado um microscópio invertido Axiovert 135 (Zeiss, Jena, Alemanha), com a agulha estéril acoplada a um micromanipulador mecânico (Zeiss, Jena, Alemanha). A agulha foi cuidadosamente deslocada até uma posição logo acima do cromossomo alvo, possibilitando que fragmentos fossem progressivamente coletados até a microdissecção completa. Os fragmentos cromossômicos foram transferidos para uma micropipeta contendo uma solução coletora (10 mM de Tris-HCl pH 7,5; 10 mM de NaCl; SDS 0,1 %; 1 mM EDTA pH 7,5-8,0; 1 % Triton X-100; 1.44 mg/ml de proteinase K e 30 % de glicerol) e inserida em uma câmara úmida a 60 ^{o}C por 1 hora.

3.6.3 Geração de sondas cromossômicas por DOP-PCR

A ponta das micropipetas foi quebrada em um tubo tipo *Eppendorf* contendo 100 mM de dNTPs, 5mM de primer DOP GMW (5 '-CCGACTCGAGNNNNNNATGTGG-3') e tampão de diluição Sequenase (24 mM Tris HCl pH 7,5, 12 mM $MgCl_2$ e 30 mM NaCl). O processo de amplificação foi realizado em termociclador (Axygen Therm-1000) de acordo com Telenius *et al.* (1992). Os oito primeiros ciclos de amplificação foram realizados utilizando a DNA polimerase T7 (USB, Cleveland, EUA) com a seguinte ciclagem: 90 ^{o}C por 1 minuto, 25 ^{o}C por 2minutos, 34 ^{o}C por 2 minutos. Um passo inicial de desnaturação da 92 ^{o}C por 5 minutos foi adicionado a cada ciclo para inativar a atividade da proteinase K. Foi adicionada 0,3 U de Sequenase a cada ciclo durante a etapa de reanelamento. Em seguida, foram adicionados 50 µL de um mix contendo 0.1 U Taq polimerase, 0.2 mM dNTPs, 20 µM DOP primer, 25 mM $MgCl_2$ e 34.23 µl de água em

um programa de amplificação de 92 ºC por 1 minuto, 56 ºC por 2 minutos, 72 ºC por 2 minutos seguido de uma etapa de extensão final de 5 minutos a 72 ºC.

3.6.4 Marcação das sondas para pintura cromossômica

Todas as seis sondas foram marcadas com Spectrum Orange-dUTP e Spectrum Green-dUTP (Vysis, Downers Grove, USA) em uma DOP-PCR secundária usando 1 µl do produto primário amplificado como DNA molde de acordo com Yang e Graphodatsky (2009).

3.7 Fração repetitiva *C_ot*-1 DNA

C_ot-1 DNA são sequências de DNA enriquecidas com sequências alta e moderadamente repetitivas e que podem ser usadas para competir com a sonda marcada utilizada para bloquear alvos não específicos. Assim, tanto para os experimentos de WCP quanto CGH foi utilizado *C_ot*-1 DNA da espécie doadora da sonda as quais foram amplificadas de acordo com Zwick *et al*. (1997).

3.8 Hibridização *in situ* fluorescente para DNAr, telômero e microssatélites

As lâminas contendo as preparações cromossômicas foram envelhecidas à 60 ºC durante 1 hora e depois incubadas com 100 µl de solução de RNAse (1 µl RNAse 10 mg/ml + 1 ml 2x SSC) por 1 hora à 37 ºC em câmara úmida. Após esta etapa as lâminas foram lavadas durante 5 minutos em *shaker* temperatura ambiente com solução de PBS 1x e então incubadas em solução de pepsina 0,005 % (1000 µl H_2O+5 µl HCl 1M+1,5 µl de pepsina (20 mg/ml) com 100 µl por lâmina durante 10 minutos à 37 ºC em câmara úmida. Lavadas novamente em PBS 1x e desidratadas em série alcoólica 70 %, 85 % e 100 % de 2 minutos cada em temperatura ambiente.

Posteriormente as lâminas foram desnaturadas em solução de formamida 70 % (70 ml de formamida + 30 ml 2x SSC) durante 3 minutos e 15 segundos à 72 ºC. Em seguida foram desidratadas em etanol 70 % gelado por 2 minutos e depois etanol 85 % e 100 % por 2 minutos cada em temperatura ambiente e secar ao ar. Em paralelo o mix de hibridização foi desnaturado em um termociclador à 85 ºC por 10 minutos. Logo depois de secas, foi adicionado o mix de hibridização em cada lâmina e deixado overnight em câmara úmida à 37 ºC.

No dia seguinte, as lâminas foram lavadas em solução aquecida à 65 ºC de 1xSSC por 5 minutos no *shaker*, seguido de uma lavagem em Tween no *shaker* em temperatura ambiente durante 5 minutos. As lâminas passaram por uma rápida lavagem em PBS 1x por 1 minutos e desidratas em série alcoólica por 2 minutos cada. Depois de totalmente secas as lâminas com cromossomos metafásicos foram coradas com 20 µl de uma mistura de antifading contendo (1.5 µg/ml) de DAPI (Cambio, Cambridge. United Kingdom) e as lâminas analisadas em microscópio de epifluorescência.

3.9 Hibridização *in situ* fluorescente para WCP e CGH

As lâminas contendo as preparações cromossômicas foram envelhecidas à 60 ºC durante 1 hora e depois incubadas com 100 µl de solução de RNAse (1 µl RNAse 10 mg/ml + 1 ml 2x SSC) por 1 hora à 37 ºC em câmara úmida. Após esta etapa as lâminas foram lavadas durante 5 minutos em *shaker* com temperatura ambiente com solução de PBS 1x e então incubadas em solução de pepsina 0,005 % (1000 µl H_2O+5 µl HCl 1M+1,5 µl de pepsina (20 mg/ml) com 100 µl por lâmina durante 10 minutos à 37 ºC em câmara úmida. Simultaneamente a esta etapa, as sondas foram desnaturadas em termociclador a 85 ºC durante 10 minutos, 4 ºC durante 2 minutos e 37 ºC de 30 minutos – 1 hora. As lâminas foram lavadas novamente em PBS 1x e desidratadas em série alcoólica 70 %, 85 % e 100 % de 2 minutos cada em temperatura ambiente.

Posteriormente as lâminas foram desnaturadas em solução de formamida 70 % (70 ml de formamida+30 ml 2x SSC) durante 3 minutos e 15 segundos à 72 ºC. Em seguida foram desidratadas em etanol 70 % gelado por 2 minutos e depois etanol 85 % e 100 % por 2 minutos cada em temperatura ambiente e secar ao ar.

Seguido os dias de hibridização (entre 2 e 3 dias), as lâminas foram lavadas duas vezes em solução aquecida à 65 ºC de 1x SSC por 5min no shaker, seguido de duas lavagens em Tween no shaker em temperatura ambiente durante 5 minutos. As lâminas passaram por uma rápida lavagem em PBS 1x por 1 minuto e desidratas em série alcoólica por 2 minutos cada. Depois de totalmente secas as lâminas com cromossomos metafásicos foram coradas com 20 µl de uma mistura de antifading contendo (1.5 µg/ml) de DAPI (Cambio, Cambridge. United Kingdom) e as lâminas analisadas em microscópio de epifluorescência.

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4. RESULTADOS E DISCUSSÃO

Os resultados obtidos no presente estudo e suas respectivas discussões encontram-se organizados na forma de capítulos, os quais correspondem aos artigos científicos listados abaixo e que se encontram publicados, submetidos ou em fase de preparação:

Capítulo 1:

Highly rearranged karyotypes and multiple sex chromosome systems in armored catfishes from the genus *Harttia* (Teleostei, Siluriformes)

Capítulo 2:

Evolutionary breakpoints regions and chromosomal remodeling in *Harttia* (Siluriformes: Loricariidae) species diversification

Capítulo 3:

Chromosomal rearrangements and origin of the multiple XX/XY_1Y_2 sex chromosome system in *Harttia* species (Siluriformes: Loricariidae)

Capítulo 4:

Microsatellite repeats accumulation in hotspots of chromosomal rearrangements in *Harttia* species (Siluriformes: Loricariidae)

Capítulo 1

Highly rearranged karyotypes and multiple sex chromosome systems in armored catfishes from the genus *Harttia* (Teleostei, Siluriformes)

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Abstract

Harttia comprises an armored catfish genus endemic to the Neotropical region, including 27 valid species with low dispersion rates that are restricted to small distribution areas. Cytogenetics data point to a wide chromosomal diversity in this genus due to changes that occurred in isolated populations, with chromosomal fusions and fissions explaining the 2n number variation. In addition, different multiple sex chromosome systems and rDNA loci location are also found in some species. However, several *Harttia* species and populations remain to be investigated. In this study, *Harttia intermontana* and two still undescribed species, morphologically identified as *Harttia* sp. 1 and *Harttia* sp. 2, were cytogenetically analyzed. *Harttia intermontana* has 2n = 52 and 2n = 53 chromosomes, while *Harttia* sp. 1 has 2n = 56 and 2n = 57 chromosomes in females and males, respectively, thus highlighting the occurrence of an XX/XY_1Y_2 multiple sex chromosome system in both species. *Harttia* sp. 2 presents 2n = 62 chromosomes for both females and males, with fission events explaining its karyotype diversification. Chromosomal locations of the rDNA sites were also quite different among species, reinforcing that extensive rearrangements had occurred in their karyotype evolution. Comparative genomic hybridization (CGH) experiments among some *Harttia* species evidenced a shared content of the XY_1Y_2 sex chromosomes in three of them, thus pointing towards their common origin. Therefore, the comparative analysis among all *Harttia* species cytogenetically studied thus far allowed us to provide an evolutionary scenario related to the speciation process of this fish group.

Keywords: chromosomal rearrangements; comparative genomic hybridization; fish mapping; fish species; karyotype evolution; sex chromosomes

1. INTRODUCTION

Fishes exhibit the greatest biodiversity among the vertebrates, constituting a useful model for studying several evolutionary questions (NELSON; GRAND; WILSON, 2016). Particularly, the large river networks found in the Neotropical region contain the world's richest biodiversity. Despite the large geographic distribution of the Neotropical fish families, different species are found inhabiting adjacent river basins split by vicariant events millions of years ago (ALBERT; TAGLIACOLLO; DAGOSTA, 2020). In the same way, species inhabiting small streams, with limited migration opportunities, tend to present an increased rate of speciation (REIS; KULLANDER; FERRARIS JR, 2003), even in parapatric populations (NASCIMENTO *et al*., 2018).

One of these examples relies on the genus *Harttia* (Siluriformes, Loricariidae, Loricariinae), an endemic and widespread group throughout many South American river basins (de OLIVEIRA; OYAKAWA, 2019). These species have a sedentary lifestyle and reduced vagility, leading them to inhabit specific sections of the river and to form small local populations (BUCKUP, 1999).

Studied species indicate a wide variation on the diploid number (2n) in *Harttia*, ranging from 2n = 52 to 62 chromosomes, with the occurrence of interstitial telomeric sites (ITS) as vestiges of chromosomal changes, different ribosomal genes distributions on the karyotypes, occurrence of B chromosomes, and multiple sex chromosome systems (Table 1). Based on the molecular phylogenetic inferences in the Harttiini tribe (COVAIN *et al*., 2016), a scenario for *Harttia* karyotype diversification was proposed by Blanco *et al*. (2017). In this scenario, a putative ancestral karyotype would have 2n = 58 chromosomes and no heteromorphic sex chromosomes, such as that found in *Harttia kronei* (BLANCO *et al*., 2017). From such a karyotype, reductions in 2n number by chromosome fusions were proposed in the diversification of lineages, until the lowest

number of chromosomes, 2n = 52, found in *H. carvalhoi* females was reached (BLANCO *et al*., 2017).

Furthermore, the chromosomal rearrangements in *Harttia* species culminated in different kinds of sex chromosome systems: (i) an XX/XY_1Y_2 system in *H. carvalhoi* (BLANCO *et al*., 2013); (ii) an $X_1X_1X_2X_2/X_1X_2Y$ system in *Harttia punctata*, *Harttia duriventris*, and *Harttia villasboas* (BLANCO *et al*., 2014; SASSI *et al*., 2020); and (iii) a neo XX/XY system in *Harttia rondoni* (SASSI *et al*., 2020). In accordance with the two branches of *Harttia* species (COVAIN *et al*., 2016), these sex chromosome systems followed independent evolutionary origins (BLANCO *et al*., 2017; SASSI *et al*., 2020). While *H. carvalhoi* is grouped with *H. kronei*, *H. longipinna*, *H. loricariformis***,** and some other species distributed in southern and southeastern Brazil, *H. punctata*, *H. rondoni*, *H. duriventris*, and *H. villasboas* are grouped in a different branch with other species from the north and northeast Brazilian drainages (COVAIN *et al*., 2016).

Simple sex chromosome systems are proposed to have originated by an inversion event or by the accumulation of transposable elements in one homologue of a proto sex chromosome pair, thus promoting a cross-over restricted region able to differentiate in a sex-specific chromosomal segment (CHARLESWORTH; CHARLESWORTH; MARAIS, 2005; STEINEMANN; STEINEMANN, 2005; SCHEMBERGER *et al*., 2019). Additionally, with regard to multiple sex chromosomes, different types of chromosomal rearrangements (such as centric fusions, centric fissions, pericentric inversions, translocations, tandem translocations), usually associated with autosomes and proto-sex chromosomes, have already been proposed to explain the origin of different types of systems (BERTOLLO; GALETTI JR, 1980; MOREIRA-FILHO; BERTOLLO *et al*., 1997; SCHEMBERGER *et al*., 2011; KITANO; PEICHEL, 2012; BLANCO *et al*., 2013; OLIVEIRA *et al*., 2017). More recently, and with the aim of discovering the

evolutionary origin of the sex chromosomes systems, molecular cytogenetics approaches such as whole chromosome painting (WCP) and comparative genome hybridization (CGH) have been successfully used in some Neotropical fish species (OLIVEIRA *et al*., 2017; CIOFFI *et al*., 2017; YANO *et al*., 2017; MORAES *et al*., 2019).

In this study, we provide a set of conventional and molecular cytogenetic approaches (Giemsa staining, C-banding, repetitive DNA mapping by FISH, comparative genomic hybridization (CGH)), in an attempt to advance the knowledge of the processes that have shaped the chromosomal evolution in the genus *Harttia*. Data allowed for a comprehensive perspective of the chromosomal diversity and evolutionary trends inside this group, in addition to a description of two other new rare occurrences of the XX/XY_1Y_2 sex chromosome system among fishes.

2. MATERIALS AND METHODS

2.1. Specimens

Three *Harttia* species not yet studied were investigated. Their collection sites, number, and sex of individuals are presented in **Figure 1** and **Table 2**. **Figure 1** also depicts the Brazilian distribution of other *Harttia* species previously studied. Fishes were collected with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-License Nso. 10538-3 and 15117-1), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09). The species were properly identified by Dr. Oswaldo Oyakawa (curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP), with expertise on *Harttia* taxonomy. One of the three species corresponds to *Harttia intermontana*, and the other two correspond to new species that have not yet been described, here named as *Harttia* sp. 1 and *Harttia* sp. 2.

2.2. Chromosome preparations and C-banding

Mitotic chromosomes were obtained from cells of the anterior region of the kidney after *in vivo* colchicine treatment according to the protocol described in Bertollo; Cioffi and Moreira-Filho (2015). The experiments followed ethical and anesthesia procedures that were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315). The C-positive heterochromatin (C-banding) was identified according to Sumner (1972) with some modifications according to Lui *et al*. (2012).

2.3. Fluorescence in situ hybridization (FISH)

Two tandemly arrayed rDNA probes were obtained by PCR from the nuclear DNA of *Harttia intermontana*. The 5S rDNA probe included 120 base pairs (bp) of the 5S rRNA transcript region and 200 bp of a non-transcribed spacer (NTS), isolated according to Pendás *et al*. (1994). The 18S rDNA probe contained a 1400 bp segment of the 18S rRNA gene and was isolated following Cioffi *et al*. (2009). The probes were directly labeled with the Nick-Translation mix kit (Jena Bioscience, Jena, Germany) using ATTO550-dUTP for the 5S rDNA and AF488-dUTP for the 18S rDNA, according to the manufacturer's manual. Telomeric $(TTAGGG)_n$ sequences were also mapped using the DAKO Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Denmark). FISH experiments followed the methodology described in Yano; Bertollo and Cioffi (2017).

2.4. Comparative genomic hybridization (CGH)

The total genomic DNA (gDNA) from male and female specimens of *H. intermontana*, *Harttia* sp. 1, and *H. carvalhoi* were extracted from liver tissues by the standard phenol-chloroform-isoamyl alcohol method (SAMBROOK; RUSSELL, 2001). The CGH experiments were focused on inter and intraspecific comparisons, with special emphasis on the XY_1Y_2 sex chromosomes. In the first set of experiments (intraspecific genomic comparisons), the male-derived gDNA of *H. intermontana* and *Harttia* sp. 1was labeled by nick translation (Jena Bioscience) with ATTO550-dUTP, while female gDNA was labeled with Atto488-dUTP. Repetitive sequences were blocked in all experiments by using unlabeled C0t-1 DNA (i.e., a fraction of genomic DNA enriched for highly and moderately repetitive sequences), prepared according to Zwick *et al*. (1997). The final hybridization mixture was applied on each slide, which was composed of male- and female-derived gDNAs (500 ng each), plus 25 µg of female-derived C0t-1 DNA from the

respective species. The probe was ethanol-precipitated, and the dry pellets were resuspended in a hybridization buffer containing 50% formamide, 2x SSC, 10% SDS, 10% dextran sulfate, and Denhardt's buffer, pH 7.0. In the second set of experiments (interspecific genomic comparisons), the gDNA samples of all-male specimens now analyzed (plus the gDNA of *H. carvalhoi*, another species harboring the same multiple XY_1Y_2 sex system) were hybridized against metaphase chromosomes of *H. intermontana*. For this purpose, male-derived gDNA of *H. intermontana* was labeled with Atto550-dUTP by nick translation (Jena Bioscience), while the gDNA samples of the other two species were labeled with Atto488-dUTP (*Harttia* sp. 1) and Atto425-dUTP (*H. carvalhoi*) also by nick translation (Jena Bioscience). The three probes were hybridized simultaneously, and the final probe cocktail was composed of 500 ng of the male-derived gDNA of each *H. intermontana, Harttia* sp. 1, and *H. carvalhoi* species and 10 µg of the female-derived C0t-1 DNA of each species. The chosen ratio of probe vs. C0t-1 DNA amount was based on fish experiments previously performed in our laboratory (OLIVEIRA *et al*., 2017; MORAES *et al*., 2017; SASSI *et al*., 2019; TOMA *et al*., 2019). The CGH experiments followed the methodology described in Symonová *et al*. (2015).

2.5. Microscopic analyses and image processing

At least 30 metaphase spreads per individual was analyzed to confirm the 2n, karyotype structure, and CGH results. Images were captured using an Olympus BX50 light microscope (Olympus Corporation, Ishikawa, Japan), with CoolSNAP camera, and the images were processed using the Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified as metacentric (m); submetacentric (sm); subtelocentric (st), or acrocentric (a) according to Levan *et al*. (1964) and arranged according to decreasing size in the karyotypes. The fundamental

number (FN), or number of chromosome arms, was achieved considering just acrocentrics as having a single chromosome arm.

3. RESULTS

3.1. Karyotypes, C-banding, and sex chromosomes

All *H. intermontana* females have 2n = 52 chromosomes (14m + 12sm + 12st + 14a; NF = 90) and all males have 2n = 53 chromosomes (13m + 12sm + 13st + 15a, NF = 91). Similarly, *Harttia* sp. 1 also differs in female and male karyotypes, with 2n = 56 (14m + 14sm + 10st + 18a; NF = 94) and 2n = 57 (13m + 14sm + 10st + 20a; NF = 94), respectively. In both cases, the sex-specific karyotypes are due the occurrence of an XX/XY_1Y_2 multiple sex chromosome system, where the X chromosome corresponds to a large metacentric, and the Y_1 to a medium-size acrocentric. In its turn, the Y_2 chromosome corresponds to a medium-size subtelocentric in *H. intermontana* and to an acrocentric chromosome in *Harttia* sp. 1 **(Figure 2a,c)**. Additionally, *Harttia* sp. 2 has 2n = 62 chromosomes (16m + 14sm + 12st + 20a; NF = 104) in both sexes, without morphologically differentiated sex chromosomes **(Figure 2e)**.

A small amount of C-positive heterochromatin was found in the three species, mostly in the centromeric/pericentromeric regions of some chromosome pairs **(Figure 2b,d,f)**, without specific accumulation in the sex chromosomes of *H. intermontana* and *Harttia* sp. 1 **(Figure 2b,d)**.

3.2. Chromosomal distribution of rDNAs and telomeric repeats

Differentiation in number and location of the 5S and 18S rDNA sequences was found among the three species. In *H. intermontana* and *Harttia* sp. 2, a single locus of 5S rDNA occurs, but in different chromosomes, i.e., in the submetacentric pairs 11 and 9, respectively. In *Harttia* sp. 1, there are two 5S rDNA loci, one of which is located in the

submetacentric pair 12, and the other in the acrocentric pair 20, with a syntenic location with the 18S rDNA in the latter **(Figure 3)**.

The 18S rDNA probe was detected in a single locus in all species but was also found in different chromosomal locations as follows: in the short arms of the second metacentric pair in *H. intermontana*; in the long arms of the acrocentric pair 20 in *Harttia* sp. 1, and in the long arms of the acrocentric pair 22 in *Harttia* sp. 2. No differences in the number and site positions of rDNA were detected between males and females **(Figure 3)**.

Hybridization with the (TTAGGG)n probe evidenced signals only in the telomeric regions of all chromosomes, without ITS in *H. intermontana* and *Harttia* sp. 1 **(Figure 3b,d)**. However, in *Harttia* sp. 2, four ITS were located in the long arms of the chromosome pairs 1, 9, 16, and 22. A double-FISH using both telomeric and 18S rDNA probes revealed that these sequences present a syntenic location in the chromosome pair 22 **(Figure 3f)**.

3.3. Intraspecific and interspecific comparative genomic hybridizations

Intraspecific genomic comparisons between males **(Figure 4b,f)** and females **(Figure 4c,g)** of *H. intermontana* and *Harttia* sp. 1 showed an overlapped hybridization, mainly in the centromeric and pericentromeric regions of almost all chromosomes **(Figure 4d,h)**. A strong binding preference for the 18S rDNA cluster occurs in *H. intermontana* **(Figure 4b,c)** and no sex-specific region was evidenced in both experiments. Interspecific comparisons of the gDNA of *H. intermontana*, *H. carvalhoi*, and *Harttia* sp. 1, all of them bearing an XY_1Y_2 sex system, did not detect species-specific regions in the sex chromosomes **(Figure 5)**.

4. DISCUSSION

4.1. Numerical chromosome changes in *Harttia* species

The Loricariidae family is an outstanding group to investigate chromosomal breaks and rearrangements that gave rise to extremely diverse karyotypes among its representatives (MARIOTTO *et al*., 2011; BARROS *et al*., 2017; GLUGOSKI *et al*., 2018, 2020). These fishes are characterized by a sedentary lifestyle, with rare migratory events (PINNA, 1998). Their species occur in small and isolated populations (BUCKUP, 1999) where the fixation of chromosomal rearrangements could occur at higher rates (WRIGHT, 1940; KING, 1993; RIESEBERG, 2001; FARIA; NAVARRO, 2010). In fact, the Loricariinae subfamily shows extensive numerical chromosome variation (36 to 74), which is attributed to chromosomal rearrangements, mainly to Robertsonian fusions (Rb fusion) and fissions (ROSA *et al*., 2012; PORTO *et al*., 2014; PRIMO *et al*., 2017; GLUGOSKI *et al*., 2018). The *Harttia* genus, in which several cryptic and undescribed species are believed to occur, displays the second-largest chromosomal variation among the Loricariinae (52 to 62, **Table 1, Figure 6**). In addition, there is also strong evidence for evolutionary breakpoint regions (EBRs) promoting intrachromosomal remodeling, which are still being studied (RUIZ-HERRERA *et al*., 2008).

A putative ancestral karyotype, probably with 2n = 58 chromosomes, is attributed to the *Harttia* lineage, and this same 2n number occurs in its sister group *Farlowella* (MARAJÓ *et al*., 2018) and in basal species from *Harttia* phylogenetic relationships (COVAIN *et al*., 2016; BLANCO *et al*., 2017). However, *Harttia* presents different pathways in relation to the 2n diversification, some species keeping 58 chromosomes, some others increasing this chromosome number by centric fissions (i.e., *H. absaberi* and

Harttia sp. 2 now studied), with others decreasing this number due to Rb fusions **(Table 1, Figure 6)**.

ITS generally reveal chromosomal rearrangements, such as Rb fusions or in tandem fusions (OCALEWICZ, 2013). In previous studies, ITS were identified in three *Harttia* species (*H. loricariformis*, 2n = 56; *H. torrenticola*, 2n = 56, and *H. carvalhoi*, 2n = 52/53), as vestiges of Robertsonian rearrangements (BLANCO *et al*., 2013, 2017). It was proposed that fusion events were responsible for originating the largest metacentric pair found in *H. torrenticola* (pair 1) and *H. carvalhoi* (X chromosome), due to the presence of a proximal ITS on their short arms (Blanco et al., 2017). *Harttia intermontana* and *Harttia* sp. 1 also share a similar large metacentric X chromosome, but no ITS were detected. It is likely that this absence is due to the fact that not all chromosome fusions retain some telomeric DNA repeats at the fusion points (GARAGNA *et al*., 1995). Moreover, the occurrence of different chromosomal rearrangements and modifications of the non-functional telomeric arrays can be also considered (SLIJEPCEVIC, 1998). In the last situation, a successive loss and degeneration of the non-functional telomeric repeats that were retained at the fusion sites leads to their gradual shortening, and, consequently, to an insufficient amount to be highlighted by FISH (OCALEWICZ, 2013; BOLZÁN, 2017).

To date, the first largest metacentric pair of *Harttia* is shared by all species that have 2n = 56 chromosomes or a smaller number, except for *H. loricariformis*, and this could be considered as being derived from an Rb fusion chromosome. In *Harttia* sp. 2 the first chromosome pair is also a large metacentric-bearing ITS, however, this chromosome has a small size compared to the chromosome 1 of *H. carvalhoi*, *H. intermontana*, *H. torrenticola*, and *Harttia* sp. 1, thus indicating that additional rearrangements probably

played a role on its origin. Noteworthy, *Harttia* sp. 2 presents four bi-armed chromosome pairs bearing ITS at the proximal regions of the long arms. According to the instability genomic proposal, ITS are hotspots for chromosomal breakage (SLIJEPCEVIC *et al*., 1997), and telomeric DNA damage can be irreparable, causing persistent activation in response to DNA damage (FUMAGALLI *et al*., 2012) or remaining as EBRs on the genome (RUIZ-HERRERA *et al*., 2008; SFEIR *et al*., 2009). This suggests that both ITS and terminal telomeric sequences are naturally prone to breakage, leading to chromosome plasticity (BOLZÁN, 2017; PERRY *et al*., 2004; SLIJEPCEVIC, 2016). Here, we propose that *Harttia* sp. 2 increased its chromosome number by centric fissions from an ancestral ITS bearing lineage, which acted as instable sites and promoted double strand breaks (DSBs) triggering further chromosomal rearrangements. This proposal is corroborated by the extensive FN modification among *Harttia* species **(Table 1)**, since only Robertsonian rearrangements keep the FN unchanged throughout the karyotype evolution. It is known that chromosomal rearrangements might play an important role in speciation (FARIA; NAVARRO, 2010; NAVARRO, 2003). In this sense, the expressive rearranged karyotypes that are found among *Harttia* species may have acted as significant post-zygotic isolating mechanisms throughout the evolutionary history of this group.

4.2. Heterochromatin and rDNA sites rearrangements in *Harttia* species

The presence of small amounts of heterochromatin is probably an intrinsic characteristic of the *Harttia* species (BLANCO *et al*., 2017). Indeed, *H. intermontana* and *Harttia* sp. 1 present the same pattern already described for other species of the genus, while in *Harttia* sp. 2, some more prominent pericentromeric bands are collocated with the ITS in the chromosome pairs 1, 9, 16, and 22. The epigenetic regulation of repetitive sequences, such as histone modifications and DNA methylation to form heterochromatin,

is proposed to protect ITS from breakages and play important roles in regulation of gene expression (BOLZÁN, 2017; LIN; YAN, 2008). In this way, the colocalization of the heterochromatin and ITS may be an expression of an epigenetic property of the *Harttia* sp. 2 genome. In addition, the rDNA loci colocalization with ITS (5S in pair 9 and 18S in pair 22) indicates that these multigene families are also probably associated with chromosomal rearrangements in *Harttia* sp. 2. In the same way, the wide differentiation of the chromosomes carrying the rDNA sequences among *Harttia* species demonstrates that these repetitive sequences may also be closely related to deep chromosomal changes that have occurred within the genus. In fact, in some groups of Loricariidae, the involvement of rDNA pseudogenes colocalized to ITS in chromosomal rearrangements have been demonstrated (BARROS *et al*., 2017; PRIMO *et al*., 2017; GLUGOSKI *et al*., 2018).

As a whole, three general conditions are found concerning the location of the rDNA genes among *Harttia* species: In the first, a syntenic condition for both 5S and 45S rDNAs occurs, as found in *H. carvalhoi*, *H. loricariformis*, and *Harttia* sp. 1, where the first acrocentric chromosome pair is the carrying one, although with *Harttia* sp. 1 showing a particular syntenic configuration **(Figures 3** and **6)**. Yet, *H. carvalhoi* and *Harttia* sp. 1 present an extra 5S rDNA locus. The second condition includes *H. gracilis*, *H. kronei*, *H. longipinna*, *H. punctata*, *H. villasboas*, *H. duriventris*, *H. torrenticola*, and *Harttia* sp. 2, in which the first acrocentric carries the 18S rDNA site, while the 5S occurs in variable locations of different meta/submetacentric chromosomes (except for *H. rondoni* that has 18S rDNA site in the largest sm). In the third pattern, the 5S locus is found in a submetacentric pair, while the chromosome that carries the 45S rDNA is a large metacentric resulting from a fusion event, as found in *H. intermontana* and *H. absaberi* karyotypes **(Figures 3** and **6)**.

EBRs are DNA clustered regions that are more prone to break and reorganize into genomes, and these specific regions have been described to be re-used during the evolution among related species (RUIZ-HERRERA; CASTRESANA; ROBINSON, 2006; CARBONE *et al*., 2009; LONGO *et al*., 2009; FARRÉ *et al*., 2011). According to the model, the evolutionary re-use of DSB regions and multiple locus repositioning among karyotypes corroborate to probable EBR occurrences adjacent to rDNA sites in the *Harttia* lineage, similar to those described in other loricariids, such as *Ancistrus* (BARROS *et al*., 2017) and *Rineloricaria* (GLUGOSKI *et al*., 2018).

4.3. The rare XX/XY_1Y_2 system in fish species

Based on an overview of available fish karyotype data (ARAI, 2011), only about 5% of the analyzed species possess heteromorphic sex chromosomes, including approximately 47 cases of multiple sex chromosomes (PENNELL *et al*., 2015). Among them, some different systems, such as ♀$X_1X_1X_2X_2$/♂X_1X_2Y; ♀XX/♂XY_1Y_2; ♀$X_1X_1X_2X_2$/♂$X_1Y_1X_2Y_2$; ♀ZZ/♂ZW1W2, and ♀$Z_1Z_1Z_2Z_2$/♂$Z_1W_1Z_2W_2$, were already identified as scattered on the fish phylogeny and independently evolved in many lineages and, sometimes, even within a same genus or species (MANK; AVISE, 2009).

In the *Harttia* genus, two multiple sex chromosome systems were previously described, the $X_1X_1X_2X_2/X_1X_2Y$ one in *H. punctata*, *H. duriventris*, and *H. villasboas* and the XX/XY_1Y_2 system in *H. carvalhoi* (BLANCO *et al*., 2017; SASSI *et al*., 2020). While the first one is well-represented among a variety of fish families (KITANO; PEICHEL, 2012), the XX/XY_1Y_2 system is found in only a few fish species **(Table 3)**. Here, like in *H. carvalhoi* (BLANCO *et al*., 2017), two additional cases were identified in *H. intermontana* and *Harttia* sp. 1.

Multiple XX/XY_1Y_2 sex chromosome systems are proposed to have originated by one bi-armed chromosome fission leading to Y_1 and Y_2 formation (FROLOV, 1990; OZOUF-COSTAZ; HUREAU; BEAUNIER, 1991; SEMBER *et al*., 2015) or by X-autosome fusion forming a large bi-armed X chromosome and subsequent centric fission in the origination of the Y_1 and Y_2 chromosomes (BERTOLLO; MOREIRA-FILHO; TAKAHASHI, 1983; OLIVEIRA, 2008; ALMEIDA *et al*., 2015; FAVARATO *et al*., 2016). In *Harttia* species, the large metacentric 1 observed in *H. torrenticola* is comparable to X chromosome in *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 2 and was proposed to be originated from an Rb fusion (BLANCO *et al*., 2017).

To date, *Harttia* lineages from the south/southeast Brazilian drainages have no proto-sex or XY chromosomes identified, which would corroborate the proposal of an X-autosome fusion acting in the origin of the XY_1Y_2 system. However, the occurrence of *H. torrenticola* (without differentiated sex chromosomes) and *H. carvalhoi* (XY_1Y_2) in the same branch of the phylogenetic relationship (COVAIN *et al*., 2016) and the same CGH pattern among *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 1 concerning sex chromosomes, point to an Rb fusion leading to their large metacentric X-chromosome, as well as to the similar large metacentric pair 1 of *H. torrenticola*.

Although *H. intermontana* and *H. carvalhoi* possess the same 2n and sex chromosome system (XX/XY_1Y_2), significant differences occur between the karyotype structure of these two species. The absence of several large submetacentric pairs in *H. intermontana* as well as the occurrence of its large second metacentric pair carrying 18S rDNA cistrons are remarkable. Besides that, the morphology of their Y_2 chromosome also differs, corresponding to a subtelocentric in *H. intermontana* and to an acrocentric chromosome in *H. carvalhoi*. By comparing the chromosomal morphology and the distribution of the ribosomal sites, it is possible to infer that some additional

rearrangements, such as Rb fusion and/or reciprocal translocation, pericentric inversion, and loss or gain of 5S sequences, took place in the chromosome evolution of these species. All data corroborate EBRs occurrence in adjacent regions to rDNA loci and in the pericentromeric region of the largest metacentric pair in the chromosomal diversification of the *Harttia* species inhabiting south and southeast Brazilian drainages.

5. CONCLUSIONS

Our study provided additional evidence on the evolutionary pathways followed by fish species of the genus *Harttia*, highlighting both shared and specific chromosomal features that have emerged throughout their life story. We were also able to identify two new cases of the rare XX/XY_1Y_2 multiple sex chromosomes systems among fishes, displaying a significant particular incidence in the *Harttia* lineages from south/southeast Brazil. The species in this branch, which include the *H. intermontana*, *Harttia* sp. 1, and *Harttia* sp. 2 here studied, experienced different ways of chromosome diversification, such as 2n reduction and increase by Rb fusions and centric fissions, respectively, and the emergence of a XX/XY_1Y_2 sex chromosome system in different species, in contrast to what occurred with the lineages from north Brazilian regions where the $X_1X_1X_2X_2/X_1X_2Y$ system stands out. The occurrence of deeply reorganized karyotypes in the species here studied are in accordance with EBRs present in the *Harttia* genome, which could be reused for chromosome speciation in this group. As a whole, the present study highlights the importance of cytogenetics as a tool for evolutionary studies and, particularly in the present case, detaching the highly differentiated patterns followed by the *Harttia* lineages from two main Brazilian geographic regions.

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TABLES AND FIGURES

Table 1: Comparative cytogenetic data of *Harttia* species.

Species	2n	Karyotype	NF	References
Harttia absaberi	♀♂62	13m+23sm+16st+10a	114	Rodrigues (2010)
Harttia carvalhoi	52♀/	16m+16sm+12st+8a	96	Blanco *et al*. (2013)
	53♂	15m+16sm+12st+10a	96	
Harttia duriventris	56♀/	16m+16sm+16st+8a ♀	104	Sassi *et al.* (2020)
	55♂	17m+16sm+16st+6a ♂	104	
Harttia gracilis	♀♂58	20m+22sm+8st+8a	108	Blanco *et al*. (2017)
H. intermontana	52♀/	14m+12sm+12st+14a ♀	90	Present study
	53♂	13m+12sm+13st+15a ♂	91	
Harttia kronei	♀♂58	16m+16sm+16st+10a	106	Blanco *et al*. (2017)
Harttia longipinna	♀♂58 + 0-2 Bs	16m+12sm+16st+14a	102	Blanco *et al*. (2012)
Harttia loricariformis	♀♂56	16m+22sm+10st+8a	104	Kavalco *et al*. (2004)
Harttia punctata	58♀/	16m+20sm+12st+10a ♀	106	Blanco *et al*. (2014)
	57♂	16m+21sm+12st+8a ♂	106	
Harttia rondoni	♀♂54	20m+26sm+4st+4a	104	Sassi *et al.* (2020)
Harttia torrenticola	♀♂56	16m+10sm+16st+14a	98	Blanco *et al*. (2013)
Harttia villasboas	56♀/	18m+24sm+6st+8a ♀	104	Sassi *et al.* (2020)
	55♂	19m+24sm+6st+6a ♂	104	
Harttia sp. 1	56♀/	14m+14sm+10st+18a ♀	94	Present study
	57♂	13m+14sm+10st+20a ♂	94	
Harttia sp. 2	♀♂62	16m+14sm+12st+20a	104	Present study

Table 2: Collection sites, sample sizes (N), and sex of the *Harttia* species analyzed.

