UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(Biologia Celular, Molecular e Microbiologia)

ANÁLISES CITOGENÔMICAS EM *Proceratophrys* (Amphibia, Odontophrynidae): UMA ABORDAGEM CROMOSSÔMICA E POPULACIONAL

MARCELO JOÃO DA SILVA

Rio Claro – SP
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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia). Área de Concentração: Estrutura, Função e Produção de Biomoléculas.

Orientador: Prof. Dra. Patricia Pasquali Parise-Maltempi

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Dedico esse trabalho aos meus pais, João e Inês, aos meus irmãos, Mara e Marlon e a toda minha família, pelo amor e apoio a mim dedicados.

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“A ciência será sempre uma busca, jamais uma descoberta.
É uma viagem, nunca uma chegada”.
Karl Popper

RESUMO

Neste trabalho, foram realizadas análises citogenéticas, genômicas e bioinformáticas de alto rendimento para investigar a estrutura, a composição molecular, os mecanismos de origem, os padrões de evolução e a possível funcionalidade de elementos repetitivos presentes no genoma da espécie de sapo, *Proceratophrys boiei*. Devido à alta quantidade de heterocromatina C-positiva e ao cromossomo sexual heteromórfico W, *P. boiei* destaca-se como um excelente modelo para estudos citogenéticos evolutivos. Após todas as análises, foi possível caracterizar o satelitoma de *P. boiei* com 226 famílias de DNAs satélites (satDNAs), se tornando a espécie de sapo com o maior número de satélites descritos até o momento. Consistente com a observação de grandes blocos centroméricos de heterocromatina C-positiva, o genoma é enriquecido com um alto número de DNAs repetitivos, com abundância total de satDNAs compreendendo 16,87% do genoma. Dentre eles, o satélite PboSat01-176 despertou o interesse por ter alta abundância genômica e ser caracterizado como relevante repeat centromérico. Dessa forma, visando investigar a presença e compartilhamento genômico, bem como seu potencial para marcador de centrômeros dos cromossomos em espécies de *Proceratophrys*, foram empregadas abordagens citogenômicas envolvendo o satélite PboSat01-176 em quatro diferentes espécies do gênero. Além disso, a análise comparativa de elementos repetitivos em diferentes populações de *P. boiei* permitiu observar que o acúmulo de satDNAs e elementos transponíveis (TEs) pode contribuir para a diferenciação dos cromossomos sexuais observada na espécie. Como resultado, este estudo revelou uma grande diversidade de repetições de satélites e elementos transponíveis que impulsionam a organização genômica nesta espécie. Foi possível notar que pesquisas envolvendo citogenética combinadas com citogenômica em sapos são fascinantes, com potencial para desvendar e contribuir para o entendimento da evolução cromossômica em diversas espécies de anfíbios anuros. Particularmente em *Proceratophrys*, os estudos realizados neste trabalho demonstraram essa dinâmica e importância de abordagens integrativas para investigar evolução cromossômica em um grupo de organismos subexplorado, com uma gama de possibilidades a serem descobertas a partir da diversidade de espécies de anfíbios brasileiros.

Palavras-chave: DNA repetitivo; satelitoma; evolução; anfíbios; cromossomos sexuais; elementos transponíveis.

ABSTRACT

In this study, high-throughput cytogenetic, genomic, and bioinformatic analyses were conducted to investigate the structure, molecular composition, origin mechanisms, evolutionary patterns, and potential functionality of repetitive elements present in the genome of the frog species, *Proceratophrys boiei*. Due to the high amount of C-positive heterochromatin and the heteromorphic W sex chromosome, *P. boiei* stands out as an excellent model for evolutionary cytogenetic studies. Following all analyses, it was possible to characterize the satellitome of *P. boiei* with 226 families of satellite DNAs (satDNAs), making it the frog species with the highest number of described satellites to date. Consistent with the observation of large centromeric blocks of C-positive heterochromatin, the genome is enriched with a high number of repetitive DNAs, with the total abundance of satDNAs comprising 16.87% of the genome. Among them, the satellite PboSat01-176 aroused interest for its high genomic abundance and characterization as a relevant centromeric repeat. Thus, aiming to investigate the presence and genomic sharing, as well as its potential as a centromere marker of chromosomes in *Proceratophrys* species, cytogenomic approaches involving the satellite PboSat01-176 were employed in four different species of the genus. Additionally, the comparative analysis of repetitive elements in different populations of *P. boiei* allowed the observation that the accumulation of satDNAs and transposable elements (TEs) may contribute to the differentiation of the observed sex chromosomes in the species. As a result, this study revealed a great diversity of satellite repeats and transposable elements that drive genomic organization in this species. It was possible to notice that research involving cytogenetics combined with cytogenomics in frogs is fascinating, with the potential to unravel and contribute to the understanding of chromosomal evolution in various species of anuran amphibians. Particularly in *Proceratophrys*, the studies conducted in this work demonstrated this dynamic and importance of integrative approaches to investigate chromosomal evolution in an underexplored group of organisms, with a range of possibilities to be discovered from the diversity of Brazilian amphibian species.

Keywords: repetitive DNA; satellitome; evolution; amphibians; sex chromosomes; transposable elements.

SUMÁRIO

1 INTRODUÇÃO

1.1 O gênero *Proceratophrys*

Em consonância com Frost (2024), a nomenclatura atribuída aos membros do gênero *Proceratophrys* Miranda-Ribeiro, 1920 é *"Smooth Horned Frogs"* (Frank e Ramus, 1995), que se traduz literalmente como "sapos de chifres lisos", porém é mais frequentemente referido como "sapos de chifres". Os sapos *Proceratophrys* demonstram uma camuflagem altamente eficaz ao adotar uma postura de sentar e esperar para caçar, permanecendo invisíveis graças à sua coloração marrom irregular, corpo verrugoso e "chifres" pontiagudos, que se misturam habilmente com as folhas mortas no solo. Seu perfil escondido evita a detecção por predadores durante essa estratégia de emboscada.

Com dimensões adultas atingindo 5 a 7,5 cm de comprimento, possuem uma boca consideravelmente ampla, capaz de engolir presas relativamente grandes, incluindo besouros, grilos, aranhas, baratas e até sapos menores (Figura 1). Estes anfíbios anuros têm sua distribuição geográfica abrangendo regiões do Leste e Sul do território brasileiro, bem como áreas do Nordeste da Argentina e Paraguai, com a possibilidade de sua presença se estender à Bolívia, próximo à fronteira com o Brasil (Figura 2) (Frost, 2024). Estes anfíbios, de natureza endêmica ao Brasil, habitam as zonas costeiras da Mata Atlântica brasileira, colonizando áreas montanhosas e serranas de florestas primárias e secundárias, inclusive áreas com degradação florestal recente (Figura 2)

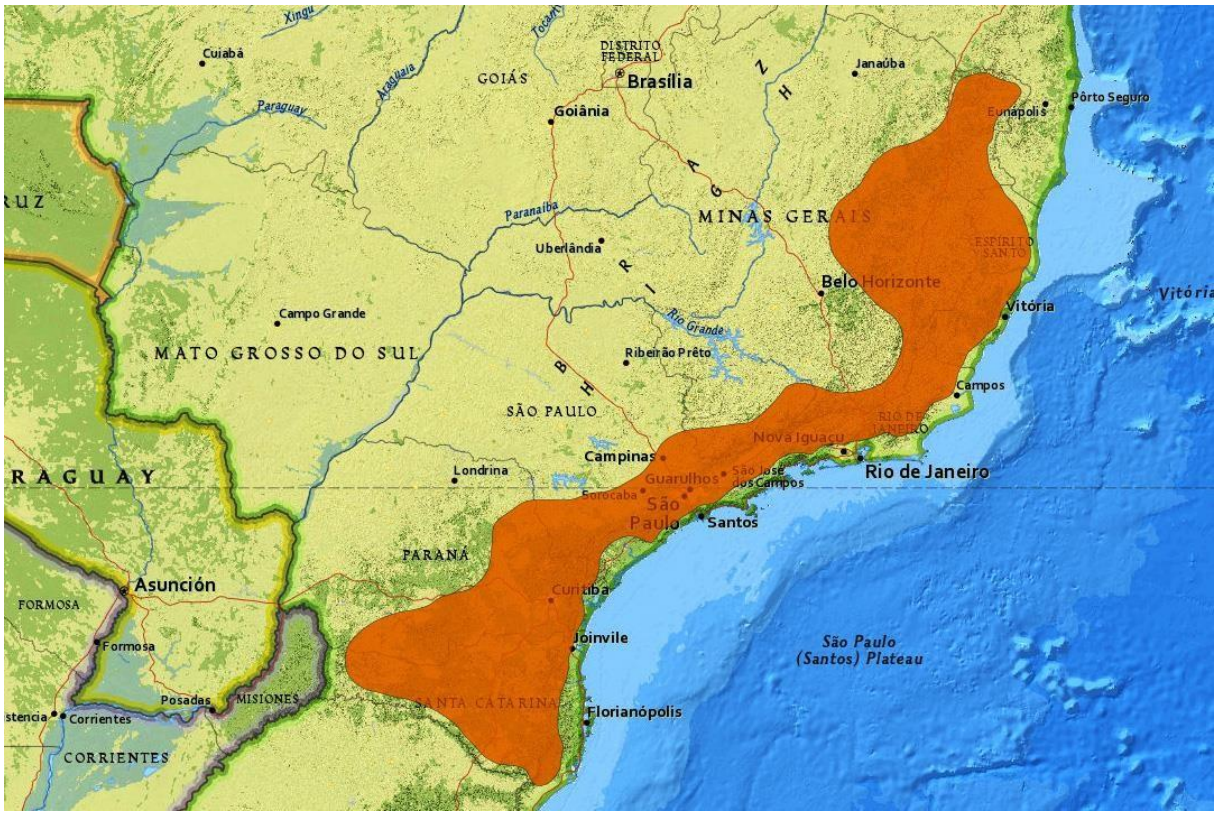
Figura 1. Indivíduo fêmea de *Proceratophrys boiei* proveniente de Tijucas do Sul, Paraná, Brasil.

Fonte: Célio F. B. Haddad.

Quando provocados, adotam uma postura defensiva que envolve o achatamento do corpo, a extensão das patas traseiras para trás e das patas dianteiras para frente, permanecendo imóveis por longos períodos. Essa tática confunde predadores, pois sua aparência lembra ainda mais as folhas caídas, tornando-os mais difíceis de serem identificados como presas (Giaretta *et al.*, 1998; Heyer *et al.*, 1990; Toledo; Zina, 2004; Rocha *et al.*, 2007).

Apesar de sua distribuição abranger uma vasta área e de sua classificação pela IUCN (União Internacional para a Conservação da Natureza) como “Pouco Preocupante”, os sapos de chifres enfrentam a ameaça da perda de habitat devido ao desmatamento. Há um leve envolvimento da espécie no comércio internacional de animais de estimação, mas em níveis baixos, minimizando impactos nas populações (IUCN Red List website, 2024).

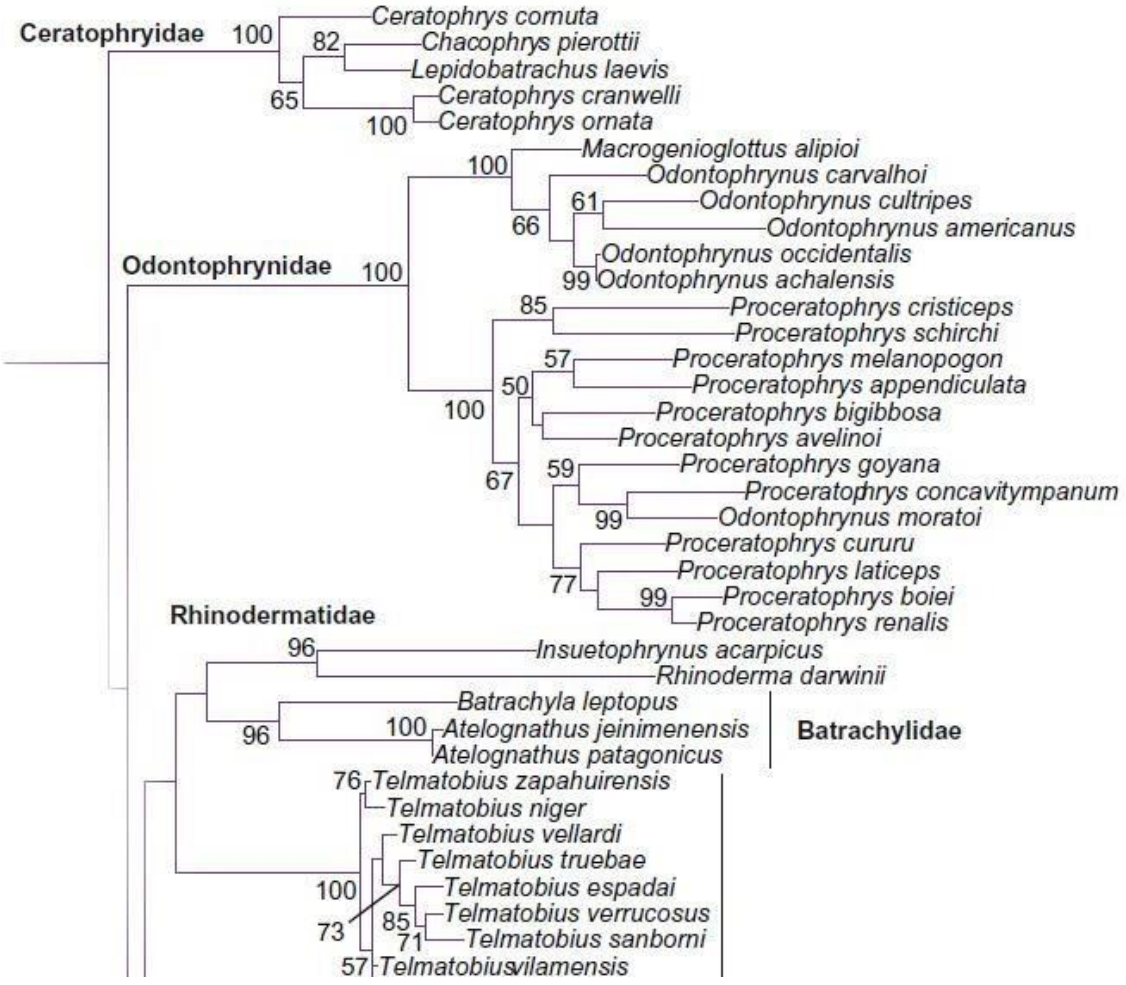
Figura 2. Área de distribuição geográfica da ocorrência de *Proceratophrys boiei*. Note a distribuição predominante em áreas litorâneas correspondentes à Mata Atlântica e remanescentes.



Fonte: Adaptado de IUCN (International Union for Conservation of Nature) 2021. *Proceratophrys boiei*. The IUCN Red List of Threatened Species. Version 2023-1. Sem escala.

Atualmente, o gênero *Proceratophrys* engloba 43 espécies (Frost, 2024), distribuídas em diferentes biomas e domínios morfoclimáticos (como Amazônia, Caatinga, Cerrado, Chaco, Mata Atlântica e Pampas). As espécies foram categorizadas em quatro agrupamentos/complexos fenéticos: a. complexo *P. bigibbosa* (Peters, 1872); b. complexo *P. cristiceps* (Müller, 1883); c. complexo *P. boiei* (Wied-Neuwied, 1824) e d. complexo *P. appendiculata* (Günther, 1873) . Ressalta-se que em contextos de inferência filogenética molecular, com exceção do grupo de espécies *P. bigibbosa*, que foi sustentado como monofilético, os outros três grupos são parafiléticos (Izecksohn *et al.*, 1999; Giaretta *et al.*, 2000; Kwet; Faivovich, 2001; Prado; Pombal Jr., 2008; Amaro *et al.*, 2009; Mângia *et al.*, 2014; Mângia *et al.*, 2018, 2022; Magalhães *et al.*, 2020 (Ver Figura 3).

Figura 3. Árvore filogenética mostrando representantes do gênero *Proceratophrys* e suas relações filogenéticas.



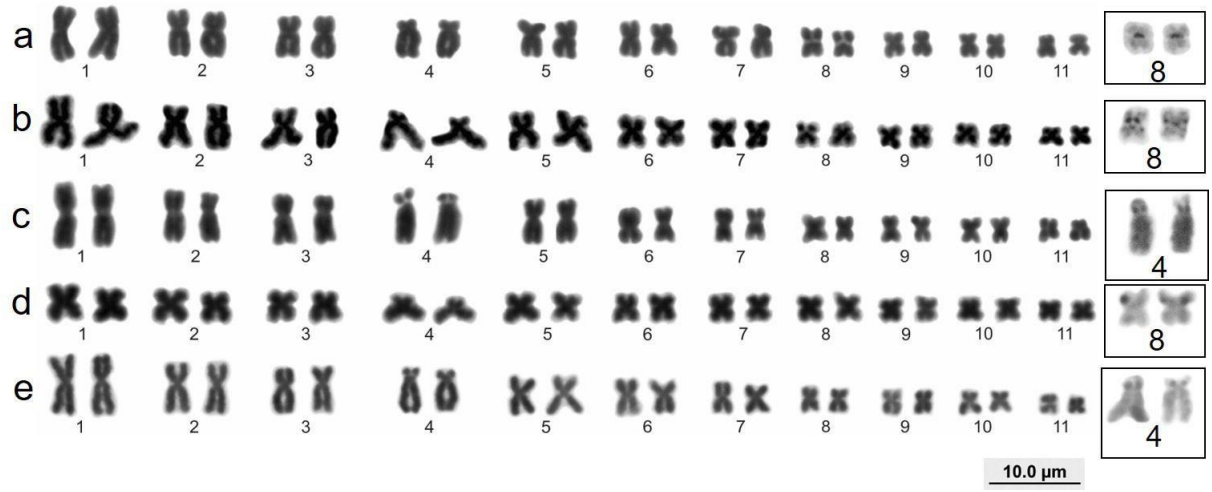
Fonte: Adaptado de Pyron; Wiens, (2011).

Embora o gênero *Proceratophrys* apresente algumas espécies não afiliadas a quaisquer agrupamentos morfológicos específicos listados acima, a exemplo de *P. minuta* Napoli, Cruz,

Abreu, e Del Grande, 2011, *P. redacta* Teixeira, Amaro, Recoder, Vechio, e Rodrigues, 2012, *P. rondonae* Prado e Pombal, 2008 e *P. schirchi* (Miranda-Ribeiro, 1937), não se verificou até o momento a proposição de qualquer reestruturação taxonômica para esses conjuntos de espécies. Importa ressaltar que o gênero *Proceratophrys* persiste sem ter passado por uma revisão sistemática integral e abrangente (Mângia *et al.*, 2022), destacando a necessidade de uma integração de dados moleculares, citogenéticos, citogenômicos, bioacústica, morfologia, para uma interpretação e definição mais clara e precisa sobre as relações filogenéticas envolvendo os representantes desse gênero (Mângia *et al.*, 2020).

Com relação a dados citogenéticos e citogenômicos de *Proceratophrys*, revisões mais antigas como King (1990) e Kuramoto (1990) já reportavam alguns dados para espécies do gênero, no entanto, restritos às espécies *P. boiei* e *P. appendiculata*, com número diploide de 2n = 22 cromossomos. Quase duas décadas depois, Ananias *et al.* (2007) e Amaro *et al.* (2012) revisitaram os dados citogenéticos de *P. boiei* e inferiram questões filogenéticas e citogenéticas interessantes para esta espécie, sobretudo levantando hipóteses para uma reformulação filogenética do gênero. Mais recentemente, algumas espécies de *Proceratophrys* foram submetidas a cariotipagem pela primeira vez (Silva *et al.*, 2021) (Ver figura 4).

Figura 4. Cariótipos de espécies de *Proceratophrys* com coloração de Giemsa. a) *P. schirchi*; b) *P. laticeps*; c) *P. melanopogon*; d) *P. boiei* (sudeste do Brasil) e e) *P. boiei* (sul do Brasil). As caixas referem-se à Região Organizadora Nuclear impregnada com Nitrato de Prata.



Fonte: Silva *et al.* (2021).

Dentre as espécies estudadas por esses autores, *P. melanopogon* (Miranda-Ribeiro, 1926), *P. schirchi* e *P. laticeps* Izecksohn e Peixoto, 1981 chamaram a atenção por seu número diploide conservado, corroborando com o mesmo número cromossômico de *P. boiei*, todos com

2n = 22 cromossomos e padrões de bandas característicos, com bandas de heterocromatina visíveis, localizadas nas regiões centroméricas de seus cromossomos (Silva *et al.*, 2021). Dentro deste grupo, *P. boiei* (2n = 22) destaca-se citogeneticamente com seus cromossomos sexuais W heteromórficos em estágio inicial de diferenciação evolutiva, exibindo um sistema de cromossomos sexuais ZZ/ZW (Ananias *et al.*, 2007; Amaro *et al.*, 2012; Silva *et al.*, 2021).

Essa diferenciação visível é observada especificamente em exemplares do sudeste do Brasil. Em contraste, indivíduos do sul do Brasil não apresentam essa diferenciação baseada em heterocromatina, sugerindo potenciais eventos de especiação envolvendo esta espécie em diferentes regiões (Amaro *et al.*, 2012; Silva *et al.*, 2020). Ainda em *Proceratophrys*, quanto à presença de cromossomos sexuais, *P. schirchi* apresenta evidências de diferenciação de cromossomos sexuais indicada pelo heteromorfismo observado nas análises de heterocromatina após a técnica de bandamento C (Silva *et al.*, 2021). Estudos citogenéticos em *Proceratophrys* refletem a necessidade de análises aprofundadas, principalmente para outras espécies do gênero, que possam trazer dados cromossômicos e detecção de variações para auxiliar no diagnóstico de espécies.

A espécie *P. boiei* vêm se mostrando um interessante modelo para estudos cromossômicos, principalmente pelo notável par cromossômico heteromórfico associado à diferenciação de cromossomos sexuais. Estudos iniciais de investigação da relação de sequências repetitivas e essa diferenciação sexual foram iniciados por Da Silva e colaboradores (2020) e mais recentemente por João da Silva e colaboradores (2023), que apresentaram todo o conteúdo de satDNA de uma população de *P. boiei* do sudeste do Brasil, utilizando análises genômicas, bioinformáticas e citogenéticas abrangentes, revelando um elevado e abundante número de sequências de DNAs satélites (satDNAs). Avanços e acessibilidade ao sequenciamento de DNA e nas análises de dados usando programas de bioinformática estão permitindo análises genômicas comparativas de DNAs repetitivos de maneiras nunca antes consideradas, mesmo para espécies chamadas de não-modelos.

1.2 Considerações sobre DNAs repetitivos: satélites e elementos transponíveis

As sequências de DNA satélites (satDNAs), tradicionalmente, se caracterizam como longas séries de unidades repetidas em tandem de megabases, compostas por muitos milhares de monômeros altamente similares, e são tipicamente encontradas em regiões cromossômicas heterocromáticas (Charlesworth *et al.*, 1994; Plohl *et al.*, 2012; Garrido-Ramos, 2017; Satovic-Vuksic; Plohl, 2023). A outra classe compreende repetições intercaladas que surgem de

processos de transposição, os elementos transponíveis (TEs), se introduzindo em novos locais, provocando mudanças na estrutura, adaptabilidade e evolução dos genomas (Marsano; Dimitri, 2022). Tanto as sequências de satDNA quanto os TEs são considerados elementos fundamentais dos genomas eucarióticos, exercendo papel crucial na evolução (Lopéz-Flores; Garrido-Ramos, 2012; Biscotti *et al.*, 2015).

A variabilidade das repetições de satDNAs é muito interessante do ponto de vista evolutivo, pois as repetições dentro da mesma família de satDNA evoluem de forma não independente, apresentando baixas taxas de divergência entre monômeros, por meio de um processo conhecido como "evolução concertada" (Dover, 1982; Elder; Turner, 1995; Garrido-Ramos, 2017; Thakur *et al.*, 2021). A evolução concertada ocorre através do "impulso molecular", um processo evolutivo que emerge das atividades de uma série de mecanismos onipresentes de renovação do DNA, como *crossing-over* desigual, deslizamento de replicação, replicação de círculo rolante e múltiplas inserções de TEs (Dover, 1982; Dover, 1986; Elder; Turner, 1995; Ugarković; Plohl, 2002; Thakur *et al.*, 2021). No entanto, existem níveis de variação nas taxas de expansão, homogeneização e fixação entre sequências. Essa dinâmica depende de vários fatores, como taxa de mutação, tamanho e estrutura da matriz de repetição (*array*), estrutura cromossômica e taxas de recombinação (López-Flores; Garrido-Ramos, 2012; Plohl *et al.*, 2012; Garrido-Ramos, 2017).

A variação de abundância de famílias de DNAs satélites entre espécies proximamente relacionadas pode ser explicada pelo modelo de bibliotecas de DNAs satélites. Tal modelo explica que espécies relacionadas compartilham uma coleção de sequências de DNAs satélites que era presente no ancestral comum, entretanto a abundância das diferentes famílias nos genomas de cada espécie pode ter um perfil espécie-específico, que é consequência das flutuações no número de cópias das sequências nessa biblioteca (Ugarković; Plohl, 2002; Acosta *et al.*, 2010; Tsoumani *et al.*, 2013).

SatDNAs e TEs têm sido estudados por sua variabilidade e papel na estruturação e funcionamento dos cromossomos, incluindo sua contribuição para a estabilidade genômica. Esses elementos repetitivos podem ter funções variadas, desde manter a estrutura dos cromossomos até influenciar a expressão gênica. No entanto, a compreensão completa de sua função e evolução ainda é um campo ativo de pesquisa na genômica envolvendo diversos grupos de organismos modelos e não-modelos. É, portanto, cada vez mais evidente que uma compreensão completa de cada genoma eucarioto é possível com uma visão detalhada da sua

fração repetitiva. Esta não é uma tarefa fácil e, em geral, ainda estamos longe da plena compreensão sobre a genômica repetitiva do DNA e sua diversidade. No entanto, a explosão de abordagens metodológicas nos últimos anos acelerou significativamente o acúmulo de dados anteriormente inacessíveis, ampliou o número de espécies estudadas e detectou famílias repetitivas de DNA, mudando as visões e conceitos pré-estabelecidos (Garrido-Ramos, 2017; Lower *et al.*, 2018; Satovic *et al.*, 2020; Louzada *et al.*, 2020; Thakur *et al.*, 2021; Sproul *et al.*, 2022).

Um número crescente de espécies estudadas e dados acumulados na escala genômica enfatizaram variações na arquitetura geral dos satDNAs, não apenas no número e abundância de famílias, mas também em sua distribuição genômica, localização de heterocromatina/eucromatina, comprimento de *array* e associação com TEs. As diferenças indicam especificidades conceituais na organização repetitiva do DNA, em particular, grupos taxonômicos e a necessidade de expandir o número de sistemas modelo (Satovic-Vuksic; Plohl, 2023).

Entre os vertebrados, os anfíbios são a classe com maior variedade de tamanhos de genoma e ainda assim estão sub-representados em análises genômicas para elucidar sequências repetitivas (Liedtke *et al.*, 2018; Gregory, 2022). Análises de leituras de sequenciamento genômico combinadas com programas de bioinformática, por exemplo, o *RepeatExplorer* (Novák *et al.*, 2013, 2020), têm mostrado um grande potencial na caracterização de sequências repetitivas em diferentes organismos (Palacios-Gimenez *et al.*, 2017; Silva *et al.*, 2017; Ruiz-Ruano *et al.*, 2018; Utsunomia *et al.*, 2019; Ferretti *et al.*, 2020; Crepaldi *et al.*, 2021).

Em anfíbios, no entanto, essa abordagem ainda é escassa, e foi realizada apenas em *Proceratophrys boiei*, no qual análises preliminares já revelaram um grande número de satDNAs provavelmente influenciando a evolução genômica nesta espécie de sapo (Da Silva *et al.*, 2020). Antes do advento do sequenciamento genômico, ainda que com limitações, a digestão genômica por endonucleases de restrição foi usada com sucesso para caracterizar sequências repetitivas em anfíbios, principalmente alguns satDNAs, como o satélite PcP190 em Hyloidea e Hylidae (Vittorazzi *et al.*, 2011, 2014; Gatto *et al.*, 2019, respectivamente) e mais recentemente, o satélite BamHI-800, uma nova família satDNA em caracterizada em sapos Bufonidae (Guzmán *et al.*, 2022).

1.3 Considerações sobre cromossomos sexuais em anfíbios

Nos genomas, é comum o acúmulo de sequências repetitivas de DNA nos cromossomos sexuais. Os cromossomos sexuais, notavelmente heterocromáticos, exemplificam entidades genômicas sujeitas a expansões e contrações da heterocromatina durante a evolução. O acréscimo de sequências repetitivas, como as famílias de satDNA, contribui para a diferenciação progressiva desses cromossomos, levando à formação de cromossomos sexuais heteromórficos, que exibem rápida diversificação (Chalopin *et al.*, 2015; Palacios-Gimenez *et al.*, 2015; Wright *et al.*, 2016; Yano *et al.*, 2017; Sember *et al.*, 2018; Charlesworth, 2021; Kratochvíl *et al.*, 2021). Os cromossomos sexuais heteromórficos frequentemente apresentam sinais de degeneração, como o acúmulo extensivo de elementos transponíveis e outras repetições, resultando em cromossomos sexuais diferenciados (Y ou W). A intensificação da heterocromatização, a redução do tamanho e a perda de material genético são efeitos da supressão prolongada da recombinação entre os cromossomos sexuais (Charlesworth, 1991; Bergero; Charlesworth, 2009; Bachtrog *et al.*, 2011; Schartl *et al.*, 2016).

Os cromossomos sexuais manifestam uma evolução independente em várias ocasiões, exibindo distintos níveis de divergência no sexo heterogamético, como em machos XY ou fêmeas ZW (Bachtrog, 2008; Bachtrog *et al.*, 2014). Diferentemente de mamíferos, pássaros e insetos, a maioria das espécies de anfíbios (~75%) apresenta cromossomos sexuais homomórficos, com casos tanto de heterogamia XY em machos, quanto ZW em fêmeas (Graves, 2008; Schmid *et al.*, 2015; Ma; Veltsos, 2021). Notavelmente, a heterogamia nos machos ocorre com maior frequência (26,7%, XX/XY) em comparação às fêmeas (8,8%, ZW/ZZ). Cerca de 41,5% dessas espécies possuem um sistema não identificado (Ma; Veltsos, 2021).

Existem grupos específicos com sistemas de cromossomos sexuais únicos, exemplificados por *Leptodactylus pentadactylus* (Laurenti, 1768) (X1Y1X2Y2X3Y3X4Y4X5Y5X6Y6) e *Leiopelma hochstetteri* Fitzinger, 1861 (WO/OO) (Green *et al.*, 1993; Gazoni *et al.*, 2018). Essas características fazem dos sapos um sistema intrigante para a investigação da diversidade e evolução dos cromossomos sexuais, dada a presença de múltiplos estágios de diferenciação e diversos sistemas de determinação sexual entre espécies, assim como variações dentro de populações da mesma espécie (Nakamura, 2009; Malcom *et al.*, 2014; Perrin, 2020; Ma; Veltsos, 2021).

O modelo canônico da evolução dos cromossomos sexuais postula que os genes

sexualmente antagônicos (SA) desempenham um papel central na degeneração progressiva desses cromossomos (Charlesworth; Crow, 1978; Bull, 1983). A teoria sugere que os cromossomos sexuais surgem de um par de autossomos, iniciando com uma mutação determinante do sexo. Mutações benéficas masculinas (ou femininas no caso do cromossomo W) são favorecidas por acumular-se nas proximidades do locus determinante do sexo no cromossomo Y (ou W). Essas mutações são selecionadas para interromper a recombinação na região, uma vez que ela contém o haplótipo mais apto possível para o sexo heterogamético. A chegada de novas mutações com efeitos benéficos específicos do sexo promove a expansão da região de supressão da recombinação para um haplótipo mais extenso. Ao longo da evolução, a parada da recombinação conduz à diferenciação gradual e à degeneração dos cromossomos sexuais, envolvendo mecanismos, como a catraca de Muller, carona genética, seleção purificadora reduzida e deriva genética mais forte (Bull, 1983; Fischer, 1931; Charlesworth; Crow, 1978). Esse modelo é amplamente aceito para explicar a supressão e degeneração da recombinação em cromossomos sexuais altamente diferenciados, comum em mamíferos, aves e a maioria dos insetos (Beukeboom; Perrin, 2014; Bachtrog *et al.*, 2014).

1.4 *Proceratophrys boiei* como modelo para estudos de DNAs repetitivos

Assim como a maioria dos anfíbios anuros, a maioria dos representantes do gênero de sapos *Proceratophrys* permanecem pouco conhecidos quanto ao seu conjunto cromossômico e organização genômica, fato este que assim como em outros organismos, merece uma maior compreensão desses mecanismos com estudos que poderiam ajudar a elucidar questões evolutivas, genômicas, filogenéticas e até geográficas, pela tão destruída e compactada Mata Atlântica, área de ocorrência da maioria das espécies do gênero. Dados obtidos sob esses aspectos podem gerar conhecimento sobre a biodiversidade e a necessidade de conservação e preservação de espécies, além de observação de áreas de ocorrência de endemismos.

Aspectos taxonômicos de *Proceratophrys* ainda são complexos e pouco explorados, e apesar do esforço de alguns pesquisadores em resolver essas questões, sua taxonomia permanece confusa, devido à falta de estudos filogenéticos completos e abrangentes. Com relação à citogenética, a distribuição da heterocromatina foi o que chamou atenção em *P. boiei* desde o início, com estudos prévios realizados por Ananias e colaboradores (2007) e Amaro e colaboradores (2012). O padrão atípico de localização, quantidade e diferenças entre populações, quando comparados com outros anfíbios anuros, com grandes blocos pericentroméricos em todos os cromossomos, além de um evidente heteromorfismo de

cromossomos sexuais fez de *P. boiei* um excelente modelo para estudos cromossômicos, genômicos e evolutivos, estendendo e reforçando a possibilidade de estudos semelhantes em outras espécies do gênero e até em outros anfíbios anuros.

Um fato interessante é que para diferentes populações de *P. boiei* já foram relatadas diferenças citogenéticas significativas, principalmente em relação ao número de blocos heterocromáticos nos cromossomos, à presença do cromossomo sexual diferenciado W e localização de regiões organizadoras de nucléolo (Amaro *et al.*, 2012; Silva *et al.*, 2020).

Análises genômicas complementadas com citogenética clássica, a chamada citogenômica, foram realizadas em indivíduos de uma população de *P. boiei* do sul do Brasil, localizada em Tijucas do Sul, Paraná. Nessa população a espécie não possui grandes blocos de heterocromatina e nem cromossomo sexual W diferenciado (Da Silva *et al.*, 2020; Silva *et al.*, 2021). Em análises prévias, foi descoberto que mesmo sendo uma população com pouca heterocromatina nos cromossomos, havia um grande número de sequências repetitivas em seu genoma, especialmente satDNAs, possivelmente envolvidas na formação e manutenção da heterocromatina na espécie (Da Silva *et al.*, 2020). Embora estes estudos tenham fornecido dados sobre a composição e dinâmica evolutiva de satDNAs, poucas informações acerca da origem e função dessas sequências foram geradas, principalmente se levarmos em conta a falta de conhecimento sobre a composição dos cromossomos, bem como do cromossomos sexual W.

Com a necessidade de aprofundar as análises, sobretudo com a possibilidade de usar diferentes genomas sequenciados de outras populações de *P. boiei*, além de ferramentas de bioinformática cada vez mais específicas e avançadas para análises genômicas e comparações com outras espécies do gênero, no presente trabalho foram realizadas investigações que ajudaram a caracterizar de forma mais abrangente e fidedigna todo o conteúdo de DNA do satélite para a espécie *P. boiei*, bem como suas possíveis implicações para a formação de heterocromatina, diferenciação de cromossomos sexuais, satélites envolvidos com função centromérica, dinâmica de TEs e comparativo de sequências em diferentes populações.

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral deste trabalho foi investigar a estrutura, a composição molecular, os padrões de evolução e a possível funcionalidade de elementos repetitivos presentes no genoma da espécie de sapo *Proceratophrys boiei*, integrando dados genômicos e cromossômicos em uma abordagem genômica comparativa.

2.2 Objetivos específicos

a) Caracterizar o satelitoma de *P. boiei*, avaliando a abundância e divergência entre os sexos, com o objetivo de elucidar a possível interferência dessas sequências na diferenciação de cromossomos sexuais observada para a espécie;
b) Fazer uma comparação genômica de composição de sequências repetitivas entre indivíduos de *P. boiei* de duas populações diferentes.
c) Analisar a presença e compartilhamento do DNA satélite PboSat01-176 em espécies filogeneticamente relacionadas do gênero *Proceratophrys*.

3 MATERIAL E MÉTODOS

3.1 Levantamento de amostras e análises cromossômicas

Para a espécie *P. boiei*, um total de 28 indivíduos foram analisados, sendo nove machos e uma fêmea coletados em Mogi das Cruzes, São Paulo; um macho e cinco fêmeas coletados em Camanducaia, Minas Gerais; uma fêmea coletada em Tijucas do Sul, Paraná; cinco machos e duas fêmeas coletados em Morretes, Paraná; dois machos e duas fêmeas coletados em São José dos Pinhais, Paraná. Além disso, foram analisadas e estudadas outras espécies de *Proceratophrys*, sendo um macho de *P. laticeps*, um macho e uma fêmea de *P. schirchi*, coletados em Santa Teresa, Espírito Santo e um macho e uma fêmea de *P. melanopogon*, coletados em Mogi das Cruzes, São Paulo.

Todos os indivíduos foram coletados na natureza sob licenças de coleta emitidas pelo Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) protocolos nº 70213–1, 70213–2 e 70213–3. A utilização de animais silvestres, bem como os tecidos biológicos utilizados, foram registrados no SisGen – Sistema Nacional de Gestão do Patrimônio Genético e Conhecimentos Tradicionais Associados (código de registro: AEF54D5). Além disso, todos os procedimentos de amostragem, manuseio de material e análise foram autorizados e aprovados pelo Comitê de Ética no Uso de Animais (CEUA - permissão 21/2019), Instituto de Biociências, UNESP, Rio Claro, SP, Brasil. Por fim, os animais foram depositados na coleção de anfíbios Célio F. B. Haddad (CFBH), localizada no Departamento de Biodiversidade, Instituto de Biociências, UNESP, Rio Claro, SP, Brasil.