Species	Locality	N
1 - Harttia intermontana	Piranga river, Carandaí-MG (Brazil) (20°59'34.0"S, 43°43'30.0"W)	20♀,13♂
2 - *Harttia* sp. 1	Macacos stream, Silveira-SP (Brazil) (22°40'43.0"S, 44°51'25.0"W)	10♀, 7♂
3 - *Harttia* sp. 2	Barra Grande river, Prudentópolis-PR (Brazil) (24°58'40.72"S, 51°7'34.25"W)	17♀,11♂

Table 3: Multiple XX/XY_1Y_2 sex chromosome systems currently found in teleost fishes.

Species	2n	Mechanism of origin	Reference
Bathydraco marri	38♀, 39♂	Fission of the Y chromosome	Ozouf-Costaz; Hureau; Beaunier (1991)
Coregonus sardinella	80♀, 81♂	Fission of the Y chromosome	Frolov (1990)
Schistura cf. *fasciolata*	50♀, 51♂	Fission of the Y chromosome	Sember *et al*. (2015)
Hoplias malabaricus (karyomorph G)	40♀, 41♂	Tandem fusion X-A	Bertollo *et al*. (1983), Bertollo *et al*. (2000), Oliveira *et al*. (2018)
Gymnotus bahianus	36♀, 37♂	Tandem fusion X-A	Almeida *et al*. (2015)
Ancistrus dubius	38♀, 39♂	Fusion of sex chromosome pair to autosomes and then fission of neo-Y	Oliveira *et al*. (2008), Favarato *et al*. (2016)
Harttia carvalhoi	52♀, 53♂	Fission of the Y chromosome	Centofante; Bertollo; Moreira-Filho (2006), Blanco *et al*. (2013, 2017), Traldi *et al*. (2019)
Harttia intermontana	52♀, 53♂	Fission of the Y chromosome	Present work
Harttia sp.1	56♀, 57♂	Fission of the Y chromosome	Present work

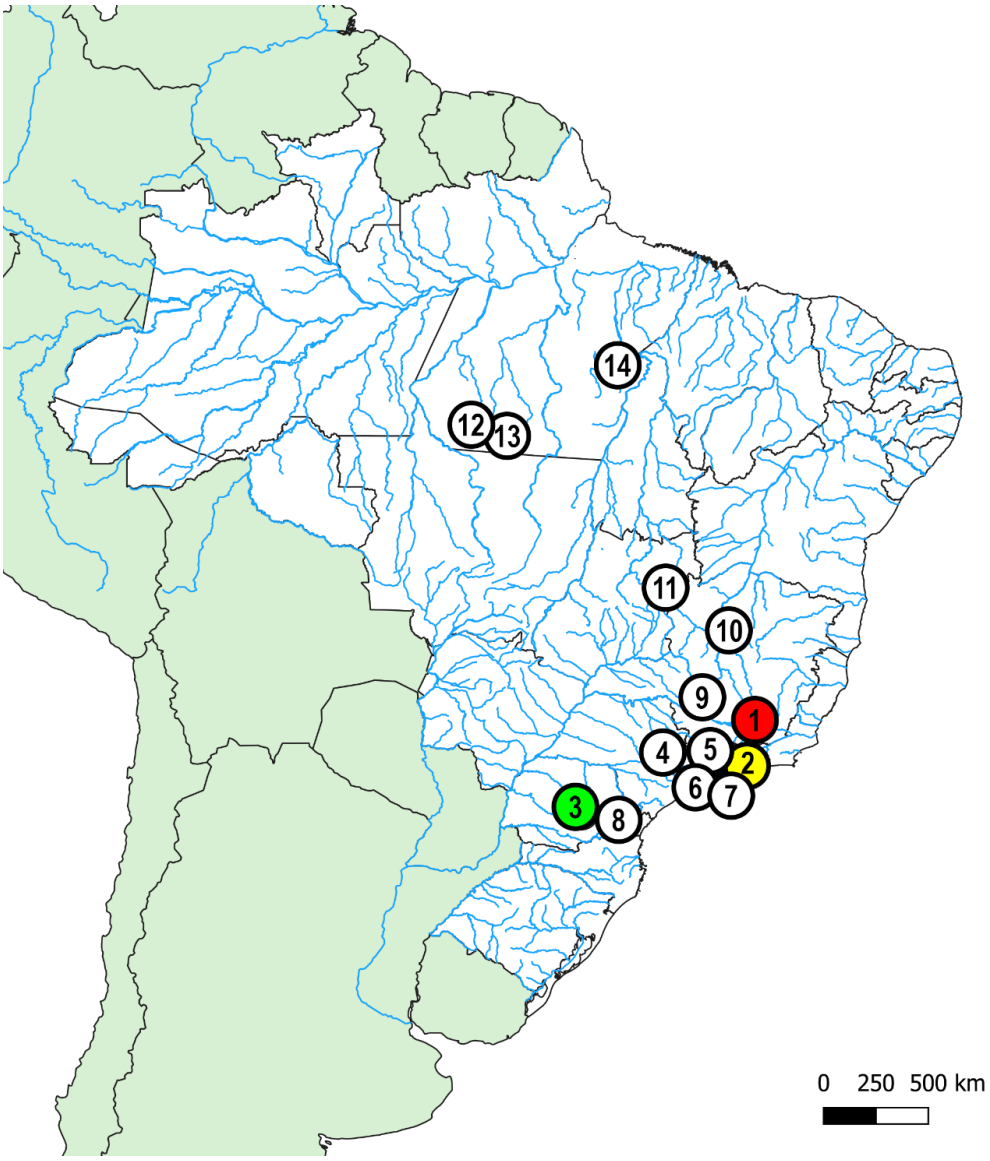

Figure 1: Partial map of South America highlighting the Brazilian collection sites of the three *Harttia* species analyzed in the present work (color circles) named: 1. *H. intermontana* (red circle); 2. *Harttia* sp. 1 (yellow circle); and 3. *Harttia* sp. 2 (green circle). The white circles represent other *Harttia* species previously studied: 4. *H. absaberi*, 5. *H. carvalhoi*, 6. *H. gracilis*, 7. *H. loricariformis*, 8. *H. kronei*, 9. *H. torrenticola*, 10. *H. longipinna*, 11. *H. punctata*, 12. *H. rondoni*, 13. *H. villasboas*, and 14. *H. duriventris*. Map created using QGis 3.4.3.

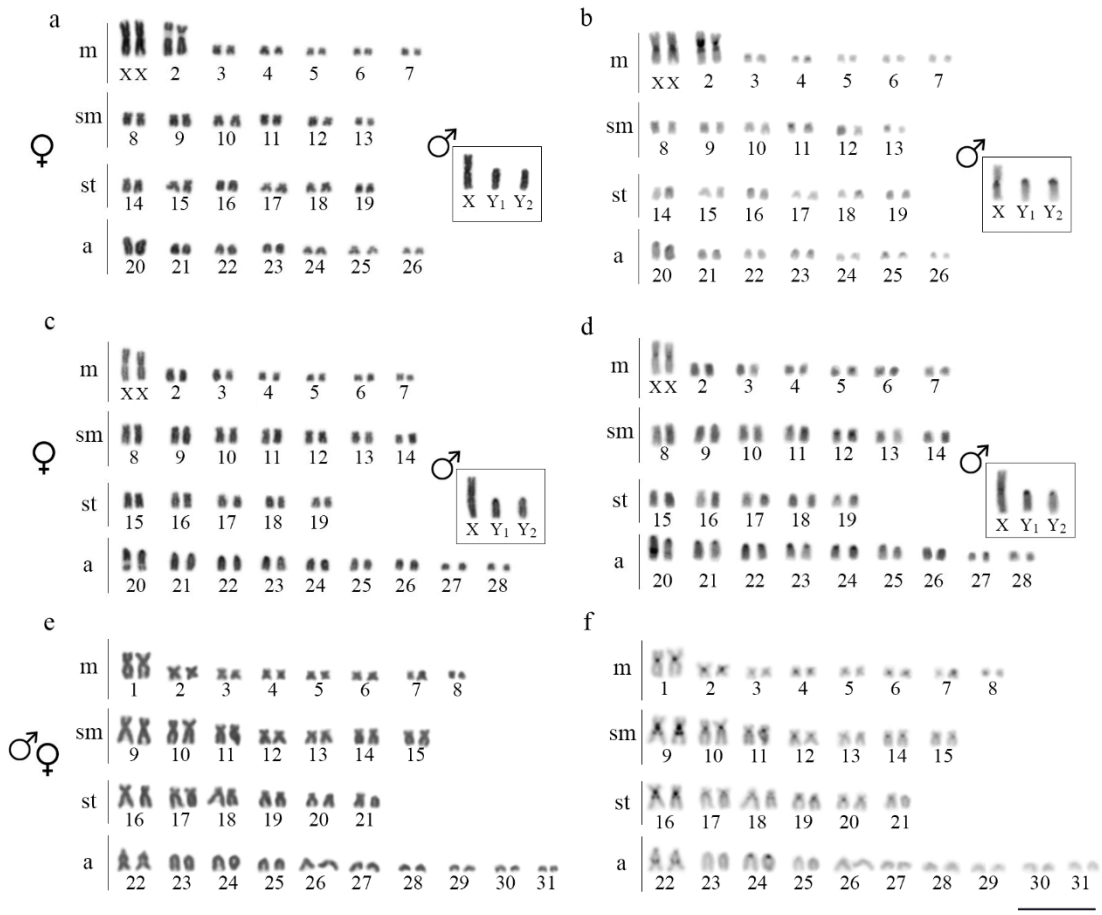

Figure 2: Karyotypes of *H. intermontana* (a,b), *Harttia* sp. 1 (c,d), and *Harttia* sp. 2 (e,f), showing sequentially Giemsa-stained (a,c,e) and C-banded (b,d,f) chromosomes. Insets depict the male sex chromosomes. Bar = 5 µm.

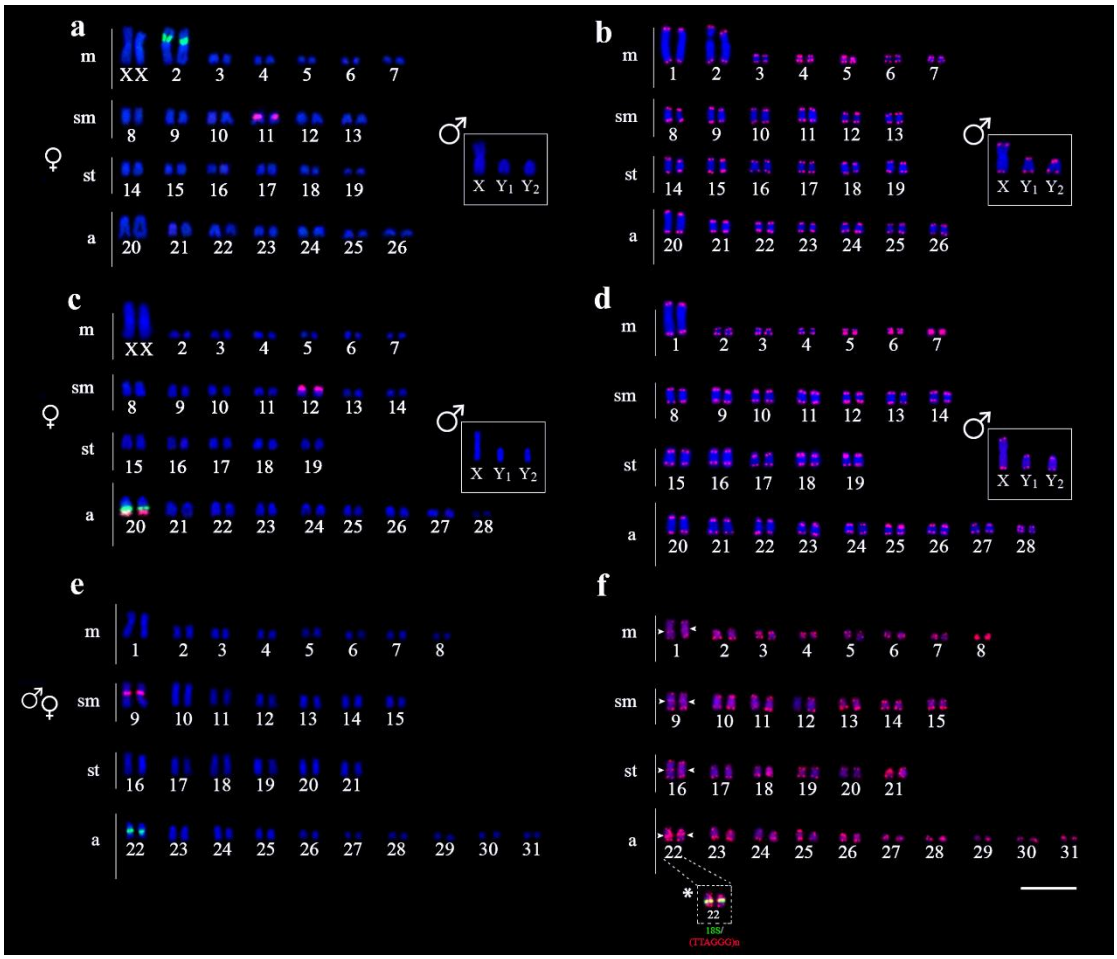

Figure 3: Karyotypes of *H. intermontana* (**a**,**b**), *Harttia* sp. 1 (**c**,**d**), and *Harttia* sp. 2 (**e**,**f**) arranged after FISH with 5S rDNA (red) and 18S rDNA (green) probes (**a**,**c**,**e**), and telomeric $(TTAGGG)_n$ probe (**b**,**d**,**f**). Inserts depict the male sex chromosomes. Arrowheads indicate the interstitial telomeric sites (ITS) locations and the (*) signals the joint localization of the 18S and ITS sites. Bar = 5 µm.

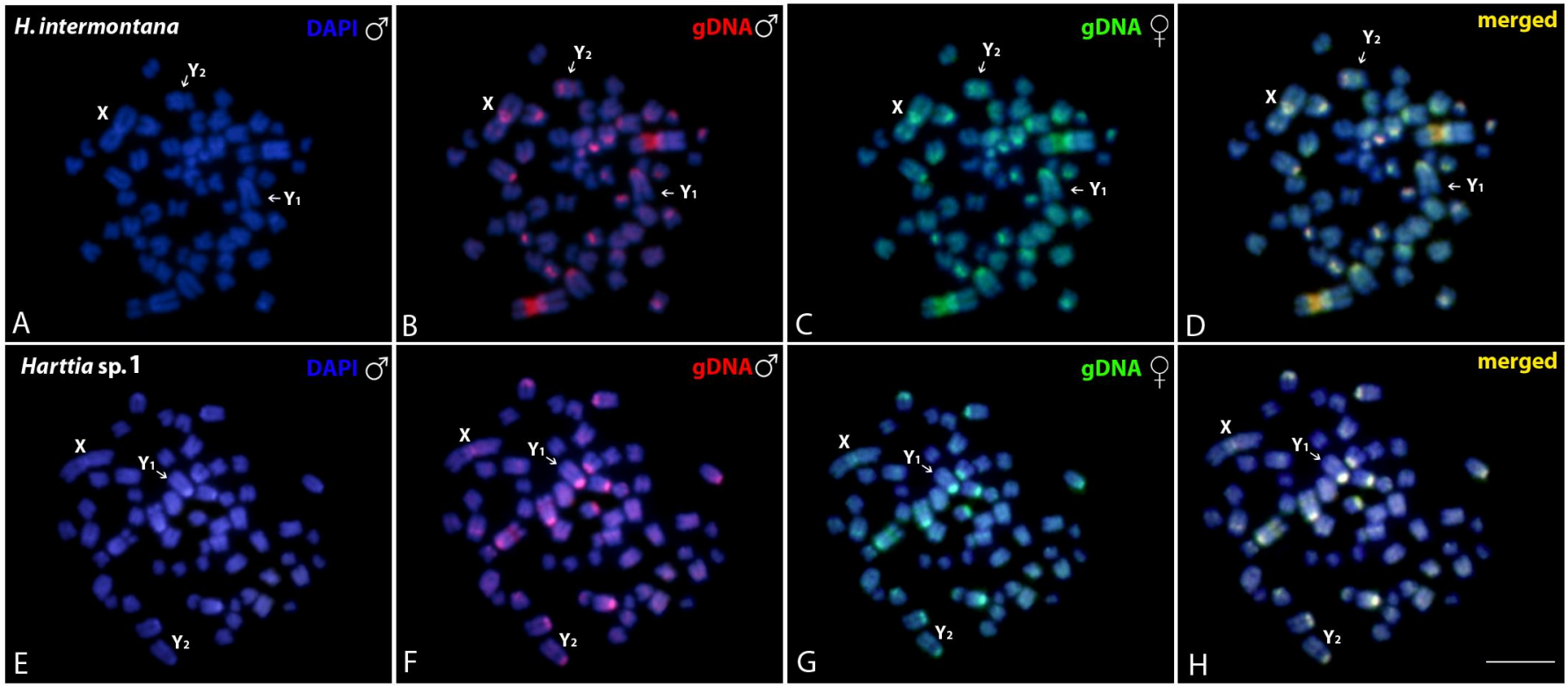

Figure 4: Mitotic chromosome spreads of males *H. intermontana* **(A-D)** and *Harttia* sp. 1 (**E**–**H**) after intraspecific genomic hybridizations, with male- and female-derived genomic probes hybridized together for each species. The first column (**A**,**E**): DAPI images (blue); Second column (**B**,**F**): hybridization pattern for the male-derived probe (red); Third column (**C**,**G**): hybridization pattern for the female-derived probe (green); Fourth column (**D**,**H**): merged images for both genomic probes and DAPI staining. The common genomic regions for males and females are depicted in yellow. Arrows indicate the sex chromosomes. Bar = 5 µm.

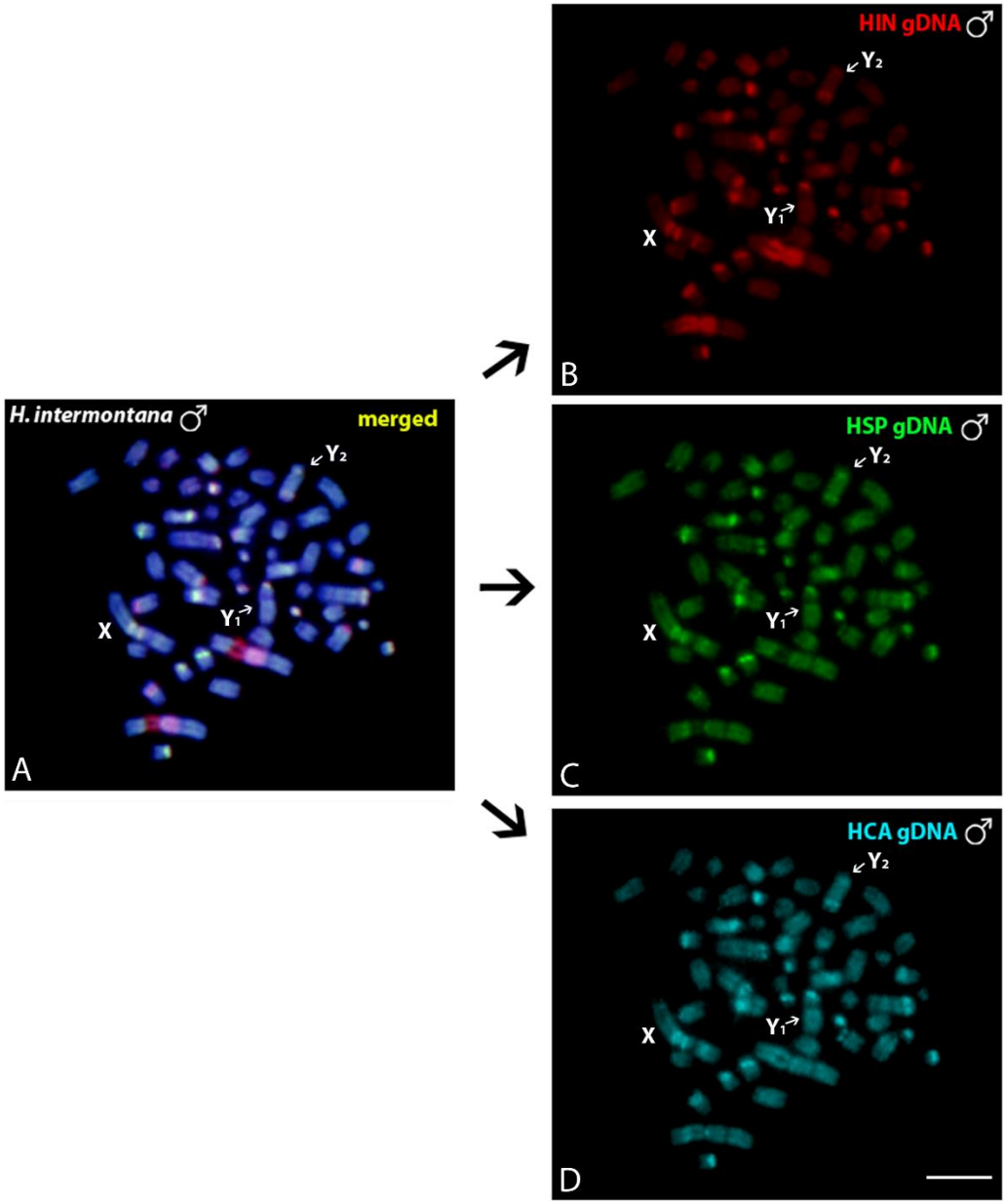

Figure 5: Mitotic chromosome spreads of males from *H. intermontana* (**A**–**D**) after comparative genomic hybridization (CGH): interspecific hybridizations probed with a male-derived genomic probe from *H. intermontana* (**B**), *Harttia* sp. 1 (**C**), and *H. carvalhoi* (**D**). (**A**) depicts the merged images of the genomic probes and DAPI staining. The common genomic regions for male and female are depicted in yellow. Sex chromosomes are indicated. Bar = 5 µm.

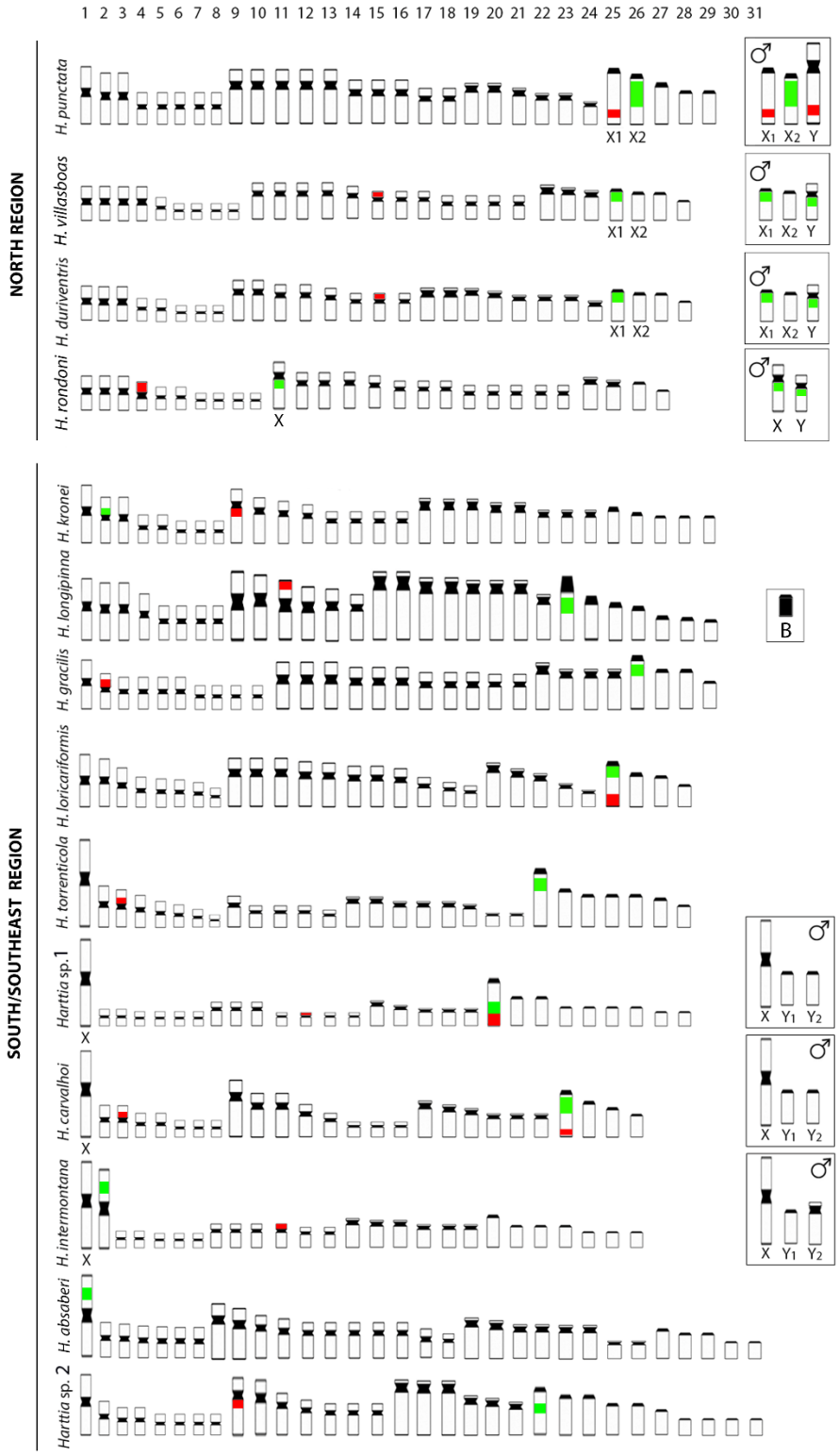

Figure 6: Representative idiograms of *Harttia* species from distinct Brazilian regions based on the distribution of rDNA sequences in their karyotypes, according to the present study, Blanco *et al*. (2017), and Sassi *et al*. (2020) data. The location of the 18S and 5S rDNA sites on the chromosomes are indicated in green and red, respectively. Inserts depict the male sex chromosomes.

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Capítulo 2

Evolutionary breakpoints regions and chromosomal remodeling in *Harttia* (Siluriformes: Loricariidae) species diversification

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Abstract

The Neotropical armored catfish *Harttia* presents a wide variation of chromosomal rearrangements among their representatives. Studies indicate that translocation and Robertsonian rearrangements have triggered the karyotype evolution in the genus, including differentiation of sex chromosome systems. However, few studies use powerful tools, such as comparative whole chromosome painting, to clarify this highly diversified scenario. Here, we isolated probes from the X_1 (a 5S rDNA carrier) and the X_2 (a 45S rDNA carrier) chromosomes of *Harttia punctata*, which displays an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system. Those probes were applied in other *Harttia* species to evidence homeologous chromosome blocks. The resulting data reinforce that translocation events played a role in the origin of the X_1X_2Y sex chromosome system in *H. punctata*. The repositioning of homologous chromosomal blocks carrying rDNA sites among ten *Harttia* species has also been demonstrated. Anchored to phylogenetic data was possible to evidence some events of the karyotype diversification of the studied species and prove an independent origin for the two types of multiple sex chromosomes, XX/XY_1Y_2 and $X_1X_1X_2X_2/X_1X_2Y$, that occur in *Harttia* species. The results point to evolutionary breakpoint regions in the genomes within or adjacent to rDNA sites that were widely reused in *Harttia* chromosome remodeling.

Keywords: chromosomal rearrangements; fish species; sex chromosome systems; whole chromosome painting.

1. INTRODUCTION

Chromosome painting is a good tool for evolutionary investigation, once it may reveal how karyotypes have changed along with their evolutionary history (RIED *et al*., 1998). Chromosome painting is based on fluorescence in situ hybridization (RIED *et al*., 1998). Thus, the generation of probes from whole chromosomes or specific chromosomal regions obtained primarily by microdissection can be established (GUAN; MELTZER; TRENT, 1994, GUAN *et al.,* 1996). Chromosome painting can be used to identify homeologous segments and rearrangements during karyotype evolution (YANG; O'BRIEN; FERGUSON-SMITH, 2000; VENTURA *et al*., 2009; SCHEMBERGER *et al*., 2011; DENG *et al*., 2013; GOKHMAN *et al*., 2019; TARGUETA *et al*., 2021). In teleosts, where banding patterns are not easily induced, a series of chromosomal rearrangements can be underestimated (SHARMA; TRIPATHI; SHARMA, 2002). Accordingly, comparative chromosomal mapping can be an appropriate method to reveal genomic rearrangements than the conventional cytogenetic bands in fishes (NAGAMACHI *et al*., 2010, 2013; CIOFFI *et al*., 2011; PUCCI *et al*., 2014; OLIVEIRA *et al*., 2018).

Chromosome breakage in evolution can be a nonrandom event, and it has been observed that specific genomic regions are more propensity to break and trigger rearrangements than others (PEVZNER; TESLER, 2003; LARKIN *et al*., 2009). Genomic regions where the gene order has been conserved among species correspond to homologous synteny blocks (MURPHY *et al*., 2005; RUIZ-HERRERA; CASTRESANA; ROBINSON, 2006). In this pathway, those small regions where the synteny has been disrupted by chromosomal reorganization may be named evolutionary breakpoint regions (MURPHY *et al*., 2005; RUIZ-HERRERA; CASTRESANA; ROBINSON, 2006; FARRÉ *et al*., 2011). The latter are enriched with repetitive

sequences, including transposable elements, tandem repeats, and segmental duplications, providing conditions for non-allelic homologous recombination (PEVZNER; TESLER, 2003; BAILEY *et al*., 2004; MURPHY *et al*., 2005). It is suggested that these specific sites have been repeatedly used (i.e., reused) during chromosomal evolutionary processes (RUIZ-HERRERA; CASTRESANA; ROBINSON, 2006; CARBONE *et al*., 2009; LONGO *et al*., 2009; FARRÉ *et al*., 2011).

Loricariidae is one of the largest families of freshwater fishes, with over 1,000 valid species grouped in more than 100 genera and distributed throughout South and Central America (REIS; KULLANDER; FERRARIS JR., 2003; FRICKE *et al*., 2021). This family shows a substantial numerical and structural variation in karyotypes, mainly due to Robertsonian rearrangements (ARTONI; BERTOLLO, 2001; KAVALCO *et al*., 2004; ZIEMNICZAK *et al*., 2012; SASSI *et al*., 2020; DEON *et al*., 2020), making it an outstanding group to investigate evolutionary processes (MARIOTTO *et al*., 2011; BARROS *et al*., 2017; GLUGOSKI *et al*., 2018, 2020). In some genera, the reuse of double-strand breaks suggests the occurrence of evolutionary breakpoint regions probable adjacent to rDNAs sites, as proposed for *Ancistrus* (BARROS *et al*., 2017), *Rineloricaria* (GLUGOSKI *et al*., 2018), and *Harttia* (DEON *et al*., 2020).

Harttia includes a wide chromosomal variation in their diploid numbers (2n = 52 - 62), karyotypes, number and position of the ribosomal clusters, and presence of sex chromosome systems (BLANCO *et al*., 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021). Until now, three different sex chromosome systems have been reported in *Harttia*: i) an XX/XY_1Y_2 system in *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 1 (BLANCO *et al*., 2013; DEON *et al*., 2020); ii) an $X_1X_1X_2X_2/X_1X_2Y$ system in *H. duriventris*, *H. punctata* and *H. villasboas* (BLANCO *et al*., 2014; SASSI *et al*., 2020) and iii) an XX/XY system in *H. rondoni* (SASSI *et al*., 2020). Given the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome

system, *H. punctata* presents 2n=58 chromosomes in females and 2n=57 chromosomes in males, characterized by an exclusive submetacentric chromosome in the heterogametic sex (BLANCO *et al*., 2014). In this species, both ribosomal cistrons are related to sex chromosomes, with 5S rDNA sites found in the terminal region of the X_1 pair in females and the X_1 and Y chromosome in males, and with 45S rDNA sites being present in both X_2 chromosomes in females and the single one in males (BLANCO *et al*., 2014). Chromosomal breaks and translocation events spanning the chromosomes 25 (X_1) and 26 (X_2) were proposed as ancestors of the Y chromosome (BLANCO *et al*., 2014).

In this study, two probes for the whole X_1 and X_2 chromosomes of *H. punctata* (HPU-X_1 and HPU-X_2, respectively) were obtained by microdissection. So, the probes were used for comparative whole chromosome paintings (WCP) among ten *Harttia* species to characterize homologous chromosome blocks and probable evolutionary breakpoint regions promoting karyotype differentiation.

2. MATERIAL AND METHODS

2.1 Specimens and chromosome preparation

Representatives of ten *Harttia* species from South and Southeast Brazilian drainages here analyzed **(Table 1, Figure 1)**. Fish were collected with the authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-License Nos. 10538-3 and 15117-2), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09). All species, including two taxonomically undescribed species in the scientific literature, *Harttia* sp. 1 and *Harttia* sp. 2, were identified based on their morphological features by Dr. Oswaldo Oyakawa (curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo - MZUSP). *Harttia* sp. 1 and *Harttia* sp. 2 karyotypes have already been published by Deon *et al*. (2020).

Mitotic chromosomes were obtained from kidney cells, according to Bertollo; Cioffi and Moreira-Filho (2015). The experiments were conducted under the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos approval (Process number CEUA 1853260315). Cell preparations were dropped onto clean glass slides at 55 °C and stained with Giemsa solution 5%.

2.2 Chromosome microdissection, probes, and labeling

Fifteen copies of the X_1 and X_2 chromosomes of *H. punctata* were isolated by microdissection and amplified using the procedure described in Yang *et al*. (2009). Their obtained probes HPU-X_1 and HPU-X_2 were then labeled with Spectrum Orange-dUTP and Spectrum Green-dUTP (Vysis, Downers Grove, USA), respectively, in a secondary

DOP-PCR, using 1 µl of the primarily amplified product as a template DNA, following Yang and Graphodatsky (2009). All the microdissection procedures were performed in the Molecular Cytogenetics Laboratory at the Institut für Humangenetik at Universitätsklinikum Jena, Germany.

2.3 Fluorescence *in situ* hybridization (FISH) for WCP

Slides were prepared and pre-treated according to Yang *et al*. (2009) and denatured in 70 % formamide/2x SSC for 3 min at 72 °C. For each slide, 12 µl of hybridization solution (containing 0.2 µg of each labeled probe, 50 % formamide, 2x SSC, and 10 % dextran sulfate) were denatured for 10 minutes at 75 °C and allowed to pre-hybridize for 1h at 37 °C. To block the hybridization of high-copy repeat sequences, 20 µg of C_0t-1 DNA, directly isolated from *H. punctata* male genome, were prepared according to Zwick *et al*. (1997). Hybridization was done for 48 h at 37 °C in a moist chamber. Post-hybridization washes were performed in 1x SSC for 5 min at 65 °C and 5min in 4xSSC/Tween at room temperature. Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector, Burlingame, CA, USA).

2.4 FISH for 5S and 18S rDNA

Two tandemly arrayed rDNA probes were obtained by PCR from the nuclear DNA of *Harttia intermontana*. The 5S rDNA probe included 120 base pairs (bp) of the 5S rRNA transcript region and 200 bp of a non-transcribed spacer, isolated according to Martins and Galetti Jr (1999) using the primers A (5'-TCAACCAACCACAAAGACATTGGCAC-3') and B (5'-TAGACTTCTGGGTGGCCAAAGGAATCA-3'). The 18S rDNA probe contained a

1,400 bp segment of the 18S rRNA gene and was isolated following Cioffi *et al*. (2009) using the primers 18SF (5'-CCGAGGACCTCACTAAACCA-3') and 18SR (5'-CCGCTTTGGTGACTCTTGAT-3'). Both probes were directly labeled with the Nick-Translation mix kit (Jena Bioscience, Jena, Germany): the 5S rDNA with ATTO550-dUTP (Jena Bioscience) and the 18S rDNA with AF488-dUTP (Jena Bioscience), following the manufacturer's manual. FISH experiments followed the methodology described in Yano; Bertollo and Cioffi (2017).

2.5 Images capture and processing

Metaphase plates were captured using an Olympus BX50 light microscope (Olympus Corporation, Ishikawa, Japan) with a CoolSNAP camera. The images were processed using the Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA).