As células para visualização de cromossomos metafásicos foram obtidas de epitélio intestinal, de acordo com o protocolo estabelecido por Schmid (1978), bem como de medula óssea e fígado, obtidas de acordo com Baldissera *et al.* (1993). As preparações mitóticas foram armazenadas a -20°C até serem utilizadas em experimentos posteriores. Para análises convencionais e visualização/identificação da presença de cromossomos sexuais, as preparações mitóticas foram gotejadas em lâminas e corados com Giemsa 10% para observação de cromossomos.

3.2 Extração de DNA genômico (gDNA) e sequenciamento Illumina

Amostras de fígado e músculo previamente armazenadas em etanol 100% a -20°C, foram utilizadas para extração de gDNA. As extrações foram obtidas utilizando o kit de

purificação Wizard® Genomic DNA (Promega®, WI, Estados Unidos), com adição de RNAse (20 mg/mL) (Roche®), conforme recomendações do fabricante. As amostras obtidas foram usadas posteriormente para sequenciamento genômico e experimentos de amplificação de sequências de DNA, usando reação em cadeia da polimerase (PCR).

Para *P. boiei* fêmea de Tijucas do Sul, Paraná, foi realizado o sequenciamento do genoma de baixa cobertura através da plataforma Illumina® (Illumina Inc., San Diego, CA, EUA) da Macrogen Inc. (Seul, República da Coreia), utilizando o Illumina® HiSeq™ 4000 (2 × 101 pb *paired-end*). Para *P. boiei* de Mogi das Cruzes, São Paulo, um indivíduo de cada sexo foi usado para sequenciamento genômico (2 × 101 pb *paired-end*), através do Illumina® Hiseq™ 2000 da Macrogen Inc. (Seul, República da Coreia). Ainda, foram sequenciados o genoma de um macho e uma fêmea de *P. schirchi*, um macho de *P. laticeps* e um macho e uma fêmea de *P. melanopogon*, todos usando sequenciamento Illumina® HiSeq™ 4000 (2 × 150 pb *paired-end*) pela Novogene (HK) Co., Ltda.., (Hong Kong, China).

3.3 Processamento dos *reads* sequenciados e identificação de sequências repetitivas

Os *reads* provenientes do sequenciamento para todos os indivíduos foram verificadas quanto à qualidade usando FastQC (Andrews, 2012). O pré-processamento dos *reads* foi realizado seguindo parâmetros padrão utilizando a plataforma pública *online* RepeatExplorer (RE) (https://repeatexplorerelixir.cerit-sc.cz/galaxy/). As leituras foram processadas seguindo as etapas de "ferramenta de corte de qualidade", "FASTQ interlacer" em *paired-end reads* e conversor "FASTQ para FASTA" usando FASTX-Toolkit (Gordon e Hannon, 2010) com as opções padrão.

Nas análises de alto rendimento, para caracterizar o satellitoma de *P. boiei*, foi utilizado o protocolo de bioinformática *satMiner* para prospecção de satDNA em ambas as bibliotecas, macho e fêmea, de acordo com Ruiz-Ruano *et al.* (2016), disponível no GitHub (https://github.com/fjruizruano/satminer, acessado em 1 abril de 2021). O protocolo *satMiner* usa várias rodadas de *clustering* no RepeatExplorer (RE) (Novák *et al.*, 2013) e mais recentemente no RepeatExplorer2 (Novák *et al.*, 2020) para identificar e extrair sequências de satDNA, e cada rodada inclui a filtragem de leituras correspondentes previamente montadas *contigs* com deconseq 0.4.3 (Schmieder e Edwards, 2011), a fim de identificar e extrair o máximo possível de sequências repetitivas, mesmo com baixa abundância no genoma, conforme mostrado no capítulo 1 desta tese.

Clusters do RE supostamente contendo satDNAs foram selecionados para cada rodada por inspeção gráfica visual para identificar formas esféricas ou em anel que são características deste tipo de sequência de DNA em tandem. Cada cluster foi analisado manualmente quanto à sua estrutura interna de *contigs* e as repetições em tandem foram investigadas usando a ferramenta *dotplot* implementada em Geneious v4.8 (Drummond *et al.*, 2015) e Tandem Repeat Finder (TRF) (https//tandem.bu.edu /trf/trf.html, acessado em 1 de abril de 2021) (Benson, 1999).

Além disso, para cada rodada, foi analisado o *output* gerado pela ferramenta TAREAN (Novák *et al.*, 2017) acoplada ao RE para identificação automática de satDNAs, com os mesmos parâmetros e definições para cada rodada no RE, e todas as possíveis DNAs satélites, com alta ou baixa confiabilidade, foram considerados e analisados manualmente, integrando o satelitoma final. As etapas de agrupamento e filtragem foram repetidas seis vezes para as bibliotecas da fêmea e do macho, adicionando novas leituras filtradas em cada iteração. A sequência consenso dos satDNAs foi comparada à busca de homologia usando múltiplos alinhamentos de sequência com *Muscle* (Edgar, 2004) implementado no *software* Geneious v4.8 (Drummond *et al.*, 2009) e executando um teste de homologia baseado em RepeatMasker (Smit *et al.*, 2013) com "rm_homology.py" (https://github.com/fjruizruano/ngsprotocols, acessado em 1 de abril de 2021).

3.4 Bioinformática e análise genômica de sequências repetitivas

As sequências de satDNA encontradas foram classificadas em subfamílias, famílias e superfamílias; todas as famílias de satDNA foram numeradas em ordem decrescente de abundância, seguindo como referência o genoma da fêmea de *P. boiei*, segundo critério de identidade proposto por Ruiz-Ruano *et al.* (2016). Foram consideradas superfamílias (SF) com pelo menos 50% de identidade, famílias atingindo 80% e subfamílias ou componentes (variantes) de uma família com pelo menos 95% de identidade (Ruiz-Ruano *et al.*, 2016). Este teste de homologia entre as diferentes famílias de satDNA foi realizado baseado em RepeatMasker (Smit *et al.*, 2017) usando o *script* "rm_homology.py" (https://github.com/fjruizruano/ngs-protocols).

Além disso, foi verificado todas as sequências consenso de satDNAs usando múltiplos alinhamentos de sequências com *Muscle* (Edgar, 2004) implementado no software Geneious v4.8.5 (Drummond *et al.*, 2009) para buscar homologias de sequência. Ainda, foram realizadas

buscas para cada família de DNA de satélite usando a ferramenta Censor Repbase (https://www.girinst.org/, acessada em 1 de abril de 2022). Ainda, nas análises do *satMiner* já vem acoplada a ferramenta *RepeatMasker*, que busca automaticamente semelhanças com possíveis elementos transponíveis e outras sequências depositadas nesta base de dados. Em seguida, foi pesquisado quaisquer semelhanças com sequências de consenso de satDNAs usando a ferramenta BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi, acessado em 1 de abril de 2022). Todas as sequências de consenso dos satDNAs de *P. boiei* caracterizadas neste trabalho foram depositadas no GenBank (NCBI), sob os números de acesso OP223503 - OP223728.

RepeatMasker (Smit *et al.*, 2013) com *rmblast engine* foi usado para determinar a abundância e a divergência média de nucleotídeos (parâmetro Kimura-2, K2P) para cada família de satDNA em *P. boiei* e em todas as outras espécies estudadas nos capítulos específicos desta tese, bem como em sequências específicas, tanto de satDNAs quanto de elementos transponíveis, usando servidor computacional para depósito e análise de dados (ver capítulos específicos). A abundância genômica para cada sequência foi estimada como o número de nucleotídeos alinhados ao consenso de referência dividido pelo tamanho da biblioteca (em pb). Com esses dados, foram gerados gráficos *landscapes* informativos para a abundância relativa (eixo Y) em intervalos de 1% da distância K2P do consenso (eixo X), usando o *script* calcDivergencFromAlign.pl (dos utilitários RepeatMasker). Todos os gráficos *landscapes* foram construídos utilizando programação R (R Core Team, 2021 - versão 4.1.2).

3.5 Amplificação de DNA e mapeamento cromossômico de DNAs repetitivos

Primers foram desenhados manualmente para famílias de satDNAs mais abundantes em *P. boiei* e para outras famílias específicas (ver capítulo 1) e fabricados pela Exxtend Biotechnology Ltd. (Paulínia, São Paulo, Brasil). As condições para experimentos de PCR para estas sequências seguiram o mesmo protocolo descrito em Da Silva *et al.* (2020). A mistura de PCR foi composta por 10× PCR Rxn Buffer, 0,2 mM MgCl2, 0,16 mM dNTPs, 2 mM de cada *primer*, 1 U de Taq Platinum DNA Polymerase (Invitrogen, San Diego, CA, EUA) e 50–100ng/µL do DNA molde. As condições de PCR incluíram uma desnaturação inicial a 94°C por 5 min e 30 ciclos a 94°C (30 s), 55°C (30 s) e 72°C (80 s), além de uma extensão final a 72°C por 5 min. Para sequências de satDNA, os *primers* foram projetados levando em consideração o tamanho dos monômeros para evitar erros de amplificação, ou seja, conjuntos divergentes para sequências menores que 500 pb e convergentes para sequências mais longas.

As bandas monoméricas foram isoladas e purificadas utilizando o kit Zymoclean™ Gel DNA Recovery (Zymo Research Corp., The Epigenetics Company, CA, EUA) de acordo com as recomendações do fabricante. Todas as sequências amplificadas foram sequenciadas pelo método Sanger para confirmar sua real amplificação.

A hibridização fluorescente *in situ* (FISH) foi realizada em cromossomos mitóticos de indivíduos das espécies usando uma ou duas sondas simultaneamente. Com exceção do PboSat03-25 (capítulo 1), que foi marcado com biotina-14-dATP (Invitrogen®) na extremidade 5' durante sua síntese, as sequências de cada satDNA obtidas por PCR foram marcadas por *nick-translation* com digoxigenina-11-dUTP (Roche®) e detectado por antidigoxigenina-rodamina (Roche®); as sequências PboSat03-25 foram detectadas por Alexa Fluor 488 conjugado (Invitrogen®), seguindo o método previamente descrito por Pinkel *et al.* (1986), com ajustes descritos por Da Silva *et al.* (2020) e Cabral-de-Mello e Marec (2021).

Os cromossomos foram contrastados com dicloridrato de 4 ', 6-diamidina-20-fenilindol (DAPI) e as lâminas foram montadas em VECTASHIELD (Vector, Burlingame, CA, Estados Unidos). As lâminas resultantes foram visualizadas em microscópio de fluorescência Olympus® BX51, com câmera digital Olympus® DP71 acoplada, e as imagens foram capturadas utilizando o software de câmera DP Controller.

4 RESULTADOS

Os dados resultantes dessa tese serão apresentados e discutidos em capítulos, na forma de manuscritos que estão publicados ou foram submetidos para publicação em revistas especializadas da área. Como segue:

Capítulo 1: Analysis in *Proceratophrys boiei* genome illuminates the satellite DNA content in a frog from the Brazilian Atlantic Forest (Análises no genoma de *Proceratophrys boiei* ilumina o conteúdo de DNA satélite em um sapo da Mata Atlântica brasileira).

Capítulo 2: Interspecific cytogenomic comparison reveals a potential chromosomal centromeric marker in *Proceratophrys* frog species (Comparação citogenômica interespecífica revela um potencial marcador cromossômico centromérico em espécies de sapos *Proceratophrys*).

Capítulo 3: Comparative analysis of repetitive DNA in frogs *Proceratophrys boiei* and its impact on population karyotypic evolution (Análise comparativa de DNA repetitivo em sapos *Proceratophrys boiei* e seu impacto na evolução cariotípica populacional).

4.1 Capítulo 1 - Analysis in *Proceratophrys boiei* genome illuminates the satellite DNA content in a frog from the Brazilian Atlantic forest

Marcelo João Da Silva[1], Thiago Gazoni[1], Célio Fernando Baptista Haddad[2] and Patricia Pasquali Parise-Maltempi[1]

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Resumo

Os DNA satélites (satDNAs) são um dos elementos mais abundantes nos genomas. Caracterizadas como sequências organizadas em tandem que podem ser amplificadas em múltiplas cópias, principalmente em regiões heterocromáticas. O sapo *Proceratophrys boiei* (2n = 22, ZZ/ZW) é encontrado na Mata Atlântica brasileira e apresenta um padrão atípico de distribuição de heterocromatina quando comparado a outros anfíbios anuros, com grandes blocos pericentroméricos em todos os cromossomos. Além disso, as fêmeas de *P. boiei* possuem um cromossomo sexual W metacêntrico, mostrando heterocromatina em toda a extensão cromossômica. Neste trabalho, realizamos análises genômicas, bioinformáticas e citogenéticas de alto rendimento para caracterizar o conteúdo de DNA satélite (satelitoma) em *P. boiei*, principalmente devido à alta quantidade de heterocromatina C-positiva e ao cromossomo sexual W altamente heterocromático. Após todas as análises, é notável que o satelitoma é composto por um alto número de famílias de satDNA (226), tornando a espécie de sapo com o maior número de satélites descritos até agora. Consistente com a observação de grandes blocos centroméricos de heterocromatina C-positiva, o genoma de *P. boiei* é enriquecido com um alto número de cópias de DNAs repetitivos, com a abundância total de satDNA correspondendo a 16,87% do genoma. Mapeamos com sucesso, por hibridização *in situ* por fluorescência, os dois satélites repetitivos mais abundantes no genoma, PboSat01-176 e PboSat02-192, destacando a presença de certas sequências de satDNAs em regiões cromossômicas estratégicas (por exemplo, centrômero e região pericentromérica), o que leva à sua participação em processos cruciais para a organização e manutenção do genoma. Nosso estudo revela uma grande diversidade de repetições satélite que estão impulsionando a organização genômica nesta espécie de sapo. A caracterização e abordagens relacionadas a satDNAs nesta espécie permitiram a confirmação de algumas informações da biologia de satélites e uma possível relação com a evolução de cromossomos sexuais, especialmente em anfíbios anuros, incluindo *P. boiei*, para os quais dados não estavam disponíveis.

Palavras-chave: DNA repetitivo; satelitoma; citogenética; evolução; citogenômica.

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Analysis in *Proceratophrys boiei* genome illuminates the satellite DNA content in a frog from the Brazilian Atlantic forest

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Satellite DNAs (satDNAs) are one of the most abundant elements in genomes. Characterized as tandemly organized sequences that can be amplified into multiple copies, mainly in heterochromatic regions. The frog *P. boiei* (2n = 22, ZZ♂/ZW♀) is found in the Brazilian Atlantic forest and has an atypical pattern of heterochromatin distribution when compared to other anuran amphibians, with large pericentromeric blocks on all chromosomes. In addition, females of *Proceratophrys boiei* have a metacentric sex chromosome W showing heterochromatin in all chromosomal extension. In this work, we performed high-throughput genomic, bioinformatic, and cytogenetic analyses to characterize the satellite DNA content (satellitome) in *P. boiei*, mainly due to high amount of C-positive heterochromatin and the highly heterochromatic W sex chromosome. After all the analyses, it is remarkable that the satellitome of *P. boiei* is composed of a high number of satDNA families (226), making *P. boiei* the frog species with the highest number of satellites described so far. Consistent with the observation of large centromeric C-positive heterochromatin blocks, the genome of *P. boiei* is enriched with high copy number of repetitive DNAs, with total satDNA abundance comprising 16.87% of the genome. We successfully mapped *via* Fluorescence in situ hybridization the two most abundant repeats in the genome, PboSat01-176 and PboSat02-192, highlighting the presence of certain satDNAs sequences in strategic chromosomal regions (e.g., centromere and pericentromeric region), which leads to their participation in crucial processes for genomic organization and maintenance. Our study reveals a great diversity of satellite repeats that are driving genomic organization in this frog species. The characterization and approaches regarding satDNAs in this species of frog allowed the confirmation of some insights from satellite biology and a possible relationship with the evolution of sex chromosomes, especially in anuran amphibians, including *P. boiei*, for which data were not available.

KEYWORDS

repetitive DNA, satellitome, cytogenetics, evolution, cytogenomics

1 Introduction

Most eukaryotic genomes contain large blocks of heterochromatin surrounding centromeres and telomeres (Plohl et al., 2012; Garrido-Ramos, 2017; Sneideman and Meller, 2021), which are regions mainly composed of satellite DNAs repeats (satDNAs) and transposable elements (TEs). It is already known that satellite repeats make up sizable portions of genomes. SatDNAs participate in several processes, including gene regulation, stress response, and nuclear organization in many different organisms, making them prominent actors in the evolution of genomes, mainly because of their high plasticity (Plohl et al., 2012; Garrido-Ramos, 2017; Sneideman and Meller, 2021; Thakur et al., 2021).

Among vertebrates, amphibians are the class with the greatest variety of genome sizes (Liedtke et al., 2018; Gregory, 2022). Analysis of genomic sequencing reads combined with computer programs [e.g., RepeatExplorer (Novák et al., 2013)] have shown potential in the characterization of repetitive sequences in different organisms, including amphibians (Palacios-Gimenez et al., 2017; Silva et al., 2017; Ruiz-Ruano et al., 2018; Utsunomia et al., 2019; Da Silva et al., 2020; Ferretti et al., 2020; Crepaldi et al., 2021). Prior to that, with limitations, genomic digestion by restriction endonucleases was successfully used to characterize repetitive sequences in amphibians, mainly satDNAs, such as the PcP190 satellite in Hyloidea and Hylidae (Vittorazzi et al., 2011, 2014; Gatto et al., 2019, respectively) and more recently, the BamHI-800 satellite, a new satDNA family in Bufonidae (Guzmán et al., 2022).

The variability of satellite repeats is very interesting from an evolutionary point of view, as repeats within the same satDNA family evolve non-independently, showing low rates of divergence between monomers, through a process known as "concerted evolution" (Dover, 1982; Elder and Turner, 1995; reviewed by Thakur et al. (2021)). The concerted evolution occurs *via* "molecular drive", an evolutionary process emerging from the activities of a number of ubiquitous mechanisms of DNA turnover, such as gene conversion, unequal crossing over, replication slippage, rolling circle replication, and multiple TE insertions (Dover, 1982; Dover, 1986; Elder and Turner, 1995; Ugarković and Plohl, 2002; Thakur et al., 2021). However, there are levels of variation in the rates of expansion, homogenization, and fixation between sequences. These dynamics depend on several factors, such as mutation rate, array size and structure, chromosome structure, and recombination rates (López-Flores and Garrido-Ramos, 2012; Plohl et al., 2012; Garrido-Ramos, 2017).

In genomes, sex chromosomes frequently accumulate satDNAs. Due to their differentiated and highly heterochromatic nature, sex chromosomes are a good example of genomic entities with expansions and contractions of heterochromatin throughout evolution. The gain of repetitive sequences, such as satDNA families, contributes to gradually differentiate from their homologs becoming heteromorphic sex chromosomes, which carry rapid diversification (Chalopin et al., 2015; Palacios-Gimenez et al., 2015; Wright et al., 2016; Yano et al., 2017; Sember et al., 2018; Charlesworth, 2021; Kratochvíl et al., 2021). Heteromorphic sex chromosomes show signs of degeneration such as extensive accumulation of transposable elements and other repeats, resulting in an enlargement of the sex-limited chromosomes (Y or W). Increased heterochromatinization or a diminishment of their size and gene loss are consequences of the long-term recombination suppression between the sex chromosomes (Charlesworth, 1991; Bergero and Charlesworth, 2009; Bachtrog et al., 2011; Schartl et al., 2016).

Sex chromosomes have independently evolved multiple times and show varied levels of divergence from each other in the heterogametic sex in XY males or ZW females (Bachtrog, 2008; Bachtrog et al., 2014). Unlike other organisms, like mammalians, birds, and insects, the majority of amphibian species (~75%) has homomorphic sex chromosomes with both male and female heterogamy cases (Graves, 2008; Schmid et al., 2015; Ma and Veltsos, 2021), but a higher male heterogametic occurrence (26.7%, XX/XY) than female (8.8%, ZW/ZZ) system. About 41.5% of these species have an unknown system [Reviewed by Ma and Veltsos (2021)] and there are specific groups with unique sex chromosome systems, such as *Leptodactylus pentadactylus* (X1Y1X2Y2X3Y3X4Y4X5Y5X6Y6) and *Leiopelma hochstetteri* (WO/OO) (Green et al., 1993; Gazoni et al., 2018). These characteristics make frogs an interesting system to study sex chromosome diversity and evolution, as they harbor multiple stages of differentiation, with diverse sex determination system across species, as well as between and within populations of the same species (Nakamura, 2009; Malcom et al., 2014; Perrin, 2020; Ma and Veltsos, 2021).

Surprisingly, the Brazilian frog *P. boiei* (2n = 22, ZZ♂/ZW♀) has an atypical pattern of heterochromatin distribution, when compared to other anuran amphibians, with large pericentromeric blocks on all chromosomes. In addition, *Proceratophrys boiei* females have a metacentric W chromosome showing heterochromatin in all chromosomal extension defined by the C-banding technique, e.g., C-positive blocks, being the W chromosome smaller than Z in females (Ananias et al., 2007; Amaro et al., 2012; Da Silva et al., 2020; Silva et al., 2021). So far, it is the only specie of the genus with these atypical chromosomal characteristics, even for amphibians. However, for different populations of *P. boiei*, significant cytogenetic differences have already been reported, mainly in relation to the number of heterochromatic blocks in the chromosomes and the presence of the differentiated sex chromosome W (Amaro et al., 2012; Da Silva et al., 2020).

Genomic and cytogenetics analyzes were performed for the first time in a population of *P. boiei* from southern Brazil, which does not have large blocks of heterochromatin and does not have a differentiated W chromosome. We uncovered a large number of repetitive sequences on its genome, especially satDNAs, possibly involved in heterochromatin formation and maintenance in the species (Da Silva et al., 2020). Now, intrigued by the high amount of C-positive heterochromatin in *P. boiei* from populations of southeastern Brazil and the highly heterochromatic W chromosome in females, we performed here a high-throughput genomic, bioinformatic and cytogenetic analyses to characterize the entire satellite DNA content and complement the satellitome for *P. boiei*, as well as its possible implications for heterochromatin formation and sex chromosome differentiation, and thus carry out the most complete quantification of the satellitome in a frog genome so far.

2 Materials and methods

2.1 Species sampling, biological materials, and chromosome preparation

Chromosomal preparations and tissue samples from ten males and five females from *P. boiei* were analyzed. The individuals were collected in the wild under collection licenses issued by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) protocol Nos. 70213–1, 70213–2 and 70213–3, in the Brazilian cities of Mogi das Cruzes, state of São Paulo and Camanducaia, state of Minas Gerais. The use of wild animals, as well as the biological tissues used, were registered in the SisGen–National System for the Management of Genetic Heritage and Associated Traditional Knowledge (registration code: AEF54D5). In addition, we used cytogenetic preparations of *P. boiei* already available at the Animal Cytogenetics Laboratory in UNESP, Rio Claro, São Paulo, Brazil, from previous studies (Da Silva et al., 2020; Silva et al., 2021).

Metaphasic chromosomes were obtained from intestinal epithelial cells according to the protocol proposed by Schmid (1978), and the bone marrow and liver were collected according to Baldissera et al. (1993). All procedures for sampling, material handling, and analysis were authorized and approved by the Animal Ethics Committee (Comitê de Ética no Uso de Animais - CEUA - permission 21/2019), Biosciences Institute, UNESP, Rio Claro, SP, Brazil. Finally, the animals were deposited in the Célio F. B. Haddad (CFBH) amphibian collection, housed in the Department of Biodiversity, Biosciences Institute, UNESP, Rio Claro, SP, Brazil.

2.2 Genomic DNA extraction, genome sequencing, and satellitome analysis

Genomic DNA (gDNA) extraction was obtained from liver or muscle samples using the Wizard® Genomic DNA purification kit (Promega, WI, United States), according to the manufacturer's recommendations. This gDNA was later used for genomic sequencing and polymerase chain reaction (PCR) assays. One individual of each sex was used for genome paired-end sequencing (2 × 101 bp) through Illumina® Hiseq™ 2000 by Macrogen Inc. (Seoul, Republic of Korea).

From sequenced libraries of both female and male individual, satDNAs sequences were recovered using different approaches for a complete search for the satellitome of the species. In addition, a comparative approach was given, focusing on the possible differences between the sexes, in search of satDNAs that could be more representative in the female genome and probably enriched in the heteromorphic sex chromosome W.

To perform a high-throughput analysis, the satMiner bioinformatics protocol for satDNA prospection in both libraries was used (Ruiz-Ruano et al., 2016), available at GitHub (https://github.com/fjruizruano/satminer, accessed on 1 April 2021). The satMiner protocol uses several rounds of clustering in RepeatExplorer (RE) (Novák et al., 2013) and most recently RepeatExplorer2 (Novák et al., 2020) to identify and extract satDNA sequences, and each round includes filtering out reads matching previously assembled contigs with deconseq 0.4.3 (Schmieder and Edwards, 2011), in order to identify and extract as many repetitive sequences as possible, even with low abundance in the genome. It started with a library sampling of 200,000 reads, incrementing this number by two in each consequent round of RE clustering.

RE clusters putatively containing satDNAs were selected for each round by visual graph inspection to identify spherical or ring shapes which are characteristic of this type of tandem DNA sequence. Each cluster was manually analyzed for their internal contigs structure and tandem repetitions were investigated using the dotplot tool implemented in Geneious v4.8 (Drummond et al., 2015) and Tandem Repeats Finder (TRF) (https://tandem.bu.edu/trf/trf.html, accessed on 1 April 2021) (Benson, 1999). In addition, for each run, the output generated by the TAREAN tool (Novák et al., 2017) coupled to the RE for automatic identification of satDNAs was analyzed, with the same parameters and definitions for each run in the RE, and all possible satellite DNAs, with high or low reliability, were considered and analyzed manually, integrating the final satellitome. The clustering and filtering steps were repeated six times for the female and male libraries, adding new filtered reads in each iteration until we could no longer detect new satDNAs in neither.

The satDNAs consensus were compared to search for homology using multiple sequence alignments with Muscle (Edgar, 2004) implemented in Geneious v4.8 software (Drummond et al., 2015) and running a homology test based on RepeatMasker (Smit et al., 2013) with "rm_homology.py" (https://github.com/fjruizruano/ngs-protocols, accessed on 1 April 2021). The results of these analyses were used to classify the satDNA collection into superfamilies, families and/or subfamilies, and all satDNA families were numbered in order of decreasing abundance in the female genome, following the identity criterion proposed by Ruiz-Ruano et al. (2016).

Also, searches were performed for each satellite DNA family using the Censor tool (http://www.girinst.org/, accessed on 1 April 2022) against Repbases. Furthermore, in satMiner analyses, the RepeatMasker tool is already coupled, which automatically searches for similarities with possible transposable elements and other sequences deposited in this database. Then, we searched all databases for any similarities to satDNAs consensus sequences using the BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 April 2022). We BLAST-searched specially for satDNAs previously detected and deposited for *P. boiei* (Da Silva et al., 2020), to check the presence of conserved satDNAs in different populations for this species. All consensus sequences of the satDNAs characterized in this work are deposited in GenBank (NCBI), under accession numbers OP223503 - OP223728.

2.3 Estimation of SatDNAs sequence abundances and divergences in the genome

RepeatMasker (Smit et al., 2013) with rmblast engine was used to determine abundance and average nucleotide divergence (Kimura-2- parameter, K2P) for each satDNA family in both sexes. Genomic abundance for every satDNA in the male and female libraries was estimated as the number of nucleotides aligned to the reference consensus divided by the library size (in bp). With this data repeat landscapes were generated for the relative

abundance (*Y*-axis) at 1% intervals of K2P distance from the consensus (*X*-axis), using the script calcDivergencFromAlign.pl (from RepeatMasker utils). A subtractive landscape was subsequently generated to evaluate which satDNA families differ between both libraries to provide the first indications of which satDNA are more prominent in one sex in comparison to the other. All landscapes graphics were built using R programming (R Core Team, 2021 - version 4.1.2).

The different enrichment of all satDNAs across the sexes was determined by generating a female to male ratio as we calculated the quotient between the abundance values of each satDNA family. This data complemented the subtractive landscape by providing more between-sexes differences, as satDNA families with Female/Male (F/M) ratio higher than one was considered more abundant in females (as the threshold to determine it as more prevalent in this sex). The 59 satDNA families that are most enriched in each sex considering the F/M ratio were selected for profiling and we generated individual landscapes for each selected female-biased satDNAs to confirm different amplification and divergence between the sexes.

2.4 DNA amplification and chromosomal mapping of repetitive DNAs

Primers were manually designed for the ten most abundant satDNA families in *P. boiei* and manufactured by Exxtend Biotechnology Ltd. (Paulínia, São Paulo, Brazil). The PCR conditions for these sequences followed the same protocol described in Da Silva et al. (2020). All amplified sequences were sequenced by the Sanger method to confirm their actual amplification.

Fluorescence *in situ* hybridization (FISH) was performed on mitotic chromosome spreads from adults using one or two probes simultaneously. With the exception of PboSat03-25, which was labelled with biotin-14-dATP (Invitrogen®) at 5′end during its synthesis, the sequences of each satDNA obtained through PCR were labeled by nick-translation with digoxigenin-11-dUTP (Roche®) and detected by antidigoxigenin-rhodamine (Roche); PboSat03-25 sequences were detected by Alexa Fluor 488-conjugated (Invitrogen), following the method previously described by Pinkel et al. (1986), with adjustments described by Da Silva et al. (2020) and Cabral-de-Mello and Marec (2021).

The chromosomes were counterstained using 4′,6-diamidine-20-phenylindole dihydrochloride (DAPI) and slides were mounted in VECTASHIELD (Vector, Burlingame, CA, United States). The resulting slides were visualized under an Olympus® BX51 fluorescence microscope, with a digital camera Olympus® DP71 attached, and the images were captured using the DP Controller camera software. For each slide, a minimum of 10 metaphases were analyzed and photographed to confirm the FISH results.

3 Results

3.1 High-throughput analysis of the satellitome

The female library sequencing provided about 2.0 Gb of sequence data (1,957,610,886 reads), yielded 19,382,286 paired-end trimmed reads and for the male library, about 1.4 Gb of sequence data (1,446,983,166 reads) and 14,326,566 paired-end trimmed reads. The six iterations performed by the satMiner protocol on male and female genome of *P. boiei* uncovered 226 different satDNA families for both sexes. The predominance of repeat unit lengths (RUL) ranging from 20 to 986 bp (average of 69 bp) and the total satDNA abundance comprised 16.87% of *P. boiei* genome, with abundance per family ranging from 0.00002% to 10.49%.

The A+ T content of consensus satDNA sequences varied between 29.3% and 76.9% (55% on average), which indicated a slight bias towards A+ T rich satellites (Supplementary Table S1). Homology tests between all satDNA families revealed the occurrence of 15 superfamilies (SFs), with homologies between 50.2% and 80%, and as expected, the families belonging to each SF showed highly similar sequence properties (RUL and A+ T content), as the superfamilies 03, 08, 09, 10, 11, 12, and 15, in which they have sequences with similar characteristics (Supplementary Figure S1).

Interestingly, PboSat01-176 comprises 93.62% of the total amount of satDNAs families, being the most abundant satDNA in the genome of *P. boiei*, comprising 10.49% of genomic abundance. The second most abundant satDNA corresponds only to 0.97% of total satDNA content and all other satDNAs are in very low abundances. The values determined by RepeatMasker for divergence were relatively variable for the species as a whole, ranging from 1.93% to 24.01% (average divergence for the species was 10.96%).

For PboSat01-176 and PboSat02-192, the genomic abundance in the present work refers to the values determined by the RepeatMasker which applied a substantial number of reads compared to the set of randomly selected reads previously analyzed for another population of *P. boiei* (and only *via* the RepeatExplorer output) by Da Silva et al. (2020). Thus, PboSat01-176 remained the most abundant satDNA family, also, PboSat02-192 (formerly PboSat03-189) came in second in abundance after this thorough analysis, and the previously described satDNA PboSat02-173 was discarded, as it was highly similar to PboSat01-176, redescribed in the present work.

As expected, GenBank searches resulted in similarities with some families of satDNAs previously deposited for *P. boiei* by Da Silva et al. (2020). BLAST results and subsequent alignments for other satDNAs showed high and low similarities to other repetitive sequences, mainly microsatellites, from other related organisms. The search in Censor Repbase showed that some consensus sequences of satDNA families share a certain degree of similarity with transposable elements, among other sequences; however, all with non-significant sequence identity. Also, most satDNA families, 144 in total, does not show any similarity with transposable elements. However, surprisingly, 82 families show some similarity, low or high, with retrotransposons, mainly Ty3/Gypsy and Tc/Mariner (Supplementary Table S1). Similarities with other types of retrotransposons were also found, as well as DNA transposons.

3.2 Satellitome differences between the sexes

Bioinformatic analysis revealed that all 226 satDNA families found in the *P. boiei* genome are shared between both sexes. These,

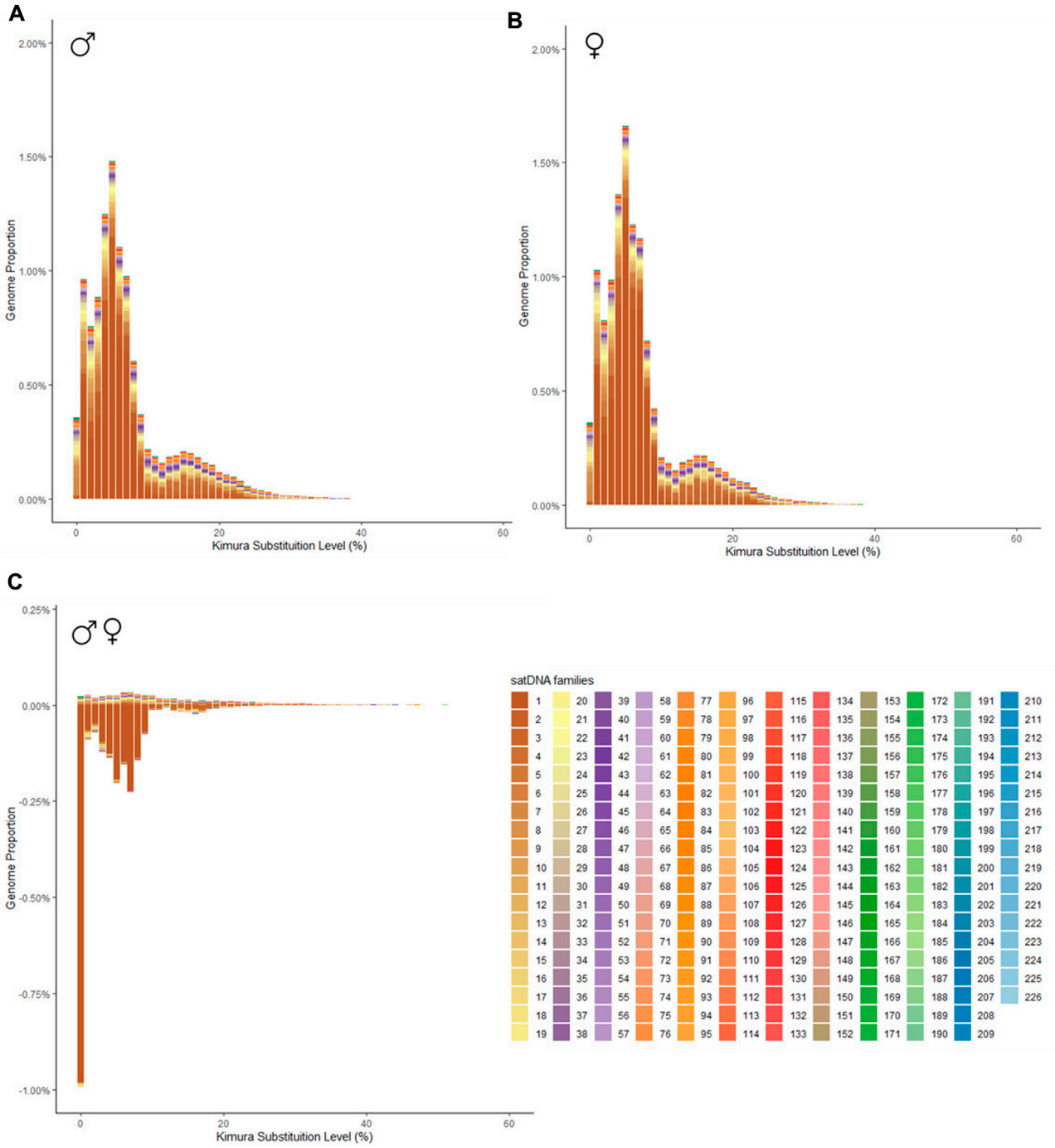

FIGURE 1
Repeat landscape (abundance vs divergence) for satDNAs identified in *Proceratophrys boiei*. The graphs show, for each color-coded element, the sequence divergence according to Kimura distance (*x*-axis) in relation to their copies in the genome (*y*-axis). **(A)** male genome; **(B)** female genome; **(C)** Subtractive repetitive landscape. The graphs show, for each color-coded element, the sequence divergence according to Kimura distance (*x*-axis) in relation to their copies in the genome (*y*-axis). Copies clustered to the left (lower divergence) potentially correspond to recent copies occurring in the genome. In the subtractive graphic, abundance values show the difference between the male minus the female genomes, thus, positive values indicate overabundance in the male, and negative values indicate overabundance in the female genome.

however, are differently enriched between them, 59 satDNAs had a F/M ratio greater than 1, suggesting an enrichment in the female library, while 159 were considered male (F/M ratio less than 1), and, eight of them had an F/M ratio equal to 1, having the same abundance on both genomes. To show differences in satellitome between the sexes, individual repeat landscapes for male and female were generated (Figures 1A, B, respectively), in addition to a subtractive landscape (Figure 1C), comparing the two genomes.