3. RESULTS

HPU-X_1 and HPU-X_2 probes hybridized in *H. punctata* X_1 and X_2 chromosomes, and results revealed that a DNA segment in common was present in their proximal regions **(Figure 2a)**. In male karyotype, HPU-X_1 and HPU-X_2 probes detected the monovalent X_1 and X_2, and also the Y chromosome was stained by HPU-X_1 probe in its distal region of the long arm (q) and by HPU-X_2 signal in the short arm (p) **(Figure 2b)**. A sequential FISH using the 5S and 18S rDNA probes efficiently detected 5S rDNA on X_1 and Y chromosomes and 18S rDNA on X_2 chromosome **(Figure 2 c**,**d)**.

Cross-species FISH using the two WCPs was performed among all the nine other species from Table 1 **(Figures 3** and **4)**, and their signals were compared to *H. punctata* karyotype **(Figure 5a)**. In *H. kronei*, the HPU-X_1 painted chromosome 9q and distal region of chromosome 2p, while HPU-X_2 painted chromosome 9p **(Figures 3a**, **4a**, and **5b)**. The 5S and 18S rDNAs were mapped in the proximal regions of chromosomes 9q and 2p, respectively **(Figures 3a**, **4a**, and **5b)**. In *H. longipinna*, the HPU-X_1 probe hybridized in chromosome 24q and the adjacent regions to the secondary constriction of chromosome 23, while the HPU-X_2 probe hybridized in chromosome 26 **(Figures 3b**, **4b**, and **5c)**. Besides that, the 5S and 18S rDNAs were detected in proximal regions of chromosomes 11p and 23q, respectively **(Figures 3b**, **4b**, and **5c)**. In *H. gracilis*, HPU-X_1 and HPU-X_2 hybridized in pairs 27 and 29, respectively **(Figures 3c**, **4c**, and **5d)**. The 5S and 18S rDNAs sites were in situ localized in the proximal regions of chromosomes 2p and 26q, respectively **(Figures 3c**, **4c**, and **5d)**.

Harttia loricariformis showed the HPU-X_1 probe hybridized in chromosome 25q, the HPU-X_2 in chromosome 10p, the 5S rDNA in the distal region of 25q, and the 18S rDNA probe located in the distal region of 25p **(Figures 3d**, **4d**, and **5e)**. *Harttia torrenticola* showed the HPU-X_1 hybridized in chromosome 24, the HPU-X_2 probe in

chromosome 21, and the 5S and 18S rDNAs in the proximal regions of chromosomes 3p and 22q, respectively **(Figures 3e**, **4e**, and **5f)**. In *Harttia* sp. 1, the HPU-X_1 probe hybridized in chromosome 22 and the HPU-X_2 probe in chromosome 14 **(Figures 3f**, **4f**, and **5g)**. The 5S rDNA was detected in the proximal region of chromosome 12p and the distal region of chromosome 20q, the last chromosome also bearing the 18S rDNA cluster **(Figures 3f**, **4f**, and **5g)**.

Harttia carvalhoi showed the HPU-X_1 probe hybridized in chromosome 25 and the HPU-X_2 probe in chromosome 15 **(Figures 3g**, **4g**, and **5h)**. The 5S rDNA probe hybridized in the proximal region of chromosome 3p and the distal region of chromosome 23q, while the 18S rDNA probe hybridized in the proximal region of 23q **(Figures 3g**, **4g**, and **5h)**. In *H. intermontana*, the HPU-X_1 and the HPU-X_2 probes hybridized in the same chromosome, i.e., 22p and 22q regions, respectively **(Figures 3h**, **4h**, and **5i)**. The 5S and 18S rDNA probes hybridized in the proximal regions of the chromosomes 11p and 2p, respectively **(Figures 3h**, **4h**, and **5i)**. *Harttia* sp. 2 showed the HPU-X_1 probe hybridized in the distal middle region of chromosome 9q and in the proximal region of the 22q, while the HPU-X_2 probe hybridized in chromosome 30 **(Figures 3i**, **4i**, and **5j)**. The 5S and 18S rDNAs were evidenced in the proximal regions of chromosomes 9q and 22q, respectively **(Figures 3i**, **4i**, and **5j)**. All the results obtained with the HPU-X_1, HPU-X_2, 5S rDNA and 18S rDNA probes location were summarized in Table 2.

4. DISCUSSION

A combined molecular and morphological phylogeny of the Harttiini and Farlowellini tribes recognized three distinct clades for the *Harttia* genus (LONDOÑO-BURBANO; REIS, 2021). These clades grouped species according to their South American distribution: (i) from the Guyana shield rivers; (ii) from the northern Brazilian rivers; and (iii) from the Brazilian south/southeast rivers (LONDOÑO-BURBANO; REIS, 2021). Karyotype evolution scenarios have been proposed in *Harttia* lineage, anchoring the chromosomal data to Harttiini phylogeny (BLANCO *et al*., 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021). In all scenarios, extensive events of chromosomal remodeling have been identified in *Harttia*, changing the 2n, chromosome morphologies and triggering sex chromosome systems origin independently in each clade (BLANCO et al., 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021), as also identified in this study.

Both *H. punctata* derived probes (HPU-X_1 and HPU-X_2) were able to detect homeologous chromosome blocks in *Harttia* species highlighting chromosomal rearrangements that occurred during the lineage evolution. WCP has also been used for genomic comparisons to detect homeologous blocks among different species (VENTURA *et al*., 2009). Regarding the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system origin in *H. punctata*, the HPU-X1 and XPU-X2 hybridizations corroborate the proposal of Blanco *et al.* (2014). In this proposal, one translocation event involving chromosomes 25 and 26 (nowadays representing chromosomes X_1 and X_2, respectively), with proximal segments lost, gave rise to the Y chromosome (BLANCO *et al*., 2014, Figure 5k). It is also relevant to point out that no positive signs of the HPU-X_1 and HPU-X_2 probes were found on the XY_1Y_2 chromosomes of *H. carvalhoi*, *Harttia* sp.1, and *H. intermontana* from the Brazilian south/southeast clade (Figures 5 and 6). This data indicates an independent origin for the two models of the multiple sex chromosome systems - X_1X_2Y

and XY_1Y_2 - that occur in the *Harttia* genus, an evolutionary route also proposed for some other teleost groups (DEVLIN; NAGAHAMA, 2002; CIOFFI *et al*., 2013; SEMBER *et al*., 2018).

An ancestral karyotype with 2n=58 chromosomes and without a differentiated sex chromosome system is proposed to the *Harttia* lineage (BLANCO *et al*., 2017). Based on phylogenetic data (COVAIN *et al*., 2016; LONDOÑO-BURBANO; REIS, 2021) and the description of the 2n=58 chromosomes in the sister group *Farlowella* (MARAJÓ *et al*., 2018), the data reinforces the proposal of a putative ancestral karyotype with 2n=58 chromosomes for the *Harttia* clade iii (Figure 6). *Harttia punctata* belongs to the clade (ii), and their X_1 and X_2 chromosomes were applied in WCP in species from the clade (iii) of *Harttia* to evaluate the chromosomal diversification. Following a probable diversification scenario in species from the clade (iii), *H. kronei* presented *H. punctata* X_1 in the distal regions of chromosome 2p and 9q, while the arm 9p represents chromosome X_2 (Figures 5 and 6). Besides that, the proximal regions of the chromosomes 2p and 9q are arranged by 45S and 5S rDNAs, respectively (Figures 5 and 6). The WCPs and rDNA in situ localization suggest sites prone to break within or adjacent to the rDNA sites were widely reused throughout the chromosomal evolution of *Harttia*, as can be observed in species from the clade iii.

Chromosomal breaks in the centromere region of chromosomes 2 and 9 from *H. kronei* followed by rearrangements could originate the chromosomes 10 and 25 in *H. loricariformis*. Since double-strand breaks close to rDNA sites have occurred, the chromosome arm 10p from *H. loricariformis* keeps a homeologous block with 9p of the *H. kronei* (**Figures 5** and **6**). At the same time, a fusion of the chromosome arms 2p and 9q from *H. kronei* could organize the acrocentric pair 25 bearing 5S and 45S rDNA sites of the *H. loricariformis* (**Figures 5** and **6**). In this pathway, the chromosomes 10 and 25

are not evolved in the 2n reduction to 56 chromosomes in *H. loricariformis*. A Robertsonian fusion could explain the 2n decrease in this species once an interstitial telomeric site was proposed in a large subtelocentric pair (BLANCO *et al*., 2017).

In *H. longipinna* lineage, chromosomal breaks close to rDNA sites rearranged 5S rDNA and 45S rDNA clusters to chromosomes 11 and 23, respectively **(Figures 5** and **6)**. In addition, chromosome fission could originate acrocentrics 24 and 26 carrying the HPU-X_1 and HPU-X_2 homeologous blocks, respectively **(Figures 5** and **6)**. Thus, the 2n=58 chromosomes in *H. longipinna* and *H. gracilis* could be an evolutionary recurrence feature. It is interesting to note, although additional chromosomal changes occurred in chromosomes possessing 5S rDNA, 45 rDNA, HPU-X_1 and HPU-X_2 homeologous blocks, these four chromosomes were kept in *H. longipinna*, *H. gracilis*, *H. torrenticola*, *Harttia* sp.1, and *H. carvalhoi* **(Figures 5** and **6)**. Besides that, the 2n=56 of *H. torrenticola* had an independent mechanism once a Robertsonian fusion was proposed in the origin of its pair 1 (BLANCO *et al*., 2017).

Harttia sp. 1 and *H. carvalhoi* karyotypes presented an extra 5S rDNA site that could emerge by gene units gain and rearrangements. In these species, a transposition could rearrange the extra site to the syntenic condition with 45S rDNA **(Figures 5** and **6)**. In addition, comparing *H. carvalhoi* and *Harttia* sp. 1 karyotypes is possible to detect an inversion relocating the syntenic 5S and 18S rDNA sites **(Figures 5** and **6)**. *Harttia intermontana* lineage showed probable translocations to originate the metacentric 2 bearing the 45S rDNA site and the chromosome 22 bearing the HPU-X_1 and HPU-X_2 homeologous blocks **(Figures 5** and **6)**. Yet, transpositions or translocations rearranged rDNA sites and HPU-X_1 and HPU-X_2 homeologous blocks in the *Harttia* sp. 2 karyotype **(Figures 5** and **6)**. All data demonstrating extensive chromosomal remodeling involving

double-strand breaks and rearrangements reinforce the proposal of evolutionary breakpoint regions close to rDNA sites in *Harttia* lineage (DEON *et al*., 2020).

Ribosomal clusters as promoters of chromosomal reorganization, mainly those located in the pericentromeric regions, have been the focus of previous studies on Robertsonian rearrangements (SULLIVAN *et al*., 1996; ROSA *et al*., 2012; BARROS *et al*., 2017; GLUGOSKI *et al*., 2018). The rDNA sites have been associated with critical chromosomal breakpoints given some features, as follow: tandem arrangements, usually pericentromeric or subterminal locations; ability to transpose; high rates of intra- and inter-chromosomal recombination (CAZAUX *et al*., 2011), in addition to intense gene expression activity (HUANG *et al*., 2008). Several types of rearrangements may result from chromosomal breaks, leading to rapid changes in the distribution of the rDNA sites among closely related species (DATSON; MURRAY, 2006; DEGRANDI *et al*., 2014). Our WCP data in *Harttia* species also indicate that adjacent regions to the rDNAs sites have been extensively reused in the chromosomal diversification of this genus.

The association between chromosomal breaks and rDNA sites is well documented in rodents, especially in *Mus* species (CAZAUX *et al*., 2011). In fish, although highly diverse karyotypes occur among its representatives, few studies portray chromosomal remodeling and its causes. Some of them, using *in situ* hybridization with rDNA probes, indicated that the distribution and dispersion of these sequences may have contributed to genomic diversification among Loricariidae species (KAVALCO *et al*., 2004; ROSA *et al*., 2012; ERRERO-PORTO *et al*., 2014; BARROS *et al*., 2017; PRIMO *et al*., 2017; GLUGOSKI *et al*., 2018, 2020). In *Harttia*, the present data evidences the occurrence of evolutionary breakpoint regions inside or adjacent to the 5S and 18S rDNA sites and their reuse triggering several chromosomal rearrangements during the evolutionary story of this lineage.

The current results cannot explain several chromosomal rearrangements that had occurred during the karyotype evolution of *Harttia*. Among them, the diversified diploid number in *Harttia* sp. 2, the origin of the largest metacentric pair in *H. carvalhoi, H. intermontana, H. torrenticola* and *Harttia* sp.1, and the differentiation of the XY_1Y_2 sex chromosome system in species from the Brazilian south/southeast region. However, our data were able to clarify the reuse of evolutionary breakpoint regions inside or to surround rDNA sites in promoting several rearrangements of homeologous chromosome blocks, and so triggering an extensive chromosomal remodeling among *Harttia* species.

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TABLES AND FIGURES

Table 1: Collection sites of *Harttia* species, with their diploid number (2n) and sample sizes (N).

Species/ Sex chromosome system	2n	Sample collection in the map/ Locality	N
***H. punctata* (X_1X_2Y)**	58♀/57♂	1. Bandeirinha river, Formosa – GO (15º19'25"S 47º25'26"W)	18♀,25♂
H. longipinna	58♀♂	2. São Francisco river, Pirapora – MG (17º21'22.8"S 44º51'0.2"W)	13♀,16♂
H. torrenticola	56♀♂	3. Araras stream, Piumhi – MG (20º16'15"S 45º55'39"W)	8♀, 6♂
***H. intermontana* (XY_1Y_2)**	52♀/53♂	4. Piranga river, Carandaí – MG (20°59'34.0"S 43°43'30.0"W)	20♀, 13♂
H. gracilis	58♀♂	5. Machadinho stream, Santo Antônio do Pinhal – SP (22º48'31"S 45º41'21"W)	18♀,15♂
***Harttia* sp. 1 (XY_1Y_2)**	56♀57♂	6. Macacos stream, Silveira – SP (22°40'43.0"S 44°51'25.0"W)	10♀, 7♂
H. loricariformis	56♀♂	7. Paraitinga river, Cunha – SP (22º52'22"S 44º51'0.2"W)	7♀, 3♂
***H. carvalhoi* (XY_1Y_2)**	52♀/53♂	8. Grande stream, Pindamonhangaba – SP (22º47'8"S 45º27'19"W)	17♀, 12♂
H. kronei	58♀♂	9. Açungui river, Campo Largo – PR (25º22'44"S 49º39'0.8"W)	10♀, 5♂
***Harttia* sp. 2**	62♀♂	10. Barra Grande river, Prudentópolis – PR (24°58'40.72"S 51°7'34.25"W)	17♀, 11♂

SP = São Paulo; MG = Minas Gerais; PR = Paraná; GO = Goiás Brazilian States.

Table 2: Localization of WCP and rDNA probes analyzed in *Harttia* species.

Species	HPU-X_1 probe	HPU-X_2 probe	5S rDNA probe	18S rDNA probe
H. punctata ♂	Chr. 25 (X_1) and Y	Chr. 26 (X_2) and Y	25q distal	26q proximal
H. punctata ♀	Chr.25 (X_1)	Chr. 26 (X_2)	25q distal	26q proximal
H. longipinna ♀♂	24q and 23q proximal	Chr. 26	11p proximal	23q proximal
H. torrenticola ♀♂	Chr. 24	Chr. 21	3p proximal	22q proximal
H. intermontana ♀♂	22p	22q	11p proximal	2p proximal
H. gracilis ♀♂	Chr. 27	Chr. 29	2p proximal	26q proximal
***Harttia* sp. 1** ♀♂	Chr. 22	Chr. 14	12p proximal and 20q distal	20q proximal
H. loricariformis ♀♂	25q	10p	25q distal	25p distal
H. carvalhoi ♀♂	Chr. 25	Chr. 15	3p proximal and 23q distal	23q proximal
H. kronei ♀♂	9q and 2p distal	9p	9q proximal	2p proximal
***Harttia* sp. 2** ♀♂	9q distal	Chr. 30	9q proximal	22q proximal

p = short arms; q = long arms; Chr. = chromosome.

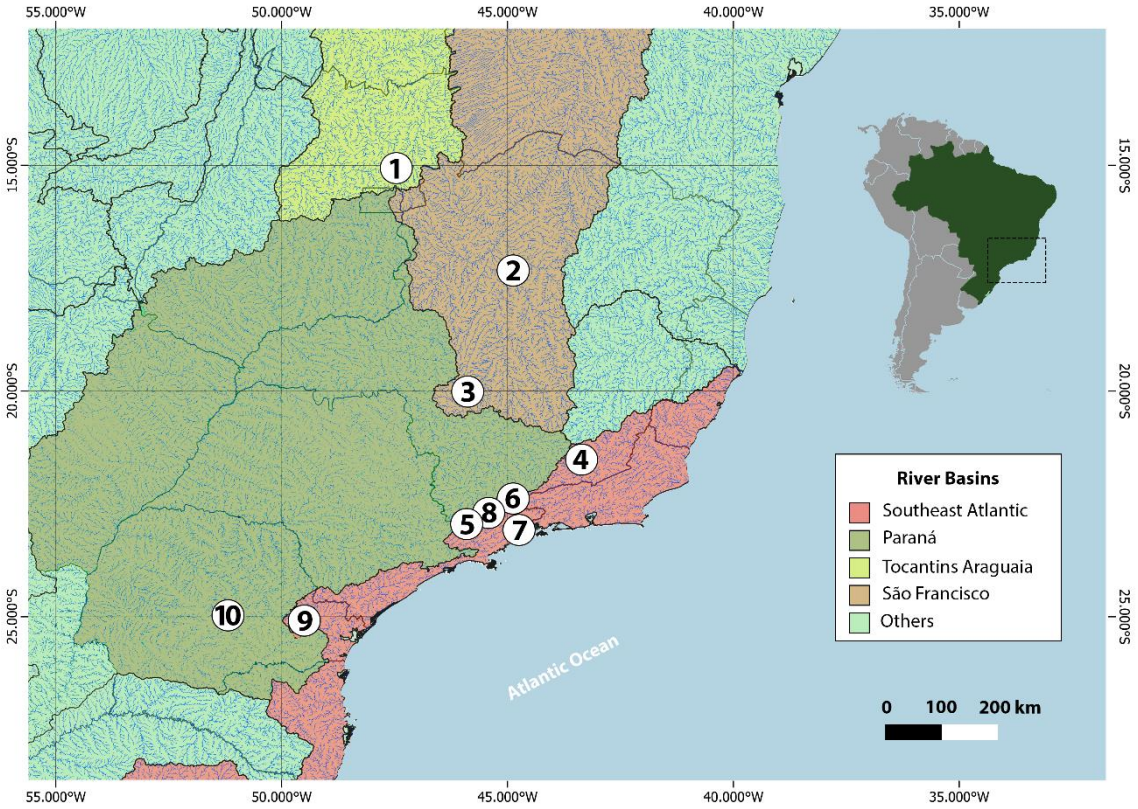

Figure 1: Partial map of South America highlighting the collection sites of *Harttia* species analyzed in this work: 1. *H. punctata*, 2. *H. longipinna*, 3. *H. torrenticola*, 4. *H. intermontana*, 5. *H. gracilis*, 6. *Harttia* sp. 1, 7. *H. loricariformis*, 8. *H. carvalhoi*, 9. *H. kronei*, and 10. *Harttia* sp. 2. Map created using QGis 3.4.3.

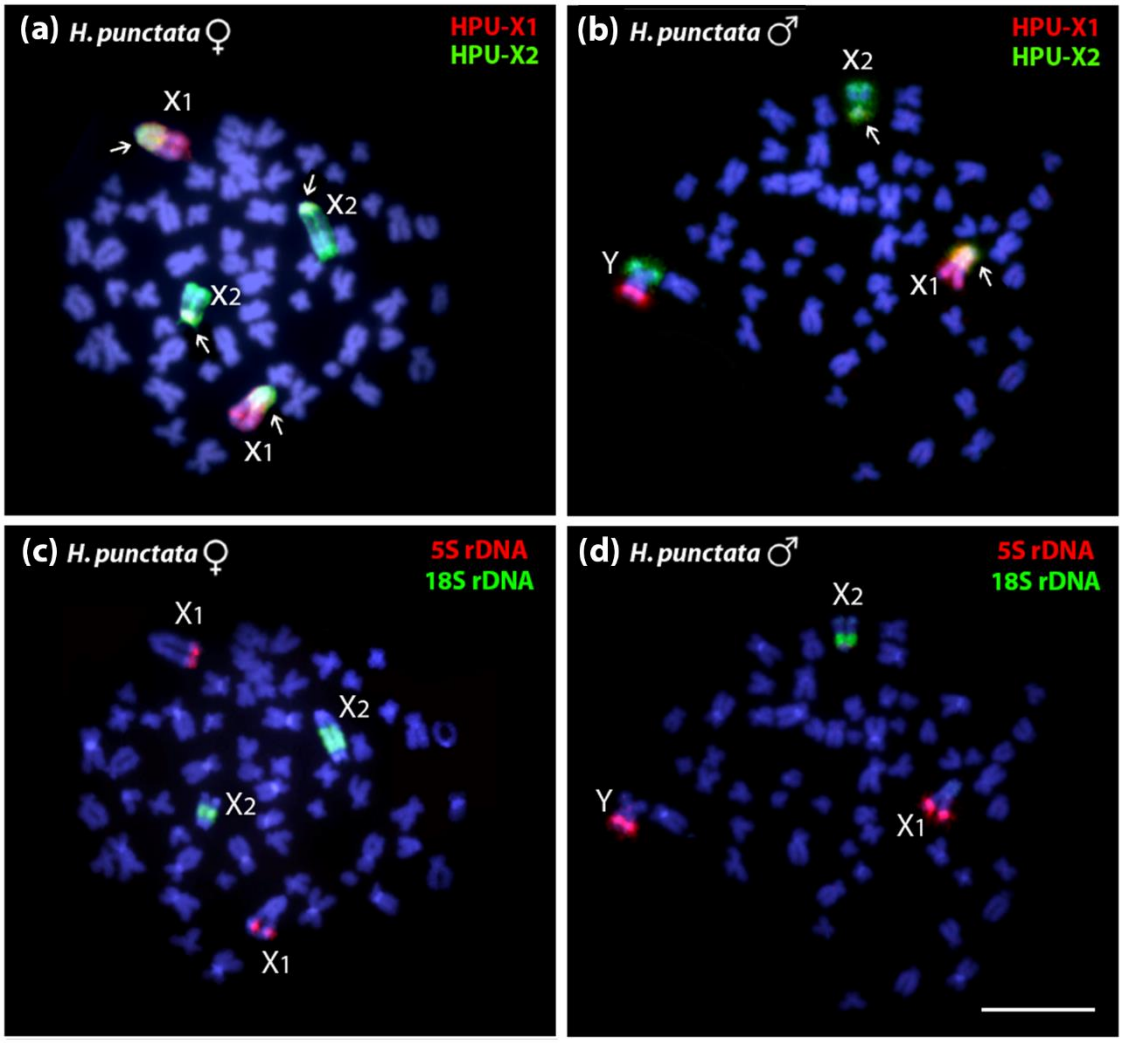

Figure 2: Fluorescence in situ hybridization results using the HPU-X_1 (red) and HPU-X_2 (green) probes in female (2n=58) and male (2n=57) chromosomes of *H. punctata*, and sequential FISH with 5S rDNA (red) and 18S rDNA (green) probes. The white arrows indicate overlapping signals and represent DNA segments in common. Chromosomes were counterstained with DAPI (blue). Bar = 5 µm.

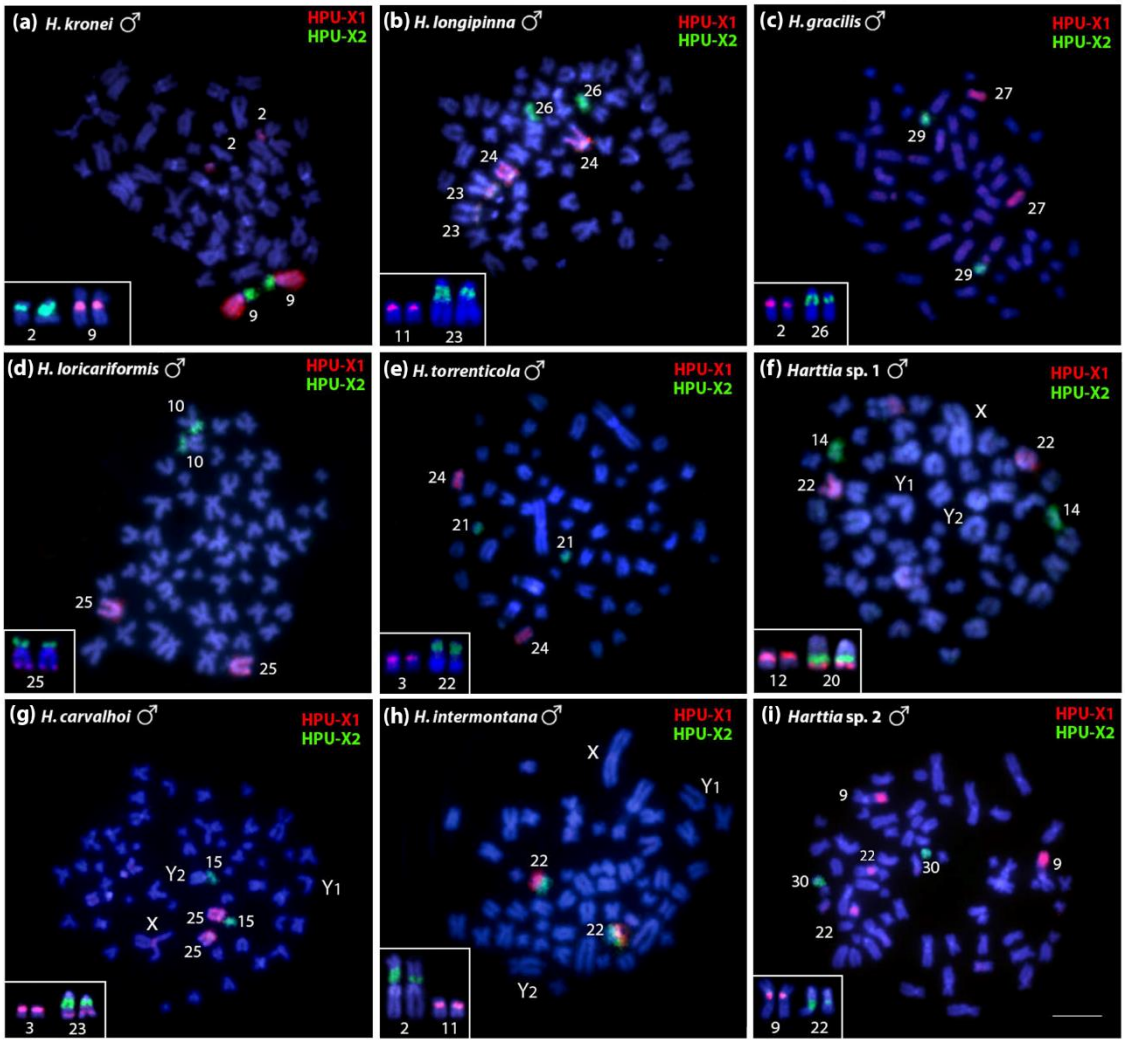

Figure 3: WCP using the HPU-X_1 (red) and HPU-X_2 (green) probes from *Harttia punctata* for comparative analyses in nine other *Harttia* species (male metaphases): (a) *H. kronei* (2n=58), (b) *H. longipinna* (2n=58), (c) *H. gracilis* (2n=58), (d) *H. loricariformis* (2n=56), (e) *H. torrenticola* (2n=56), (f) *Harttia* sp. 1 (2n=57), (g) *H. carvalhoi* (2n=53), (h) *H. intermontana* (2n=53), and (i) *Harttia* sp. 2 (2n=62). The chromosomes displaying the 5S rDNA (red) and 18S rDNA (green) sites are highlighted in the boxes. Chromosomes were counterstained with DAPI (blue). Bar = 5 µm.

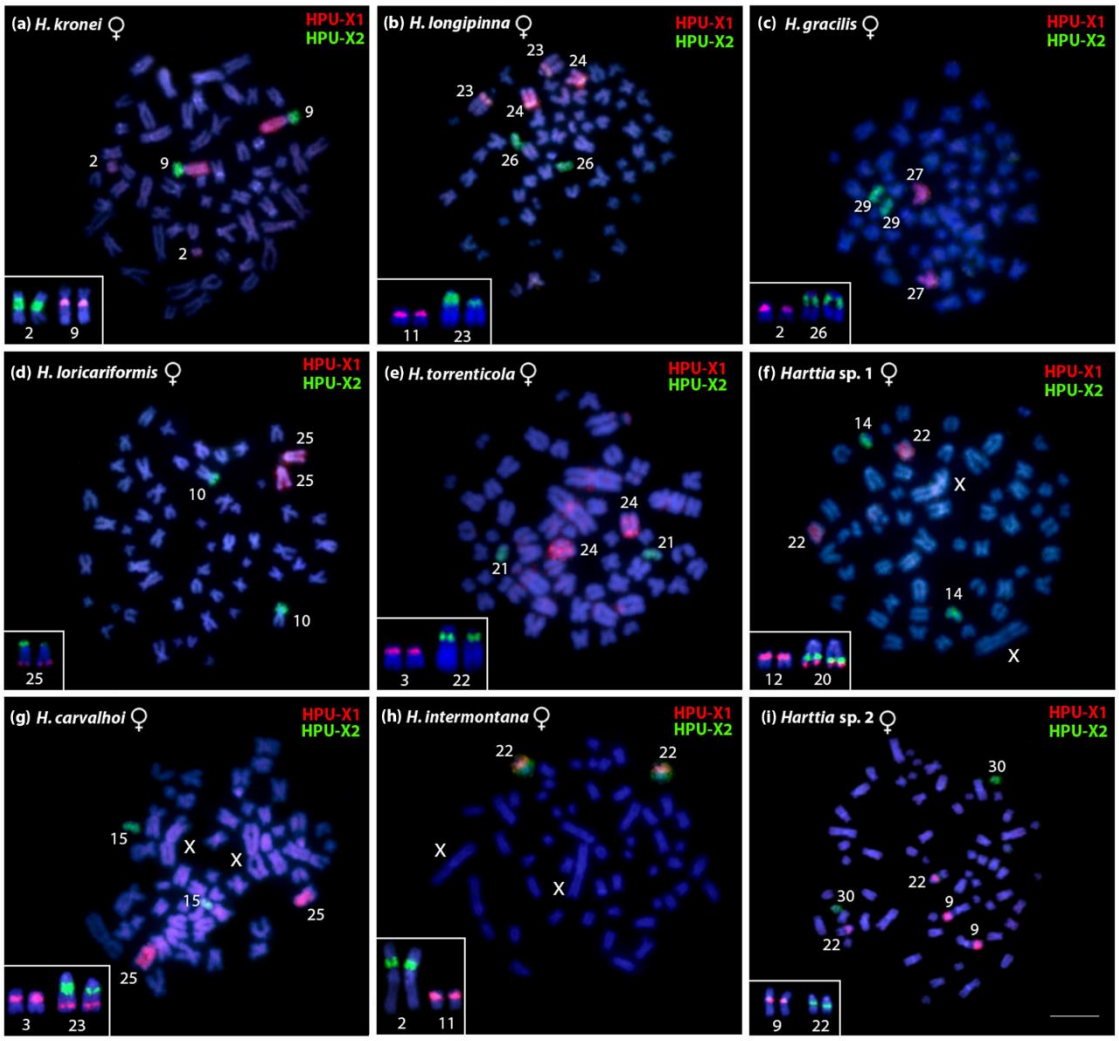

Figure 4: WCP using the HPU-X_1 (red) and HPU-X_2 (green) probes from *Harttia punctata* for comparative analyses in nine other *Harttia* species (female metaphases): (a) *H. kronei* (2n=58), (b) *H. longipinna* (2n=58), (c) *H. gracilis* (2n=58), (d) *H. loricariformis* (2n=56), (e) *H. torrenticola* (2n=56), (f) *Harttia* sp. 1 (2n=56), (g) *H. carvalhoi* (2n=52), (h) *H. intermontana* (2n=52), and (i) *Harttia* sp. 2 (2n=62). The chromosomes displaying the 5S rDNA (red) and 18S rDNA (green) sites are highlighted in the boxes. Chromosomes were counterstained with DAPI (blue). Bar = 5 µm.

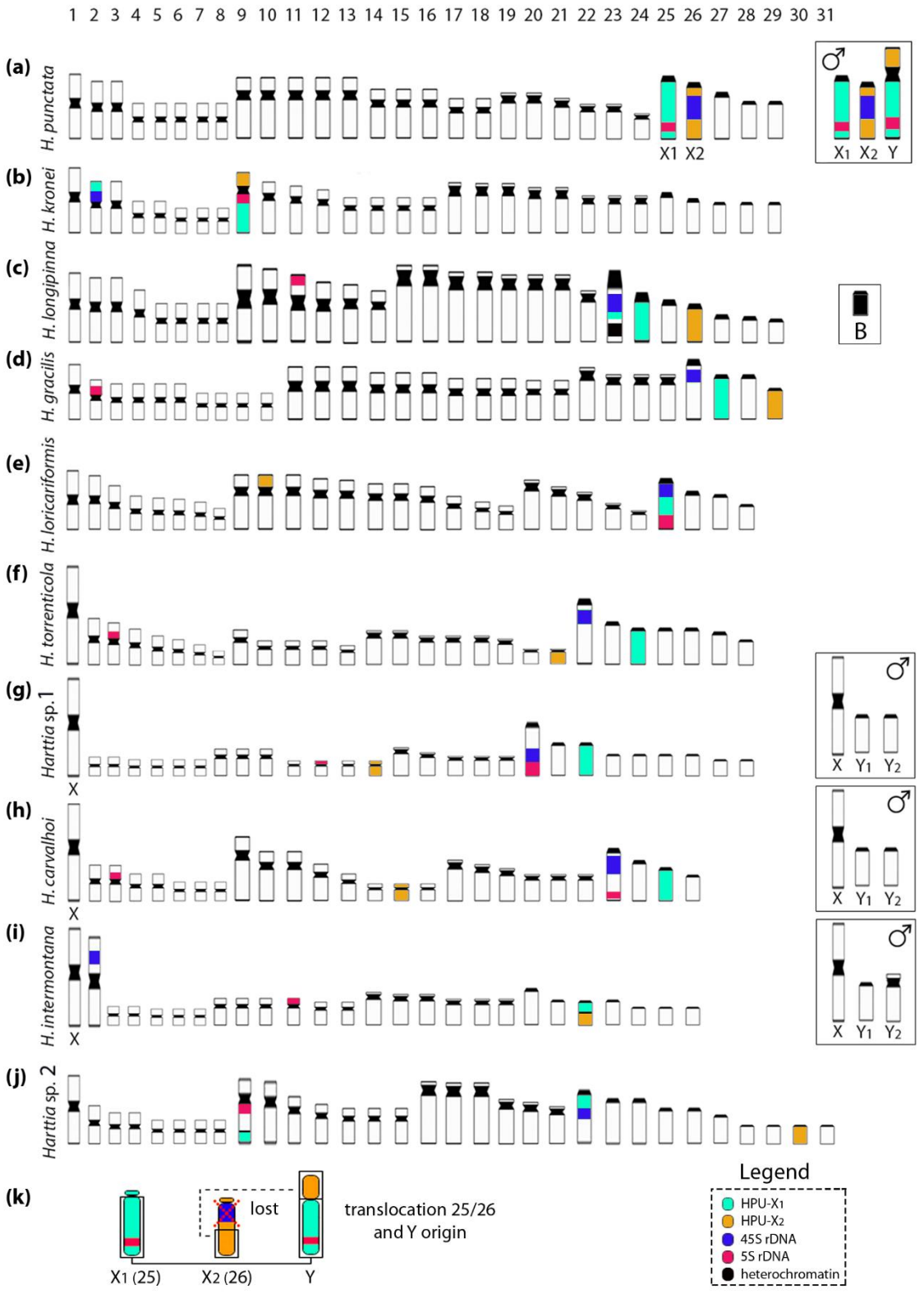

Figure 5: Idiograms representatives of the *Harttia* species analyzed in this study with HPU-X_1, HPU-X_2, 5S rDNA, and 18S rDNA probes. In (a) *H. punctata* idiogram demonstrating the structure of the X_1 and X_2 chromosome probes used for comparative whole chromosome paintings in this study (5S rDNA site on X_1 and 45S rDNA on X_2);

(b-j) idiograms representatives of the nine *Harttia* species (*H. kronei*, *H. longipinna*, *H. gracilis*, *H. loricariformis*, *H. torrenticola*, *Harttia* sp. 1, *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 2, respectively) from South and Southeast of Brazil demonstrating the HPU-X_1 and HPU-X_2 homeologs blocks; and (k) schematic representation based on Blanco *et al*. (2014) of the rearrangements between the 25 and 26 male chromosomes giving rise to the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system of *Harttia punctata*, as clarified by chromosomal painting.