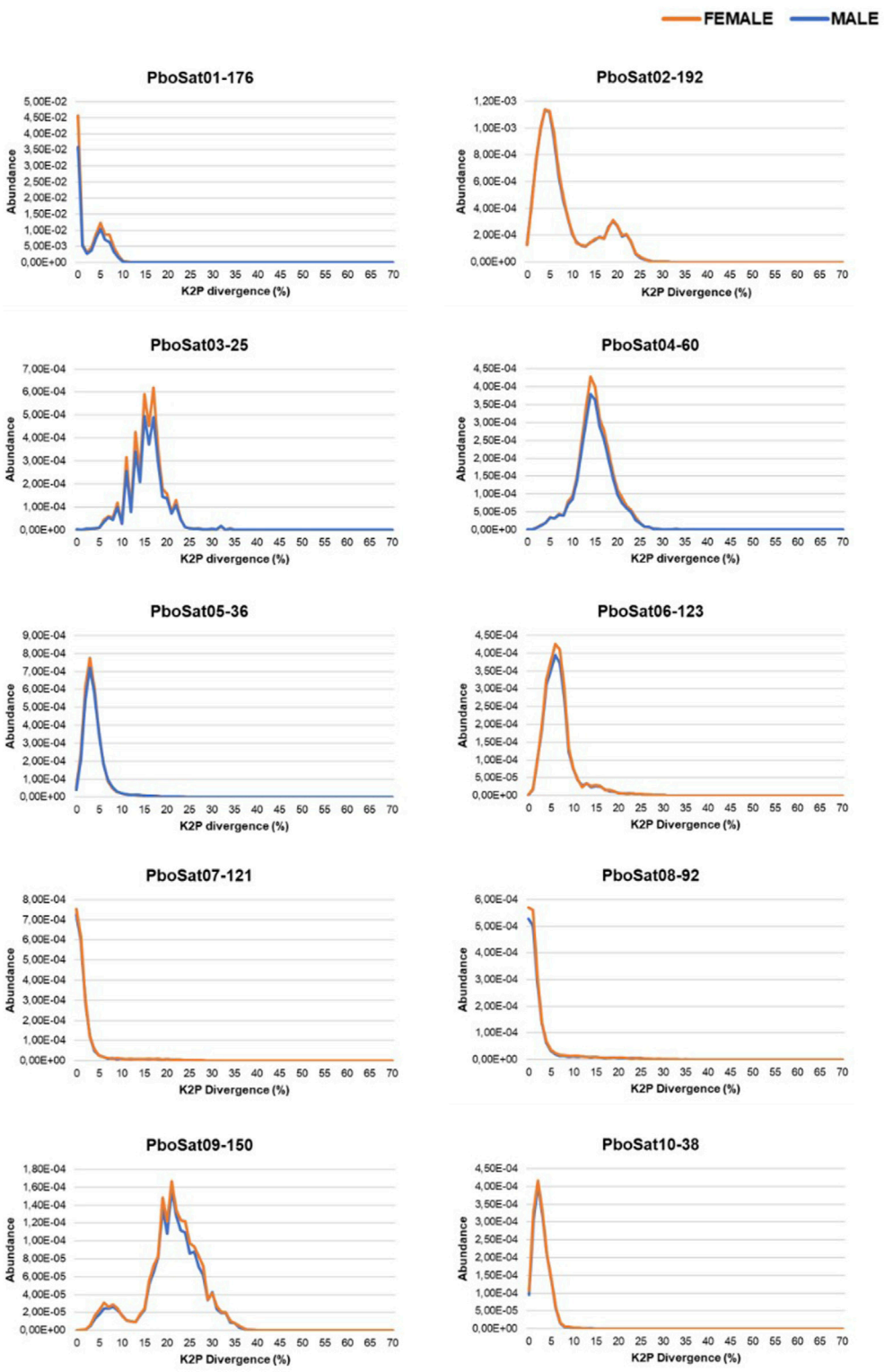

FIGURE 2
Individual satDNA landscapes of male (blue) and female (orange) repeats for ten more abundant satDNAs families identified in the *Proceratophrys boiei* genome.

In order to verify families of satDNAs that could be highly biased towards females, the F/M ratio revealed 59 satDNAs biased for the female library and, consequently, putatively clustered on the W sex chromosome. To demonstrate the abundance and divergence for the ten most abundant satDNA families identified in the genome of Proceratophrys boiei, individual landscapes were constructed,

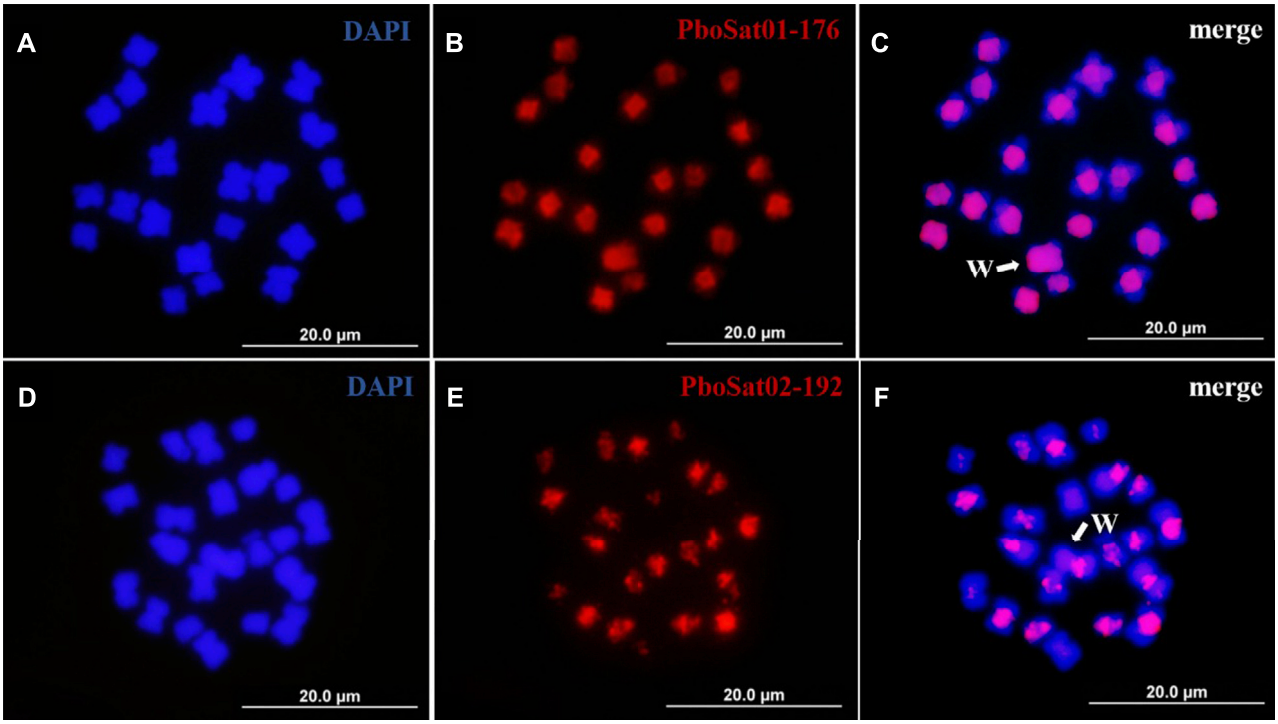

FIGURE 3
FISH mapping of two more abundant satDNAs (red) and in mitotic metaphase of Proceratophrys boiei female individual. **(A,D)** chromosomes counterstained with DAPI; **(B)** PboSat01-176; **(E)** PboSat02-192; **(C,F)** merge. Note that both satDNAs showed a high clustering in centromeric and pericentromeric region of chromosomes. Sex chromosome W is highlighting in C and F (arrows).

showing an evolutionary dynamic for each satDNA repeat analyzed (Figure 2). However, the total composition of the satellitome in *P. boiei* corresponded to 16.87% and 14.91% of the female and male genomes, respectively. Almost all satDNAs showed very similar divergence values for both males and females, with exception of PboSat221-28 (23.86% and 17.88%, for female and male respectively), PboSat223-28 (19.54% and 16.16%), PboSat224-38 (30.32% and 21.89%), PboSat225-31 (13.86% and 16.76%), and PboSat226-20 (14.52% and 18.4%), interestingly, the least abundant satDNAs (Supplementary Table S1).

3.3 Chromosome mapping of the most abundant SatDNAs families

The physical mapping by FISH on female and male chromosomes of *P. boiei* for the ten most abundant satDNAs families was performed to study the organization of those families in the genome and analyze whether the genomic results reflect at the cytogenetic level. FISH was performed for ten most abundant satellites; however, satisfactory hybridization results were only found for the satellites PboSat01-176 and PboSat02-192, with visible signs of clustering on chromosomes (Figure 3).

The chromosomal locations for these satDNAs families indicate that the FISH bands found in the female and male *P. boiei* chromosomes were located in pericentromeric regions surrounding the centromeres. It is evident that the pericentric heterochromatic regions are enriched in satDNA in *P. boiei*, as they contained the most abundant first families, representing 81% of all satDNA content in the species genome (Supplementary Table S1). It was expected that the most abundant satellites in the female genome would be found by FISH on the W sex chromosome; however, none of them was found exclusively on this chromosome.

4 Discussion

The complete satellitome of *P. boiei* provided in this work is composed of a high number of satellite families. The in-depth characterization of 226 satDNAs makes *P. boiei* the frog species with the highest number of satellites described so far. Similarity analyzes showed that 67 of the satDNAs found can be grouped into 15 superfamilies. Notably, satDNA sequences are considered crucial factors in genomic and karyotypic evolution, perhaps most importantly, they may constitute rapidly evolving genome sequences (Garrido-Ramos, 2017; Ahmad et al., 2020).

By performing several successive filtering steps and searches with satMiner and RepeatExplorer, in each step subtracting those repetitive elements found in previous steps, the chance of finding other poorly represented satDNAs is substantially increased (Ruiz-Ruano et al., 2016). This genomic mining approach has been used efficiently for the analysis of TEs and satDNA content in distinct species in the last few years, e.g., plants, insects and fish, providing an opportunity to uncover satDNA families whose isolation was elusive

by other methods (Ruiz-Ruano et al., 2018; Utsunomia et al., 2019; Crepaldi et al., 2021; Camacho et al., 2022). In this sense, the discovery of a large number of satellite families, with several of them grouped into superfamilies, draws attention to an expansion of satDNAs through duplications of existing repeats, followed by substitutions/deletions/insertion events in the genome of *P. boiei* (Ruiz-Ruano et al., 2016; Utsunomia et al., 2019). In previous analyzes by Da Silva et al. (2020), using the methodology available at the time, the authors found a large amount of satDNAs from another population for *P. boiei*. However, in the more accurate analyzes carried out in this work, the previous data are contrasted and complemented, revealing the need to update the genomic content data of satDNAs for *P. boiei*.

Studies on repetitive DNA, and particularly on satDNA, are of high interest to better understand genome structure and dynamics (Ruiz-Ruano et al., 2018). In this work, valuable information was retrieved regarding the evolutionary dynamics of these sequences and their genomic organization in *P. boiei* frog. The fact that the satellitome presents as mostly small and highly diversified sequences, with low divergence, suggests that recent amplification and diversification events occurred in the *P. boiei* genome, which may have followed the heterochromatinization events as evidenced by C-banding (Ananias et al., 2007; Amaro et al., 2012; Da Silva et al., 2020). In terms of recent diversification of satDNA sequences, this is well shown for fish, such as *Astyanax* (Silva et al., 2017), *Megaleporinus macrocephalus* (Utsunomia et al., 2019), *Characidium gomesi* (Serrano-Freitas et al., 2020) and *Megaleporinus elongatus* (Crepaldi et al., 2021), making it clear that evolutionary mechanisms for these repetitive sequences behave similarly in distinct groups of organisms.

It is well known that novel families of satDNAs can arise from the independent duplication of different genomic sequences such as intergenic spacers, portions of transposable elements, or even those derived from other satellite DNAs (Ruiz-Ruano et al., 2016; Garrido-Ramos, 2017; Ahmad et al., 2020; Thakur et al., 2021). About satDNAs grouped into superfamilies in the *P. boiei* genome, it is remarkable that these satellites actually belong to sequence groups and may be subject to similar genome duplication and amplification, as they have common characteristics, such as monomer size and low genomic abundance. More studies are needed to understand the evolutive dynamics specifically of these repetitions.

Consistent with its richness of centromeric C heterochromatin, it became apparent that the genome of *P. boiei* is enriched in high copy repetitive DNAs. However, in relation to the highly heterochromatic W sex chromosome, unlike expected, specific satDNAs to this chromosome were not evidenced, leading to the belief that there was still no clustering of repeats directly related to the differentiation of sex chromosomes in this species. We suggest that this W chromosome in females of *P. boiei* may be a young element currently in the initial phase of heterochromatinization and differentiation. Our bioinformatics/FISH analyzes showed that the absence of sex-specific signals may be due to negligible absence, loss or gain, or due to non-differentiation of the DNA content of the sex chromosomes in *P. boiei*. Although the FISH technique has limitations for detecting less abundant sequences, this may still suggest an early stage of differentiation of the content of the ZZ/ZW sex chromosome system in *P. boiei*, with no evident molecular differentiation between the heteromorphic sex chromosomes.

As expected, strategic mapping by FISH for certain sequences revealed that most satDNAs were not detectable by the technique in either sex, since in previous analyzes it was also not detectable, especially those satDNAs with very low abundance. In high-throughput analysis, it has been easy to characterize a large amount of satDNAs; however, to show positive signals in FISH, there is some difficulty, mainly due to the low abundance and the difficulty in performing the technique (Ruiz-Ruano et al., 2016; Silva et al., 2017; Cabral-de-Mello and Marec, 2021; Crepaldi et al., 2021). Also, the majority of the satellitome already described is arranged in small arrays below the detection threshold of FISH, and for the *P. boiei* satellitome analyzed here it is no different (Ruiz-Ruano et al., 2016; Bardella et al., 2020; Ferretti et al., 2020; Crepaldi et al., 2021). Thus, in this work we can suggest that the detection of low-abundant satDNAs signals in amphibian genomes should be improved with adaptations of FISH protocols and more specific experiments, which could lead to a more accurate knowledge of these less abundant genomic repeats.

For the highly abundant PboSat01-176 and PboSat02-192 satellites, the successful mapping by FISH highlights the presence of certain satDNAs in strategic regions of the chromosomes of *P. boiei* (e.g., centromere and pericentromeric regions), which leads to their supposedly part in crucial processes for genomic organization and maintenance in this species. It is well highlighted that the abundance of satDNAs is prone to change rapidly due to evolutionary molecular mechanisms such as scattering and amplification, and can result in rapid repatterning as they expand or shrink their arrays (Plohl et al., 2008; Garrido-Ramos, 2017; Palacios-Gimenez et al., 2020; Sproul et al., 2020; Thakur et al., 2021); this rapid evolution is probably also occurring in *P. boiei*. In amphibians, abundant repeats of satDNAs seem not to be dispersed in the karyotype, and even in specific chromosomes, but rather clustered in peri/centromeric regions, and possibly playing a role in the organization, regulation and maintenance of these chromosomal regions. In summary, this feature was detailed for the anuran genera *Physalaemus*, *Proceratophrys*, and *Bufo* (Vittorazzi et al., 2011; Da Silva et al., 2020; Guzmán et al., 2022, respectively), and, for all cases, the chromosomal location of abundant satDNAs indicates a role in centromere function or in the formation and maintenance of heterochromatin in these regions.

Previously, our selection of satDNAs *via* analysis of relative abundance values (female/male) revealed the presence of 58 satDNAs that possible had differentially accumulated within the heteromorphic sex chromosomes of *P. boiei*. We show here a great diversity of satellites in the genome of *P. boiei*; however, this high amplification does not seem to be the main factor that leads to the heterochromatic expansion of the W chromosome, since none of the satDNAs was mapped exclusively on this chromosome, being always shared equally between the both chromosomes pairs. Unlike the fish *M. elongatus*, in which high rates of amplification and homogenization of some satDNAs particularly abundant in the female genome effectively contributed to the heterogametic differentiation of the sex chromosomes of this species (Crepaldi et al., 2021).

Different studies have already shown that TEs are abundant in sex chromosomes. TEs may play a significant role in sex chromosome differentiation by allowing W/Y chromosomes to achieve a state of beneficial non-homology/non-recombination

via TE insertions in a brief time (McDonald, 1993; Charlesworth and Charlesworth, 2000; Hua-Van et al., 2005; Bachtrog, 2008). Here, surprisingly, it was found that of the 226 satellite families found for *P. boiei*, 82 of them show some similarity, low or high, with retrotransposons, mainly Ty3/Gypsy and Tc/Mariner, being an indication of plasticity and mobility of these repetitive elements for the organization, regulation, and enlargement of *P. boiei* genome. As mobile elements, TEs can replicate and spread within genomes through transposition, leading to an increase in copy number by intrinsic replication, as in the case of retrotransposons, or the repair of double-strand breaks generated during transposition (Eickbush and Malik, 2002).

The insertion of TEs has been postulated to be one of the earliest triggers causing recombination suppression (Kent et al., 2017; Drost and Sanchez, 2019). Although a more in-depth analysis of this relationship in *P. boiei* has not been done, it is notable that TEs are driving genomic functions in this species. Whether plant and animal sex chromosomes preferentially accumulate specific TEs compared to the other chromosomes remains unclear. This suggests that the non-random distribution of Ty3/Gypsy in the genome may drive sex chromosome differentiation. However, investigating the possible existence of Ty3/Gypsy elements in other organisms, using fluorescence *in situ* hybridization mapping, bioinformatics analysis, and whole-genome sequencing may further substantiate this hypothesis, particularly in *P. boiei*.

In summary, our study reveals the complete satellitome of *P. boiei*, showing a great diversity of repeats that are driving genomic organization in this frog species. The characterization and approaches regarding satDNAs in this species of frog allowed the confirmation of some insights from satellite biology and a possible relationship about the evolution of sex chromosomes, especially in anuran amphibians, which did not yet have complete data from this species so far. When combined with future analyses, these data will be useful for the accurate characterization of specific satellites, as well as transposable elements, their relationship with centromeric functions, and their possible influence on sex chromosome differentiation in *Proceratophrys*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee (Comitê de Ética no Uso de Animais - CEUA - permission 21/2019), Biosciences Institute, UNESP, Rio Claro, SP, Brazil.