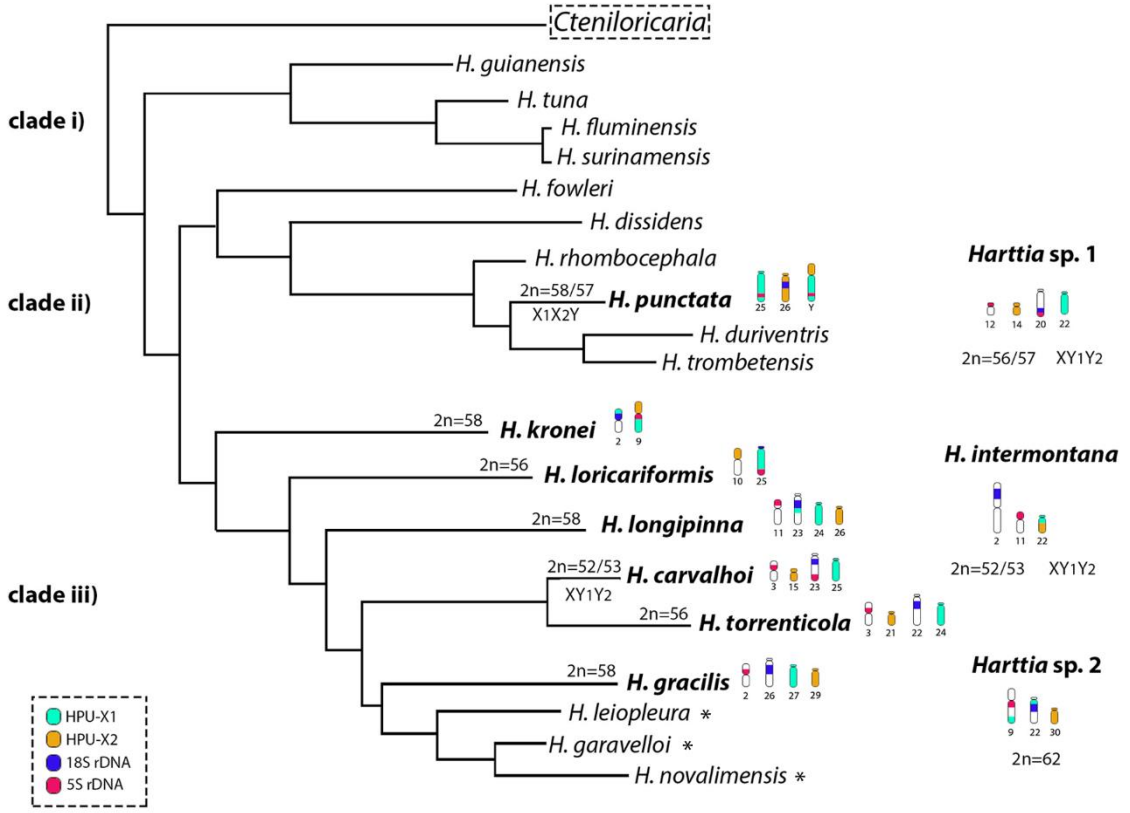

Figure 6: Schematic representation of the phylogenetic relationships among *Harttia* species from the Londoño-Burbano and Reis (2021). On the branches side, idiogramatic representation of the chromosomes bearing 5S rDNA, 45 rDNA, HPU-X_1 and HPU-X_2 homeologous blocks. These regions triggered extensive chromosomal remodeling in the *Harttia* lineage. On the right side, an idiogramatic representatiton of the chromosomes bearing 5S rDNA, 45 rDNA, HPU-X_1 and HPU-X_2 homeologous blocks in species not present on original phylogeny (*Harttia* sp. 1, *H. intermontana*, and *Harttia* sp. 2) * Species without cytogenetic characterization in *Harttia* clade iii.

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Capítulo 3

Chromosomal rearrangements and origin of the multiple XX/XY$_1$Y$_2$ sex chromosome system in *Harttia* species (Siluriformes: Loricariidae)

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Abstract

The Neotropical genus *Harttia* comprises species with extensive chromosomal remodeling and distinct sex chromosome systems (SCSs). So far, three different SCSs with male heterogamety have been characterized in the group. In some species, the presence of the XX/XY$_1$Y$_2$ SCS is associated with a decrease in diploid numbers and several chromosomal rearrangements, although a direct relation to sex chromosome differentiation has not been shown yet. Here, we aimed to investigate the differentiation processes that have led to the establishment of the rare XX/XY$_1$Y$_2$ SCS and track its evolutionary history among other *Harttia* species. For that, four whole chromosome painting probes derived from chromosome 1 of *H. torrenticola* (HTO-1), chromosomes 9 and X of *H. carvalhoi* (HCA-9 and HCA-X), and chromosome X from *H. intermontana* (HIN-X) were applied in nine *Harttia* species. Homeologous chromosome blocks were located in *Harttia* species and demonstrated that Robertsonian (Rb) fusions originated HTO-1, HCA-9, and HCA-X chromosomes, while Rb fissions explain Y$_1$ and Y$_2$ sex chromosomes. Specifically, in *H. intermontana*, HCA-X, HCA-9, and the NOR-bearing chromosome demonstrated that homeologous blocks were used in the HIN-X and metacentric pair 2 origins. Consequently, diploid numbers changed between the studied species. Overall, the data also reinforce the existence of unstable genomic sites promoting chromosomal differentiation and remodeling within the genus *Harttia*.

Keywords: fish; karyotype evolution; molecular cytogenetics; unstable genomic sites; whole chromosome painting.

1. INTRODUCTION

Although sex determination can be environmentally determined in some species, it is usually genetically regulated, and often associated with the presence of sex chromosomes (FURMAN *et al*., 2020). According to a widely accepted model, sex chromosomes arise from an autosomal pair due to the emergence of a sex-specific locus in one of the homologous (BULL, 1983; CHARLESWORTH, 2002). Over time, the ancestral homologous pair undergoes divergences in its genetic composition, recombination rate, and morphology, leading to sex chromosomes differentiation (CHARLESWORTH; CHARLESWORTH; MARAIS, 2005; BACHTROG *et al*., 2014). Thus, sex chromosomes can be recognized according to their size and shape in a karyotype (GHIGLIOTTI; CHENG; PISANO, 2016). However, sometimes sex chromosomes are indistinguishable concerning their gross morphology, and so defined as homomorphic ones (CHALOPIN *et al*., 2015). The most common examples of heteromorphic systems are the XX/XY, where the Y chromosome is restricted to males, and the ZZ/ZW, where the W chromosome is restricted to females (GRAVES, 2006). Being observed in most mammals and birds, respectively, both systems show different levels of genetic divergence (GRAVES, 2006; CHALOPIN *et al*., 2015). Although little is known about why and how different SCSs have evolved (TREE OF SEX CONSORTIUM, 2014), the processes associated with their evolutionary origin and differentiation among vertebrates have awoken considerable interest (DEVLIN; NAGAHAMA, 2002). In fishes, a high diversity of sex-determining mechanisms and SCSs with independent origins can be found (DEVLIN; NAGAHAMA, 2002; SEMBER *et al*., 2021), thus making comparative evolutionary analyzes somewhat difficult. Fishes' species often present high plasticity concerning sex chromosomes, including none or only

subtle changes between the sex pair to major chromosomal rearrangements and size differences (DEVLIN; NAGAHAMA, 2002).

Among the multiple SCSs, the following types were already identified in fishes $X_1X_1X_2X_2/X_1X_2Y$, $X_1X_1X_2X_2/X_1Y_1X_2Y_2$, XX/XY_1Y_2, $Z_1Z_1Z_2Z_2/Z_1Z_2W_1W_2$, and ZZ/ZW_1W_2 (KITANO; PEICHEL, 2012). While the $X_1X_1X_2X_2/X_1X_2Y$ system is well-represented among several fish families, the XX/XY_1Y_2 system is found only in a few (KITANO; PEICHEL, 2012; SEMBER *et al*., 2021). In contrast to simple SCSs, where repetitive DNAs play an essential role in sex chromosome differentiation (YANO *et al*., 2014; SCHEMBERGER *et al*., 2019), multiple SCSs appear forced by divergent evolutionary trends. It appears that chromosomal rearrangements are more relevant to the evolutionary process of multiple SCSs than the accumulation of repetitive sequences (ALMEIDA *et al*., 2015). For this reason, molecular cytogenetic procedures based on fluorescence *in situ* hybridization (FISH), e.g., using whole chromosome painting (WCP) probes, has been successfully applied in different fish groups, providing new insights into the differentiation of sex chromosomes, especially for multiple ones (CIOFFI *et al*., 2011; BLANCO *et al*., 2014; OLIVEIRA *et al*., 2018; MORAES *et al*., 2019).

Harttia is a Neotropical fish group comprising species with distinct diploid numbers and karyotypic variations emerged by extensive evolutionary conserved chromosomal rearrangements (BLANCO *et al*., 2013, 2014, 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021). The chromosomal number ranges from 2n=52 to 62, including B chromosomes and different SCSs (BLANCO *et al*., 2012, 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021). In phylogenetic reconstructions, three distinct clades were proposed for the genus, thus reinforcing the extensive diversification experienced by the lineage; also, it is grouping the species according to their South American distribution: (i) from the Guyana shield rivers; (ii) from the northern Brazilian rivers; and (iii) from

the Brazilian south/southeast rivers (LONDOÑO-BURBANO; REIS, 2021). Three SCSs were detected so far: (1) the $X_1X_1X_2X_2/X_1X_2Y$ system, present in *H. punctata*, *H. duriventris,* and *H. villasboas*, and (2) a proto/neo-XX/XY system in *H. rondoni*, both belonging to clade ii, and (3) the XX/XY_1Y_2 system in *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 1, species which belong to clade iii (CENTOFANTE; BERTOLLO; MOREIRA-FILHO, 2006; BLANCO *et al*., 2017; SASSI *et al*., 2020, 2021; DEON *et al*., submitted). Chromosomal data compared to a phylogenetic framework indicate that ancestral karyotype with 2n=58 chromosomes and without a differentiated SCS could represent a plesiomorphic condition for clade iii (DEON *et al*., submitted). Belonging to the same clade iii, the species *H. torrenticola* has a karyotype composed by 2n=56, undifferentiated sex chromosomes (BLANCO *et al*., 2013), and a large metacentric pair being morphologically similar to the X chromosome of *H. carvalhoi*. WCP-FISH experiments using X_1 and X_2 probes derived from *H. punctata*, confirmed that chromosomes that gave rise to the X_1X_2Y and the XY_1Y_2 systems are evolutionary independent (DEON *et al*., submitted).

Here, we aimed to investigate the differentiation processes that have led to the establishment of the rare XX/XY_1Y_2 SCS and to track its evolutionary history among other *Harttia* species. For that, we performed a WCP-FISH investigation using four distinct sex chromosome-specific probes hybridized in several species. The results allowed us to identify the main rearrangements involved in the origin of this unique SCS. Besides, the data provide new insights into the origin and evolution of such a rare XY-derived SCS, consequently increasing our knowledge about the evolution of vertebrate sex chromosomes.

2. MATERIAL AND METHODS

2.1 Individuals and chromosome preparation

Representatives of *Harttia* species analyzed in this study are summarized in **Table 1.** Specimens were collected with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-Licenses No. 10538-3 and 15117-2), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09), Brazil. Species were identified based on their morphological features by Dr. Oswaldo Oyakawa (curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP), Brazil.

Mitotic chromosomes were obtained from kidney cells, according to Bertollo; Cioffi and Moreira-Filho (2015). All procedures agreed with the Ethics Committee of Animal Usage of the Universidade Federal de São Carlos (Process number CEUA 1853260315), Brazil.

2.2 Chromosome microdissection, probe preparation, and labeling

Fifteen copies of each target chromosome were isolated by glass-needle-based microdissection, and obtained DNA was by oligonucleotide primed-polymerase chain reaction (DOP-PCR) as described in Yang *et al*. (2009). Chromosomes were chosen based on their morphology - bi-armed chromosomes that were suspected to be originated from Robertsonian fusions were targeted: the largest metacentric (HCA-X), and the largest submetacentric (HCA-9) from *H. carvalhoi*; the largest metacentric (HIN-X) from *H. intermontana*, and the largest metacentric (HTO-1) from *H. torrenticola* (**Figure 1**). Probes were labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis,

Downers Grove, USA) in a secondary DOP-PCR, using 1µl of the primarily amplified product as a template DNA (YANG; GRAPHODATSKY, 2009).

2.3 Fluorescence *in situ* hybridization (FISH)

Slides were prepared and pre-treated according to Yang *et al*. (2009) and denatured in 70% formamide/2x SSC for 3 min at 72°C. For each slide, 12 µl of hybridization solution (containing 0.2 µg of each labeled probe, 50% formamide, 2×SSC, 10% dextran sulfate, and 5µg of salmon sperm DNA) was denatured for 10 minutes at 75ºC and incubated to pre-hybridize for 1h at 37ºC. To block the hybridization of high-copy repeat sequences, 20 µg of C0t-1 DNA, directly prepared from *H. carvalhoi, H. torrenticola*, and *H. intermontana* male genomes were used, according to Zwick *et al*. (1997). Hybridization took place for 48h at 37°C in a moist chamber. Post-hybridization washes were performed in 1×SSC for 5min at 65°C, and 5min in 4×SSC/Tween at room temperature. Finally, the slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector, Burlingame, CA, USA).

2.4 Image analyses and processing

Metaphase plates were captured using an Olympus BX50 light microscope (Olympus Corporation, Ishikawa, Japan) coupled with a CoolSNAP camera. The images were processed using Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA).

3. RESULTS

Results obtained by HCA-X and HCA-9 probes are summarized in **Figure 2** and **Table 2**. In *H. carvalhoi* (52♀/53♂ - XX/XY_1Y_2), the HCA-X probe successfully identified their X chromosomes in females and the X, Y_1, and Y_2 chromosomes in males. Small centromeric signals in both acrocentric pairs 23 and 24 were also evidenced. In agreement, the HCA-9 probe correctly recognized the submetacentric pair 9 **(Figure 2a**, **b)**. Similarly, in *Harttia* sp. 1 (56♀/57♂ - XX/XY_1Y_2), the HCA-X probe detected the X chromosome pair in females and the X, Y_1, and Y_2 chromosomes in males, besides small centromeric signals in both 20 and 24 acrocentric pairs **(Figure 2c**, **d)**. The HCA-9 hybridized to 21 and 26 acrocentric pairs **(Figure 2c**, **d)**. In females of *H. intermontana* (52♀/53♂ - XX/XY_1Y_2), the HCA-X probe stained the long (q) arms of the chromosomes X and 2 **(Figure 2e)**. In males, this probe gave signals on Xq, the Y_2 chromosome, and the 2q **(Figure 2f)**, as well as in the centromeric region of the pair 24 in both males and females **(Figure 2e, f)**. The HCA-9 probe detected the short (p) arms of the X chromosome and the 20q distal region in females, and the Xp arms, the Y_1 chromosome, and the 20q distal region in males **(Figure 2e, f)**. In *H. punctata* (58♀/57♂ - $X_1X_1X_2X_2/X_1X_2Y$), the HCA-X probe hybridized on the submetacentric pairs 9 and 11, while the HCA-9 probe showed signals on the metacentric pair 8 and subtelocentric pair 19 **(Figure 2g**, **h)**.

The HTO-1 probe, derived from *H. torrenticola*, showed the same results obtained with the HCA-X probe when tested on those species with an identical large metacentric pair **(Supplementary Figure 1)**. In contrast, the HIN-X probe, from *H. intermontana*, showed different results than those obtained applying HCA-X and HTO-1 probes **(Figure 3)**. In *H. carvalhoi*, HIN-X hybridized on the Xq and 9q in females **(Figure 3a)**, and on the Xq, Y_2 chromosome, and 9q in males **(Figure 3b)**. In *Harttia* sp. 1, HIN-X labeled

the Xq and the acrocentric 21 pair in females **(Figure 3c)**, and these same chromosomes, as well as the Y_2 chromosome, in males **(Figure 3d)**. In *H. torrenticola*, HIN-X hybridized in the 1q and the acrocentric 23 pair **(Figure 3e, f)**.

Harttia species without the heteromorphic sex chromosomes were also used as targets for the comparative WCP-FISH using the HCA-X and HCA-9 probes **(Figure 4, Table 2)**. In *H. kronei* (58♀♂) HCA-X hybridized in the subtelocentric pairs 17 and 19, while the HCA-9 hybridized in chromosome pairs 8 and 13 **(Figure 4a)**. In *H. gracilis* (58♀♂), HCA-X marked the submetacentric 11 and the subtelocentric 22, besides centromeric signals in the acrocentric pairs 26 and 28 **(Figure 4b)**, and the HCA-9 probe was detected in chromosomes 10 and 21 **(Figure 4b)**. In *H. longipinna* (58♀♂), HCA-X hybridized in the subtelocentric pairs 15 and 17, besides the centromeric region of the acrocentric pairs 23 and 25, and HCA-9 hybridized in the metacentric 7, and submetacentric 10 (Figure 4c). In *H. loricariformis* (56♀♂), HCA-X was detected in the submetacentric pair 9, subtelocentric 20, and the centromeric region of the chromosome 25 **(Figure 4d)**, while the HCA-9 probe hybridized in chromosome pairs 8 and 11 **(Figure 4d)**. Finally, in *H. torrenticola* (56♀♂), HCA-X hybridized in chromosome 1 and the centromeric region of the acrocentric pairs 22 and 25, while the HCA-9 probe presents signals of hybridization in pairs 8 and 23 **(Figure 4e)**.

4. DISCUSSION

In *Harttia* species, diploid numbers range from 52 to 62 chromosomes (DEON *et al*., 2020; SASSI *et al*., 2020, 2021). Data from phylogeny reconstructions indicate that 58 chromosomes and no large biarmed chromosomes could correspond to a plesiomorphic karyotype condition for species distributed on south and southeast Brazilian drainages – the clade iii (DEON *et al*., submitted). These chromosomal features (**Figure 5**) include the absence of morphologically differentiated sex chromosomes and a single location of the 5S and 45S rDNA sites in medium-sized bi-armed chromosomes (DEON *et al*., 2020, submitted). Here, the ancestral reconstructions of the *Harttia* karyotype, using both HCA-X and HCA-9 probes, demonstrated that two chromosome pairs were probably related to the *H. carvalhoi* chromosomes X and 9 (**Figure 6**). Thus, *in situ* localizations also reaffirm the role of Robertsonian fusions as the main rearrangements responsible for reducing the diploid number in *H. carvalhoi*. These homeologous chromosome pairs (unfused chromosomes) are shared by *H. kronei*, *H. loricariformis*, *H. longipinna*, and *H. gracilis* (**Figure 6**). As common features, *H. kronei*, *H. longipinna*, and *H. gracilis* kept 2n=58 chromosomes and the absence of morphologically differentiated SCSs (BLANCO *et al*., 2017), with chromosomal diversification events mainly occurring by repositioning of the rDNA sites in their karyotypes (DEON *et al*., 2020 submitted). Although *H. loricariformis* decreased the diploid number to 2n=56, this species shares the homologous chromosome pairs to HCA-X and HCA-9 as highlighted in *H. kronei* by WCP-FISH. The presence of interstitial telomeric sites in a subtelocentric chromosome of *H. loricariformis* karyotype suggests an origin by Robertsonian fusions (BLANCO *et al*., 2017). The current data thus support the hypothesis on the occurrence of a chromosomal fusion event in *H. loricariformis*

karyotype, and that this corresponds to an independent evolutionary event being not associated with the chromosomes X and 9 of *H. carvalhoi*.

Data also showed that the chromosomal rearrangements that led to the XX/XY_1Y_2 SCS were triggered within the branch with *H. torrenticola* (**Figure 5**). The phylogenetic branch grouping *H. carvalhoi* and *H. torrenticola* (COVAIN *et al*., 2016; LONDOÑO-BURBANO; REIS, 2021; **Figure 5**) was diversified by Robertsonian fusions, initially giving rise to a large metacentric pair, like that found in the *H. torrenticola* karyotype. Indeed, the large homeologous chromosome regions shared between the chromosomes 1 of *H. torrenticola* (HTO-1) and the X chromosome of *H. carvalhoi* and *Harttia* sp. 1, corroborate that a single evolutionary event of chromosomal fusion would have generated the large metacentric pair in these species. Although *H. torrenticola* does not show sex chromosome heteromorphism related to the metacentric chromosome 1, in *H. carvalhoi* and *Harttia* sp. 1, this chromosome corresponds to the X one, with additional rearrangements triggering the origin of the Y_1 and Y_2 chromosomes. According to former suggestions (BLANCO *et al*., 2017; DEON *et al*., 2020) centric fission on the largest metacentric formed the Y_1 and Y_2 chromosomes in *Harttia* sp. 1, which are also shared by *H. carvalhoi*. The Y_1 and Y_2 positive hybridizations using the HCA-X probe reiterate that centric fission is the main rearrangement related to the origin of the multiple XX/XY_1Y_2 SCS of *H. carvalhoi* and *Harttia* sp. 1.

However, different from *Harttia* sp. 1 (2n=56 in females and 57 in males), *H. carvalhoi* diversified its karyotype by other chromosomal fusions, reducing the diploid number to 2n=52 in females and 2n=53 in males. Based on the HCA-9 WCP-FISH experiments a chromosome fusion between the subtelocentric pairs 21 and 26, like those found in *Harttia* sp. 1, triggered the origin of pair 9 of *H. carvalhoi* (**Figure 5**, **6**). In fact, despite some morphological alterations, this chromosome pair is represented by two

other, homologous pairs in *H. kronei, H. loricariformis, H. longipinna, H. gracilis*, and *H. torrenticola*. Based on conventional cytogenetic studies, Deon *et al*. (2020) proposed the same origin of the XX/XY_1Y_2 system in *H. carvalhoi*, *Harttia* sp. 1, and *H. intermontana*. However, the use of the HCA-X, HCA-9, and HIN-X probes enabled now to evidence that additional rearrangements are associated with the XX/XY_1Y_2 system of *H. intermontana*. The X chromosome of this species comprises the 9q and one arm of the X chromosome of *H. carvalhoi*, indicating a reciprocal translocation between these two chromosome pairs in its origin **(Figure 6)**. After that, centric fission in one of the X chromosomes, followed by a pericentric inversion in one of the resulted elements, generated the Y_1 and Y_2 chromosomes in males **(Figure 7)**. It is relevant to notice that the Y_1 chromosome of *H. intermontana* is derived from chromosome 9 of *H. carvalhoi*, thus different from the Y_1 chromosome of *H. carvalhoi* and *Harttia* sp. 1. In the same way, the metacentric pair 2 of *H. intermontana* was originated from species-specific chromosomal rearrangements, implying a translocation between the acrocentric chromosome bearing the 45S site and one X chromosome arm. Indeed, the chromosome pair of *H. intermontana* bears the 45S rDNA locus (DEON *et al*., 2020), a site prone to breaks in *Harttia* karyotypes, leading to extensive chromosomal remodeling events (DEON *et al*., 2020, submitted).

According to molecular-phylogenetic reconstructions, *H. punctata* - 2n=58♀/57♂, $X_1X_1/X_2X_2/X_1X_2Y$ (BLANCO *et al*., 2014) - belongs to *Harttia*'s clade ii (COVAIN *et al*., 2016; LONDOÑO-BURBANO; REIS, 2021), and the WCP results here obtained evidenced a similar hybridization condition to those found in *H. kronei*, i.e., the HTO-1, HCA-X, HCA-9, and HIN-X chromosomes were not related to the karyotype diversification of *H. punctata*, highlighting a probable plesiomorphic condition.

It was demonstrated that sex chromosomes could emerge independently and follow distinct differentiation patterns, even among closely related species (CIOFFI *et al*., 2013). Our WCP-FISH data also indicated independent origins for the X_1X_2Y and XY_1Y_2 SCSs of *Harttia* lineage, as previously proposed (SASSI *et al*., 2020; DEON *et al*., 2020). The X chromosome of the XX/XY_1Y_2 system originated by fusion of two autosome pairs, leading to the largest metacentric in the karyotype. This fusion could set up a putative homomorphic XX/XY SCS, with subsequent centric fission originating the Y_1 and Y_2 chromosomes, as proposed by Blanco *et al*. (2013). Thus, a set of diverse chromosomal rearrangements probably triggered the differentiation of the same or different SCSs within the *Harttia* lineage, suggesting that sex chromosome turnover may play an important role in the speciation processes of this group.

Evolutionarily conserved breakpoint regions (ECBRs), inside or adjacent to rDNA clusters, were proposed to occur in some Loricariidae lineages, leading to extensive chromosomal remodeling (BARROS *et al*., 2017; GLUGOSKI *et al*., 2018; DEON *et al*., 2020, DEON *et al*., submitted). In the *Harttia* clade iii from the south/southeast Brazilian region, several rearrangements adjacent to the rDNAs sites have been extensively reused in the chromosomal diversification (DEON *et al*., 2020, submitted), including the origin of the $X_1X_1X_2X_2/X_1X_2Y$ SCS in *Harttia* clade ii (DEON *et al*., submitted). In contrast, as rDNAs were not involved in the origin of the XX/XY_1Y_2 system, other unstable sites likely occur in the genomes of the species from *Harttia* clade iii, as well.

In addition, some hybridization signals besides those indicating the discussed main rearrangements were also detected in small chromosomal regions. They correspond to a highly rearranged repetitive DNA unit shared among *Harttia* species. In *H. carvalhoi*, *H. gracilis, H. longipinna*, *H. torrenticola,* and *Harttia* sp. 1, they localize close to the

nucleolar organizer region (NOR) and in a small acrocentric pair. In *H. loricariformis* and *H. intermontana* only the signal close to the NOR site and in the small acrocentric chromosome were detected, respectively. The mechanisms responsible for the instability of *Harttia* genome are not fully understood (DEON et al., 2020, submitted). However, repetitive DNA clusters scattered at some genome locations are likely candidates for chromosomal breaks and rearrangements. Cytogenetic data indicate that these sequences are reused in several chromosome rearrangements, including the Robertsonian ones responsible for the origin of the SCSs and a 2n decrease in *Harttia*.

Interstitial telomeric sites (ITS) are common features in some *Harttia* genomes (BLANCO *et al*., 2017; DEON *et al*., 2020). *Harttia carvalhoi* and *H. torrenticola*, for example, present an ITS in the large metacentric chromosome (BLANCO *et al*., 2017), indicating its origin by Robertsonian fusion. In contrast, this ITS was lost during the chromosomal evolution of *Harttia* sp. 1. It is known that ITS are hotspots for breakages (SLIJEPCEVIC *et al*., 1997) and that telomeric DNA damages can be irreparable, causing persistent DNA-damage-response activation (FUMAGALLI *et al*., 2012), or remaining as fragile sites (SFEIR *et al*., 2009). According to Slijepcevic (2016), both ITS and terminal telomeric sequences are naturally prone to breakage, leading to chromosome plasticity. Therefore, the rearrangements observed in the X and 2 chromosomes of *H. intermontana* may have been triggered by the instability generated by the ITS in the X chromosome of *H. carvalhoi*.

5. CONCLUSION

Data obtained by WCP-FISH allowed to highlight small pieces of the complex chromosomal evolution that has taken place in *Harttia* species, with a particular emphasis on the origin of a rare multiple SCS and diploid number decrease. We demonstrated the existence of unstable genomic sites promoting chromosomal differentiation and remodeling, where homeologous chromosome blocks were identified after WCP experiments. Besides, we highlighted the distinct Robertsonian fusions and fissions that were involved in the origin the sex chromosomes. In this context, the genus *Harttia* has proved to be an excellent model for the study of evolution of sexual chromosome systems among Neotropical fish species. Next steps now will include a fine-scale analysis of the genetic content of the sex chromosomes in this group aiming to discover novel sex-determining genes, which is an inevitable next step towards fully understating this puzzling scenario.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Osvaldo Oyakawa for the contributions in the animal´s identification.

TABLES AND FIGURES

Table 1: Collection sites of the *Harttia* species, diploid number (2n), and the sample sizes (N).

Species	2n	Locality	N
H. carvalhoi	♀52, XX ♂53, XY_1Y_2	Grande stream, Pindamonhangaba – SP (22º47'8"S 45º27'19"W)	17♀, 12♂
Harttia sp. 1	♀56, XX ♂57, XY_1Y_2	Macacos stream, Silveira – SP (22°40'43.0"S 44°51'25.0"W)	10♀, 7♂
H. intermontana	♀52, XX ♂53, XY_1Y_2	Piranga river, Carandaí – MG (20°59'34.0"S 43°43'30.0"W)	20♀, 13♂
H. punctata	♀58, X_1X_1 X_2X_2 ♂57, X_1X_2Y	Bandeirinha river, Formosa – GO (15º19'25"S 47º25'26"W)	18♀,25♂
H. kronei	58♀♂	Açungui river, Campo Largo – PR (25º22'44"S 49º39'08"W)	10♀, 5♂
H. gracilis	58♀♂	Machadinho stream, Santo Antônio do Pinhal – SP (22º48'31"S 45º41'21"W)	18♀,15♂
H. longipinna	58♀♂	São Francisco river, Pirapora – MG (17º21'22.8"S 44º51'0.2"W)	13♀,16♂
H. loricariformis	56♀♂	Paraitinga river, Cunha – SP (22º52'22"S 44º51'0.2"W)	7♀, 3♂
H. torrenticola	56♀♂	Araras stream, Piumhi – MG (20º16'15"S 45º55'39"W)	8♀, 6♂

Table 2: Localization of WCP probes analyzed in *Harttia* species. Some small signals were not considered.

	Species	HCA-X probe	HCA-9
XX/XY_1Y_2 system	***H. carvalhoi*** ♀	X Chr.	Chr. 9
	H. carvalhoi ♂	X, Y_1 and Y_2 Chr.	Chr. 9
	***Harttia* sp. 1** ♀	X Chr.	Chr. 21 and 26
	***Harttia* sp. 1** ♂	X, Y_1 and Y_2 Chr.	Chr. 21 and 26
	H. intermontana ♀	Xq and 2q	Xp and 20q distal
	H.intermontana ♂	Xq, Y_2 and 2q	Xp, Y_1 and 20q distal
$X_1X_1X_2X_2/X_1X_2Y$ system	***H. punctata*** ♀♂	Chr. 8 and 19	Chr. 9 and 11
Without differentiated SCSs	***H. kronei***	Chr. 17 and 19	Chr. 8 and 13
	H. gracilis	Chr. 11 and 22	Chr. 10 and 21
	H. longipinna	Chr. 15 and 17	Chr. 7 and 10
	H. loricariformis	Chr. 9 and 20	Chr. 8 and 11
	H. torrenticola	Chr. 1	Chr. 8 and 23

p = short arms; q = long arms; Chr. = chromosome; SCSs = sex chromosome system.

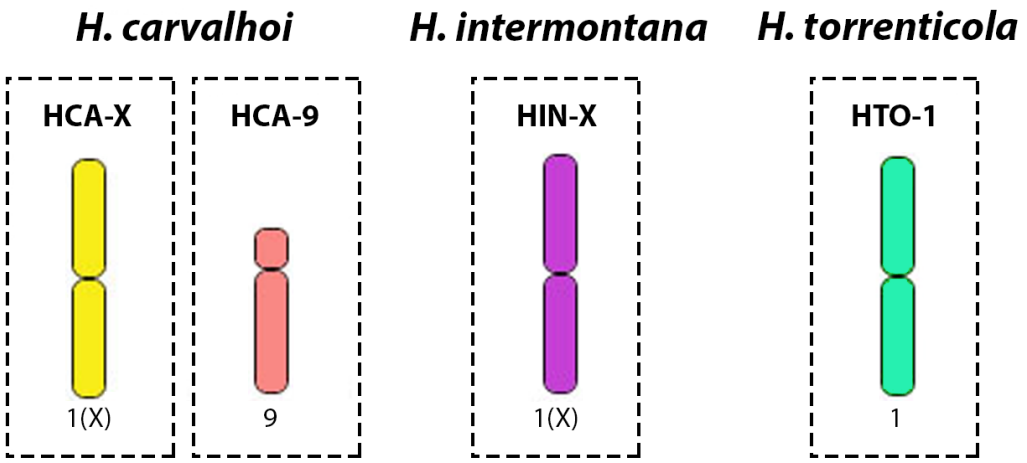


Figure 1: Schematic representation of the microdissected chromosomes used for probe construction in comparative WCP-FISH analyses. 1) the X chromosome of *H. carvalhoi* (HCA-X, in yellow); 2) chromosome 9 of *H. carvalhoi* (HCA-9, in light pink); 3) the X chromosome of *H. intermontana* (HIN-X, in purple); 4) chromosome 1 of *H. torrenticola* (HTO-1, in green).

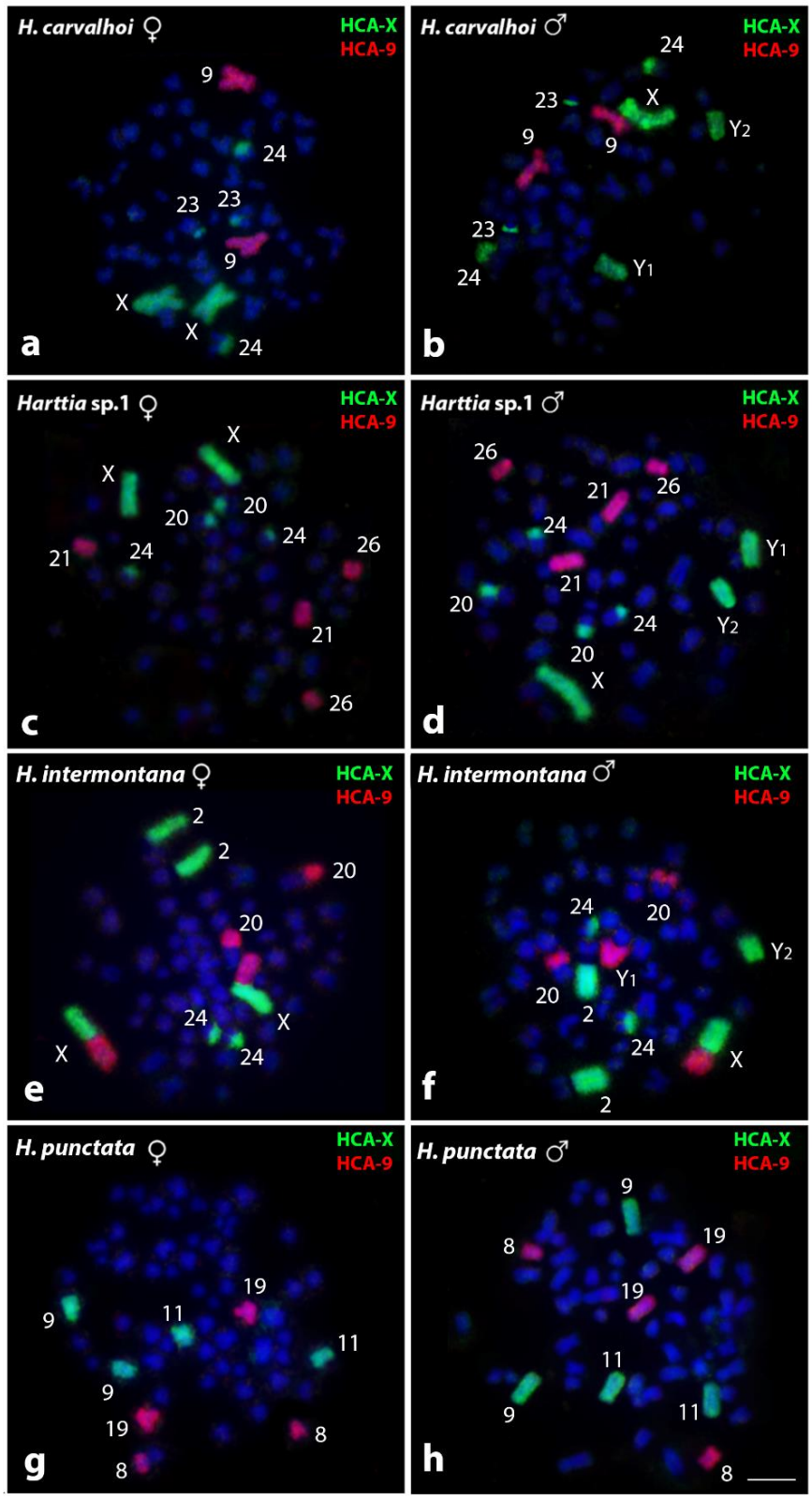


Figure 2: Whole chromosome painting by FISH using the HCA-X (green) and HCA-9 (red) probes among *Harttia* species that possess SCSs. Bar = 5 µm.