Author contributions

MJ contributed to the work's conception, data acquisition, analysis and interpretation, and wrote the manuscript. TG and CH contributed to the acquisition of specimens for biological and genomic material. MJ conducted cytogenetic analysis. PP-M conceptualized, funded and coordinated the research. All authors contributed to manuscript revision, read and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1101397/full#supplementary-material

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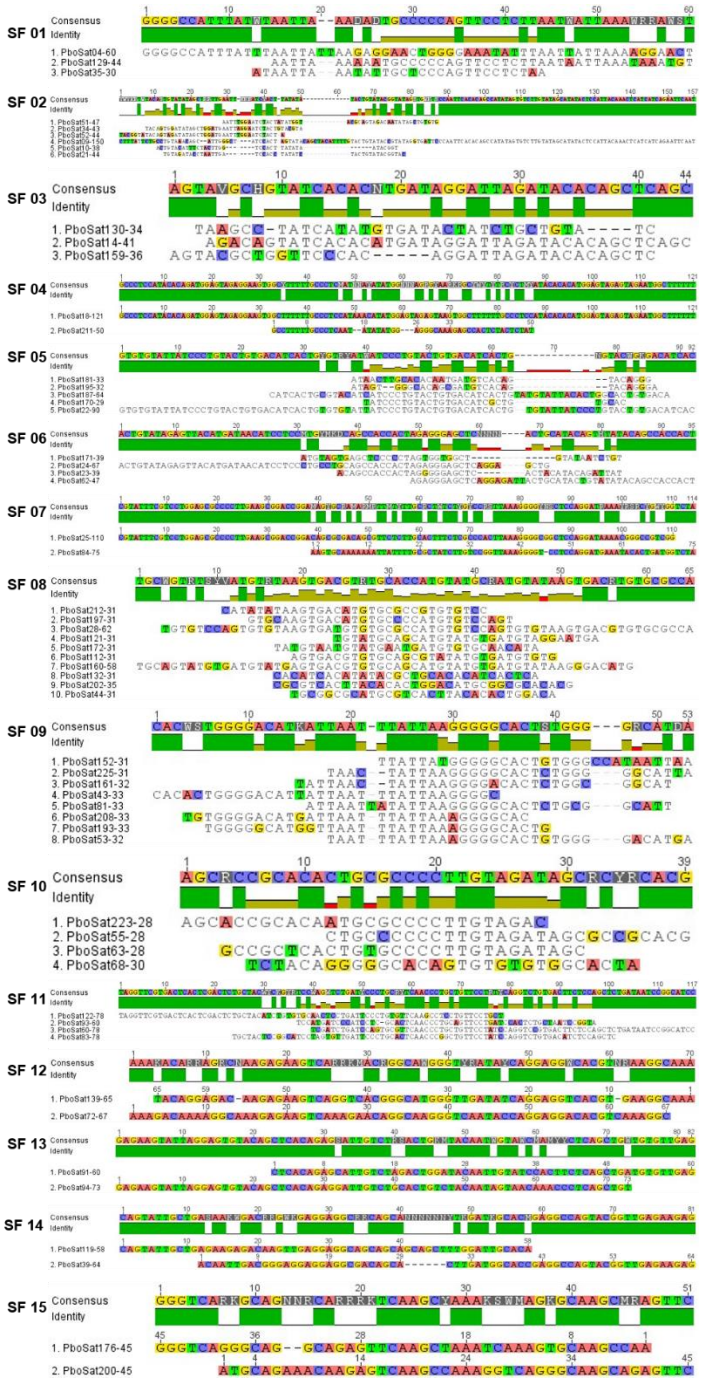
Supplementary Material

1 Supplementary Figures and Tables



Supplementary Figure S1. Alignments between sequences from the characterized superfamilies in the *Proceratophrys boiei* genome.

SF	Satellite DNA family	Length	A+T	Abundance		Divergence		F/M
				F	M	F	M	
	PboSat01-176	176	54,5	10,4973	8,4003	3,10	3,08	1,25
	PboSat02-192	192	53,6	0,9736	0,9894	8,61	8,65	0,98
	PboSat03-25	25	48,0	0,4102	0,3421	15,98	16,01	1,20
01	PboSat04-60	60	71,6	0,3294	0,2975	15,42	15,40	1,11
	PboSat05-36	36	67,7	0,3123	0,2932	4,0	4,15	1,06
	PboSat06-123*	123	64,3	0,2463	0,2634	7,12	7,13	0,93
	PboSat07-121*	121	71,1	0,1961	0,2040	2,05	2,10	0,96
	PboSat08-92	92	57,6	0,1743	0,1913	2,85	2,86	0,91
02	PboSat09-150	150	63,3	0,1635	0,1786	21,42	21,39	0,92
02	PboSat10-38	38	65,8	0,1557	0,1624	2,76	2,74	0,96
03	PboSat11-72*	72	66,7	0,1427	0,1667	9,46	9,84	0,86
	PboSat12-52	52	50,9	0,1313	0,1479	7,56	7,65	0,89
	PboSat13-181	181	29,3	0,1162	0,1459	8,95	9,39	0,80
04	PboSat14-41	41	58,3	0,1155	0,1254	6,03	6,42	0,92
	PboSat15-166	166	38,0	0,1118	0,0883	3,26	3,45	1,27
	PboSat16-25*	25	52,0	0,1063	0,0873	15,68	15,74	1,22
	PboSat17-90	90	50,0	0,1057	0,1084	5,71	5,75	0,97
05	PboSat18-121	121	56,2	0,0971	0,0884	11,98	11,98	1,10
	PboSat19-178	178	52,2	0,0931	0,0995	1,90	2,01	0,94
	PboSat20-52	52	47,1	0,0855	0,0971	7,60	7,60	0,88
02	PboSat21-44*	44	63,6	0,0853	0,0918	6,92	6,91	0,93
06	PboSat22-90	90	57,8	0,0827	0,0891	9,12	9,10	0,93
07	PboSat23-39	39	51,2	0,0753	0,0716	4,86	5,07	1,05
07	PboSat24-67	67	47,8	0,0746	0,0776	6,23	6,28	0,96
08	PboSat25-110	110	38,2	0,0733	0,0580	2,46	3,03	1,26
	PboSat26-115	115	60,7	0,0680	0,0706	8,28	8,29	1,04
	PboSat27-24*	24	62,5	0,0507	0,0541	26,88	26,75	0,94
09	PboSat28-62	62	46,7	0,0499	0,0573	8,66	8,70	0,87
	PboSat29-68	68	73,5	0,0473	0,0237	2,74	2,48	2,0
	PboSat30-91	91	69,3	0,0464	0,0486	2,25	2,25	0,96
	PboSat31-33	33	57,5	0,0419	0,0420	11,45	11,55	1,0
	PboSat32-43*	43	58,2	0,0407	0,0446	4,63	4,91	0,91
	PboSat33-986	986	67,6	0,0390	0,0422	1,93	2,03	0,92
02	PboSat34-43	43	62,8	0,0374	0,0379	9,13	9,14	0,99
01	PboSat35-30*	30	69,9	0,0358	0,0324	12,05	11,73	1,10
	PboSat36-39	39	59,0	0,0330	0,0351	10,47	10,74	0,94
	PboSat37-31	31	58,1	0,0319	0,0341	13,78	14,01	0,94
	PboSat38-96	96	33,3	0,0311	0,0356	11,99	12,27	0,87
17	PboSat39-64*	64	42,2	0,0311	0,0352	8,34	7,87	0,88
03	PboSat40-40	40	55,0	0,0296	0,0361	11,90	11,71	0,82

	PboSat41-84*	84	64,3	0,0292	0,0333	20,13	20,19	0,89
	PboSat42-51*	51	53,9	0,0282	0,0293	11,04	11,09	0,96
10	PboSat43-33*	33	60,4	0,0235	0,0235	15,13	15,02	1,0
09	PboSat44-31*	31	42,0	0,0225	0,0252	10,01	9,94	0,89
	PboSat45-110	110	60,9	0,0214	0,0219	10,91	10,90	0,98
11	PboSat46-106*	106	48,1	0,0202	0,0211	6,96	6,95	0,96
	PboSat47-91	91	69,3	0,0199	0,0211	1,35	1,62	0,94
	PboSat48-93	93	63,4	0,0196	0,0205	5,29	5,31	0,96
	PboSat49-37*	37	54,0	0,0195	0,0205	21,20	21,34	0,95
	PboSat50-30	30	50,0	0,0194	0,0224	14,89	15,03	0,87
02	PboSat51-47	47	63,8	0,0193	0,0215	8,52	8,59	0,90
02	PboSat52-44*	44	65,9	0,0184	0,0202	9,29	9,18	0,91
10	PboSat53-32*	32	59,3	0,0184	0,0185	19,91	19,86	1,0
	PboSat54-39	39	61,6	0,0183	0,0182	5,94	6,50	1,0
12	PboSat55-28*	28	32,2	0,0181	0,0223	11,02	11,88	0,81
	PboSat56-54*	54	59,2	0,0180	0,0161	8,55	9,86	1,11
	PboSat57-88	88	54,6	0,0171	0,0272	11,67	11,69	0,63
	PboSat58-75	75	61,3	0,0170	0,0163	16,29	16,64	1,04
	PboSat59-50	50	52,0	0,0160	0,0139	10,70	11,20	1,15
13	PboSat60-78	78	46,2	0,0157	0,0182	4,97	5,18	0,86
	PboSat61-74	74	59,4	0,0154	0,0165	4,17	4,58	0,93
07	PboSat62-47	47	53,2	0,0154	0,0164	7,40	7,40	0,94
12	PboSat63-28*	28	39,3	0,0152	0,0186	11,1	11,14	0,82
	PboSat64-29	29	51,7	0,0151	0,0160	9,50	9,30	0,94
	PboSat65-45	45	53,3	0,0150	0,0148	12,71	12,79	1,01
	PboSat66-31	31	58,1	0,0145	0,0159	21,0	21,08	0,91
	PboSat67-44	44	54,5	0,0143	0,0142	3,95	3,85	1,01
12	PboSat68-30	30	43,3	0,0142	0,0154	18,97	18,74	0,92
	PboSat69-62*	62	59,7	0,0138	0,0142	6,51	6,58	0,97
	PboSat70-88*	88	59,1	0,0138	0,0154	3,56	3,53	0,90
	PboSat71-46	46	50,0	0,0137	0,0139	3,56	3,97	0,99
14	PboSat72-67*	67	53,8	0,0133	0,0150	24,75	25,14	0,89
	PboSat73-34*	34	57,8	0,0132	0,0152	10,65	10,65	0,87
	PboSat74-88*	88	62,5	0,0131	0,0135	13,21	12,79	0,97
	PboSat75-43	43	30,3	0,0127	0,0143	17,12	16,98	0,89
	PboSat76-61*	61	55,8	0,0126	0,0121	17,28	17,42	1,04
15	PboSat77-33*	33	42,4	0,0125	0,0136	10,82	10,89	0,93
	PboSat78-29	29	48,2	0,0124	0,0139	11,33	11,32	0,89
	PboSat79-69	69	50,7	0,0124	0,0159	13,71	12,75	0,78
	PboSat80-77*	77	58,5	0,0122	0,0134	7,52	7,80	0,91
10	PboSat81-33	33	60,5	0,0122	0,0105	11,5	11,88	1,16
	PboSat82-34*	34	55,9	0,0116	0,0111	8,84	8,72	1,04
13	PboSat83-78	78	44,9	0,0115	0,0123	23,64	23,76	0,93

08	PboSat84-75	75	60,0	0,0113	0,0125	13,57	13,48	0,91
	PboSat85-24	24	66,7	0,0110	0,0123	28,02	28,35	0,92
	PboSat86-25	25	48,0	0,0110	0,0094	20,35	20,16	1,18
	PboSat87-33*	33	57,6	0,0110	0,0150	10,16	12,50	0,73
	PboSat88-45*	45	44,4	0,0110	0,0118	14,77	14,82	0,93
	PboSat89-38*	38	60,5	0,0104	0,0097	14,67	13,73	1,06
18	PboSat90-31	31	51,6	0,0103	0,0110	19,88	19,80	0,94
16	PboSat91-60	60	56,7	0,0101	0,0104	11,0	10,82	0,97
	PboSat92-93	93	32,2	0,0098	0,0073	0,27	0,26	1,30
13	PboSat93-60	60	46,7	0,0094	0,0107	11,49	11,48	0,88
16	PboSat94-73	73	56,2	0,0094	0,0103	18,46	18,01	0,91
	PboSat95-44	44	61,3	0,0090	0,0088	6,52	6,65	1,02
	PboSat96-30	30	56,7	0,0090	0,0093	15,52	15,12	0,97
	PboSat97-41	41	21,9	0,0088	0,0106	14,89	14,51	0,83
	PboSat98-30*	30	50,0	0,0088	0,0101	12,78	13,30	0,87
	PboSat99-131*	131	61,8	0,0086	0,0082	4,46	4,48	1,06
	PboSat100-36	36	50,0	0,0086	0,0083	21,58	21,89	1,04
	PboSat101-351	351	67,2	0,0085	0,0135	13,96	11,95	0,63
	PboSat102-37	37	64,8	0,0084	0,0230	15,66	13,69	0,37
	PboSat103-97	97	50,5	0,0084	0,0085	10,85	10,79	0,99
	PboSat104-84*	84	60,6	0,0083	0,0089	16,02	15,12	0,94
	PboSat105-141	141	62,4	0,0082	0,0098	14,66	14,34	0,84
	PboSat106-405	405	53,8	0,0081	0,0113	5,59	5,88	0,72
	PboSat107-34*	34	57,7	0,0078	0,0080	6,54	6,56	0,98
	PboSat108-435	435	56,3	0,0078	0,0077	3,76	3,87	1,02
	PboSat109-88	88	60,3	0,0078	0,0081	15,11	14,79	0,96
	PboSat110-742	742	60,6	0,0076	0,0090	1,34	1,12	0,84
	PboSat111-25*	25	48,0	0,0076	0,0082	14,07	13,90	0,92
09	PboSat112-31	31	51,6	0,0075	0,0079	10,62	11,05	0,95
	PboSat113-27*	27	70,3	0,0074	0,0082	4,42	4,20	0,91
	PboSat114-27	27	55,5	0,0073	0,0077	14,26	14,69	0,94
	PboSat115-20	20	70,0	0,0073	0,0077	8,96	8,02	0,94
	PboSat116-44	44	36,3	0,0073	0,0076	4,44	4,46	0,96
	PboSat117-543	543	53,6	0,0072	0,0063	6,13	6,82	1,13
	PboSat118-73	73	57,5	0,0071	0,0082	4,08	4,33	0,87
17	PboSat119-58*	58	50,0	0,0067	0,0074	9,26	8,74	0,90
	PboSat120-21*	21	47,7	0,0066	0,0076	7,28	6,90	0,88
09	PboSat121-31*	31	61,3	0,0066	0,0060	6,30	6,49	1,09
13	PboSat122-78	78	48,7	0,0064	0,0073	21,73	22,56	0,89
	PboSat123-45	45	46,6	0,0063	0,0067	8,01	8,34	0,94
	PboSat124-62	62	74,2	0,0063	0,0068	16,39	16,68	0,92
	PboSat125-47	47	63,8	0,0062	0,0043	7,20	7,20	1,43

	PboSat126-52*	52	50,0	0,0062	0,0068	17,88	19,27	0,91
	PboSat127-44*	44	45,5	0,0061	0,0062	12,24	12,04	0,98
	PboSat128-68*	68	50,4	0,0060	0,0062	8,61	9,20	0,97
01	PboSat129-44	44	75,0	0,0059	0,0054	8,66	8,89	1,09
04	PboSat130-34*	34	64,7	0,0059	0,0064	21,52	22,42	0,93
	PboSat131-41	41	48,8	0,0059	0,0073	9,28	9,68	0,81
09	PboSat132-31*	31	54,9	0,0056	0,0066	14,35	14,89	0,84
	PboSat133-78	78	43,6	0,0054	0,0061	6,43	6,43	0,89
	PboSat134-39	39	61,5	0,0053	0,0061	15,43	15,35	0,88
	PboSat135-68	68	44,4	0,0053	0,0060	17,84	17,93	0,88
	PboSat136-59*	59	40,7	0,0053	0,0056	4,03	4,26	0,93
	PboSat137-34	34	55,9	0,0051	0,0050	11,66	11,47	1,03
	PboSat138-29*	29	48,3	0,0047	0,0049	11,69	11,72	0,95
14	PboSat139-65*	65	49,2	0,0047	0,0047	21,78	21,97	0,99
	PboSat140-36*	36	50,0	0,0044	0,0042	7,44	8,06	1,05
	PboSat141-39*	39	64,1	0,0043	0,0051	11,02	11,80	0,85
	PboSat142-60	60	66,7	0,0043	0,0040	10,80	10,95	1,07
	PboSat143-38*	38	43,8	0,0041	0,0049	4,96	4,86	0,84
	PboSat144-98	98	55,1	0,0041	0,0045	8,59	8,39	0,90
	PboSat145-33*	33	47,6	0,0040	0,0035	10,51	12,12	1,12
	PboSat146-27*	27	51,7	0,0039	0,0035	5,58	6,02	1,09
	PboSat147-21	21	76,2	0,0038	0,0024	7,88	10,13	1,60
	PboSat148-22	22	45,5	0,0038	0,0048	3,85	4,04	0,81
	PboSat149-25*	25	48,0	0,0038	0,0038	3,64	3,91	1,0
	PboSat150-51*	51	54,9	0,0038	0,0040	12,98	12,86	0,95
	PboSat151-52	52	63,4	0,0037	0,0028	5,78	5,81	1,30
10	PboSat152-31	31	58,1	0,0036	0,0039	17,68	17,16	0,92
	PboSat153-75	75	80,0	0,0035	0,0036	4,01	4,31	0,97
	PboSat154-25*	25	80,0	0,0035	0,0043	8,31	7,62	0,82
	PboSat155-20*	20	75,0	0,0035	0,0050	11,04	8,86	0,71
	PboSat156-40*	40	67,5	0,0035	0,0039	14,43	14,49	0,86
18	PboSat157-30*	30	50,0	0,0035	0,0039	16,48	16,65	0,88
	PboSat158-41	41	41,5	0,0034	0,0037	13,22	12,90	0,91
04	PboSat159-36*	36	50,0	0,0033	0,0035	19,21	19,17	0,94
09	PboSat160-58	58	56,9	0,0032	0,0039	13,74	13,77	0,83
10	PboSat161-32	32	56,2	0,0032	0,0031	11,82	12,68	1,03
	PboSat162-87	87	80,4	0,0032	0,0032	3,34	3,32	1,0
	PboSat163-39	39	51,3	0,0031	0,0032	8,76	8,65	0,96
	PboSat164-30	30	59,0	0,0030	0,0034	14,15	13,71	0,89
	PboSat165-452	452	48,2	0,0029	0,0056	0,35	0,27	0,53
	PboSat166-25	25	80,0	0,0028	0,0029	8,71	9,69	0,97
	PboSat167-25	25	60,0	0,0028	0,0035	10,42	10,66	0,80
	PboSat168-77	77	54,4	0,0028	0,0047	3,17	3,27	0,59