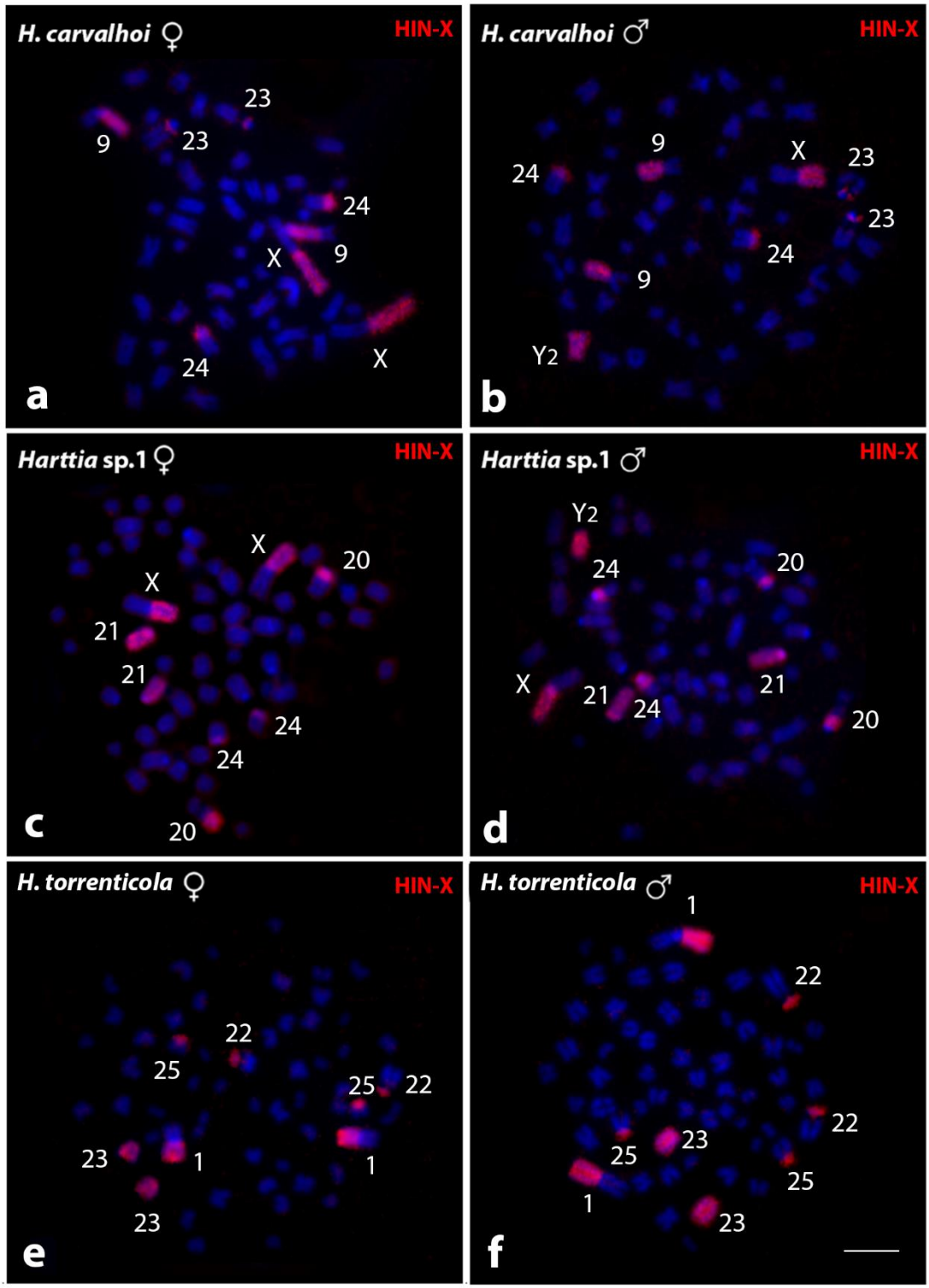


Figure 3: Whole chromosome painting by FISH using the HIN-X probe (red) among *Harttia* species that shared a large metacentric pair. Bar = 5 µm.

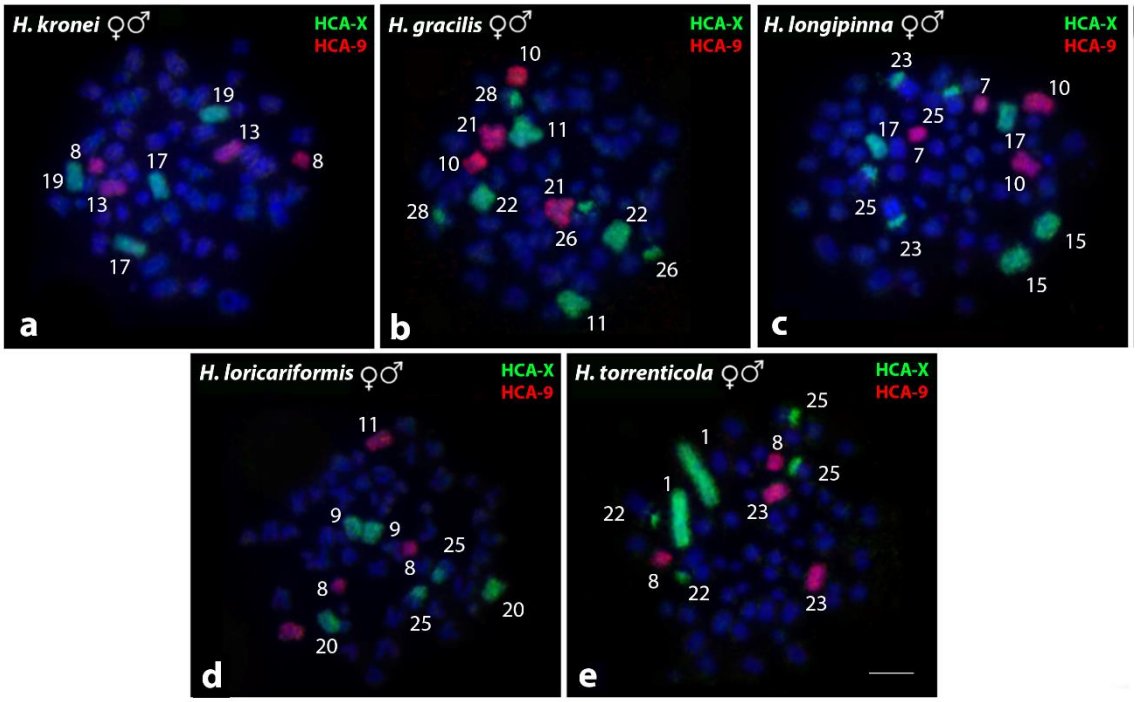


Figure 4: Whole chromosome painting by FISH using the HCA-X (green) and HCA-9 probes (red) in *Harttia* species without heteromorphic SCS. Bar = 5 µm.

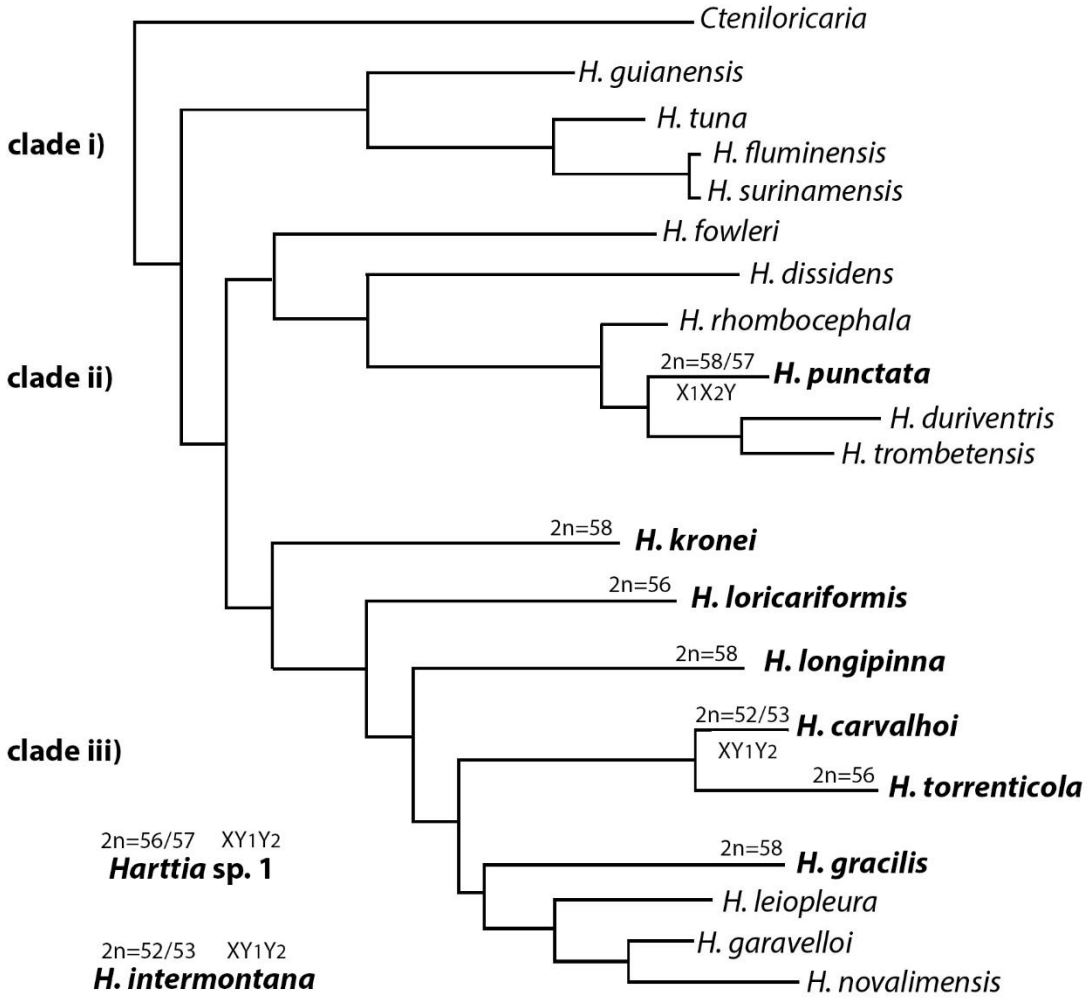


Figure 5: Schematic representation of the phylogenetic relationships among *Harttia* species adapted from the Londoño-Burbano and Reis (2021). *Harttia* sp. 1 and *H. intermontana* are not being represented since they were not included in such previous analysis.

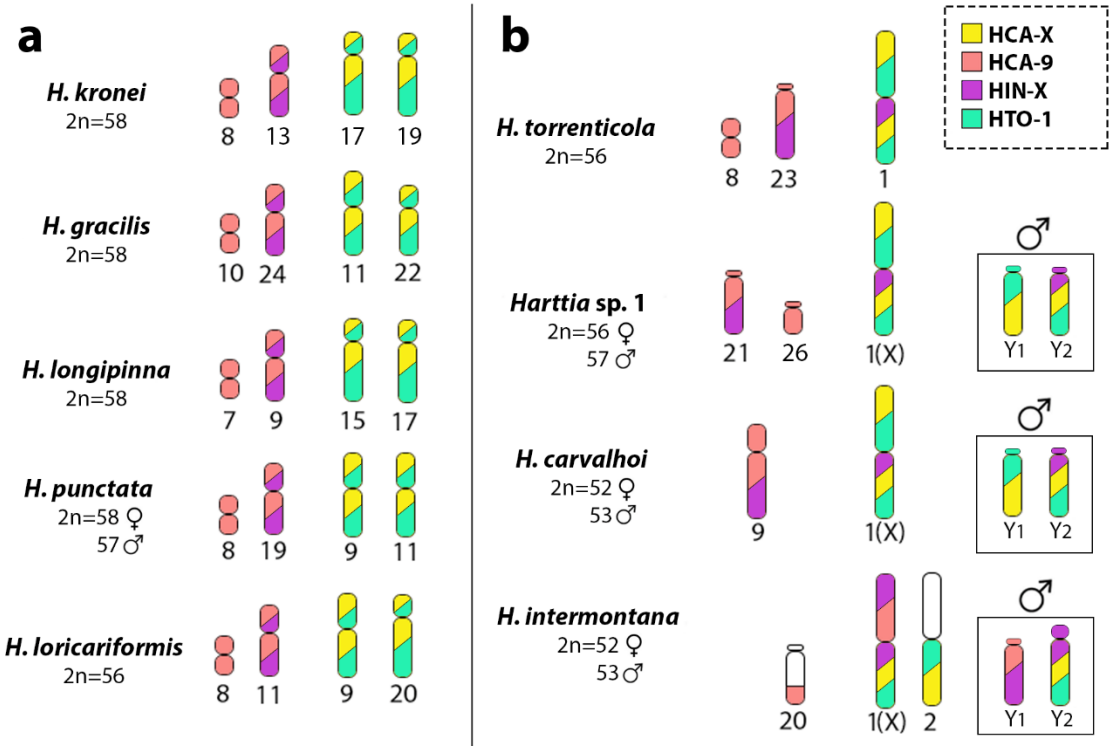


Figure 6: Schematic representation summarizing the distribution of the WCP probes obtained in this study: HCA-X (yellow), HCA-9 (light pink), HIN-X (purple) and HTO-1 (green) in *Harttia* species (a) without the largest metacentric chromosome pair -*H. kronei*, *H. gracilis*, *H. longipinna*, *H. loricariformis*, and *H. punctata*- and (b) with the largest metacentric pair in the karyotype -*H. carvalhoi*, *H. torrenticola*, *H. intermontana* and *Harttia* sp. 1. The highlighted boxes show the male condition and the different composition of the Y_1 and Y_2 chromosomes in *H. carvalhoi*, *H. intermontana* and *Harttia* sp. 1. Note the overlapping of the HCA-9 and HIN-X probes (light pink and purple), and the HCA-X and HTO-1 (yellow and green) probes.

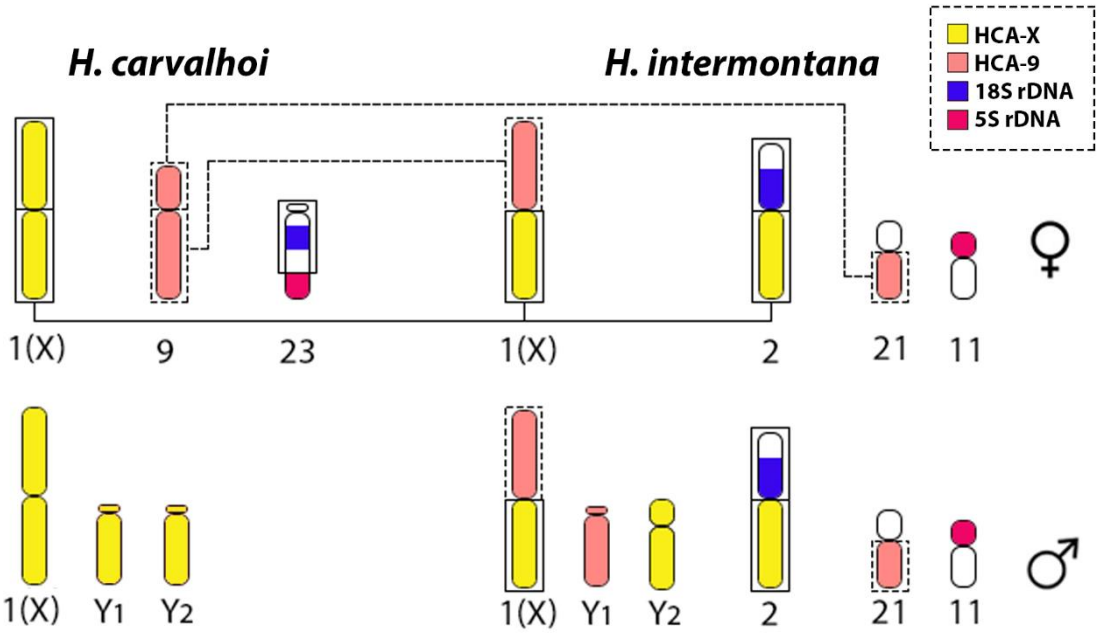
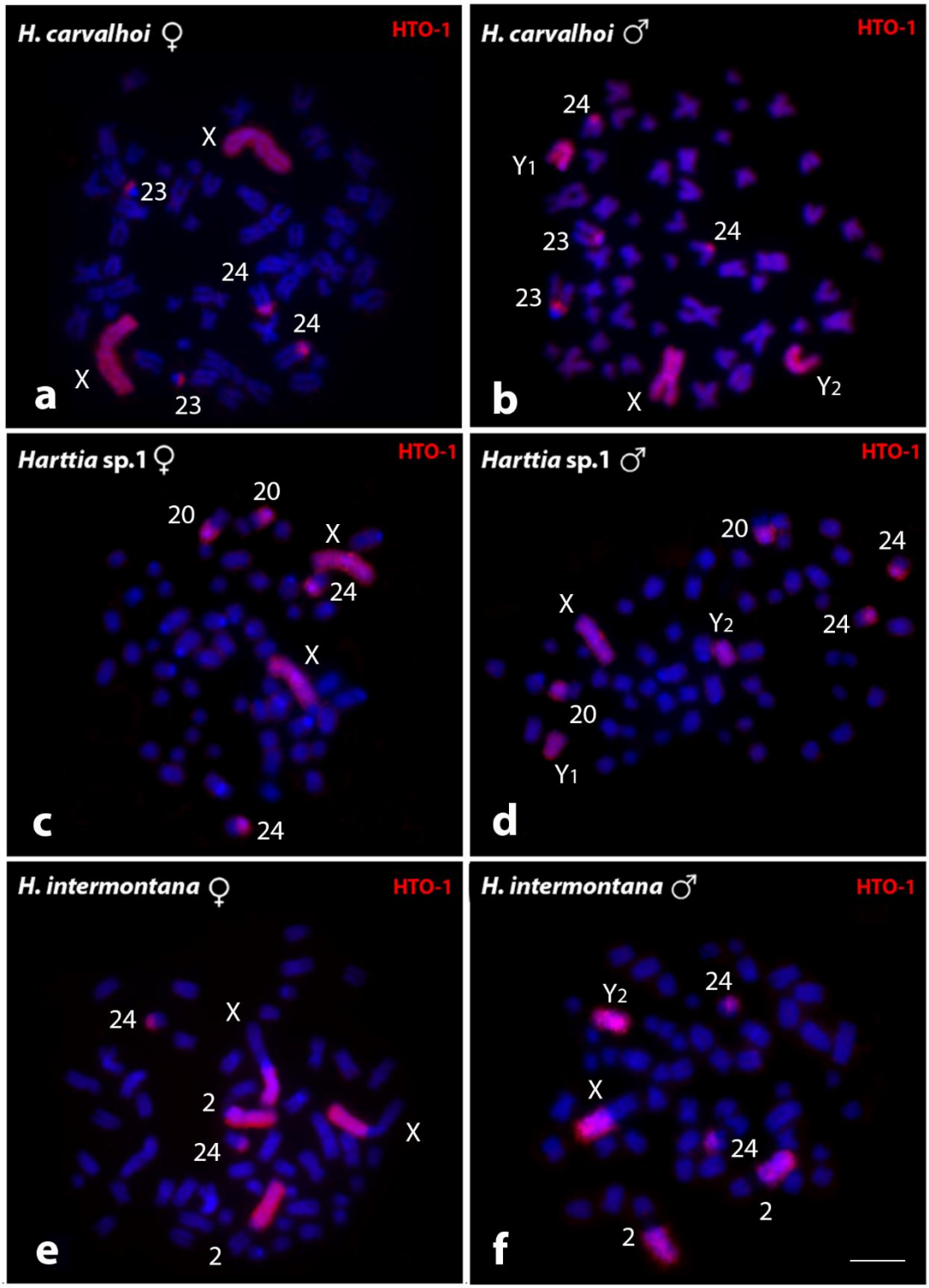


Figure 7: Schematic model representing the rearrangements occurred from *H. carvalhoi* to *H. intermontana*, and the evolvement of HCA-X (yellow) and HCA-9 probes (light pink) on the origin of the XX/XY_1Y_2 SCS. Considering *H. intermontana*, a derived species from *H. carvalhoi*, a centric fusion between the long arms of the chromosome 1 (X) and the long arms of the chromosome 9 of *H. carvalhoi*, lead the origin of the X chromosome in *H. intermontana*. An additional fusion with part of the X chromosome and part of the chromosome 23 (bearing the 18S rDNA site) gave rise to the second-largest metacentric chromosome pair in *H. intermontana* (pair 2). It is worth mentioning that the different origin of the X chromosome directly reflects on the genomic composition of the Y chromosomes: while the Y_1 chromosome corresponds to the long arms of the chromosome 9 and the Y_2 one corresponds to a part of the X chromosome of *H. carvalhoi*.



Supplementary figure 1: Hybridizations using the HTO-1 probe (red) in species that present a large metacentric chromosome pair in the karyotype: a) *H. carvalhoi* female, b) *H. carvalhoi* male, c) *Harttia* sp.1 female, d) *Harttia* sp. 1 male, e) *H. intermontana* female and f) *H. intermontana* male. Bar = 5 µm.

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Capítulo 4

Microsatellite repeats accumulation in hotspots of chromosomal rearrangements in *Harttia* species (Siluriformes: Loricariidae)

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Artigo em preparação

Abstract

Some genomic regions are propensity to chromosomal instability and rearrangements. Studies have demonstrated that chromosomal break regions are enriched with different types of repetitive sequences surrounding or inside these regions, including in tandem arrays of genic families and microsatellite sequences. *Harttia* genus is a fish group characterized for highly rearranged karyotypes evidenced by whole chromosome painting studies. Unstable sites surrounding ribosomal genes or the pericentromeric heterochromatin of sex chromosomes have been proposed to trigger chromosomal remodeling in the *Harttia* genomes. However, the characterization of the repetitive units flanking ribosomal genes or the pericentromeric heterochromatin is still absent in *Harttia* chromosomes. So, aiming to analyze the microsatellite accumulation in recognized rearranged chromosome regions in *Harttia*, here we performed in situ hybridization using the $(A)_{30}$, $(CA)_{15}$, $(GA)_{15}$, and $(CGG)_{10}$ probes in ten *Harttia* species. The $(CA)_{15}$ and $(GA)_{15}$ probes showed scattered distribution with preferential accumulation in terminal regions in most species. In addition, the data showed that GA motifs could be associated with the chromosomal rearrangements points in *H. torrenticola* and *H. intermontana*. On the other hand, microsatellites A and CGG are collocated with the 5S rDNA site in *Harttia* karyotypes. *Harttia* sp. 2 showed that microsatellites A and CGG are colocalized with 5S and 45S rDNA sites, regions originating from rearrangements in this karyotype. Still, the CGG microsatellite showed accumulation on the centromeric region of chromosome X, a region assumed to be originated by fusion. In conclusion, this study highlights the enrichment of different microsatellite motifs in remarkable hotspots to chromosomal rearrangement regions in *Harttia* species.

Keywords: repetitive DNA; unstable sites; cytogenetic; karyotype evolution; in situ hybridization.

1. INTRODUCTION

Repetitive DNA comprises a substantial portion of the eukaryotes genome and can be generally classified into two major classes: tandem repeats (such as multigene families and satellite DNA) and transposable elements (TEs), according to their structural organization and sequence composition (CHARLESWORTH; SNIEGOWSKI; STEPHAN, 1994; KUBIS; SCHMIDT; HESLOP-HARRISON, 1998; JURKA *et al*., 2007). Based on the size of the unit repeat length, satellite DNA has grouped as: satellites (100-1000 bp), minisatellites (>7 bp), and microsatellites or short tandem repeats (1-6 bp) (TAUTZ, 1993; NASLUND *et al.*, 2005). More recently, it has been proposed that short tandem repeats, when present and organized in arrays of many hundred or thousand copies in heterochromatin, constitute satellite DNA sequences (GARRIDO-RAMOS, 2015, 2017). Satellite DNA is the main component of the heterochromatin, while microsatellites occur randomly located into genomes in coding and non-coding regions (TAUTZ, 1993; TÓTH; GÁSPARI; JURKA, 2000).

Chromosomal rearrangements contribute to the genetic diversification and speciation process, and your occurrence is associated with chromosomal breaks in highly repetitive regions in the genomes (CAZAUX *et al*., 2011; BRUSCHI *et al*., 2014). This propensity to chromosomal instability in specific genomic regions was considered a hotspot to double-strand break and chromosomal rearrangements (PEVZNER; TESLER, 2003). Chromosomal break regions are enriched in different types of repetitive sequences like SINEs (*short interspaced nuclear elements*), LINEs (*long interspaced nuclear elements*), and LTRs (*long terminal repeats*), located surrounding or inside of the segmental supplication (SD) (CARBONE *et al*., 2009), or organizing genomic regions named evolutionary breakpoint regions (EBRs) (RUIZ-HERRERA; CASTRESANA; ROBINSON, 2006).

Tandem repeats can be considered an important source of DNA variation and mutation (ARMOUR, 2006) and shown to be fundamental in studies related to genomic evolution (CHARLESWORTH; SNIEGOWSKI; STEPHAN, 1994). The accumulation of tandem repeats, including multigenic families and microsatellites, could change the chromatin state and generate unstable chromosome sites (FARRÉ *et al.*, 2011). These repeats can form non-B DNA structures (i.e., DNA structures different from the Watson-Crick configuration), such as hairpins, cruciform or triplex conformations (BACOLLA *et al.*, 2008), promoting DNA instability and giving rise to chromosomal reorganizations (KOLB *et al.*, 2009).

In Loricariidae, a Neotropical fish family, cytogenetic studies indicate the presence of several chromosomal rearrangements and chromosomal reorganizations (ROSA *et al*., 2012; BARROS *et al*., 2017; PRIMO *et al*., 2017; GLUGOSKI *et al*., 2018). Some of these events were associated with the presence of EBRs inside or close to rDNAs sites, which triggered chromosomal breaks (BARROS *et al*., 2017; GLUGOSKI *et al*., 2018). However, the characterization of repetitive DNA sequences compounding EBRs in the Loricariidae genomes remains uncertain (PRIMO *et al*., 2018).

Harttia comprises a Loricariidae genus presenting several chromosomal reorganizations in their representatives (DEON *et al.*, submitted a). Their species present diploid numbers ranging from 2n = 52 to 62 chromosomes, variable number and position of the ribosomal clusters, and presence of multiple and simple sex chromosome systems (BLANCO *et al*., 2017; DEON *et al*., 2020; SASSI *et al*., 2020, 2021). Concerning the EBRs occurrence in *Harttia* genomes, repetitive regions inside or flanking ribosomal clusters were widely reused in the repositioning of homologous chromosomal blocks in this lineage chromosome evolution (DEON *et al.*, submitted a).

In this way, considering that the whole chromosome painting studies indicate the extensively reused repetitive regions triggering chromosomal diversification in *Harttia* and the scarce characterization of repeat units in *Harttia*, here we evaluate, at the cytogenetic level, the participation of the microsatellites in the heterochromatin composition on proposed EBRs in the *Harttia* karyotypes.

2. MATERIALS AND METHODS

Representatives of ten *Harttia* species from South and Southeast Brazilian drainages were here analyzed. Fish were collected with the authorization of the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-License Nos. 10538-3 and 15117-2), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09). All species, including two taxonomically undescribed species in the scientific literature, *Harttia* sp. 1 and *Harttia* sp. 2, were identified based on their morphological features by Dr. Oswaldo Oyakawa (curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo - MZUSP). *Harttia* sp. 1 and *Harttia* sp. 2 karyotypes have already been published by Deon *et al*. (2020).

Mitotic chromosomes were obtained from kidney cells, according to Bertollo; Cioffi and Moreira-Filho (2015). The experiments were conducted under the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos approval (Process number CEUA 1853260315). Cell preparations were dropped onto clean glass slides and submitted to fluorescence in situ hybridization (FISH) using microsatellite probes.

The microsatellites $(A)_{30}$, $(CA)_{15}$, $(GA)_{15}$ and $(CGG)_{10}$ were selected based on their abundance and patterns of chromosome distribution encountered in previous studies in different fish species and previous studies in *Harttia* species (SASSI *et al*., 2020, 2021). The corresponding microsatellites probes were directly labeled with Cy3 (Sigma-Aldrich, Darmstadt, Germany) during their synthesis, as described in Kubat *et al*. (2018). FISH experiments followed the methodology described in Yano; Bertollo and Cioffi (2017). Metaphase plates were captured using an Olympus BX50 light microscope (Olympus

Corporation, Ishikawa, Japan) with a CoolSNAP camera. The images were processed using the Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA).

3. RESULTS

The distribution of the microsatellites $(CA)_{15}$ showed a preferential location in the terminal regions of the chromosomes, and some interstitial blocks in most chromosomes in all analyzed species **(Figure 1)**. In contrast to the $(CA)_{15}$, the hybridization pattern of $(GA)_{15}$ showed different types of distribution along *Harttia* chromosomes **(Figure 2)**. While *H. carvalhoi*, *H. punctata*, *H. gracilis*, *H. kronei*, *H. loricariformis*, *Harttia* sp. 1, and *Harttia* sp. 2 presented scattered signals with a preferential location in the terminal regions, *H. intermontana* and *H. torrenticola* presented few signals in some chromosome pairs, mainly in the centromeric region of the chromosomes 1 and 2, in *H. torrenticola* and *H. intermontana*, respectively **(Figure 2)**. In *H. longipinna* few centromeric blocks were detected in some chromosomes while in *Harttia* sp. 2, signals were detected on centromeric blocks and associated with rDNA clusters **(Figure 2)**.

The $(A)_{30}$ in situ localization showed scattered signals in most chromosomes of the analyzed species **(Figure 3)**. Mononucleotide A expansions were detected in the 45S rDNA sites in *H. carvalhoi*, *H. intermontana*, *Harttia* sp. 1, *H. gracilis*, *H. loricariformis*, and *H. torrenticola*, while in *Harttia* sp. 2, this microsatellite was located in the 5S rDNA sites. Microsatellite CGG presented similar signals to $(A)_{30}$ probe, i.e., associated with 45S rDNA sites, except for *H. carvalhoi*, that showed positive blocks on the largest metacentric (chromosome X), and *Harttia* sp. 2, with CGG expansion in both rDNA clusters **(Figure 4)**.

4. DISCUSSION

Microsatellites are helpful chromosome markers to analyze the relationship among species karyotypes, contributing to the study of fish chromosome evolution (reviewed in CIOFFI; BERTOLLO, 2012). In some fish species cases, this approach is usually performed to detect the differentiation of the heteromorphic sex chromosome systems, including XY or ZW cases (CIOFFI; KEJNOVSKY; BERTOLLO, 2011; ZIEMNICZAK *et al.*, 2014; PUCCI *et al.*, 2016; YANO *et al.*, 2016; FREITAS *et al.*, 2018; SCHEMBERGER *et al*., 2019). On the other hand, the microsatellite in situ localization has been scarcely used to recognize EBRs or chromosome regions indicative of rearrangements. Cross-species chromosome painting studies in *Harttia* species have revealed that evolutionary breakpoints (i.e., the disruption of two orthologous chromosomal segments) are not homogeneously distributed but relatively concentrated in specific regions across the genome, mainly close to ribosomal clusters and some pericentromeric heterochromatin regions (DEON *et al*., submitted a, b). In this study, some microsatellites showed accumulation in chromosome regions involved in chromosome remodeling in *Harttia* species.

The dinucleotide CA is one of the most abundant microsatellite families in vertebrate genomes, occurring on average every 15 to 30 kb (STALLINGS *et al*., 1991; ESTOUP *et al*., 1993; JURKA; PETHIYAGODA, 1995). In fish genomes, CA and GA repeats were associated with the terminal and subterminal regions of chromosomes (CIOFFI; KEJNOVSKY; BERTOLLO, 2011; ZIEMNICZAK *et al*., 2014; PUCCI *et al.*, 2016; SASSI *et al*., 2019). An accumulation of the GA and CA repeats was also found in terminal regions of the chromosomes in *Harttia* species here analyzed. Remarkably, an unusual location pattern of (GA)n was found in pericentromeric region of chromosome 1 in *H. torrenticola*, and chromosome 2 of *H. intermontana*.

Whole chromosome painting studies have already demonstrated these chromosome regions had been involved in rearrangements, including fusion and translocation events (DEON *et al*., submitted b). Chromosome 1 of *H. torrenticola* was originated by chromosome fusion (BLANCO *et al*., 2017; DEON *et al*. submitted b). Cross-species chromosome painting revealed high homeology between *H. torrenticola* chromosome 1 and *H. carvalhoi* chromosome X (DEON *et al*., submitted b). *Harttia carvalhoi* chromosome X suffered pericentromeric fission to originate Y_1, and Y_2 chromosomes (BLANCO *et al*., 2017; DEON *et al*., submitted b). So, once *H. carvalhoi* does not have an accumulation of GA repeats on the centromeric region of chromosome X, it is possible to propose that GA repeats were lost during the XY_1Y_2 origin or the centromeric region the chromosome 1 in *H. torrenticola* suffered GA accumulation stabilizing the heterochromatin area.

At the same time, *H. intermontana* has a diversified XX/XY_1Y_2 sex chromosome system from *H. carvalhoi*, triggered by translocations involving X and autosome (DEON *et al*., submitted b). The *H. intermontana* chromosome 2 also was originated by reciprocal translocations involving NOR-bearing chromosome and one X chromosome arm from *H. carvalhoi* lineage, generating a large metacentric (DEON *et al*., submitted b). The GA accumulation visualized on *H. carvalhoi* chromosome 23 (NOR-bearing), and *H. intermontana* chromosome 2 suggests this microsatellite's possible role in this region's chromosomal instability on *Harttia* chromosomes, highly reused to chromosome changes in the lineage.

On the other hand, the microsatellite role on the *H. punctata* X_1, X_2, and Y sex chromosome rearrangements is unclear. The CA and GA accumulations in *H. punctata* were visualized on the terminal chromosome regions, as observed in the closely related species *H. duriventris* and *H. villasboas* (SASSI *et al*., 2020). At the same time, A

mononucleotide accumulations were not related with X_1, X_2, or Y chromosomes in *H. punctata*, while $(A)_n$ was described on the centromeric region of the X_1 chromosome in *H. duriventris* and *H. villasboas* (SASSI *et al*., 2020).

Remarkably, the colocalization of the microsatellites kinds and ribosomal clusters has an important role in fish genomes organization. Microsatellites were proposed to stabilize a wide variety of DNA structures, including the tandemly arranged clusters of multigene families (CROSS; REBORDINOS, 2005). Hence, since the microsatellites can act as hot spots for recombination, the homogenizing mechanisms could be favored into multigene families (CHISTIAKOV; HELLEMANS; VOLCKAERT, 2006). In some *Harttia* species, both $(A)_{30}$ and $(CGG)_{10}$ were detected in association with 45S rDNA (such as *H. carvalhoi*, *H. intermontana*, *Harttia* sp. 1, *H. gracilis*, *H. loricariformis*, and *H. torrenticola*) and with both, 5S and 45S rDNA in *Harttia* sp. 2. In addition, the colocalization of CGG units and ribosomal DNA has been frequent among fishes (DITCHAROEN *et al.*, 2020; SASSI *et al*., 2019), adding a potential role to these microsatellites favoring recombination in rDNA clusters.

Distinct DNA units compounding non-transcribed regions into multigene families are observed in fish genomes (MERLO *et al*., 2012, 2013; REBORDINOS; CROSS; MERLO, 2013). Especially in the 5S rDNA, short interspersed nuclear elements (SINEs) tRNA-derived, long terminal repeats (LTRs), U small nuclear RNA, microsatellites, and fragments of transposable elements has been identified (MERLO *et al*., 2012, 2013; REBORDINOS; CROSS; MERLO, 2013; YANO *et al*. 2017, 2020; GLUGOSKI *et al*., 2018). In some Loricariidae genera with high rearranged karyotypes, studies about chromosome evolution suggest the occurrence of sites prone to break close or inside 5S rDNA sites (BARROS *et al.*, 2017; GLUGOSKI *et al*., 2018), including *Harttia* (DEON *et al*., 2020). However, the chromosomal instability could not be present in the rDNA

genes but other repeats units surrounding the multigene family. In mammalian genomes, Farré *et al*. (2011) found different microsatellites motifs significantly accumulated in EBRs. The accumulation of tandem repeats in specific genomic regions might form secondary structures in the DNA and, therefore, promotes genome instability that could lead to evolutionary chromosomal changes (FARRÉ *et al*., 2011). Thus, the microsatellite accumulation in sites recognized in the chromosome reshuffling mechanism in *Harttia* species adds a new perspective on the lineage chromosomal instability.

The effects of several microsatellites motifs, particularly trinucleotide repeats, on genome stability have been investigated in detail in *S. cerevisiae* (KIM *et al*., 2008; FREUDENREICH; KANTROW; ZAKIAN, 1998). In yeast, the microsatellites CTG and GAA may act as hotspots for mitotic double-strand breaks, which are recombinogenic or associated with chromosome rearrangements (KIM *et al*., 2008; FREUDENREICH; KANTROW; ZAKIAN, 1998). However, repetitive DNAs could only have facilitated chromosome rearrangement because they contain homologous sequences, whereas breakage sites could be located in flanking sequences of duplicated regions (KEHRER-SAWATZKI; COOPER, 2008). In *Harttia* karyotypes, CGG and A motifs are intensely accumulated in colocalization with 45S rDNA. So, since multigene families and microsatellites expansion could propitiate prone to break sites, and *Harttia* karyotypes showed the colocalization of both (i.e., rDNA and microsatellite) in suggested EBRs, these genomic regions assemble features to organize non-B DNA structures and unstable sites, but still lacking a deep genomic characterization.

In conclusion, the data added important contributions to the characterization of repetitive sequences in double-strand break regions from a cytogenetic viewpoint in *Harttia* genome. Here, the data provide evidence of the distinct profiles of the microsatellites motifs distribution and density in *Harttia* karyotypes. The main evidence

of rDNA and microsatellites colocalization reinforces their organization and participation in *Harttia* EBRs, promoting remodeling the karyotype architecture in this fish group lineage.