	PboSat169-30	30	70,0	0,0027	0,0026	11,55	10,42	1,07
06	PboSat170-29	29	48,2	0,0026	0,0039	8,18	6,70	0,68
07	PboSat171-39	39	51,2	0,0026	0,0020	4,09	4,37	1,28
09	PboSat172-31	31	71,0	0,0026	0,0026	17,81	18,58	0,98
	PboSat173-29	29	55,2	0,0024	0,0024	15,59	17,20	0,99
	PboSat174-82	82	59,7	0,0024	0,0022	8,33	8,31	1,09
	PboSat175-49	49	44,9	0,0024	0,0024	19,96	19,72	1,0
19	PboSat176-45*	45	48,9	0,0021	0,0025	21,03	21,27	0,87
	PboSat177-75*	75	56,0	0,0020	0,0016	4,78	5,21	1,23
	PboSat178-69*	69	58,0	0,0019	0,0020	3,18	2,82	0,96
	PboSat179-23	23	69,5	0,0019	0,0031	11,26	11,25	0,62
	PboSat180-42	42	54,8	0,0019	0,0023	3,14	3,27	0,84
06	PboSat181-33	33	57,6	0,0017	0,0020	19,85	19,12	0,84
	PboSat182-32	32	43,8	0,0016	0,0016	15,92	16,52	0,97
	PboSat183-97	97	49,5	0,0016	0,0017	9,91	10,38	0,95
	PboSat184-39	39	43,6	0,0016	0,0017	5,58	6,05	0,94
	PboSat185-31	31	58,1	0,0015	0,0016	14,08	12,45	0,90
	PboSat186-30	30	53,4	0,0015	0,0015	5,63	6,49	0,95
06	PboSat187-64	64	54,7	0,0014	0,0013	18,90	20,80	1,06
15	PboSat188-26	26	46,1	0,0014	0,0016	12,82	10,41	0,86
	PboSat189-20	20	70,0	0,0013	0,0014	5,42	6,39	0,98
	PboSat190-80	80	60,0	0,0013	0,0010	2,91	3,01	1,29
	PboSat191-51	51	58,8	0,0013	0,0015	11,91	12,32	0,87
	PboSat192-30	30	56,7	0,0012	0,0014	14,46	14,11	0,87
10	PboSat193-33	33	54,5	0,0011	0,0012	8,05	7,99	0,92
	PboSat194-59	59	40,7	0,0011	0,0020	5,92	5,49	0,56
06	PboSat195-32	32	46,9	0,0011	0,0011	14,58	14,87	0,98
	PboSat196-77*	77	61,1	0,0010	0,0011	11,67	12,10	0,90
09	PboSat197-31	31	45,2	0,0009	0,0010	17,66	17,76	0,90
	PboSat198-44*	44	62,2	0,0009	0,0008	3,16	3,02	1,08
	PboSat199-80*	80	55,0	0,0009	0,0013	5,22	5,54	0,67
19	PboSat200-45	45	51,1	0,0009	0,0008	24,01	24,26	1,07
	PboSat201-75	75	70,6	0,0008	0,0007	0,44	0,38	1,19
02	PboSat202-35	35	37,1	0,0008	0,0007	10,92	11,82	1,01
	PboSat203-52	52	42,3	0,0007	0,0010	4,98	4,45	0,78
	PboSat204-36	36	72,2	0,0007	0,0008	18,91	18,99	0,91
	PboSat205-27	27	62,9	0,0007	0,0006	6,57	7,89	1,16
	PboSat206-69	69	66,6	0,0007	0,0006	19,67	20,13	1,10
	PboSat207-23*	23	64,2	0,0007	0,0009	9,92	10,63	0,76
10	PboSat208-33*	33	60,6	0,0006	0,0006	7,23	6,10	1,06
	PboSat209-51	51	53,0	0,0006	0,0008	19,16	22,07	0,79
	PboSat210-30*	30	43,4	0,0006	0,0005	10,66	11,24	1,10

05	PboSat211-50*	50	56,0	0,0006	0,0005	20,68	20,57	1,17
09	PboSat212-31	31	51,6	0,0005	0,0006	16,74	18,61	0,80
	PboSat213-80*	80	58,8	0,0004	0,0005	5,08	4,22	0,91
	PboSat214-54*	54	38,9	0,0004	0,0004	7,98	8,93	1,03
	PboSat215-25*	25	44,0	0,0003	0,0003	7,76	7,87	0,99
	PboSat216-43*	43	34,9	0,0003	0,0004	20,11	21,41	0,80
	PboSat217-26*	26	65,4	0,0003	0,0005	16,36	16,34	0,64
	PboSat218-25*	25	68,0	0,0003	0,0003	13,82	13,39	1,0
	PboSat219-26*	26	76,9	0,0002	0,0002	10,74	10,67	1,32
	PboSat220-31*	31	74,2	0,0002	0,0002	19,60	17,16	1,07
11	PboSat221-28*	28	42,8	0,0001	0,0001	17,88	23,86	0,87
	PboSat222-29	29	41,4	0,0001	0,0002	12,51	12,43	0,60
12	PboSat223-28*	28	39,3	0,00009	0,0001	16,16	19,54	0,89
	PboSat224-38	38	52,6	0,00009	0,00007	21,89	30,32	1,27
10	PboSat225-31*	31	51,6	0,00008	0,0001	16,76	13,86	0,71
	PboSat226-20*	20	75,0	0,00002	0,000007	18,40	14,52	2,80
	Average	**98**	**64,75**	**5,24866**	**4,200154**	**10,75**	**8,8**	**2,025**

Supplementary Table S1. General characteristics of satDNA families recovered from male and female genomes of *Proceratophrys boiei*, such as satellite DNA families, length (bp), A+T (%), female (F) and male (M) abundance (% of the genome) and female (F) and male (M) divergence (%). Each satDNA has their own quotient for female and male abundance (F/M ratio). Superfamily (SF) is also evidenced. Averages are at the bottom of the table. *Families of satDNAs with some similarity to a transposable element.

Primer	Sequence (5'–3')
PboSat01-176F	GTCAGGCGAATGAAAACTC
PboSat01-176R	ATTCCTGCACAGAGAAATTG
PboSat02-192F	GATTGCGACATTCTACATTGG
PboSat02-192R	TACTGCGCCTGTGTATTTGG
***PboSat03-25**	ATGGGGGCACAGTGTATATGGCACT
PboSat04-60F	CCATTTATTTAATTATTAAGAG
PboSat04-60R	CCCCAGTTCCTTTTAATAA
PboSat05-36F	TACTATAATACTGCCCCCTA
PboSat05-36R	GTTATATTGTTGTAGATAGGG
PboSat06-123F	GATTGCGACATTCTACATTGG
PboSat06-123R	TACTGCGCCTGTGTATTTGG
PboSat07-121F	TAATATATAATGTAATATATAT
PboSat07-121R	CATATATACGTTATATATAT
PboSat08-92F	CTTATCCTGTACTGATCCT
PboSat08-92R	TAATGTATGTACACAGTGAC
PboSat09-150F	TGATTCCCAATTCACACAGC

PboSat09-150R	CCTATACGGTATACAGTACAA
PboSat10-38F	CTTATATAATACGGTACTGT
PboSat10-38R	TGGACCAAGTAGAAATGTAC

*Biotin-labeled primer, no amplification required.

Supplementary Table S2. List of primers assigned to the 10 most abundant satellite DNAs for *Proceratophrys boiei*.

4.2 Capítulo 2 - Interspecific cytogenomic comparison reveals a potential chromosomal centromeric marker in *Proceratophrys* frog species

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Resumo

Dentre os elementos repetitivos, o DNA satélite (satDNA) destaca-se como extensas sequências de unidades repetidas *in tandem* altamente similares, abrangendo megabases em comprimento. Dado que o satDNA PboSat01-176, previamente caracterizado em *Proceratophrys boiei*, despertou nosso interesse por sua alta abundância nessa espécie e potencial como satélite centrômero, empregamos diversas abordagens, incluindo sequenciamento genômico de baixa cobertura, seguido de análise computacional e técnicas de localização cromossômica em quatro espécies de *Proceratophrys*. Investigamos a presença genômica e compartilhamento, bem como seu potencial como marcador cromossômico centromérico em quatro espécies diferentes. Nossos achados demonstram que o PboSat01-176 exibe alta abundância em todas as quatro espécies de *Proceratophrys*, apresentando características distintas que o estabelecem como o elemento repetitivo predominante nessas espécies. O DNA satélite está agrupado de forma proeminente na região peri/centromérica dos cromossomos, especialmente em regiões heterocromáticas. A presença generalizada de PboSat01-176 em espécies de *Proceratophrys* intimamente relacionadas reforça a validade da hipótese da biblioteca para sequências repetitivas. Assim, este estudo destacou a utilidade da família de satDNA PboSat01-176 como um marcador centrômero confiável em espécies de *Proceratophrys*, com potencial para ser aplicado em outras espécies de anfíbios anuros. O compartilhamento observado e a manutenção dessa sequência dentro do gênero sugerem possibilidades para pesquisas futuras, especialmente por meio de amostragem ampliada para elucidar parâmetros que fundamentam a hipótese de biblioteca e a dinâmica evolutiva das sequências de satDNAs.

Palavras-chave: cromossomos; repeatmasker; citogenética; anfíbios; evolução.

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Abstract

Among the repetitive elements, satellite DNA (SatDNA) emerges as extensive arrays of highly similar tandemly repeated units, spanning megabases in length. Given that the satDNA PboSat01-176, previously characterized in *P. boiei*, prompted our interest for having a high abundance in *P. boiei* and potential for centromeric satellite, here, we employed various approaches, including low coverage genome sequencing, followed by computational analysis and chromosomal localization techniques in four *Proceratophrys* species and, investigating the genomic presence and sharing, as well as its potential for chromosomal centromere marker in *Proceratophrys* frog species. Our findings demonstrate that PboSat01-176 exhibits high abundance across all four *Proceratophrys* species, displaying distinct characteristics that establish it as the predominant repetitive DNA element in these species. The satellite DNA is prominently clustered in the peri/centromeric region of the chromosomes, particularly in the heterochromatic regions. The widespread presence of PboSat01-176 in closely related *Proceratophrys* species reinforces the validity of the library hypothesis for repetitive sequences. Thus, this study highlighted the utility of the satDNA family PboSat01-176 as a reliable centromeric marker in *Proceratophrys* species, with potential to be applied in other species of anuran amphibians. The observed sharing and maintenance of this sequence within the genus suggest possibilities for future research, particularly through expanded sampling to elucidate parameters that underlie the library hypothesis and the evolutionary dynamics of satDNA sequences.

Key-words: chromosome, repeatmasker, cytogenetics, Amphibia, evolution

Introduction

Among the different repetitive elements in eukaryotic genomes, satellite DNA (SatDNA) emerges as extensive arrays of highly similar tandemly repeated units, spanning megabases in length (Garrido-Ramos 2017; Louzada et al. 2020; Šatovi´c-Vukši´c and Plohl 2023).

Centromeres in most eukaryotes are typically occupied by large arrays of satellite repeats that span multiple megabases (Plohl et al. 2012; Garrido-Ramos 2017). While centromeres play a conserved role in chromosome segregation, the centromeric satellite DNA sequences are highly dynamic and rapidly evolving. This leads to significant variations in the centromere DNA sequences among closely related species (Henikoff et al. 2001; Rudd et al. 2006; Melters et al. 2013). These variations are consistent with the concept of the "library hypothesis", which suggests that related species share a set or library of satellite DNA (satDNA) families. According to this hypothesis, each species usually displays one or a few predominant satDNA families within their genome, guaranteeing specific features in each one (Garrido-Ramos 2017).

Research on the dynamics of satDNA content, organization and location of centromeric sequences in anuran amphibians remains limited. Species of *Proceratophrys* frogs have shown immense potential for studies involving these issues, as they represent the majority of the Odontophrynidae family, encompassing 43 species of small to medium-sized frogs (Frost 2023), and interesting previously cytogenetic and cytogenomic data for a subset *Proceratophrys* species.

Recently, comprehensive genomic, bioinformatic, and cytogenetic analyses were conducted in *P. boiei* (2n = 22, ZZ/ZW) to characterize its entire satellitome (João Da Silva et al. 2023). These analyses revealed a highly abundant and distinctive satDNA, named PboSat01-176, which accounts for 93.62% of the total satDNA content in the species. PboSat01-176 constitutes the most prevalent satDNA in the genome of *P. boiei*, comprising 10.49% of the genomic abundance, speculated with a probable association with the maintenance and structural organization of crucial chromosomal regions, such as the centromere and pericentromeric regions (João Da Silva et al. 2023).

Some species of *Proceratophrys* have undergone karyotyping recently, including chromosomal and banding analyses such as C-banding. Among these species, *P.*

melanopogon, *P. schirchi*, and *P. laticeps* drew attention for their conserved diploid number, all with 2n = 22 chromosomes and characteristic banding patterns, with visible heterochromatin bands localized in the centromeric regions of their chromosomes (Silva et al. 2021).

Given that the centromeric satDNA PboSat01-176, previously characterized in *P. boiei*, prompted our interest for having a high abundance in *P. boiei* and potential for centromeric satellite, here, we conducted genomic and cytogenetic analyses to characterize and determine the chromosomal localization of this satDNA in these related *Proceratophrys* species, with potential to enhance our understanding of the sharing of repetitive DNAs and their contribution to genome evolution.

Material and methods

Chromosome preparations, biological materials, genomic DNA extraction and sequencing

We utilized the cytogenetic preparations of *P. boiei*, *P. schirchi*, *P. melanopogon*, and *P. laticeps*, which had been previously studied by Da Silva et al. (2020), Silva et al. (2021), and João Da Silva et al. (2023), and were available at the Animal Cytogenetics Laboratory in UNESP, Rio Claro, São Paulo, Brazil. For molecular and bioinformatic analyses, we extracted total genomic DNA (gDNA) from liver or muscle samples of each species using the Wizard® Genomic DNA purification kit (Promega, WI, United States), following the manufacturer's instructions.

The extracted gDNA samples were employed for both genomic sequencing and polymerase chain reaction (PCR) assays. The genome of *P. boiei* was subjected to paired-end sequencing (2 × 101 bp) using the Illumina® HiSeq™ 2000 platform, performed by Macrogen Inc. (Seoul, Republic of Korea). As for *P. schirchi*, *P. laticeps*, and *P. melanopogon*, Illumina® HiSeq™ 4000 (2 × 150 bp paired-end) sequencing was conducted by Novogene (HK) Co., Ltd. (Hong Kong, China).

Search for PboSat1-176 by graph-based clustering

Before conducting graph-based clustering analysis using RepeatExplorer, quality assessment and preprocessing of the paired-end reads were performed using FastQC (Version 0.10.1, 2012). The reads were processed following standard parameters through

the public platform (https://repeatexplorer-elixir.cerit-sc.cz/) (Novák et al. 2013). Quality trimming, interlacing paired-end reads, converting FASTQ to FASTA, and RepeatExplorer clustering were executed using recommended standard options. Additionally, an additional analysis was conducted using the TAREAN tool, Tandem Repeat Analyzer (Novák et al. 2020), to enhance the search for satellites. The clusters exhibiting repeat graph density, as identified in both RepeatExplorer and TAREAN summary output, were further scrutinized to identify PboSat01-176 satellite DNA. Contigs displaying tandem sequences within each cluster were singled out using the Dotlet dotplot graphic alignment tool (Drummond et al. 2009; Junier and Pagni, 2000) to confirm their tandem organization.

Estimation of satDNAs sequence abundance and divergence in each *Proceratophrys* genome

To determine the abundance and average nucleotide divergence (Kimura-2-parameter, K2P) of PboSat01-176 in the *Proceratophrys* genomes, we employed RepeatMasker (Smit et al. 2013) with the rmblast engine. All RepeatMasker analyses were conducted remotely via the Kasahara server, hosted by the Insect Cytogenomics Group at Universidade Estadual Paulista - UNESP, Rio Claro, São Paulo, Brazil.

Using the obtained data, we generated graphs and repeat landscapes to depict the relative abundance (Y-axis) at 1% intervals of K2P distance from the consensus (X-axis). The script calcDivergenceFromAlign.pl (from RepeatMasker utils) was utilized for this purpose. Subsequently, individual and general landscapes for PboSat01-176 satDNA were generated by analyzing the data in Microsoft Excel 2016 and utilizing R programming (R Core Team 2021 - version 4.1.2). This analysis was performed to confirm any differences in amplification and divergence between the sequences of the *Proceratophrys* species.

DNA amplification and chromosomal mapping of satDNAs

In order to conduct intraspecific comparisons, we utilized previously isolated centromeric satellite DNA sequence and primers from *P. boiei* (PboSat01-176F 5'-GTCAGGCGAATGAAAACTC-3' and PboSat01-176R 5'-ATTCCTGCACAGAGAAATTG-3') as described by João Da Silva et al. (2023). The specific sequence of PboSat01-176 in FASTA format was obtained from GenBank

(accession OP223503) and employed for PCR amplifications. The PCR amplifications were conducted using 10× Taq Reaction Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 50 mM MgCl2, 2 mM dNTPs, 10 μM of each primer (forward and reverse), 1U of Taq Platinum DNA Polymerase (Invitrogen, San Diego, CA, USA), and 1 μL of a 50– 100 ng/μL template DNA.

PCR cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50–60°C for 30 s, and extension at 72°C for 1 min 20 s. A final extension was performed at 72°C for 5 min. Monomeric bands obtained from PCR amplification were isolated and purified using the Zymoclean TM Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics Company, USA) following the manufacturer's instructions. The purified bands served as a reamplification source for subsequent analyses.

For fluorescence *in situ* hybridization (FISH), mitotic chromosome spreads were prepared and labeled using one or two probes simultaneously. The PCR-amplified satDNA sequences were labeled with digoxigenin-11-dUTP (Roche®) through nick- translation and detected using antidigoxigenin-rhodamine (Roche), following the methodology described by Pinkel et al. (1986) with adjustments by Da Silva et al. (2020) and Cabral-de-Mello and Marec (2021). Chromosomes were counterstained with 4',6- diamidine-2'-phenylindole dihydrochloride (DAPI), and the slides were mounted in VECTASHIELD (Vector, Burlingame, CA, United States). Visualization of the slides was performed using an Olympus® BX51 fluorescence microscope equipped with a digital camera Olympus® DP71. Images were captured using the DP Controller camera software. A minimum of 10 metaphase cells were analyzed and photographed on each slide to confirm the FISH results.

Results

The genomic sequencing data (reads) for each of the *Proceratophrys* species are presented as paired-end trimmed reads, being 19.382.286 for *P. boiei*, 7. 498.094 for *P. schirchi*, 9.309.126 for *P. laticeps* and 5.862.520 for *P. melanopogon*.

The satellite DNA being investigated, PboSat01-176, was previously isolated from *P. boiei* and its characteristics were confirmed in the present work. The repeat unit length (RUL) of PboSat01-176 is 176 bp, which is consistent with the typical size of

centromeric satellite DNA. The consensus sequence of PboSat01-176 has an A + T content of 54.5%, indicating a slight bias towards A+T-rich satellites. Additionally, PboSat01-176 constitutes 93.62% of all satellite DNA families in *P. boiei*, making it the most abundant satellite DNA in the genome, accounting for 10.49% of the genome's abundance, as reported by João Da Silva et al. (2023). In the other three species, *P. schirchi*, *P. melanopogon* and *P. laticeps*, the consensus sequence for the PboSat01-176 satellite had an average of 176 bp, in accordance with what was already found for *P. boiei*. Alignments between the four sequences were carried out and revealed a homology between them, reinforcing a certain homogeneity between sequences (Figure 3).

To compare the abundance and divergence of PboSat01-176 in the four *Proceratophrys* species and investigate sequence sharing between closely related species, genomic quantification analyses were conducted for all species, including *P. boiei*. As expected, PboSat01-176 showed the highest abundance in *P. boiei*, with values of 10.49% abundance and 3.1 divergence (Table 1). Although less abundant in *P. schirchi*, PboSat01-176 was also present, with abundance and divergence values of 0.2% and 28.36, respectively. In *P. laticeps* and *P. melanopogon*, PboSat01-176 exhibited relatively high abundance values above 1%, namely 3.7% and 1.4%, respectively, while the divergence values were 17.2 and 11.41.

To illustrate the abundance and divergence of PboSat01-176 in the analyzed *Proceratophrys* species, individual landscapes were constructed, revealing the evolutionary dynamics of each species (Figure 1). The analysis of landscapes depicting abundance versus divergence highlighted a more recent amplification of PboSat01-476 in *P. boiei* compared to other species, as it exhibited a high abundance with lower divergence (K2P) (Figure 1). In *P. laticeps*, *P. melanopogon* and *P. schirchi*, the satellite PboSat01-176 showed less genomic abundance in these species compared to *P. boiei*, however, the divergence values are higher, suggesting that this satellite follows different patterns of homogenization and amplification (Figure 1).