FIGURES

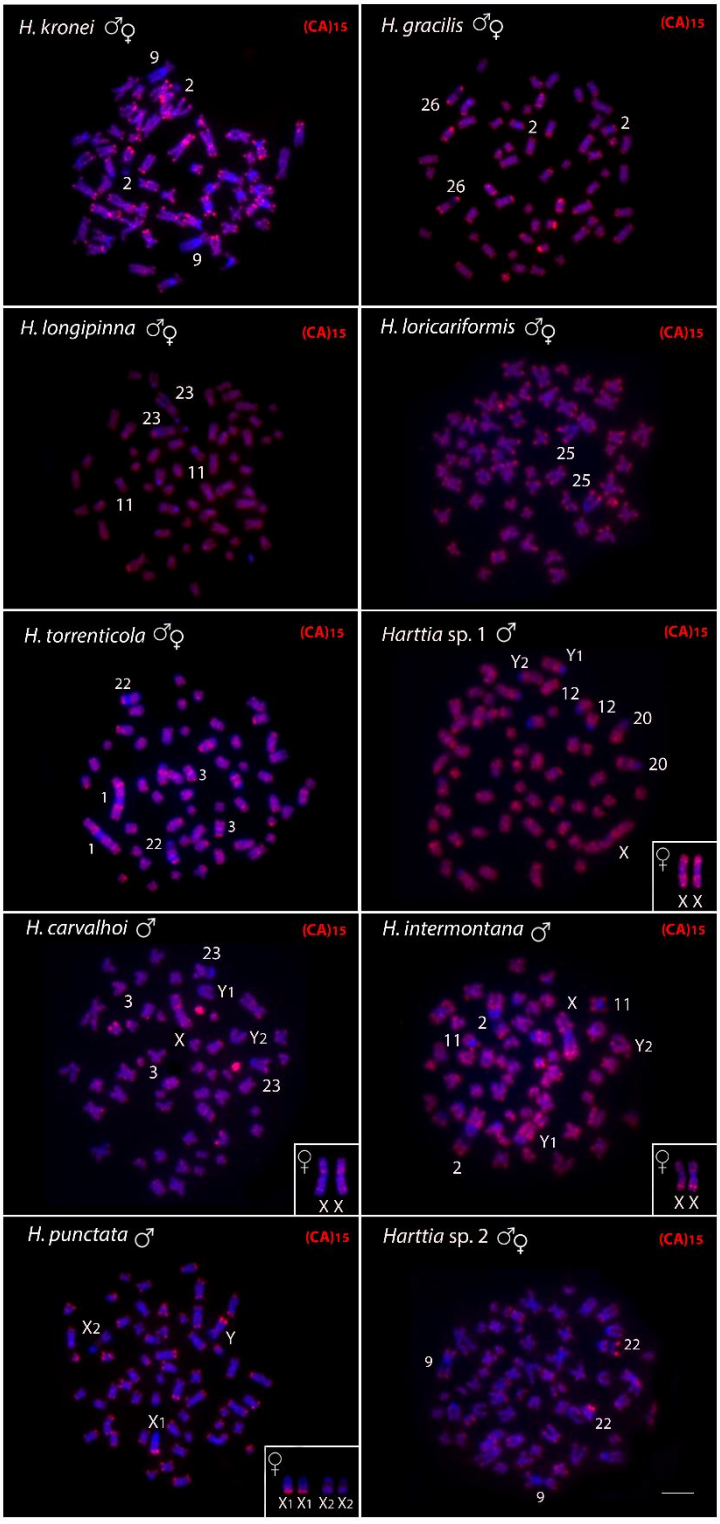

Figure 1: Male metaphases of *Harttia* species showing the distribution of $(CA)_{15}$ microsatellite (red) in: *H. kronei*, *H. gracilis*, *H. longipinna*, *H. loricariformis*, *H. torrenticola*, *Harttia* sp. 1, *H. carvalhoi*, *H. intermontana*, *H. punctata* and *Harttia* sp. 2 Boxes depict the female sex chromosomes. Chromosomes were counterstained with DAPI. Bar = 5 μm.

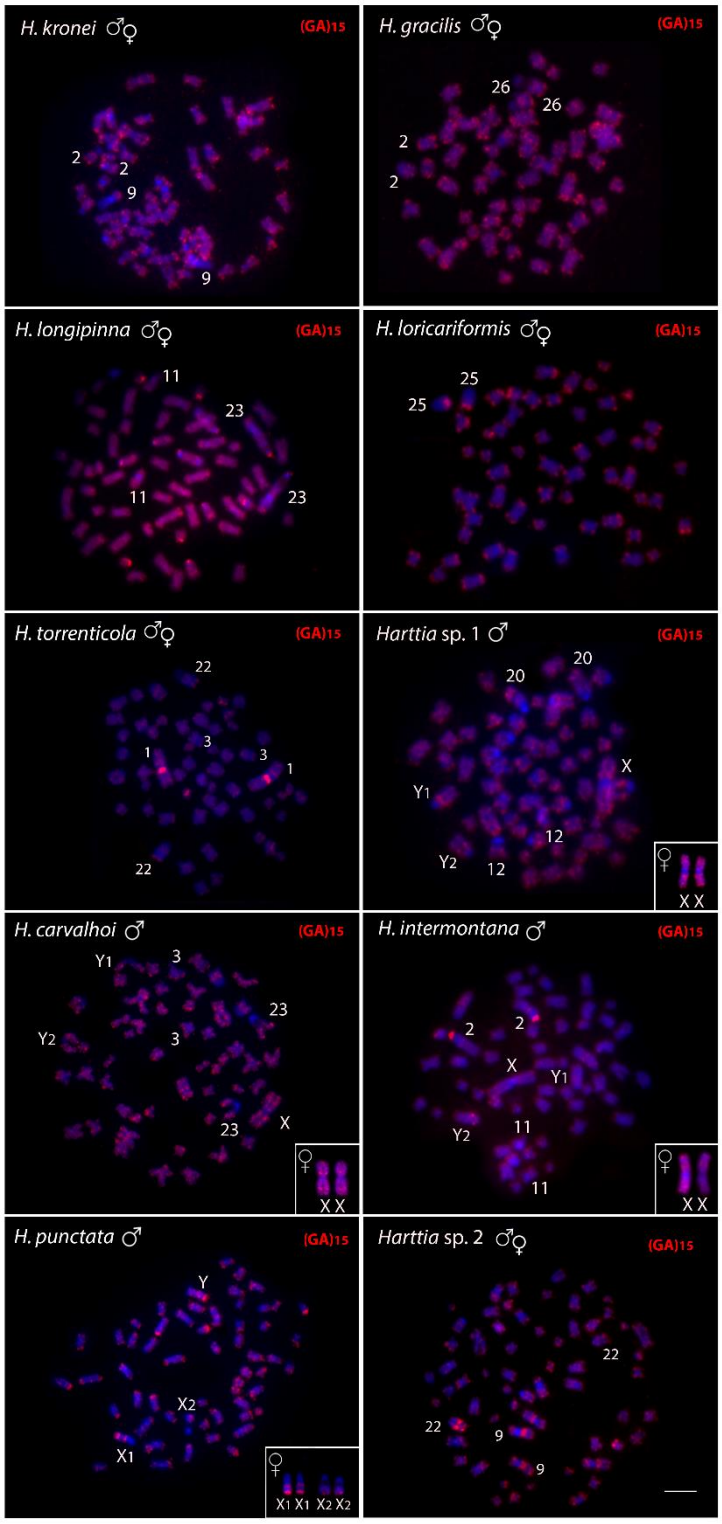

Figure 2: Male metaphases of *Harttia* species showing the distribution of $(GA)_{15}$ microsatellite (red) in: *H. kronei*, *H. gracilis*, *H. longipinna*, *H. loricariformis*, *H. torrenticola*, *Harttia* sp. 1, *H. carvalhoi*, *H. intermontana*, *H. punctata* and *Harttia* sp. 2 Boxes depict the female sex chromosomes. Chromosomes were counterstained with DAPI. Bar = 5 μm.

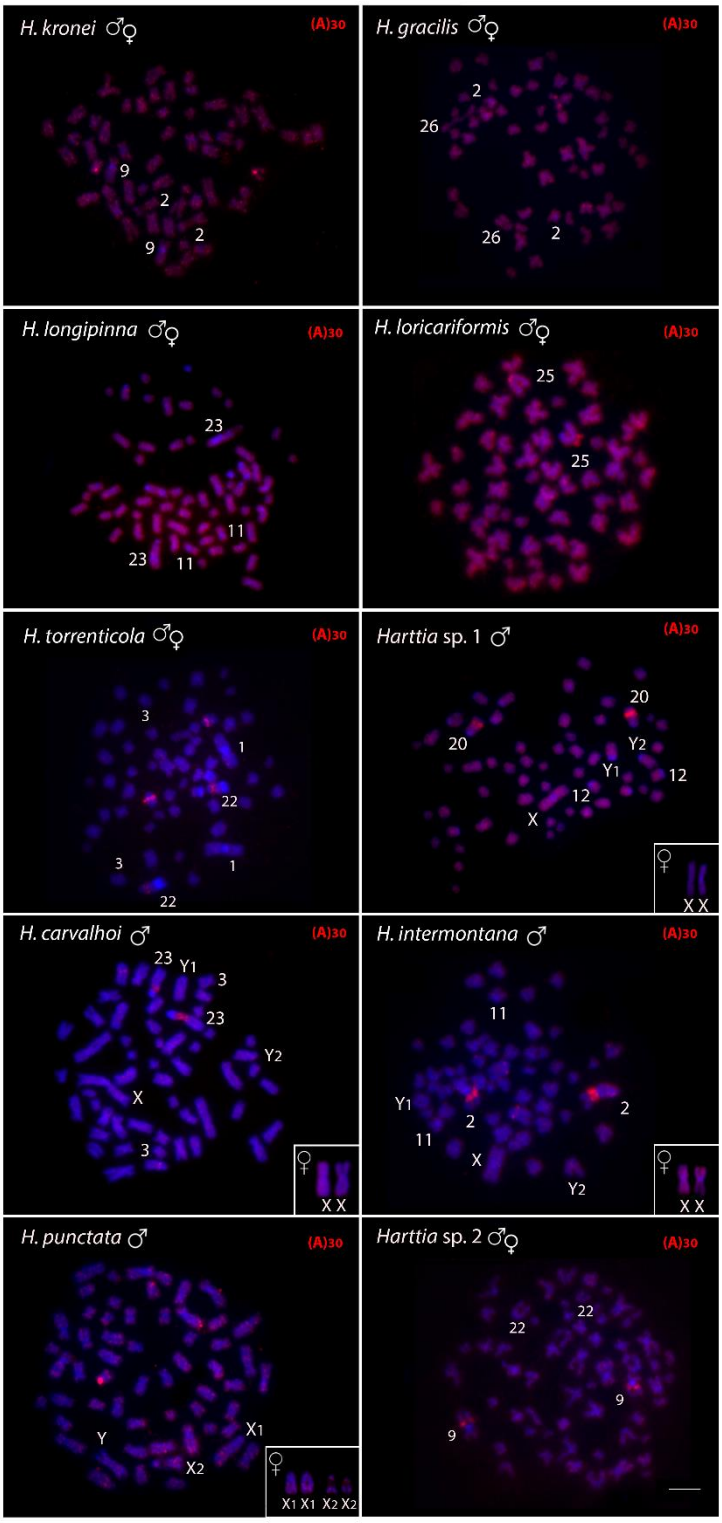

Figure 3: Male metaphases of *Harttia* species showing the distribution of $(A)_{30}$ microsatellite (red) in: *H. kronei*, *H. gracilis*, *H. longipinna*, *H. loricariformis*, *H. torrenticola*, *Harttia* sp. 1, *H. carvalhoi*, *H. intermontana*, *H. punctata* and *Harttia* sp. 2 Boxes depict the female sex chromosomes. Chromosomes were counterstained with DAPI. Bar = 5 μm.

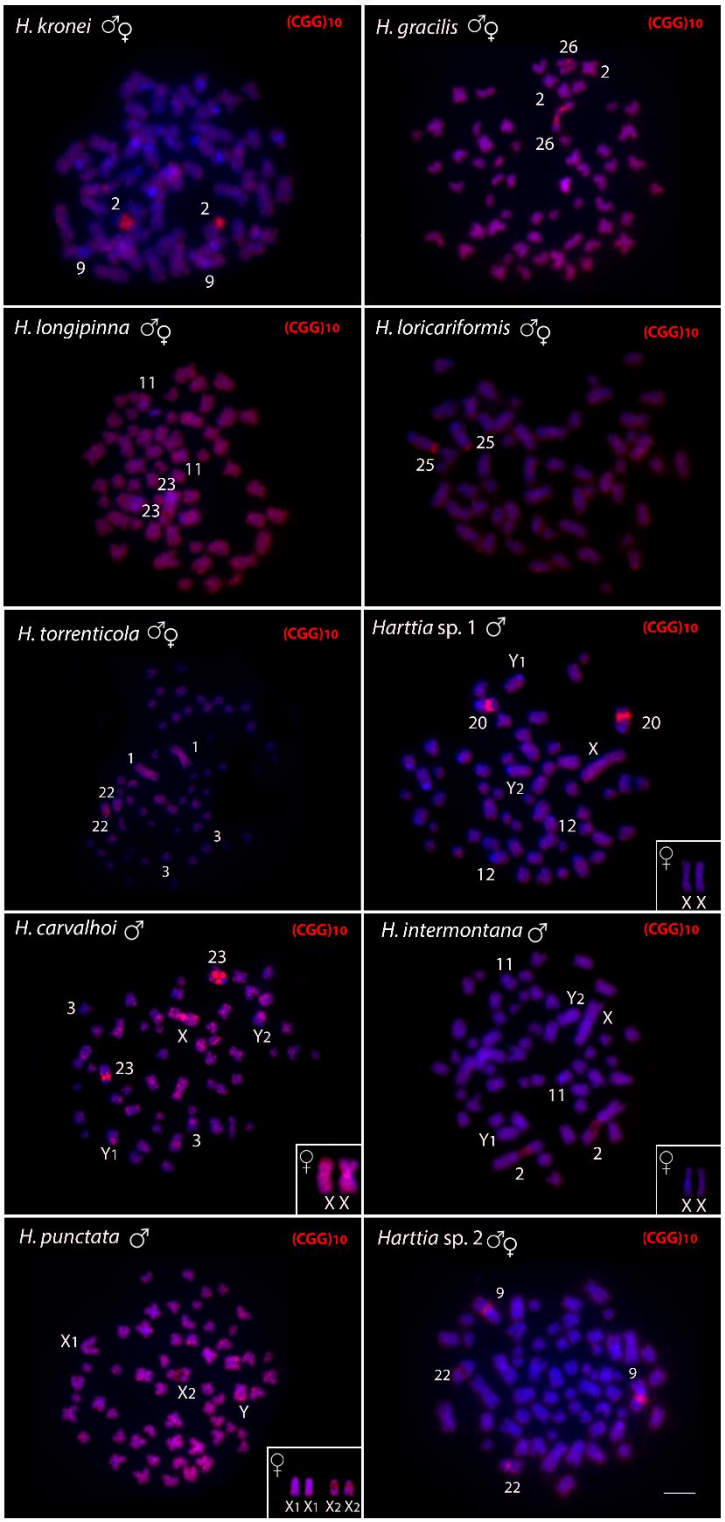

Figure 4: Male metaphases of *Harttia* species showing the distribution of $(CGG)_{10}$ microsatellite (red) in: *H. kronei*, *H. gracilis*, *H. longipinna*, *H. loricariformis*, *H. torrenticola*, *Harttia* sp. 1, *H. carvalhoi*, *H. intermontana*, *H. punctata* and *Harttia* sp. 2 Boxes depict the female sex chromosomes. Chromosomes were counterstained with DAPI. Bar = 5 μm.

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CONSIDERAÇÕES FINAIS

Estudos citogenéticos clássicos e moleculares já mostravam fortes evidências de que os cariótipos das espécies pertencentes ao gênero *Harttia* são dinâmicos e sofreram uma série de rearranjos cromossômicos ao longo de sua história evolutiva. Neste trabalho, foram caracterizadas citogeneticamente três espécies da região sul/sudeste do Brasil que enfatizam essas variações com duas novas ocorrências de sistemas de cromossomos sexuais múltiplos em peixes. Das três espécies analisadas, duas correspondem a espécies ainda não descritas na literatura, o que demonstrou uma provável diversificação de espécies no gênero *Harttia* em diferentes tributários ainda não amostrada. Além disso, também foram obtidas seis sondas por microdissecção cromossômica de cromossomos sexuais e autossomos, as quais foram utilizadas na investigação de rearranjos cromossômicos e na origem dos sistemas de cromossomos sexuais em *Harttia*.

Com relação aos rearranjos cromossômicos, foram identificados blocos cromossômicos homeólogos portadores de sítios de DNA ribossômicos, os quais ancorados em dados filogenéticos, possibilitaram evidenciar algumas rotas de diversificação cariotípica destes segmentos cromossômicos, bem como a dispersão destas sequências pelo genoma de *Harttia*. Embora os mecanismos responsáveis pela instabilidade genômica no gênero ainda não estejam bem compreendidos, foi possível notar regiões enriquecidas com clusters de DNA repetitivo em prováveis sítios propensos a quebras e rearranjos cromossômicos em *Harttia*. Assim, este trabalho abre portas para futuros estudos que venham elucidar e caracterizar essas regiões no genoma deste gênero de peixes.

A respeito da origem dos dois sistemas de cromossomos sexuais múltiplos, o uso de técnicas de citogenética molecular, como por exemplo a pintura cromossômica total, apontaram para eventos evolutivos diferentes na origem dos sistemas X_1X_2Y e Y_1Y_2. Enquanto o sistema X_1X_2Y se originou através de um evento de translocação, o sistema XY_1Y_2 foi originado pela fusão de dois pares autossômicos, provavelmente a partir de um par sexual XX/XY homomórfico. Esses resultados reforçam a ocorrência de uma série de rearranjos principalmente para a origem do sistema XY_1Y_2, além de indicar eventos de translocação envolvendo o cromossomo X.

Os dados de pintura cromossômica também trouxeram evidências concretas da origem independente dos dois sistemas de cromossomos sexuais múltiplos no gênero *Harttia*. Este fato já havia sido documentado em outros grupos de peixes, mas ainda não

havia sido documentado neste gênero. Assim, demonstrou que a origem do sistema de cromossomos sexuais XY_1Y_2 está diretamente relacionada com a diminuição do número diploide no gênero *Harttia*. Neste trabalho foi possível mapear, dois dos três eventos de fusão que ocorreram até o menor 2n já relatado para esse grupo (2n=52), sendo o primeiro para a formação do cromossomo X que corresponde ao maior par de cromossomos metacêntricos e o segundo na origem do par cromossômico submetacêntrico 9 em *H. carvalhoi.* Além disso, o evento de redução do 2n em *H. loricariformis* apresenta-se como um evento independente e não está relacionado com os mesmos cromossomos envolvidos com a diminuição do número diploide em *H. torrenticola*.

A morfologia diferente dos cromossomos Y_1 e Y_2 em *H. intermontana* comparado a *H. carvalhoi* reforçam eventos de diferenciação após a formação do sistema, possivelmente responsável por diminuir a recombinação entre os dois cromossomos, característica inerente de sistemas sexuais múltiplos. Além disso, o evento de translocação ocorrido em *H. intermontana*, indicou um possível envolvimento de sequências de DNA repetitivo acumuladas em regiões de quebras cromossômicas presentes no cromossomo X e 9 na origem do sistema XY_1Y_2 em *H. intermontana.*

Neste contexto, os resultados aqui apresentados, juntamente com os dados já publicados na literatura, apontam esse grupo de peixes como modelo para estudos de rearranjos cromossômicos, com a descrição e caracterização de novas espécies em diversas bacias hidrográficas, além da investigação e origem de novos casos de sistemas de cromossomos sexuais simples e múltiplos. Também representa um grupo interessante para futuras investigações genômicas no estudo e caracterização de sequências envolvidas em quebras cromossômicas.

ANEXOS

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Article

Highly Rearranged Karyotypes and Multiple Sex Chromosome Systems in Armored Catfishes from the Genus *Harttia* (Teleostei, Siluriformes)

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Abstract: *Harttia* comprises an armored catfish genus endemic to the Neotropical region, including 27 valid species with low dispersion rates that are restricted to small distribution areas. Cytogenetics data point to a wide chromosomal diversity in this genus due to changes that occurred in isolated populations, with chromosomal fusions and fissions explaining the 2n number variation. In addition, different multiple sex chromosome systems and rDNA loci location are also found in some species. However, several *Harttia* species and populations remain to be investigated. In this study, *Harttia intermontana* and two still undescribed species, morphologically identified as *Harttia* sp. 1 and *Harttia* sp. 2, were cytogenetically analyzed. *Harttia intermontana* has 2n = 52 and 2n = 53 chromosomes, while *Harttia* sp. 1 has 2n = 56 and 2n = 57 chromosomes in females and males, respectively, thus highlighting the occurrence of an XX/XY_1Y_2 multiple sex chromosome system in both species. *Harttia* sp. 2 presents 2n = 62 chromosomes for both females and males, with fission events explaining its karyotype diversification. Chromosomal locations of the rDNA sites were also quite different among species, reinforcing that extensive rearrangements had occurred in their karyotype evolution. Comparative genomic hybridization (CGH) experiments among some *Harttia* species evidenced a shared content of the XY_1Y_2 sex chromosomes in three of them, thus pointing towards their common origin. Therefore, the comparative analysis among all *Harttia* species cytogenetically studied thus far allowed us to provide an evolutionary scenario related to the speciation process of this fish group.

Keywords: chromosomal rearrangements; comparative genomic hybridization; fish mapping; fish species; karyotype evolution; sex chromosomes

1. Introduction

Fishes exhibit the greatest biodiversity among the vertebrates, constituting a useful model for studying several evolutionary questions [1]. Particularly, the large river networks found in the Neotropical region contain the world's richest biodiversity. Despite the large geographic distribution of the Neotropical fish families, different species are found inhabiting adjacent river basins split

by vicariant events millions of years ago [2]. In the same way, species inhabiting small streams, with limited migration opportunities, tend to present an increased rate of speciation [3], even in parapatric populations [4].

One of these examples relies on the genus *Harttia* (Siluriformes, Loricariidae, Loricariinae), an endemic and widespread group throughout many South American river basins [5]. These species have a sedentary lifestyle and reduced vagility, leading them to inhabit specific sections of the river and to form small local populations [6].

Studied species indicate a wide variation on the diploid number (2n) in *Harttia*, ranging from 2n = 52 to 62 chromosomes, with the occurrence of interstitial telomeric sites (ITS) as vestiges of chromosomal changes, different ribosomal genes distributions on the karyotypes, occurrence of B chromosomes, and multiple sex chromosome systems (Table 1). Based on the molecular phylogenetic inferences in the Harttiini tribe [7], a scenario for *Harttia* karyotype diversification was proposed by Blanco et al. [8]. In this scenario, a putative ancestral karyotype would have 2n = 58 chromosomes and no heteromorphic sex chromosomes, such as that found in *Harttia kronei* [8]. From such a karyotype, reductions in 2n number by chromosome fusions were proposed in the diversification of lineages, until the lowest number of chromosomes, 2n = 52, found in *H. carvalhoi* females was reached [8].

Furthermore, the chromosomal rearrangements in *Harttia* species culminated in different kinds of sex chromosome systems: (*i*) an XX/XY_1Y_2 system in *H. carvalhoi* [9]; (*ii*) an $X_1X_1X_2X_2/X_1X_2Y$ system in *Harttia punctata*, *Harttia duriventris*, and *Harttia villasboas* [10,11]; and (*iii*) a neo XX/XY system in *Harttia rondoni* [11]. In accordance with the two branches of *Harttia* species [7], these sex chromosome systems followed independent evolutionary origins [8,11]. While *H. carvalhoi* is grouped with *H. kronei*, *H. longipinna*, *H. loricariformis*, and some other species distributed in southern and southeastern Brazil, *H. punctata*, *H. rondoni*, *H. duriventris*, and *H. villasboas* are grouped in a different branch with other species from the north and northeast Brazilian drainages [7].

Simple sex chromosome systems are proposed to have originated by an inversion event or by the accumulation of transposable elements in one homologue of a proto sex chromosome pair, thus promoting a cross-over restricted region able to differentiate in a sex-specific chromosomal segment [12–14]. Additionally, with regard to multiple sex chromosomes, different types of chromosomal rearrangements (such as centric fusions, centric fissions, pericentric inversions, translocations, tandem translocations), usually associated with autosomes and proto-sex chromosomes, have already been proposed to explain the origin of different types of systems [9,15–19]. More recently, and with the aim of discovering the evolutionary origin of the sex chromosomes systems, molecular cytogenetics approaches such as whole chromosome painting (WCP) and comparative genome hybridization (CGH) have been successfully used in some Neotropical fish species [19–22].

In this study, we provide a set of conventional and molecular cytogenetic approaches (Giemsa staining, C-banding, repetitive DNA mapping by FISH, comparative genomic hybridization (CGH)), in an attempt to advance the knowledge of the processes that have shaped the chromosomal evolution in the genus *Harttia*. Data allowed for a comprehensive perspective of the chromosomal diversity and evolutionary trends inside this group, in addition to a description of two other new rare occurrences of the XX/XY_1Y_2 sex chromosome system among fishes.

Table 1. Available cytogenetic data for *Harttia* species.

Species	2n	Karyotype	FN [1]	References
Harttia absaberi	♀♂62	13m + 23sm + 16st + 10a	114	[23]
Harttia carvalhoi	52♀, 53♂	16m + 16sm + 12st + 8a ♀ 15m + 16sm + 12st + 10a ♂	96 96	[9]
Harttia duriventris	56♀, 55♂	16m + 16sm + 16st + 8a ♀ 17m + 16sm + 16st + 6a ♂	104 104	[11]
Harttia gracilis	♀♂58	20m + 22sm + 8st + 8a	108	[8]
Harttia intermontana	52♀, 53♂	14m + 12sm + 12st + 14a ♀ 13m + 12sm + 13st + 15a ♂	90 91	Present study
Harttia kronei	♀♂58	16m + 16sm + 16st + 10a	106	[8]
Harttia longipinna	♀♂58 + 0 – 2 Bs	16m + 12sm + 16st + 14a	102	[24]
Harttia loricariformis	♀♂56	16m + 22sm + 10st + 8a	104	[25]
Harttia punctata	58♀, 57♂	16m + 20sm + 12st + 10a ♀ 16m + 21sm + 12st + 8a ♂	106 106	[10]
Harttia rondoni	♀♂54	20m + 26sm + 4st + 4a	104	[11]
Harttia torrenticola	♀♂56	16m + 10sm + 16st + 14a	98	[9]
Harttia villasboas	56♀, 55♂	18m + 24sm + 6st + 8a ♀ 19m + 24sm + 6st + 6a ♂	104 104	[11]
Harttia sp. 1 (Macacos stream)	56♀, 57♂	14m + 14sm + 10st + 18a ♀ 13m + 14sm + 10st + 20a ♂	94 94	Present study
Harttia sp. 2 (Barra Grande river)	♀♂62	16m + 14sm + 12st + 20a	104	Present study

[1] FN = fundamental number.

2. Materials and Methods

2.1. Specimens

Three *Harttia* species not yet studied were investigated. Their collection sites, number, and sex of individuals are presented in Figure 1 and Table 2. Figure 1 also depicts the Brazilian distribution of other *Harttia* species previously studied. Fishes were collected with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-License Nsº. 10538-3 and 15117-1), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09). The species were properly identified by Dr. Oswaldo Oyakawa (curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP), with expertise on *Harttia* taxonomy. One of the three species corresponds to *Harttia intermontana*, and the other two correspond to new species that have not yet been described, here named as *Harttia* sp. 1 and *Harttia* sp. 2.

Table 2. Collection sites, sample sizes (*n*), and sex of the *Harttia* species analyzed.

	Species	Locality	*n*
1.	*Harttia intermontana*	Piranga river, Carandaí, MG (Brazil) (20°59′34.0″ S, 43°43′30.0″ W)	20♀, 13♂
2.	*Harttia* sp. 1	Macacos stream, Silveira, SP (Brazil) (22°40′43.0″ S, 44°51′25.0″ W)	10♀, 7♂
3.	*Harttia* sp. 2	Barra Grande river, Prudentópolis, PR (Brazil) (24°58′40.72″ S, 51°7′34.25″ W)	17♀, 11♂

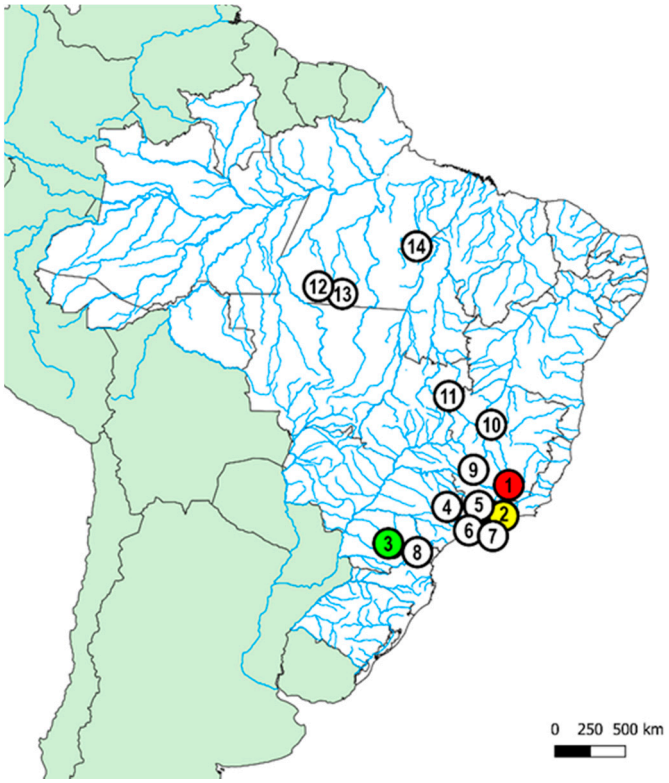

Figure 1. Partial map of South America highlighting the Brazilian collection sites of the three *Harttia* species analyzed in the present work (color circles) named: 1. *H. intermontana* (red circle); 2. *Harttia* sp. 1 (yellow circle); and 3. *Harttia* sp. 2 (green circle). The white circles represent other *Harttia* species previously studied: 4. *H. absaberi*, 5. *H. carvalhoi*, 6. *H. gracilis*, 7. *H. loricariformis*, 8. *H. kronei*, 9. *H. torrenticola*, 10. *H. longipinna*, 11. *H. punctata*; 12. *H. rondoni*; 13. *H. villasboas*, and 14. *H. duriventris*. Map created using QGis 3.4.3 and Adobe Photoshop CC 2020.

2.2. Chromosome Preparations and C-Banding

Mitotic chromosomes were obtained from cells of the anterior region of the kidney after in vivo colchicine treatment according to the protocol described in Bertollo et al. [26]. The experiments followed ethical and anesthesia procedures that were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315). The C-positive heterochromatin (C-banding) was identified according to Sumner [27] with some modifications according to Lui et al. [28].

2.3. Fluorescence In Situ Hybridization (FISH)

Two tandemly arrayed rDNA probes were obtained by PCR from the nuclear DNA of *Harttia intermontana*. The 5S rDNA probe included 120 base pairs (bp) of the 5S rRNA transcript region and 200 bp of a non-transcribed spacer (NTS), isolated according to Pendás et al. [29]. The 18S rDNA probe contained a 1400 bp segment of the 18S rRNA gene and was isolated following Cioffi et al. [30]. The probes were directly labeled with the Nick-Translation mix kit (Jena Bioscience, Jena, Germany) using ATTO550-dUTP for the 5S rDNA and AF488-dUTP for the 18S rDNA, according to the manufacturer's manual. Telomeric (TTAGGG)n sequences were also mapped using the DAKO Telomere PNA FISH Kit/FITC (DAKO, Glostrup, Denmark). FISH experiments followed the methodology described in Yano et al. [31].

2.4. Comparative Genomic Hybridization (CGH)

The total genomic DNA (gDNA) from male and female specimens of *H. intermontana*, *Harttia* sp. 1, and *H. carvalhoi* were extracted from liver tissues by the standard phenol-chloroform-isoamyl alcohol method [32]. The CGH experiments were focused on inter and intraspecific comparisons, with special emphasis on the XY_1Y_2 sex chromosomes. In the first set of experiments (intraspecific genomic comparisons), the male-derived gDNA of *H. intermontana* and *Harttia* sp. 1was labeled by nick translation (Jena Bioscience) with ATTO550-dUTP, while female gDNA was labeled with Atto488-dUTP. Repetitive sequences were blocked in all experiments by using unlabeled C_0t-1 DNA (i.e., a fraction of genomic DNA enriched for highly and moderately repetitive sequences), prepared according to Zwick et al. [33]. The final hybridization mixture was applied on each slide, which was composed of male- and female-derived gDNAs (500 ng each), plus 25 µg of female-derived C_0t-1 DNA from the respective species. The probe was ethanol-precipitated, and the dry pellets were resuspended in a hybridization buffer containing 50% formamide, 2× SSC, 10% SDS, 10% dextran sulfate, and Denhardt's buffer, pH 7.0. In the second set of experiments (interspecific genomic comparisons), the gDNA samples of all-male specimens now analyzed (plus the gDNA of *H. carvalhoi*, another species harboring the same multiple XY_1Y_2 sex system) were hybridized against metaphase chromosomes of *H. intermontana*. For this purpose, male-derived gDNA of *H. intermontana* was labeled with Atto550-dUTP by nick translation (Jena Bioscience), while the gDNA samples of the other two species were labeled with Atto488-dUTP (*Harttia* sp. 1) and Atto425-dUTP (*H. carvalhoi*) also by nick translation (Jena Bioscience). The three probes were hybridized simultaneously, and the final probe cocktail was composed of 500 ng of the male-derived gDNA of each *H. intermontana, Harttia* sp. 1, and *H. carvalhoi* species and 10 µg of the female-derived C_0t-1 DNA of each species. The chosen ratio of probe vs. C_0t-1 DNA amount was based on fish experiments previously performed in our laboratory [19,34–36]. The CGH experiments followed the methodology described in Symonová et al. [37].

2.5. Microscopic Analyses and Image Processing

At least 30 metaphase spreads per individual was analyzed to confirm the 2n, karyotype structure, and CGH results. Images were captured using an Olympus BX50 light microscope (Olympus Corporation, Ishikawa, Japan), with CoolSNAP camera, and the images were processed using the Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified as metacentric (m); submetacentric (sm); subtelocentric (st), or acrocentric (a) according to Levan et al. [38] and arranged according to decreasing size in the karyotypes. The fundamental number (FN), or number of chromosome arms, was achieved considering just acrocentrics as having a single chromosome arm.

3. Results

3.1. Karyotypes, C-Banding, and Sex Chromosomes

All *H. intermontana* females have 2n = 52 chromosomes (14m + 12sm + 12st + 14a; NF = 90) and all males have 2n = 53 chromosomes (13m + 12sm + 13st + 15a, NF = 91). Similarly, *Harttia* sp. 1 also differs in female and male karyotypes, with 2n = 56 (14m + 14sm + 10st + 18a; NF = 94) and 2n = 57 (13m + 14sm + 10st + 20a; NF = 94), respectively. In both cases, the sex-specific karyotypes are due the occurrence of an XX/XY_1Y_2 multiple sex chromosome system, where the X chromosome corresponds to a large metacentric, and the Y_1 to a medium-size acrocentric. In its turn, the Y_2 chromosome corresponds to a medium-size subtelocentric in *H. intermontana* and to an acrocentric chromosome in *Harttia* sp. 1 (Figure 2a,c). Additionally, *Harttia* sp. 2 has 2n = 62 chromosomes (16m + 14sm + 12st + 20a; NF = 104) in both sexes, without morphologically differentiated sex chromosomes (Figure 2e).

A small amount of C-positive heterochromatin was found in the three species, mostly in the centromeric/pericentromeric regions of some chromosome pairs (Figure 2b,d,f), without specific accumulation in the sex chromosomes of *H. intermontana* and *Harttia* sp. 1 (Figure 2b,d).

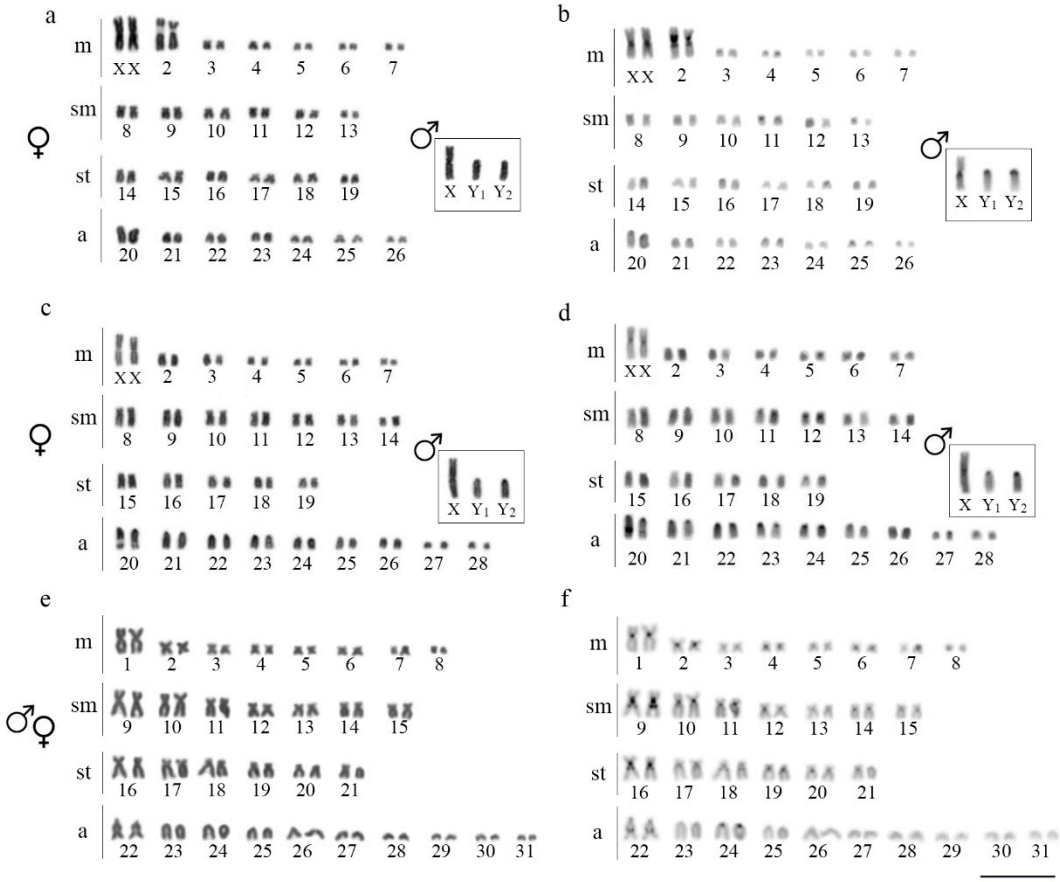

Figure 2. Karyotypes of *H. intermontana* (**a**,**b**), *Harttia* sp. 1 (**c**,**d**), and *Harttia* sp. 2 (**e**,**f**), showing sequentially Giemsa-stained (**a**,**c**,**e**) and C-banded (**b**,**d**,**f**) chromosomes. Insets depict the male sex chromosomes. Bar = 5 μm.

3.2. Chromosomal Distribution of rDNAs and Telomeric Repeats

Differentiation in number and location of the 5S and 18S rDNA sequences was found among the three species. In *H. intermontana* and *Harttia* sp. 2, a single locus of 5S rDNA occurs, but in different chromosomes, i.e., in the submetacentric pairs 11 and 9, respectively. In *Harttia* sp. 1, there are two 5S rDNA loci, one of which is located in the submetacentric pair 12, and the other in the acrocentric pair 20, with a syntenic location with the 18S rDNA in the latter (Figure 3).

The 18S rDNA probe was detected in a single locus in all species, but was also found in different chromosomal locations as follows: in the short arms of the second metacentric pair in *H. intermontana*; in the long arms of the acrocentric pair 20 in *Harttia* sp. 1, and in the long arms of the acrocentric pair 22 in *Harttia* sp. 2. No differences in the number and site positions of rDNA were detected between males and females (Figure 3).

Hybridization with the (TTAGGG)n probe evidenced signals only in the telomeric regions of all chromosomes, without ITS in *H. intermontana* and *Harttia* sp. 1 (Figure 3b,d). However, in *Harttia* sp. 2, four ITS were located in the long arms of the chromosome pairs 1, 9, 16, and 22. A double-FISH using both telomeric and 18S rDNA probes revealed that these sequences present a syntenic location in the chromosome pair 22 (Figure 3f).

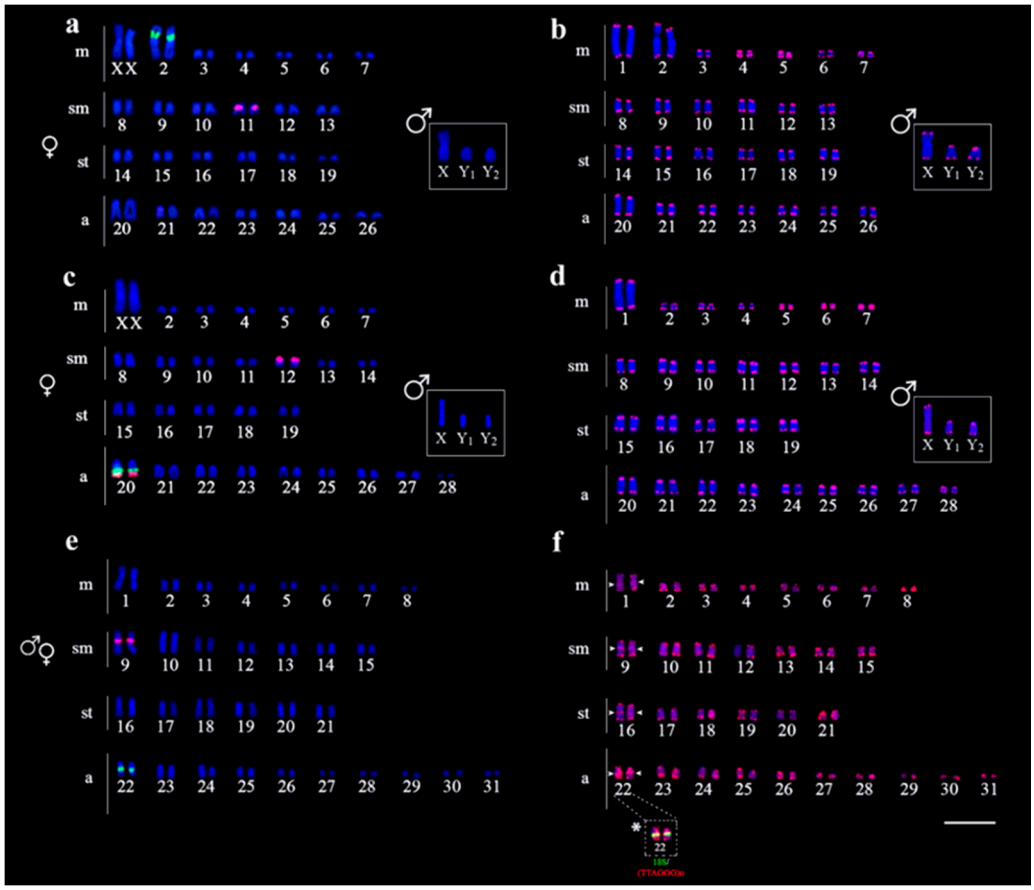

Figure 3. Karyotypes of *H. intermontana* (**a**,**b**), *Harttia* sp. 1 (**c**,**d**), and *Harttia* sp. 2 (**e**,**f**) arranged after FISH with 5S rDNA (red) and 18S rDNA (green) probes (**a**,**c**,**e**), and telomeric (TTAGGG)n probe (**b**,**d**,**f**). Inserts depict the male sex chromosomes. Arrowheads indicate the interstitial telomeric sites (ITS) locations and the (*) signals the joint localization of the 18S and ITS sites. Bar = 5 µm.

3.3. Intraspecific and Interspecific Comparative Genomic Hybridizations

Intraspecific genomic comparisons between males (Figure 4b,f) and females (Figure 4c,g) of *H. intermontana* and *Harttia* sp. 1 showed an overlapped hybridization, mainly in the centromeric and pericentromeric regions of almost all chromosomes (Figure 4d,h). A strong binding preference for the 18S rDNA cluster occurs in *H. intermontana* (Figure 4b,c) and no sex-specific region was evidenced in both experiments. Interspecific comparisons of the gDNA of *H. intermontana*, *H. carvalhoi*, and *Harttia* sp. 1, all of them bearing an XY_1Y_2 sex system, did not detect species-specific regions in the sex chromosomes (Figure 5).

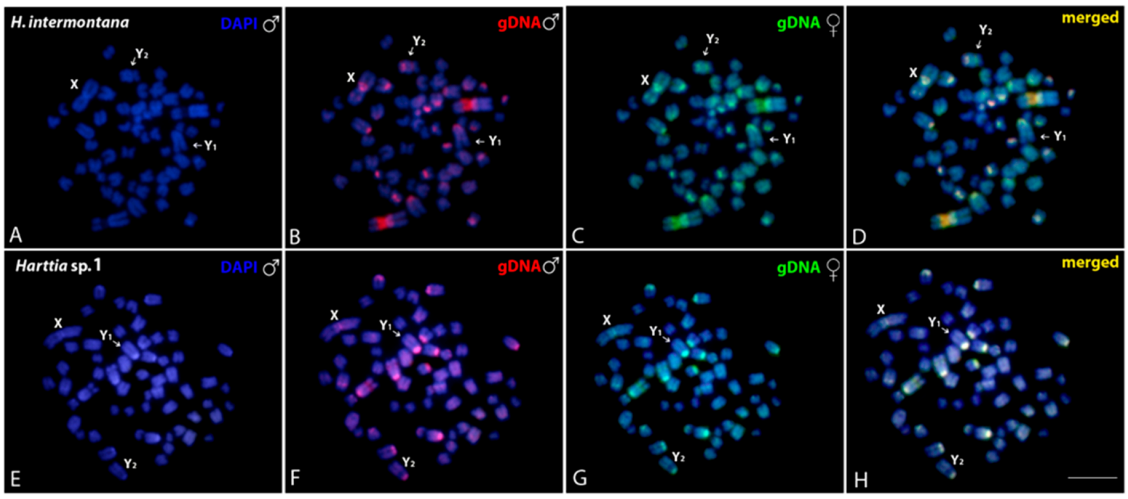

Figure 4. Mitotic chromosome spreads of males *H. intermontana* (**A**–**D**) and *Harttia* sp. 1 (**E**–**H**) after intraspecific genomic hybridizations, with male- and female-derived genomic probes hybridized together for each species. The first column (**A**,**E**): DAPI images (blue); Second column (**B**,**F**): hybridization pattern for the male-derived probe (red); Third column (**C**,**G**): hybridization pattern for the female-derived probe (green); Fourth column (**D**,**H**): merged images for both genomic probes and DAPI staining. The common genomic regions for males and females are depicted in yellow. Arrows indicate the sex chromosomes. Bar = 10 μm.

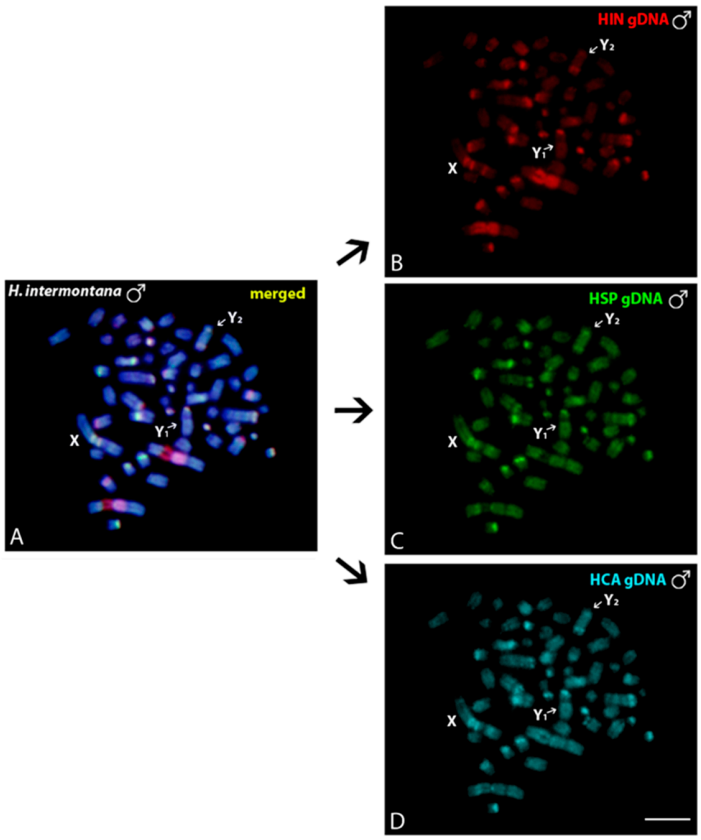

Figure 5. Mitotic chromosome spreads of males from *H. intermontana* (**A**–**D**) after comparative genomic hybridization (CGH): interspecific hybridizations probed with a male-derived genomic probe from *H. intermontana* (**B**), *Harttia* sp. 1 (**C**), and *H. carvalhoi* (**D**). (**A**) depicts the merged images of the genomic probes and DAPI staining. The common genomic regions for male and female are depicted in yellow. Sex chromosomes are indicated. Bar = 10 μm.

4. Discussion

4.1. Numerical Chromosome Changes in Harttia Species

The Loricariidae family is an outstanding group to investigate chromosomal breaks and rearrangements that gave rise to extremely diverse karyotypes among its representatives [39–42]. These fishes are characterized by a sedentary lifestyle, with rare migratory events [43]. Their species occur in small and isolated populations [6] where the fixation of chromosomal rearrangements could occur at higher rates [44–47]. In fact, the Loricariinae subfamily shows extensive numerical chromosome variation (36 to 74), which is attributed to chromosomal rearrangements, mainly to Robersonian fusions (Rb fusion) and fissions [41,48–50]. The *Harttia* genus, in which several cryptic and undescribed species are believed to occur, displays the second-largest chromosomal variation among the Loricariinae (52 to 62, Table 1, Figure 6). In addition, there is also strong evidence for evolutionary breakpoint regions (EBRs) promoting intrachromosomal remodeling, which are still being studied [51].

A putative ancestral karyotype, probably with 2n = 58 chromosomes, is attributed to the *Harttia* lineage, and this same 2n number occurs in its sister group *Farlowella* [52] and in basal species from *Harttia* phylogenetic relationships [7,8]. However, *Harttia* presents different pathways in relation to the 2n diversification, some species keeping 58 chromosomes, some others increasing this chromosome number by centric fissions (i.e., *H. absaberi* and *Harttia* sp. 2 now studied), with others decreasing this number due to Rb fusions (Table 1, Figure 6).

ITS generally reveal chromosomal rearrangements, such as Rb fusions or in tandem fusions [53]. In previous studies, ITS were identified in three *Harttia* species (*H. loricariformis*, 2n = 56; *H. torrenticola*, 2n = 56, and *H. carvalhoi*, 2n = 52♀/53♂), as vestiges of Robertsonian rearrangements [8,9]. It was proposed that fusion events were responsible for originating the largest metacentric pair found in *H. torrenticola* (pair 1) and *H. carvalhoi* (X chromosome), due to the presence of a proximal ITS on their short arms [8]. *Harttia intermontana* and *Harttia* sp. 1 also share a similar large metacentric X chromosome, but no ITS were detected. It is likely that this absence is due to the fact that not all chromosome fusions retain some telomeric DNA repeats at the fusion points [54]. Moreover, the occurrence of different chromosomal rearrangements and modifications of the non-functional telomeric arrays can be also considered [55]. In the last situation, a successive loss and degeneration of the non-functional telomeric repeats that were retained at the fusion sites leads to their gradual shortening, and, consequently, to an insufficient amount to be highlighted by FISH [53,56].

To date, the first largest metacentric pair of *Harttia* is shared by all species that have 2n = 56 chromosomes or a smaller number, except for *H. loricariformis*, and this could be considered as being derived from an Rb fusion chromosome. In *Harttia* sp. 2 the first chromosome pair is also a large metacentric-bearing ITS, however, this chromosome has a small size compared to the chromosome 01 of *H. carvalhoi*, *H. intermontana*, *H. torrenticola*, and *Harttia* sp. 1, thus indicating that additional rearrangements probably played a role on its origin. Noteworthy, *Harttia* sp. 2 presents four bi-armed chromosome pairs bearing ITS at the proximal regions of the long arms. According to the instability genomic proposal, ITS are hotspots for chromosomal breakage [57], and telomeric DNA damage can be irreparable, causing persistent activation in response to DNA damage [58] or remaining as EBRs on the genome [51,59]. This suggests that both ITS and terminal telomeric sequences are naturally prone to breakage, leading to chromosome plasticity [56,60,61]. Here, we propose that *Harttia* sp. 2 increased its chromosome number by centric fissions from an ancestral ITS bearing lineage, which acted as instable sites and promoted double strand breaks (DSBs) triggering further chromosomal rearrangements. This proposal is corroborated by the extensive FN modification among *Harttia* species (Table 1), since only Robertsonian rearrangements keep the FN unchanged throughout the karyotype evolution. It is known that chromosomal rearrangements might play an important role in speciation [47,62]. In this sense, the expressive rearranged karyotypes that are found among *Harttia* species may have acted as significant post-zygotic isolating mechanisms throughout the evolutionary history of this group.

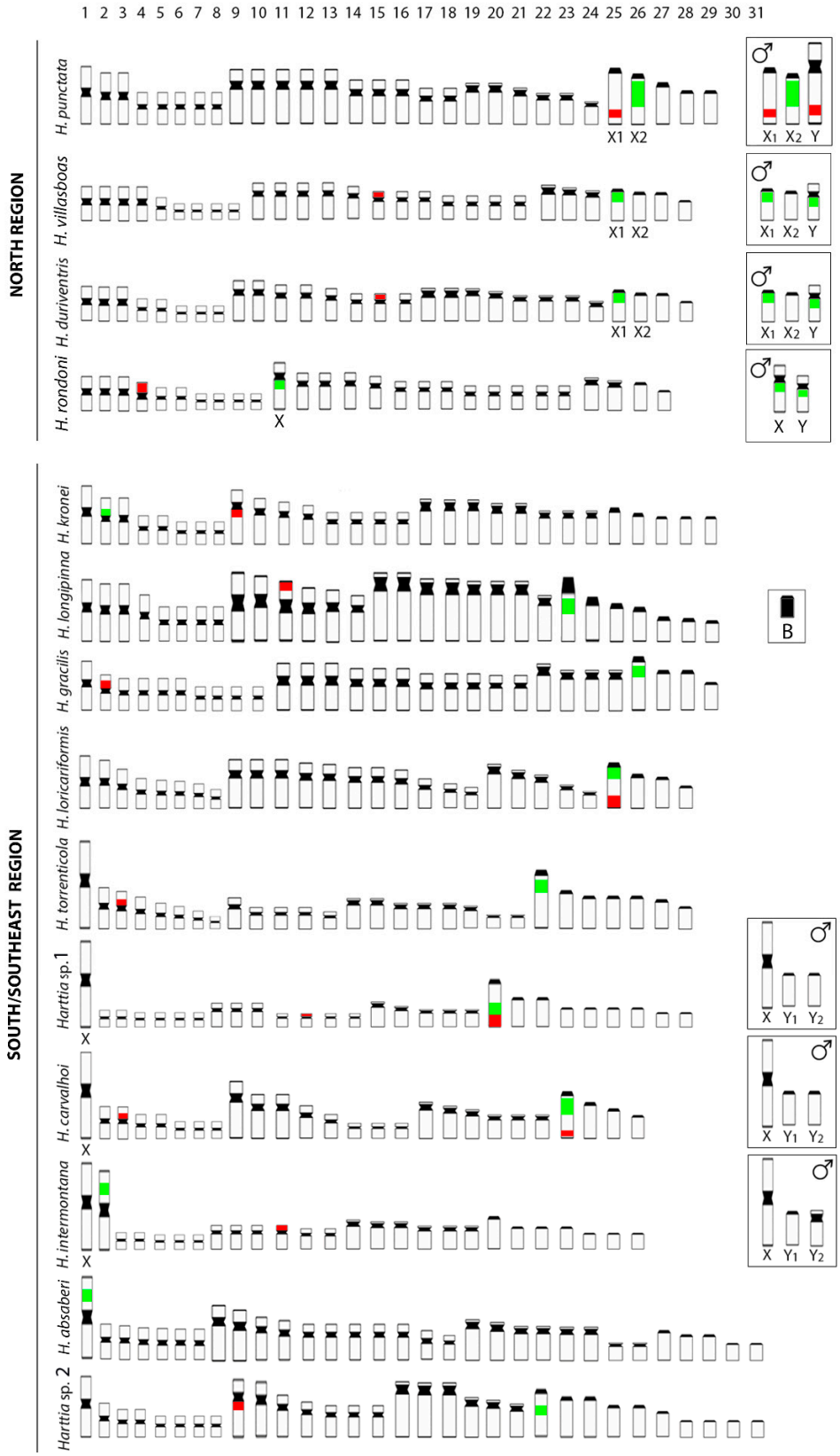

Figure 6. Representative idiograms of *Harttia* species from distinct Brazilian regions based on the distribution of rDNA sequences in their karyotypes, according to the present study, Blanco et al. [8], and Sassi et al. [11] data. The location of the 18S and 5S rDNA sites on the chromosomes are indicated in green and red, respectively. Inserts depict the male sex chromosomes.

4.2. Heterochromatin and rDNA Sites Rearrangements in Harttia Species

The presence of small amounts of heterochromatin is probably an intrinsic characteristic of the *Harttia* species [8]. Indeed, *H. intermontana* and *Harttia* sp. 1 present the same pattern already described for other species of the genus, while in *Harttia* sp. 2, some more prominent pericentromeric bands are colocated with the ITS in the chromosome pairs 1, 9, 16, and 22. The epigenetic regulation of repetitive sequences, such as histone modifications and DNA methylation to form heterochromatin, is proposed to protect ITS from breakages and play important roles in regulation of gene expression [56,63]. In this way, the colocalization of the heterochromatin and ITS may be an expression of an epigenetic property of the *Harttia* sp. 2 genome. In addition, the rDNA loci colocalization with ITS (5S in pair 9 and 18S in pair 22) indicates that these multigene families are also probably associated with chromosomal rearrangements in *Harttia* sp. 2. In the same way, the wide differentiation of the chromosomes carrying the rDNA sequences among *Harttia* species demonstrates that these repetitive sequences may also be closely related to deep chromosomal changes that have occurred within the genus. In fact, in some groups of Loricariidae, the involvement of rDNA pseudogenes colocalized to ITS in chromosomal rearrangements have been demonstrated [40,41,50].

As a whole, three general conditions are found concerning the location of the rDNA genes among *Harttia* species: In the first, a syntenic condition for both 5S and 45S rDNAs occurs, as found in *H. carvalhoi*, *H. loricariformis*, and *Harttia* sp. 1, where the first acrocentric chromosome pair is the carrying one, although with *Harttia* sp. 1 showing a particular syntenic configuration (Figures 3 and 6). Yet, *H. carvalhoi* and *Harttia* sp. 1 present an extra 5S rDNA locus. The second condition includes *H. gracilis*, *H. kronei*, *H. longipinna*, *H. punctata*, *H. villasboas*, *H. duriventris*, *H. torrenticola*, and *Harttia* sp. 2, in which the first acrocentric carries the 18S rDNA site, while the 5S occurs in variable locations of different meta/submetacentric chromosomes (except for *H. rondoni* that has 18S rDNA site in the largest sm). In the third pattern, the 5S locus is found in a submetacentric pair, while the chromosome that carries the 45S rDNA is a large metacentric resulting from a fusion event, as found in *H. intermontana* and *H. absaberi* karyotypes (Figures 3 and 6).

EBRs are DNA clustered regions that are more prone to break and reorganize into genomes, and these specific regions have been described to be re-used during the evolution among related species [64–67]. According to the model, the evolutionary re-use of DSB regions and multiple locus repositioning among karyotypes corroborate to probable EBR occurrences adjacent to rDNA sites in the *Harttia* lineage, similar to those described in other loricariids, such as *Ancistrus* [40] and *Rineloricaria* [41].

4.3. The Rare XX/XY_1Y_2 System in Fish Species

Based on an overview of available fish karyotype data [68], only about 5% of the analyzed species possess heteromorphic sex chromosomes, including approximately 47 cases of multiple sex chromosomes [69]. Among them, some different systems, such as $♀X_1X_1X_2X_2/♂X_1X_2Y$; $♀XX/♂XY_1Y_2$; $♀X_1X_1X_2X_2/♂X_1Y_1X_2Y_2$; $♂ZZ/♀ZW_1W_2$, and $♂Z_1Z_1Z_2Z_2/♀Z_1W_1Z_2W_2$, were already identified as scattered on the fish phylogeny and independently evolved in many lineages and, sometimes, even within a same genus or species [70].

In the *Harttia* genus, two multiple sex chromosome systems were previously described, the $X_1X_1X_2X_2/X_1X_2Y$ one in *H. punctata*, *H. duriventris,* and *H. villasboas* and the XX/XY_1Y_2 system in *H. carvalhoi* [8,11]. While the first one is well-represented among a variety of fish families [18], the XX/XY_1Y_2 system is found in only a few fish species (Table 3). Here, like in *H. carvalhoi* [8], two additional cases were identified in *H. intermontana* and *Harttia* sp. 1.

Multiple XX/XY_1Y_2 sex chromosome systems are proposed to have originated by one bi-armed chromosome fission leading to Y_1 and Y_2 formation [71–73] or by X-autosome fusion forming a large bi-armed X chromosome and subsequent centric fission in the origination of the Y_1 and Y_2 chromosomes [74–77]. In *Harttia* species, the large metacentric 1 observed in *H. torrenticola* is comparable to X chromosome in *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 2 and was proposed to be originated from an Rb fusion [8].

To date, *Harttia* lineages from the south/southeast Brazilian drainages have no proto-sex or XY chromosomes identified, which would corroborate the proposal of an X-autosome fusion acting in the origin of the XY_1Y_2 system. However, the occurrence of *H. torrenticola* (without differentiated sex chromosomes) and *H. carvalhoi* (XY_1Y_2) in the same branch of the phylogenetic relationship [7] and the same CGH pattern among *H. carvalhoi*, *H. intermontana*, and *Harttia* sp. 1 concerning sex chromosomes, point to an Rb fusion leading to their large metacentric X-chromosome, as well as to the similar large metacentric pair 1 of *H. torrenticola*.

Although *H. intermontana* and *H. carvalhoi* possess the same 2n and sex chromosome system (XX/XY_1Y_2), significant differences occur between the karyotype structure of these two species. The absence of several large submetacentric pairs in *H. intermontana* as well as the occurrence of its large second metacentric pair carrying 18S rDNA cistrons are remarkable. Besides that, the morphology of their Y_2 chromosome also differs, corresponding to a subtelocentric in *H. intermontana* and to an acrocentric chromosome in *H. carvalhoi*. By comparing the chromosomal morphology and the distribution of the ribosomal sites, it is possible to infer that some additional rearrangements, such as Rb fusion and/or reciprocal translocation, pericentric inversion, and loss or gain of 5S sequences, took place in the chromosome evolution of these species. All data corroborate EBRs occurrence in adjacent regions to rDNA loci and in the pericentromeric region of the largest metacentric pair in the chromosomal diversification of the *Harttia* species inhabiting south and southeast Brazilian drainages.

Table 3. Multiple XX/XY_1Y_2 sex chromosome systems currently found in teleosts.

Species	2n	Mechanism of Origin	Reference
Bathydraco marri	38♀, 39♂	Y-chromosome fission	[72]
Coregonus sardinella	80♀, 81♂	Y-chromosome fission	[71]
Schistura cf. *fasciolata*	50♀, 51♂	Y-chromosome fission	[73]
Hoplias malabaricus (karyomorph G)	40♀, 41♂	Tandem fusion X-A	[19,74,78]
Gymnotus bahianus	36♀, 37♂	Tandem fusion X-A	[76]
Ancistrus dubius	38♀, 39♂	X-A tandem fusion and further neo-Y chromosome fission	[75,77]
Harttia carvalhoi	52♀, 53♂	Y-chromosome fission	[8,9,79,80]
Harttia intermontana	52♀, 53♂	X-A tandem fusion and further neo-Y chromosome fission	Present study
Harttia sp. 1	56♀, 57♂	X-A tandem fusion and further neo-Y chromosome fission	Present study

5. Conclusions

Our study provided additional evidence on the evolutionary pathways followed by fish species of the genus *Harttia*, highlighting both shared and specific chromosomal features that have emerged throughout their life story. We were also able to identify two new cases of the rare XX/XY_1Y_2 multiple sex chromosomes systems among fishes, displaying a significant particular incidence in the *Harttia* lineages from south/southeast Brazil. The species in this branch, which include the *H. intermontana*, *Harttia* sp. 1, and *Harttia* sp. 2 here studied, experienced different ways of chromosome diversification, such as 2n reduction and increase by Rb fusions and centric fissions, respectively, and the emergence of a XX/XY1Y2 sex chromosome system in different species, in contrast to what occurred with the lineages from north Brazilian regions where the $X_1X_1X_2X_2/X_1X_2Y$ system stands out. The occurrence of deeply reorganized karyotypes in the species here studied are in accordance with EBRs present in the *Harttia* genome, which could be reused for chromosome speciation in this group. As a whole, the present study highlights the importance of cytogenetics as a tool for evolutionary studies and, particularly in the present case, detaching the highly differentiated patterns followed by the *Harttia* lineages from two main Brazilian geographic regions.

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Chromosomal Rearrangements and Origin of the Multiple XX/XY_1Y_2 Sex Chromosome System in *Harttia* Species (Siluriformes: Loricariidae)

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The Neotropical genus *Harttia* comprises species with extensive chromosomal remodeling and distinct sex chromosome systems (SCSs). So far, three different SCSs with male heterogamety have been characterized in the group. In some species, the presence of the XX/XY_1Y_2 SCS is associated with a decrease in diploid numbers and several chromosomal rearrangements, although a direct relation to sex chromosome differentiation has not been shown yet. Here, we aimed to investigate the differentiation processes that have led to the establishment of the rare XX/XY_1Y_2 SCS and track its evolutionary history among other *Harttia* species. For that, four whole chromosome painting probes derived from chromosome 1 of *H. torrenticola* (HTO-1), chromosomes 9 and X of *H. carvalhoi* (HCA-9 and HCA-X), and chromosome X from *H. intermontana* (HIN-X) were applied in nine *Harttia* species. Homeologous chromosome blocks were located in *Harttia* species and demonstrated that Robertsonian (Rb) fusions originated HTO-1, HCA-9, and HCA-X chromosomes, while Rb fissions explain Y_1 and Y_2 sex chromosomes. Specifically, in *H. intermontana*, HCA-X, HCA-9, and the NOR-bearing chromosome demonstrated that homeologous blocks were used in the HIN-X and metacentric pair 2 origins. Consequently, diploid numbers changed between the studied species. Overall, the data also reinforce the existence of unstable genomic sites promoting chromosomal differentiation and remodeling within the genus *Harttia*.

Keywords: fish, karyotype evolution, molecular cytogenetics, unstable genomic sites, whole chromosome painting

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INTRODUCTION

Although sex determination can be environmentally determined in some species, it is usually genetically regulated, and often associated with the presence of sex chromosomes (Furman et al., 2020). According to a widely accepted model, sex chromosomes arise from an autosomal pair due to the emergence of a sex-specific locus in one of the homologous (Bull, 1983; Charl-esworth, 2002). Over time, the ancestral homologous pair undergoes divergences in its genetic composition, recombination rate, and morphology, leading to sex chromosomes differentiation (Charlesworth

et al., 2005; Bachtrog et al., 2014). Thus, sex chromosomes can be recognized according to their size and shape in a karyotype (Ghigliotti et al., 2016). However, sometimes sex chromosomes are indistinguishable concerning their gross morphology, and so defined as homomorphic ones (Chalopin et al., 2015). The most common examples of heteromorphic systems are the XX/XY, where the Y chromosome is restricted to males, and the ZZ/ZW, where the W chromosome is restricted to females (Graves, 2006). Being observed in most mammals and birds, respectively, both systems show different levels of genetic divergence (Graves, 2006; Chalopin et al., 2015). Although little is known about why and how different SCSs have evolved (Tree of Sex Consortium, 2014), the processes associated with their evolutionary origin and differentiation among vertebrates have awoken considerable interest (Devlin and Nagahama, 2002). In fishes, a high diversity of sex-determining mechanisms and SCSs with independent origins can be found (Devlin and Nagahama, 2002; Sember et al., 2021), thus making comparative evolutionary analyzes somewhat difficult. Fishes often present high plasticity concerning sex chromosomes, including none or only subtle changes between the sex pair to major chromosomal rearrangements and size differences (Devlin and Nagahama, 2002).

Among the multiple SCSs, the following types were already identified in fishes $X_1X_1X_2X_2/X_1X_2Y$, $X_1X_1X_2X_2/X_1Y_1X_2Y_2$, XX/XY_1Y_2, $Z_1Z_1Z_2Z_2/Z_1Z_2W_1W_2$, and ZZ/ZW_1W_2 (Kitano and Peichel, 2012). While the $X_1X_1X_2X_2/X_1X_2Y$ system is well-represented among several fish families, the XX/XY_1Y_2 system is found only in a few (Kitano and Peichel, 2012; Sember et al., 2021). In contrast to simple SCSs, where repetitive DNAs play an essential role in sex chromosome differentiation (Yano et al., 2014; Schemberger et al., 2019), multiple SCSs appear forced by divergent evolutionary trends. It appears that chromosomal rearrangements are more relevant to the evolutionary process of multiple SCSs than the accumulation of repetitive sequences (Almeida et al., 2015). For this reason, molecular cytogenetic procedures based on fluorescence *in situ* hybridization (FISH), e.g., using whole chromosome painting (WCP) probes, has been successfully applied in different fish groups, providing new insights into the differentiation of sex chromosomes, especially for multiple ones (Cioffi et al., 2011; Blanco et al., 2014; Oliveira et al., 2018; Moraes et al., 2019).

Harttia is a Neotropical fish group comprising species with distinct diploid numbers and karyotypic variations emerged by extensive evolutionary conserved chromosomal rearrangements (Blanco et al., 2013, 2014, 2017; Deon et al., 2020; Sassi et al., 2020; Sassi et al., 2021). The chromosomal number ranges from 2n = 52 to 62, including B chromosomes and different SCSs (Blanco et al., 2012, 2017; Deon et al., 2020; Sassi et al., 2020; Sassi et al., 2021). In phylogenetic reconstructions, three distinct clades were proposed for the genus, thus reinforcing the extensive diversification experienced by the lineage; also, it is grouping the species according to their South American distribution: (I) from the Guyana shield rivers; (II) from the northern Brazilian rivers; and (III) from the Brazilian south/southeast rivers (Londoño-Burbano and Reis, 2021). Three SCSs were detected so far: (1) the $X_1X_1X_2X_2/X_1X_2Y$ system, present in *H. punctata*, *H. duriventris,* and *H. villasboas*, and (2) a proto/neo-XX/XY system in *H. rondoni*, both belonging to clade II, and (3) the XX/XY_1Y_2 system in *H. carvalhoi, H. intermontana,* and *Harttia* sp.1, species which belong to clade III (Centofante et al., 2006; Blanco et al., 2017; Sassi et al., 2020; Sassi et al., 2021; Deon et al., submitted). Chromosomal data compared to a phylogenetic framework indicate that ancestral karyotype with 2n = 58 chromosomes and without a differentiated SCS could represent a plesiomorphic condition for clade III (Deon et al., submitted). Belonging to the same clade III, the species *H. torrenticola* has a karyotype composed by 2n = 56, undifferentiated sex chromosomes (Blanco et al., 2013), and a large metacentric pair being morphologically similar to the X chromosome of *H. carvalhoi*. WCP-FISH experiments using X_1 and X_2 probes derived from *H. punctata*, confirmed that chromosomes that gave rise to the X_1X_2Y and the XY_1Y_2 systems are evolutionary independent (Deon et al., submitted).

Here, we aimed to investigate the differentiation processes that have led to the establishment of the rare XX/XY_1Y_2 SCS and to track its evolutionary history among other *Harttia* species. For that, we performed a WCP-FISH investigation using four distinct sex chromosome-specific probes hybridized in several species. The results allowed us to identify the main rearrangements involved in the origin of this unique SCS. Besides, the data provide new insights into the origin and evolution of such a rare XY-derived SCS, consequently increasing our knowledge about the evolution of vertebrate sex chromosomes.

MATERIALS AND METHODS

Individuals and Chromosome Preparation

Representatives of *Harttia* species analyzed in this study are summarized in **Table 1**. Specimens were collected with the authorization of the Chico Mendes Institute for Biodiversity Conservation (ICMBIO), System of Authorization and Information about Biodiversity (SISBIO-Licenses No. 10538-3 and 15117-2), and National System of Genetic Resource Management and Associated Traditional Knowledge (SISGEN-A96FF09), Brazil. Species were identified based on their morphological features by Dr. Oswaldo Oyakawa curator of the fish collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP), Brazil.

Mitotic chromosomes were obtained from kidney cells, according to Bertollo et al. (2015). All procedures agreed with the Ethics Committee of Animal Usage of the Universidade Federal de São Carlos (Process number CEUA 1853260315), Brazil.

Chromosome Microdissection, Probe Preparation, and Labeling

Fifteen copies of each target chromosome were isolated by glass-needle-based microdissection, and obtained DNA was amplified by oligonucleotide primed-polymerase chain reaction (DOP-PCR) as described in Yang et al. (2009). Chromosomes were chosen based on their morphology - bi-armed chromosomes that

TABLE 1 | Collection sites of the studied species, diploid chromosome number (2n), and sample size (N).

Species	2n	Locality	N
H. carvalhoi	♀52, XX ♂53, XY_1Y_2	Grande stream, Pindamonhangaba – SP (22°47′8″S 45°27′19″W)	17♀, 12♂
Harttia sp. 1	♀56, XX ♂57, XY_1Y_2	Macacos stream, Silveiras – SP (22°40′43.0″S 44°51′25.0″W)	10♀, 7♂
H. intermontana	♀52, XX ♂53, XY_1Y_2	Piranga river, Carandaí – MG (20°59′34.0″S 43°43′30.0″W)	20♀, 13♂
H. punctata	♀58, $X_1X_1X_2X_2$ ♂57, X_1X_2Y	Bandeirinha river, Formosa – GO (15°19′25″S 47°25′26″W)	18♀,25♂
H. kronei	58♀♂	Açungui river, Campo Largo – PR (25°22′44″S 49°39′08″W)	10♀, 5♂
H. gracilis	58♀♂	Machadinho stream, Santo Antônio do Pinhal – SP (22°48′31″S 45°41′21″W)	18♀,15♂
H. longipinna	58♀♂	São Francisco river, Pirapora – MG (17°21′22.8″S 44°51′0.2″W)	13♀,16♂
H. loricariformis	56♀♂	Paraitinga river, Cunha – SP (22°52′22″S 44°51′0.2″W)	7♀, 3♂
H. torrenticola	56♀♂	Araras stream, Piumhi – MG (20°16′15″S 45°55′39″W)	8♀, 6♂

SP, São Paulo; MG, Minas Gerais; PR, Paraná; GO, Goiás Brazilian states.

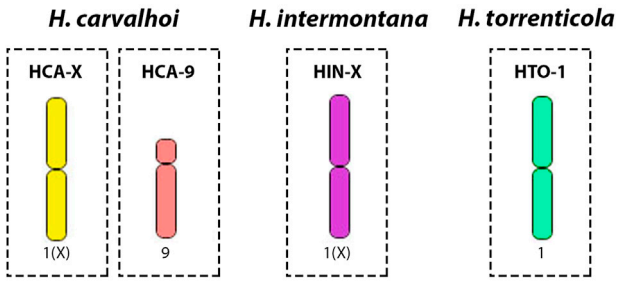

FIGURE 1 | Schematic representation of the microdissected chromosomes used for probe construction in comparative WCP-FISH analyses. 1) the X chromosome of *H. carvalhoi* (HCA-X, in yellow); 2) chromosome 9 of *H. carvalhoi* (HCA-9, in light pink); 3) the X chromosome of *H. intermontana* (HIN-X, in purple); 4) chromosome 1 of *H. torrenticola* (HTO-1, in green).

were suspected to be originated from Robertsonian fusions were targeted: the largest metacentric (HCA-X), and the largest submetacentric (HCA-9) from *H. carvalhoi*; the largest metacentric (HIN-X) from *H. intermontana*, and the largest metacentric (HTO-1) from *H. torrenticola* (**Figure 1**). Probes were labeled with Spectrum Orange-dUTP or Spectrum Green-dUTP (Vysis, Downers Grove, United States) in a secondary DOP-PCR, using 1 µL of the primarily amplified product as a template DNA (Yang and Graphodatsky, 2009).

Fluorescence *in situ* Hybridization

Slides were prepared and pre-treated according to Yang et al. (2009) and denatured in 70% formamide/2x SSC for 3 min at 72°C. For each slide, 12 µL of hybridization solution (containing 0.2 µg of each labeled probe, 50% formamide, 2×SSC, 10% dextran sulfate, and 5 µg of salmon sperm DNA) was denatured for 10 min at 75°C and incubated to pre-hybridize for 1 h at 37°C. To block the hybridization of high-copy repeat sequences, 20 µg of C_0t-1 DNA, directly prepared from *H. carvalhoi, H. torrenticola,* and *H. intermontana* male genomes were used, according to Zwick et al. (1997). Hybridization took place for 48 h at 37°C in a moist chamber. Post-hybridization washes were performed in 1×SSC for 5 min at 65°C, and 5 min in 4×SSC/Tween at room temperature. Finally, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector, Burlingame, CA, United States).

Image Analyses and Processing

Metaphase plates were captured using an Olympus BX50 light microscope (Olympus Corporation, Ishikawa, Japan) coupled with a CoolSNAP camera. The images were processed using Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, United States). The figures were edited and organized using Adobe Photoshop CC 2020 (San Jose, CA, United States) software.

RESULTS

Results obtained by HCA-X and HCA-9 probes are summarized in **Figure 2** and **Table 2**. In *H. carvalhoi* (52♀/53♂ - XX/XY_1Y_2), the HCA-X probe successfully identified their X chromosomes in females and the X, Y_1, and Y_2 chromosomes in males. Small centromeric signals in both acrocentric pairs 23 and 24 were also evidenced. In agreement, the HCA-9 probe correctly recognized the submetacentric pair 9 (**Figures 2A,B**). Similarly, in *Harttia* sp. 1 (56♀/57♂ - XX/XY_1Y_2), the HCA-X probe detected the X chromosome pair in females and the X, Y_1, and Y_2 chromosomes in males, besides small centromeric signals in both 20 and 24 acrocentric pairs (**Figures 2C,D**). The HCA-9 hybridized to 21 and 26 acrocentric pairs (**Figures 2C,D**). In females of *H. intermontana* (52♀/53♂ - XX/XY_1Y_2), the HCA-X probe stained the long (q) arms of the chromosomes X and 2 (**Figure 2E**). In males, this probe gave signals on Xq, the Y_2 chromosome, and the 2q (**Figure 2F**), as well as in the centromeric region of the pair 24 in both males and females (**Figures 2E,F**). The HCA-9 probe detected the short (p) arms of the X chromosome and the 20q distal region in females, and the Xp arms, the Y_1 chromosome, and the 20q distal region in males (**Figures 2E,F**). In *H. punctata* (58♀/57♂ - $X_1X_1X_2X_2/X_1X_2Y$), the HCA-X probe hybridized on the submetacentric pairs 9 and

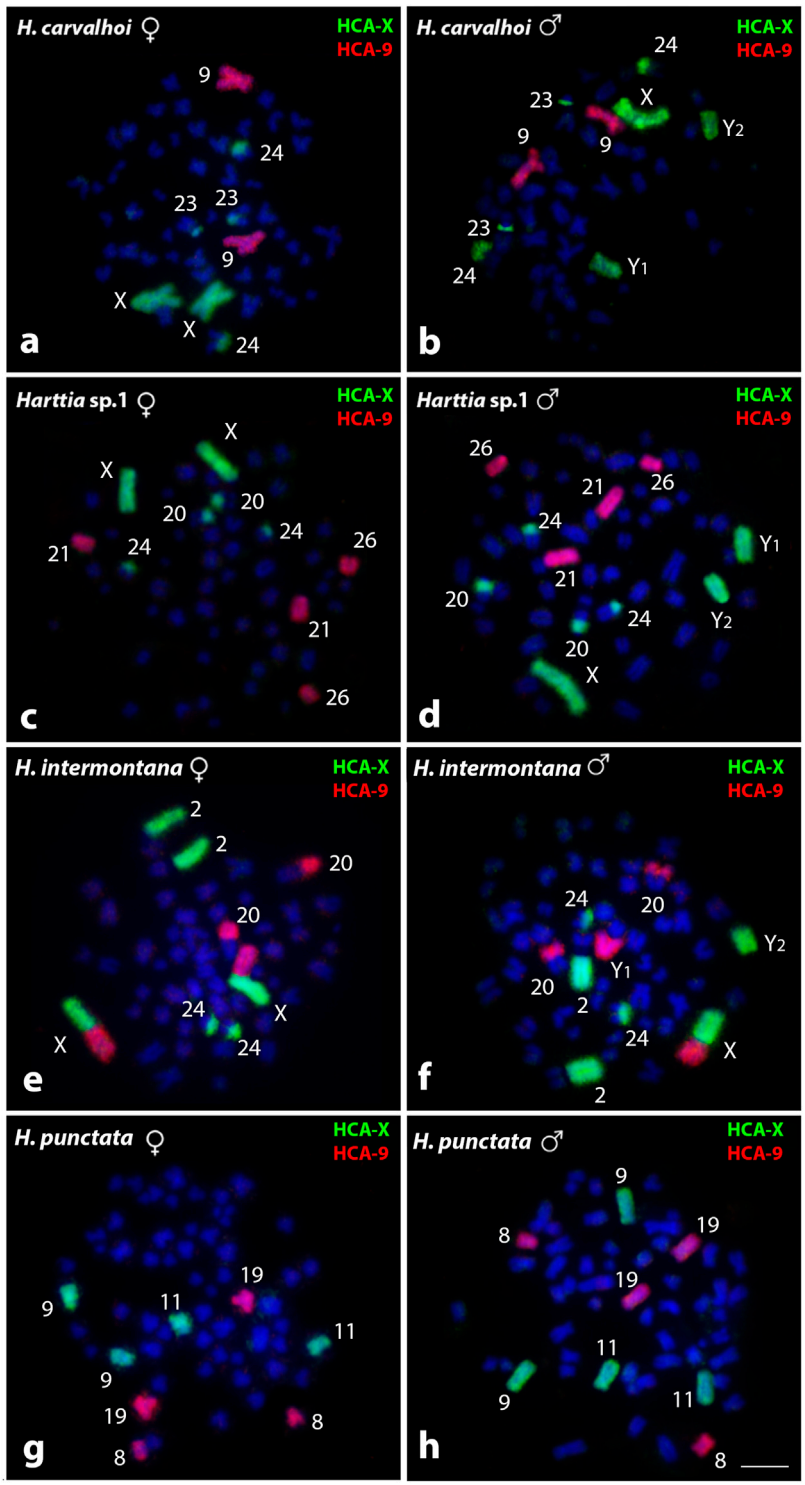

FIGURE 2 | Whole chromosome painting by FISH using HCA-X (green) and HCA-9 (red) probes among *Harttia* species that possess SCSs. The numbers of the labeled chromosome pairs are highlighted in the images. In **(A, B)** metaphases of *H. carvalhoi* female and male, respectively; **(C, D)** metaphases of *Harttia* sp. 1 female and male, respectively; **(E, F)** metaphases of *H. intermontana* female and male, respectively; and **(G, H)** metaphases of *H. punctata* female and male, respectively. Bar = 5 μm.

TABLE 2 | Main localization of the WCP probes in *Harttia* species. Some small signals were not considered. SCSs means sex chromosome systems.

	Species	HCA-Xprobe	HCA-9
XX/XY_1Y_2 system	*H. carvalhoi* ♀	X Chr	Chr. 9
	H. carvalhoi ♂	X, Y_1 and Y_2 Chr	Chr. 9
	Harttia sp. 1 ♀	X Chr	Chr. 21 and 26
	Harttia sp. 1 ♂	X, Y_1 and Y_2 Chr	Chr. 21 and 26
	H. intermontana ♀	Xq and 2q	Xp and 20q distal
	H.intermontana ♂	Xq, Y_2 and 2q	Xp, Y_1 and 20q distal
$X_1X_1X_2X_2/X_1X_2Y$ system	*H. punctata* ♀♂	Chr. 8 and 19	Chr. 9 and 11
Without differentiated *SCSs*	*H. kronei*	Chr. 17 and 19	Chr. 8 and 13
	H. gracilis	Chr. 11 and 22	Chr. 10 and 21
	H. longipinna	Chr. 15 and 17	Chr. 7 and 10
	H. loricariformis	Chr. 9 and 20	Chr. 8 and 11
	H. torrenticola	Chr. 1	Chr. 8 and 23

p, short arms; q, long arms; Chr., chromosome.

11, while the HCA-9 probe showed signals on the metacentric pair 8 and subtelocentric pair 19 (**Figures 2G,H**).

The HTO-1 probe, derived from *H. torrenticola*, showed the same results obtained with the HCA-X probe when tested on those species with an identical large metacentric pair (**Supplementary Figure S1**). In contrast, the HIN-X probe, from *H. intermontana*, showed different results than those obtained applying HCA-X and HTO-1 probes (**Figure 3**). In *H. carvalhoi*, HIN-X hybridized on the Xq and 9q arms in females (**Figure 3A**), and on the Xq arms, Y_2 chromosome, and 9q arms in males (**Figure 3B**). In *Harttia* sp. 1, HIN-X labeled the Xq arms and the acrocentric 21 pair in females (**Figure 3C**), and these same chromosomes, as well as the Y_2 chromosome, in males (**Figure 3D**). In *H. torrenticola*, HIN-X stained the 1q arms and the acrocentric 23 pair (**Figures 3E,F**).

Harttia species without the heteromorphic sex chromosomes were also used as targets for the comparative WCP-FISH using the HCA-X and HCA-9 probes (**Figure 4**; **Table 2**). In *H. kronei* (58♀♂) HCA-X hybridized in the subtelocentric pairs 17 and 19, while the HCA-9 hybridized in chromosome pairs 8 and 13 (**Figure 4A**). In *H. gracilis* (58♀♂), HCA-X marked the submetacentric 11 and the subtelocentric 22, besides centromeric signals in the acrocentric pairs 26 and 28 (**Figure 4B**), and the HCA-9 probe was detected in chromosomes 10 and 21 (**Figure 4B**). In *H. longipinna* (58♀♂), HCA-X hybridized in the subtelocentric pairs 15 and 17, besides the centromeric region of the acrocentric pairs 23 and 25, and HCA-9 hybridized in the metacentric 7, and submetacentric 10 (**Figure 4C**). In *H. loricariformis* (56♀♂), HCA-X was detected in the submetacentric pair 9, subtelocentric 20, and the centromeric region of the chromosome 25 (**Figure 4D**), while the HCA-9 probe hybridized in chromosome pairs 8 and 11 (**Figure 4D**). Finally, in *H. torrenticola* (56♀♂), HCA-X hybridized in chromosome 1 and the centromeric region of the acrocentric pairs 22 and 25, while the HCA-9 probe presents signals of hybridization in pairs 8 and 23 (**Figure 4E**).

DISCUSSION

In *Harttia* species, diploid numbers range from 52 to 62 chromosomes (Deon et al., 2020; Sassi et al., 2020; Sassi et al., 2021). Data from phylogeny reconstructions indicate that 58 chromosomes and no large biarmed chromosomes could correspond to a plesiomorphic karyotype condition for species distributed on south and southeast Brazilian drainages—the clade III (Deon et al., submitted). These chromosomal features (**Figure 5**) include the absence of morphologically differentiated sex chromosomes and a single location of the 5S and 45S rDNA sites in medium-sized bi-armed chromosomes (Deon et al., 2020; Deon et al., submitted). Here, the ancestral reconstructions of the *Harttia* karyotype, using both HCA-X and HCA-9 probes, demonstrated that two chromosome pairs were probably related to the origin of the *H. carvalhoi* chromosomes X and 9 (**Figure 6**). Thus, *in situ* localizations also reaffirm the role of Robertsonian fusions as the main rearrangements responsible for reducing the diploid number in *H. carvalhoi*. These homeologous chromosome pairs (unfused chromosomes) are shared by *H. kronei*, *H. loricariformis*, *H. longipinna,* and *H. gracilis* (**Figure 6**). As common features, *H. kronei*, *H. longipinna,* and *H. gracilis* kept 2n = 58 chromosomes and the absence of morphologically differentiated SCSs (Blanco et al., 2017), with chromosomal diversification events mainly occurring by repositioning of the rDNA sites in their karyotypes (Deon et al., 2020; Deon et al., submitted). Although *H. loricariformis* decreased the diploid number to 2n = 56, this species shares the homologous chromosome pairs to HCA-X and HCA-9 as highlighted in *H. kronei* by WCP-FISH. The presence of interstitial telomeric sites in a subtelocentric chromosome of *H. loricariformis* karyotype suggests an origin by Robertsonian fusions (Blanco et al., 2017). The current data thus support the hypothesis on the occurrence of a chromosomal fusion event in *H. loricariformis* karyotype, and that this corresponds to an independent evolutionary event being not associated with the chromosomes X and 9 of *H. carvalhoi*.

Data also showed that the chromosomal rearrangements that led to the XX/XY_1Y_2 SCS were triggered within the branch with *H. torrenticola* (**Figure 5**). The phylogenetic branch grouping *H. carvalhoi* and *H. torrenticola* (Covain et al., 2016; Londoño-Burbano and Reis, 2021; **Figure 5**) was diversified by Robertsonian fusions, initially giving rise to a large metacentric pair, like that found in the *H. torrenticola* karyotype. Indeed, the large homeologous chromosome

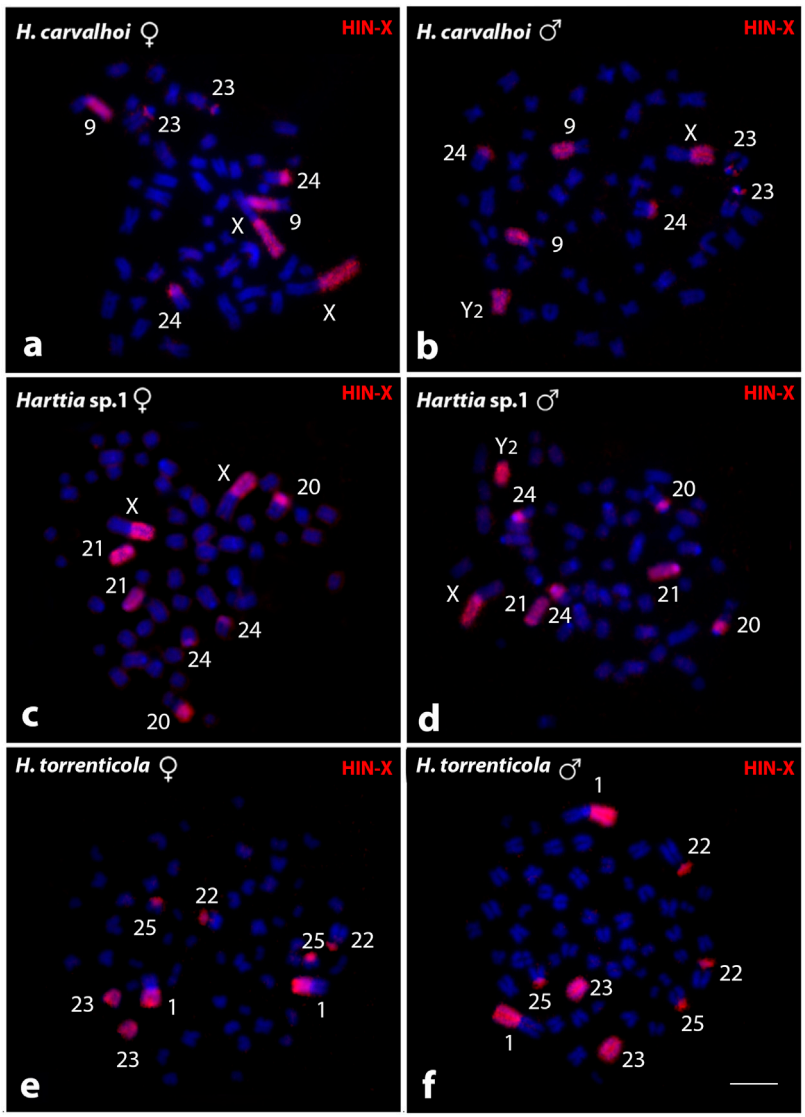

FIGURE 3 | Whole chromosome painting by FISH using the HIN-X probe (red) among *Harttia* species that shared a large metacentric pair. The numbers of the labeled chromosome pairs are highlighted in the images. In **(A, B)** metaphases of *H. carvalhoi* female and male, respectively; **(C, D)** metaphases of *Harttia* sp. 1 female and male, respectively; and **(E, F)** metaphases of *H. torrenticola* female and male, respectively. Bar = 5 μm.

regions shared between the chromosomes 1 of *H. torrenticola* (HTO-1) and the X chromosome of *H. carvalhoi* and *Harttia* sp. 1, corroborate that a single evolutionary event of chromosomal fusion would have generated the large metacentric pair in these species. Although *H. torrenticola* does not show sex chromosome heteromorphism related to the metacentric chromosome 1, in *H. carvalhoi* and *Harttia* sp. 1, this chromosome corresponds to the X sex chromosome, with additional rearrangements triggering the origin of the Y_1 and Y_2 chromosomes. According to former suggestions (Blanco et al., 2017; Deon et al., 2020) centric fission on the largest metacentric formed the Y_1 and Y_2 chromosomes in *Harttia* sp. 1, which are also shared by *H. carvalhoi*. The Y_1 and Y_2 positive hybridizations using the HCA-X probe reiterate that centric fission is the main rearrangement related to the origin of the multiple XX/XY_1Y_2 SCS of *H. carvalhoi* and *Harttia* sp. 1.

However, different from *Harttia* sp. 1 (2n = 56 in females and 57 in males), *H. carvalhoi* diversified its karyotype by other chromosomal fusions, reducing the diploid number to 2n = 52 in females and 2n = 53 in males. Based on the HCA-9 WCP-FISH experiments a chromosome fusion between the subtelocentric pairs 21 and 26, like those found in *Harttia* sp. 1, triggered the origin of pair 9 of *H. carvalhoi* (**Figures 5**, **6**). In fact, despite some morphological alterations, this chromosome pair is represented by two other, homologous pairs in *H. kronei*, *H. loricariformis*, *H. longipinna*, *H. gracilis*, and *H. torrenticola*. Based on conventional cytogenetic studies, Deon et al. (2020) proposed the same origin of the XX/XY_1Y_2 system in *H. carvalhoi*, *Harttia* sp. 1, and *H.*

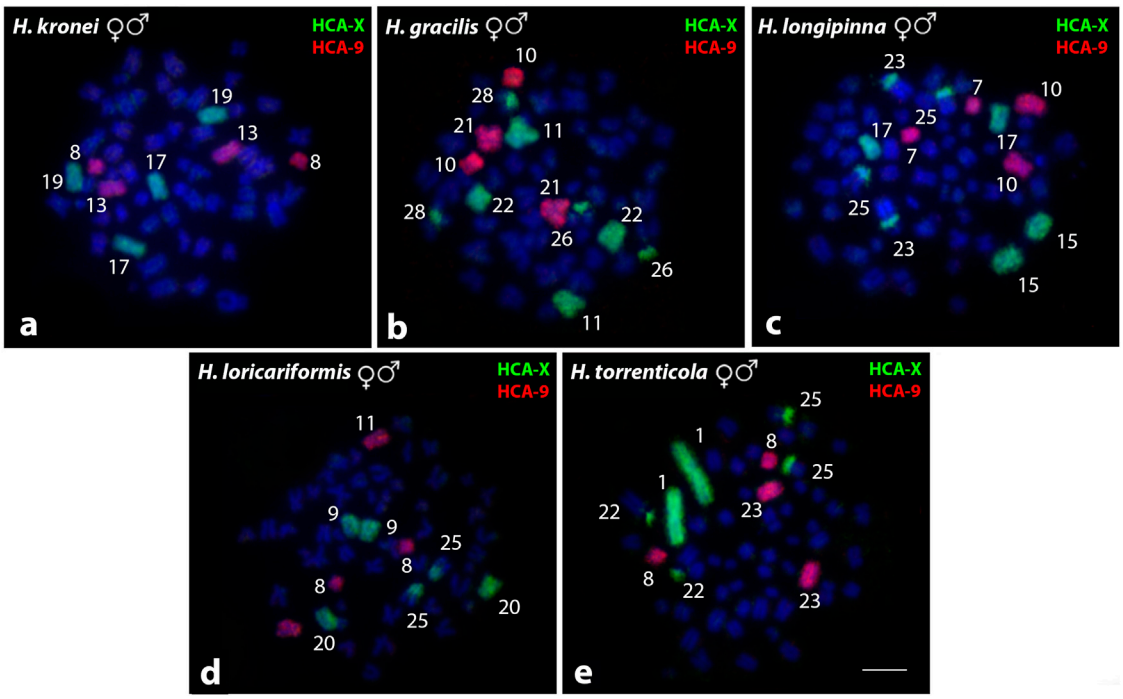

FIGURE 4 | Whole chromosome paint by FISH using HCA-X (green) and HCA-9 probes (red) in *Harttia* species without heteromorphic SCS. The numbers of the labeled chromosome pairs are highlighted in the images. Metaphases of *H. kronei* **(A)**, *H. gracilis* **(B)**, *H. longipinna* **(C)**, *H. loricariformis* **(D)**, and *H. torrenticola* **(E)**. Bar = 5 μm.

intermontana. However, the use of the HCA-X, HCA-9, and HIN-X probes enabled now to evidence that additional rearrangements are associated with the XX/XY_1Y_2 system of *H. intermontana*. The X chromosome of this species comprises the 9q arms and one arm of the X chromosome of *H. carvalhoi*, indicating a reciprocal translocation between these two chromosome pairs in its origin (**Figure 6**). After that, centric fission in one of the X chromosomes, followed by a pericentric inversion in one of the resulted elements, generated the Y_1 and Y_2 chromosomes in males (**Figure 7**). It is relevant to notice that the Y_1 chromosome of *H. intermontana* is derived from chromosome 9 of *H. carvalhoi*, thus different from the Y_1 chromosome of *H. carvalhoi* and *Harttia* sp. 1. In the same way, the metacentric pair 2 of *H. intermontana* was originated from species-specific chromosomal rearrangements, implying a translocation between the acrocentric chromosome bearing the 45S site and one X chromosome arm. Indeed, the chromosome pair of *H. intermontana* bears the 45S rDNA locus (Deon et al., 2020), a site prone to breaks in *Harttia* karyotypes, leading to extensive chromosomal remodeling events (Deon et al., 2020; Deon et al., submitted).

According to molecular-phylogenetic reconstructions, *H. punctata* - 2n = 58♀/57♂, $X_1X_1/X_2X_2/X_1X_2Y$ (Blanco et al., 2014) - belongs to *Harttia*'s clade II (Covain et al., 2016; Londoño-Burbano and Reis, 2021), and the WCP results here obtained evidenced a similar hybridization condition to those found in *H. kronei*, i.e., the HTO-1, HCA-X, HCA-9, and HIN-X chromosomes were not related to the karyotype diversification of *H. punctata*, highlighting a probable plesiomorphic condition.

It was demonstrated that sex chromosomes could emerge independently and follow distinct differentiation patterns, even among closely related species (Cioffi et al., 2013). Our WCP-FISH data also indicated independent origins for the X_1X_2Y and XY_1Y_2 SCSs of *Harttia* lineage, as previously proposed (Deon et al., 2020; Sassi et al., 2020). The X chromosome of the XX/XY_1Y_2 system originated by fusion of two autosome pairs, leading to the largest metacentric in the karyotype. This fusion could set up a putative homomorphic XX/XY SCS, with subsequent centric fission originating the Y_1 and Y_2 chromosomes, as proposed by Blanco et al. (2013). Thus, a set of diverse chromosomal rearrangements probably triggered the differentiation of the same or different SCSs within the *Harttia* lineage, suggesting that sex chromosome turnover may play an important role in the speciation processes of this group.

Evolutionarily conserved breakpoint regions (ECBRs), inside or adjacent to rDNA clusters, were proposed to occur in some Loricariidae lineages, leading to extensive chromosomal remodeling (Barros et al., 2017; Glugoski et al., 2018; Deon et al., 2020; Deon et al., submitted). In the *Harttia* clade III from the south/southeast Brazilian region, several rearrangements adjacent to the rDNAs sites have been extensively reused in the chromosomal diversification (Deon et al., 2020; Deon et al., submitted), including the origin of the $X_1X_1X_2X_2/X_1X_2Y$ SCS in *Harttia* clade II (Deon et al., submitted). In contrast, as rDNAs were not involved in the

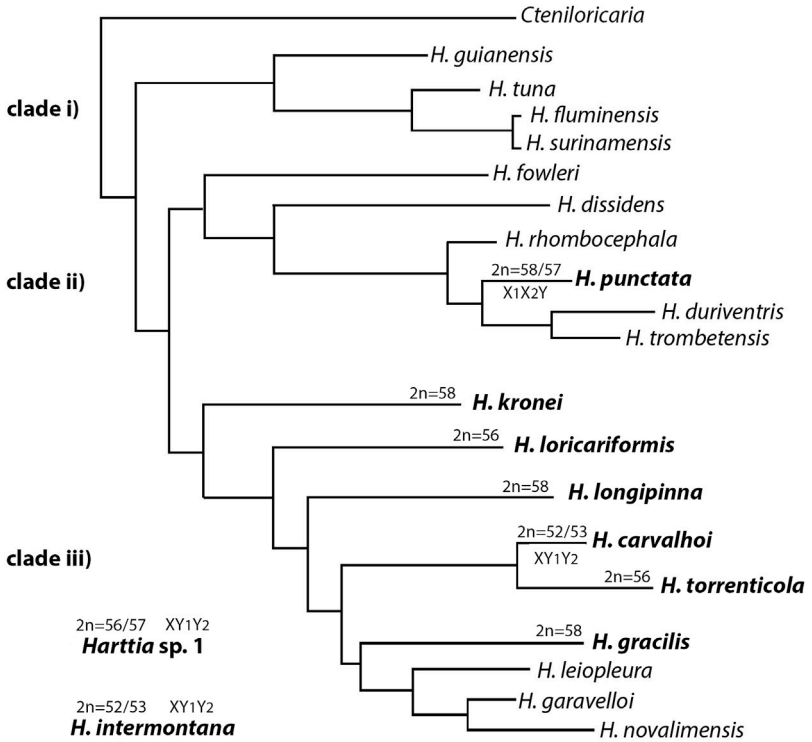

FIGURE 5 | Schematic representation of the phylogenetic relationships among *Harttia* species adapted from the Londoño-Burbano and Reis (2021). *Harttia* sp.1 and *H. intermontana* are not being represented since they were not included in such previous analysis.

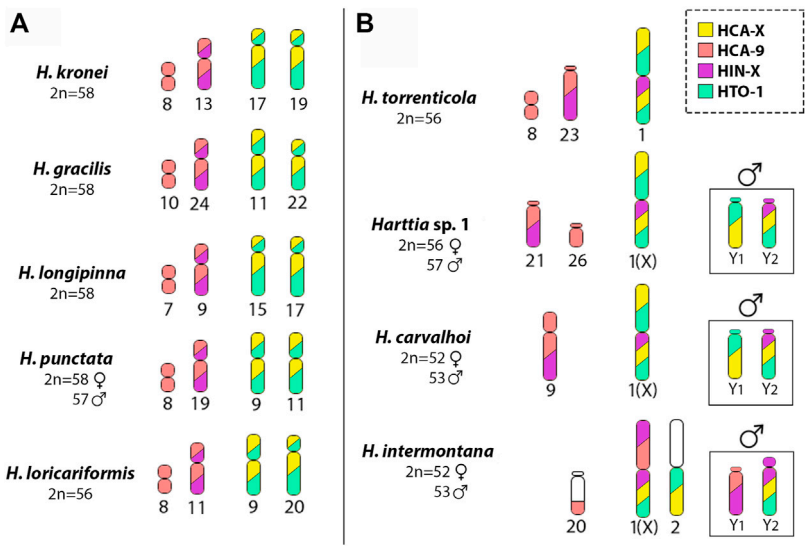

FIGURE 6 | Schematic representation summarizing the distribution of the WCP probes obtained in this study: HCA-X (yellow), HCA-9 (light pink), HIN-X (purple) and HTO-1 (green) in *Harttia* species **(A)** without the largest metacentric chromosome pair -*H. kronei, H. gracilis, H. longipinna, H. loricariformis,* and *H. punctata*- and **(B)** with the largest metacentric pair in the karyotype -*H. carvalhoi, H. torrenticola, H. intermontana* and *Harttia* sp. 1. The highlighted boxes show the male condition and the different composition of the Y_1 and Y_2 chromosomes in *H. carvalhoi, H. intermontana* and *Harttia* sp. 1. Note the overlapping of the HCA-9 and HIN-X probes (light pink and purple), and the HCA-X and HTO-1 (yellow and green) probes.

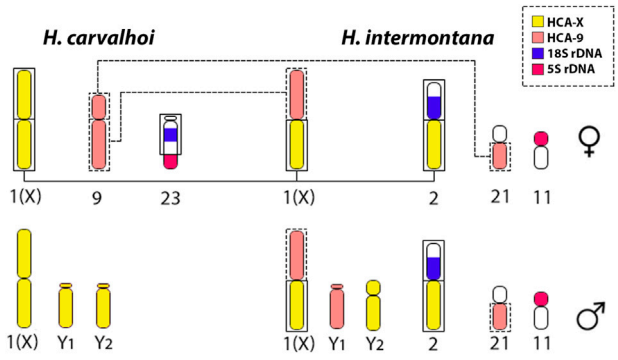

FIGURE 7 | Schematic model representing the rearrangements occurred from *H. carvalhoi* to *H. intermontana*, and the evolvement of HCA-X (yellow) and HCA-9 probes (light pink) on the origin of the XX/XY_1Y_2 SCS. Considering *H. intermontana,* a derived species from *H. carvalhoi*, a centric fusion between the long arms of the chromosome 1 (X) and the long arms of the chromosome 9 of *H. carvalhoi*, lead the origin of the X chromosome in *H. intermontana*. An additional fusion with part of the X chromosome and part of the chromosome 23 (bearing the 18S rDNA site) gave rise to the second-largest metacentric chromosome pair in *H. intermontana* (pair 2). It is worth mentioning that the different origin of the X chromosome directly reflects on the genomic composition of the Y chromosomes: while the Y_1 chromosome corresponds to the long arms of the chromosome 9, the Y2 chromosome corresponds to a part of the X chromosome of *H. carvalhoi.*

origin of the XX/XY_1Y_2 system, other unstable sites likely occur in the genomes of the species from *Harttia* clade III, as well.

In addition, some hybridization signals besides those indicating the discussed main rearrangements were also detected in small chromosomal regions. They correspond to a highly rearranged repetitive DNA unit shared among *Harttia* species. In *H. carvalhoi, H. gracilis, H. longipinna, H. torrenticola,* and *Harttia* sp. 1, they localize close to the nucleolar organizer region (NOR) and in a small acrocentric pair. In *H. loricariformis* and *H. intermontana* only the signal close to the NOR site and in the small acrocentric chromosome were detected, respectively. The mechanisms responsible for the instability of *Harttia* genome are not fully understood (Deon et al., 2020; Deon et al., submitted). However, repetitive DNA clusters scattered at some genome locations are likely candidates for chromosomal breaks and rearrangements. Cytogenetic data indicate that these sequences are reused in several chromosome rearrangements, including the Robertsonian ones responsible for the origin of the SCSs and a 2n decrease in *Harttia*.

Interstitial telomeric sites (ITS) are common features in some *Harttia* genomes (Blanco et al., 2017; Deon et al., 2020). *H. carvalhoi* and *H. torrenticola*, for example, present an ITS in the large metacentric chromosome (Blanco et al., 2017), indicating its origin by Robertsonian fusion. In contrast, this ITS was lost during the chromosomal evolution of *Harttia* sp. 1. It is known that ITS are hotspots for breakages (Slijepcevic et al., 1997) and that telomeric DNA damages can be irreparable, causing persistent DNA-damage-response activation (Fumagalli et al., 2012), or remaining as fragile sites (Sfeir et al., 2009). According to Slijepcevic (2016), both ITS and terminal telomeric sequences are naturally prone to breakage, leading to chromosome plasticity. Therefore, the rearrangements observed in the X and 2 chromosomes of *H. intermontana* may have been triggered by the instability generated by the ITS in the X chromosome of *H. carvalhoi*.

CONCLUSION

Data obtained by WCP-FISH allowed to highlight small pieces of the complex chromosomal evolution that has taken place in *Harttia* species, with a particular emphasis on the origin of a rare multiple SCS and diploid number decrease. We demonstrated the existence of unstable genomic sites promoting chromosomal differentiation and remodeling, where homeologous chromosome blocks were identified after WCP experiments. Besides, we highlighted the distinct Robertsonian fusions and fissions that were involved in the origin the sex chromosomes. In this context, the genus *Harttia* has proved to be an excellent model for the study of evolution of sexual chromosome systems among Neotropical fish species. Next steps now will include a fine-scale analysis of the genetic content of the sex chromosomes in this group aiming to discover novel sex-determining genes, which is an inevitable next step towards fully understating this puzzling scenario.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by All procedures agreed with the Ethics Committee of Animal Usage of the Universidade Federal de São Carlos (Process number CEUA 1853260315), Brazil.

AUTHOR CONTRIBUTIONS

GD, LG, FS, MC, and MV carried out the analysis and drafted the manuscript. TH, VN and AA-R helped in the analysis, drafted, and revised the manuscript. TL, LB, OM-F, MC and MV drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.877522/full#supplementary-material

Supplementary Figure S1 | Hybridizations using the HTO-1 probe (red) in species that present a large metacentric chromosome pair in the karyotype: **(A)** *H. carvalhoi* female, **(B)** *H. carvalhoi* male, **(C)** *Harttia* sp.1 female, **(D)** *Harttia* sp. 1 male, **(E)** *H. intermontana* female and **(F)** *H. intermontana* male. Bar = 5 µm.

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