We investigated the chromosomal distribution of PboSat01-176, originally isolated from *P. boiei*, in other *Proceratophrys* species including *P. laticeps*, *P. melanopogon*, and *P. schirchi*. FISH analysis revealed similar positive signals of clustered PboSat01-176 probes at specific locations in all studied species (Figure 2). The FISH results demonstrated that this satellite DNA is predominantly located in the peri-

and centromeric regions of all chromosome pairs.

Consistent with previously published data (Silva et al. 2021), centromeric heterochromatin, known as c-heterochromatin, was found to be prevalent in all centromeres of the four *Proceratophrys* species. After DAPI staining and FISH analysis, the c-heterochromatin appeared as brightly stained regions, indicating its abundance (Figure 2). In *P. boiei*, the signals formed large blocks that occupied all the c- heterochromatin of the chromosomes (Figure 2). Conversely, in *P. melanopogon*, *P. laticeps*, and *P. schirchi*, the hybridization signals were smaller than in *P. boiei*, representing smaller blocks of centromeric heterochromatin across the entire chromosome set of each species. The distinctive hybridization patterns of the PboSat01- 176 satellite DNA in each *Proceratophrys* species underscore its preferential clustering in the centromeric region, suggesting its involvement in important processes within this chromosomal region.

Discussion

Recent high-throughput genomic analysis has identified *Proceratophrys boiei* as the frog species with the highest number of satDNAs sequences reported thus far (João Da Silva et al. 2023). Cytogenetic analyzes have shown that species of *Proceratophrys* exhibit characteristics shared, such as centromeric heterochromatin, a diploid number of 2n = 22, and phylogenetic proximity (Ananias et al. 2007; Amaro et al. 2012; Silva et al. 2020; Mângia et al. 2020).

The satellite DNA PboSat01-176, emerges as a promising candidate for a centromeric chromosomal marker. Its potential suitability is primarily attributed to specific characteristics, including its precise genomic localization, high abundance, elevated A+T content, and monomer size. Moreover, the investigation of the presence and distribution of this particular satDNA family not only provides insights into the library hypothesis for *Proceratophrys* frogs but also suggests its potential applicability to other anuran amphibians.

The presence of PboSat01-176 in high abundance within the genome of *P. boiei*, as observed in this study, reinforces a potential genomic function in this species (João Da Silva et al. 2023). While the abundance of this satellite sequence in *P. schirchi* is relatively lower, reaching approximately 0.2%, it is still noteworthy and should be

considered. Interestingly, PboSat01-176 exhibits high abundance in *P. laticeps* and *P. melanopogon*, with rates of 3.7% and 1.4%, respectively. The chromosomal localization in the centromere region of this satellite sequence is crucial for characterizing it as a possible marker across these species. The clustering patterns of PboSat01-176 in these specific chromosomal regions align with observations in other organisms, such as insects, where highly abundant satellites are clustered within their centromeres (Bardella et al. 2020; Pereira et al. 2023). A centromeric chromosomal marker for anuran amphibians will be significant for this group still underestimated in relation to its repetitive sequence content and chromosomal composition. Our work shows that PboSat01-176 has potential and should be explored in other species of frogs, phylogenetically close or distant, as it has typical characteristics of centromeric sequences.

The PboSat01-176 satellite DNA displayed distinct patterns of abundance and divergence across *Proceratophrys* species. In *P. boiei*, this satellite exhibited high abundance and low divergence, suggesting a more recent amplification in its genome compared to other species. Conversely, *P. laticeps*, *P. melanopogon*, and *P. schirchi* showed lower abundance and higher divergence values. The computational and chromosomal analyses initially detected successfully PboSat01-176 sequences in *P. laticeps*, *P. melanopogon*, and *P. schirchi*. FISH data added important insights into the characteristics of chromosomal location in each genome, supporting the hypothesis of sequence sharing, as proposed by the library hypothesis, indicating that these species share certain sequences of the satellite DNA.

According to this hypothesis, closely related species inherit a shared set of satDNA sequences from their common ancestor, and certain sequences within this library undergo differential amplification. As a result, these amplified sequences become the predominant components of the satDNA pool, while others occur at lower abundance, giving rise to distinct satDNA profiles among species (Fry and Salser 1977; Palacios- Gimenez et al. 2020). The observed sharing and maintenance of this sequence within the genus suggest possibilities for future research, particularly through expanded sampling to elucidate parameters that underlie the library hypothesis and the evolutionary dynamics of satDNA sequences.

Centromeric satellite DNAs have already been documented in amphibians, as is the case of *Physalaemus cuvieri* in which the PcP190 satDNA was initially identified as

centromeric (Vittorazzi et al. 2011). However, when examined in other related and phylogenetically distant species, the sequence exhibited dynamism, highlighting the variability and dynamic nature associated with satDNA sequences (Gatto et al. 2019). Recently, Guzmán et al. (2022) characterized a novel family of centromeric satDNA in Bufonidae, which displayed an unusual monomeric size of 807 bp, deviating from the typical size range of centromeric satDNAs, typically up to 200 bp. In this study, the analyzed PboSat01-176 sequence was found to be clustered in the peri/centromeric region of all chromosomes across all analyzed species of *Proceratophrys*. These findings imply that this repetitive DNA sequence has undergone significant differentiation processes, and it holds considerable value as a tool for both genomic evolutionary studies and karyotypic investigations.

SatDNAs tend to accumulate in (peri)centromeres due to their involvement in crucial centromeric functions such as kinetochore assembly, chromosome segregation during mitosis or meiosis, and potential epigenetic regulations. Additionally, the absence of recombination-based elimination mechanisms contributes to the passive accumulation of satDNAs in these regions (Plohl et al. 2014; Catania et al. 2015). Conducting (peri)centromere composition studies in distinct species is thus vital for obtaining a more representative understanding of their organization and evolution, and this way, this study highlights the utility of the satDNA family PboSat01-176 as a reliable centromeric marker in *Proceratophrys* species, with potential to be applied in other species of anuran amphibians. Centromeric markers are fundamental tools for genetic and evolutionary studies in many organisms. Having one of them in amphibians will allow a better understanding of the chromosomal structure, genetic inheritance and evolutionary history of this group, and can be used in chromosome identification, evolution studies, genetic mapping, conservation genetics and comparative genomics.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee (Comitê de Ética no Uso de Animais - CEUA - permission 21/2019), Biosciences Institute, UNESP, Rio Claro, SP, Brazil.

Author contributions

MJS contributed to the work’s conception, data acquisition, analysis and interpretation,

and wrote the manuscript. MJS and TG contributed to the acquisition of specimens for biological and genomic material. MJS and RFD conducted cytogenetic analysis. PPM conceptualized, funded and coordinated the research. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of interest

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

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Data archiving

Not applicable.

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Figure legends

Figure 1 – Landscape (abundance versus divergence) for the PboSat01-176 satDNA on the genomes of *Proceratophrys* species. The abundance and divergence were estimated by RepeatMasker. In (A), general landscapes for PboSat01-176. (B) show landscapes in more detail for specific species concerning abundance and divergence of PboSat01-176. PBO (*P. boiei*); PSC (*P. schirchi*); PME (*P. melanopogon*); PLA (*P. laticeps*).

Figure 2 – Chromosomal distribution of PboSat01-176 detected by FISH in *Proceratophrys* species. (A–C) *P. boiei*; (D–F) *P. schirchi*; (G-I) *P. laticeps*; (J-l) *P. melanopogon*.

Figure 3 – Alignment of genomic consensus sequences from the PboSat01-176 satellite in the *Proceratophrys species* analyzed in this work. Note the approximate monomer size of 176 bp in all species.

Figure 1

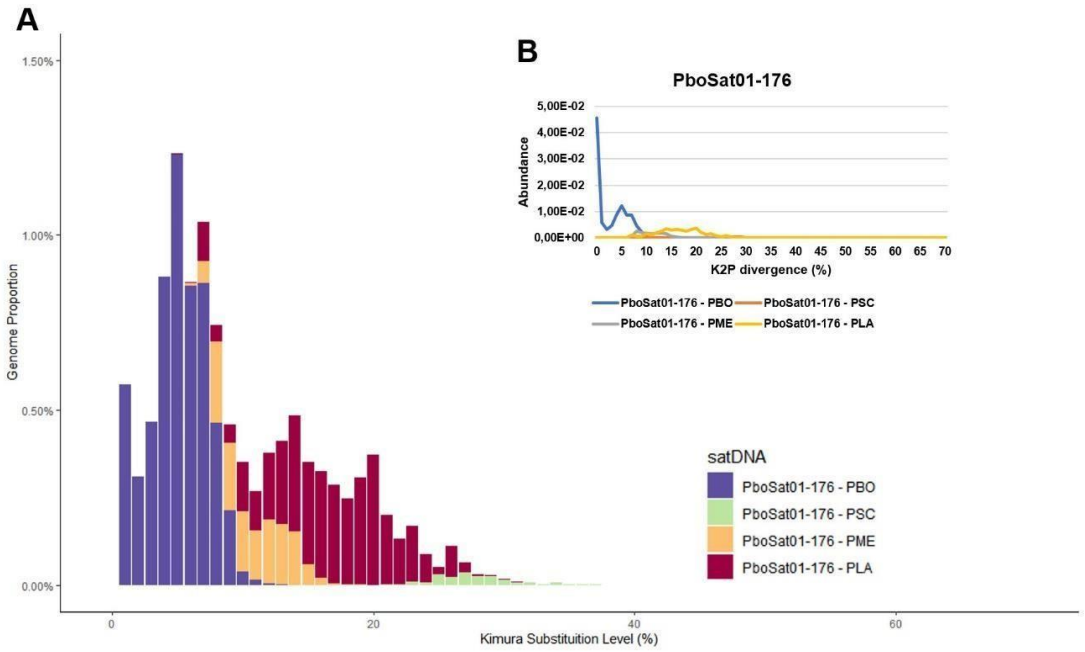
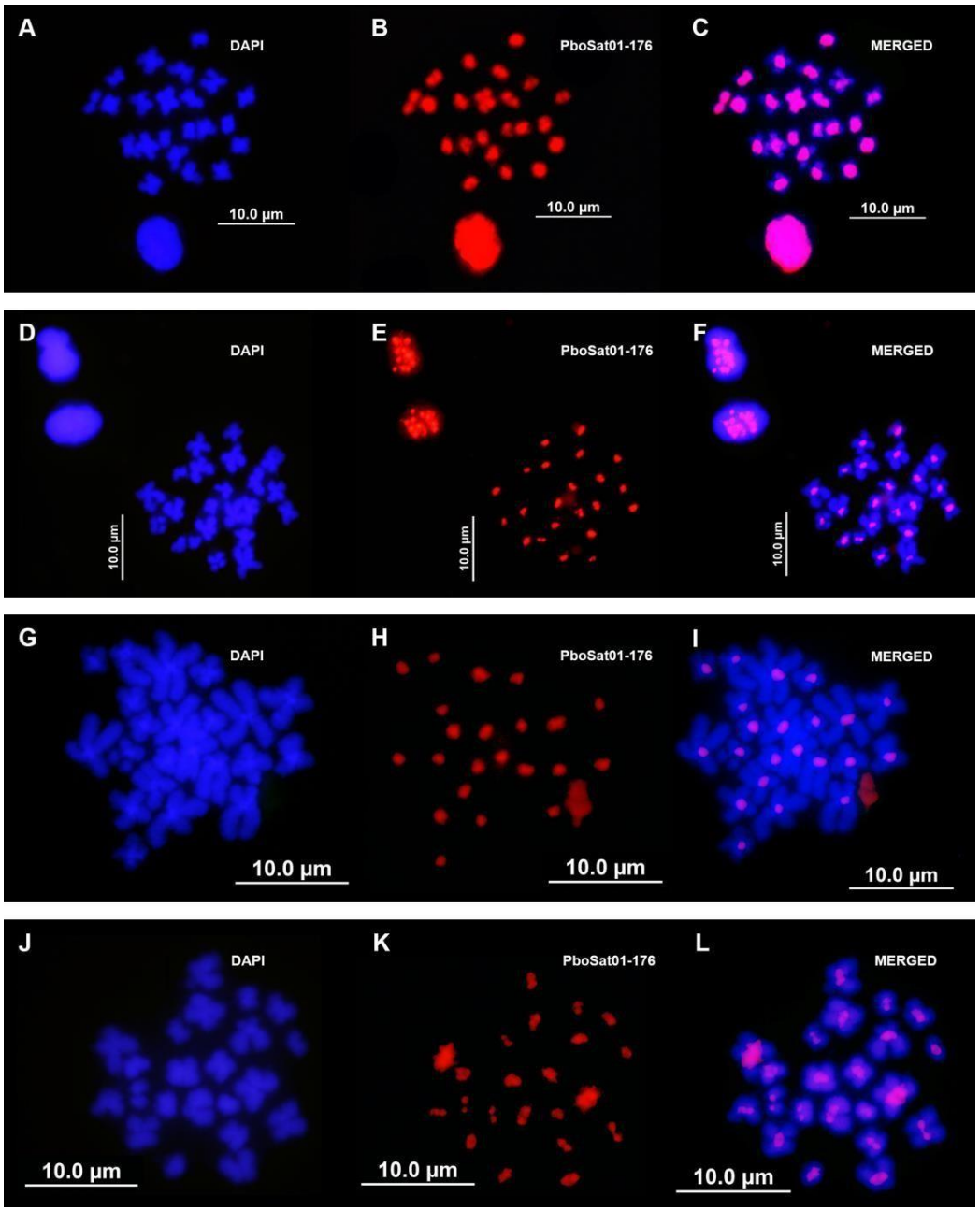

A
1.50%
1.00%
0.50%
0.00%
Genome Proportion
0
20
40
60
Kimura Substituition Level (%)
satDNA
PboSat01-176 - PBO
PboSat01-176 - PSC
PboSat01-176 - PME
PboSat01-176 - PLA
B
PboSat01-176
5,00E-02
4,00E-02
3,00E-02
2,00E-02
1,00E-02
0,00E+00
Abundance
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
K2P divergence (%)
PboSat01-176 - PBO
PboSat01-176 - PSC
PboSat01-176 - PME
PboSat01-176 - PLA

Figure 2


A
DAPI
10.0 µm
B
PboSat01-176
10.0 µm
C
MERGED
10.0 µm
D
DAPI
10.0 µm
E
PboSat01-176
10.0 µm
F
MERGED
10.0 µm
G
DAPI
10.0 µm
H
PboSat01-176
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I
MERGED
10.0 µm
J
DAPI
10.0 µm
K
PboSat01-176
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Figure 3

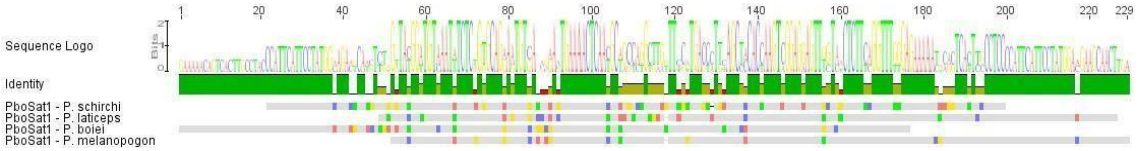

Table 1. RepeatMasker Abundance and Divergence (K2P%) data for PboSat01-176 in each of *Proceratophrys* species.

SatDNA	**Specie**	**Abundance (%)**	**Divergence (K2P %)**
PboSat01-176	*P. boiei*	10.4	3.1
	P. schirchi	0.2	28.36
	P. melanopogon	1.4	11.41
	P. laticeps	3.7	17.2

4.3 Capítulo 3 - Comparative analysis of repetitive DNA in frogs *Proceratophrys boiei* and its impact on population karyotypic evolution

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Resumo

DNAs repetitivos, incluindo DNA satélite (satDNA) e elementos transponíveis (TEs), desempenham papéis fundamentais na evolução genômica. Em *Proceratophrys boiei*, uma espécie de sapo com cromossomos sexuais heteromórficos, o acúmulo de SatDNAs e TEs pode contribuir para a diferenciação dos cromossomos sexuais. Este estudo analisa comparativamente a composição e a história evolutiva de SatDNAs e TEs em populações de *P. boiei* do sudeste e sul do Brasil. Análises genômicas e citogenéticas revelam um satelitoma rico com 226 famílias e uma considerável diversidade de TEs. Análises comparativas destacam elementos repetitivos compartilhados e específicos de cada população analisada. A população do sul, que não apresenta cromossomos sexuais diferenciados, exibe uma maior abundância de TEs em comparação com a população do sudeste. Este trabalho fornece uma compreensão abrangente dos elementos repetitivos de *Proceratophrys*, contribuindo para a compreensão da organização genômica de anfíbios e a possível evolução dos cromossomos sexuais.

Palavras-chave: cromossomo sexual; DNA satélite; satélite; elemento transponível; Ty3/Gypsy.

Abstract

Repetitive DNAs, encompassing satellite DNA (SatDNA) and transposable elements (TEs), play pivotal roles in genomic evolution. In *Proceratophrys boiei*, a frog species with heteromorphic sex chromosomes, the accumulation of SatDNAs and TEs may contribute to sex chromosome differentiation. This study analyzes the composition and evolutionary history of SatDNAs and TEs in *P. boiei* populations from southeast and south Brazil. Genomic and cytogenetic analyses reveal a rich satellitome with 226 families and substantial TE diversity. Comparative analyses highlight shared and population-specific repetitive elements. The southern population, lacking differentiated sex chromosomes, exhibits higher TE abundance. This work provides comprehensive insights into repetitive elements in *Proceratophrys*, contributing to the understanding of amphibian genomic organization and possible sex chromosome evolution.

Introduction

Sequence motifs that are repeated hundreds or thousands of times in the nuclear genome are an abundant part of all eukaryotic genomes, in most species representing more than half of the total DNA content in the cell nucleus, and overall, repeat types can be characterized as satellite DNA (i.e., sequences organized as tandem repetitions) and interspersed repeats (i.e., transposable elements) (Biscotti et al. 2015).

The satellite DNA (SatDNA) are organized as lengthy arrays and serve as the primary constituent of constitutive heterochromatin (Plohl et al. 2012; Biscotti et al. 2015; Thakur et al. 2021; Šatovi´c-Vukši´c and Plohl 2023). Transposable elements (TEs), on the other hand, are the major category of repetitive DNA, capable of mobilizing within the genome. DNA transposons, or class II TEs, propagate through DNA-based mechanisms, whereas class I TEs, known as retrotransposons, amplify via an RNA intermediate (Wicker et al. 2007).

Invasion and amplification of repetitive DNAs are common features in newly formed sex chromosomes, by allowing W/Y chromosomes to achieve a state of beneficial non-homology/non-recombination *via* SatDNA accumulation and TE insertions in a brief time (Charlesworth and Charlesworth, 2000; Hua-Van et al. 2005; Bachtrog, 2008; Charlesworth, 2019).

The frog species *Proceratophrys boiei* (2n = 22) stands out cytogenetically with its heteromorphic W sex chromosomes at the initial stage of evolutionary differentiation, exhibiting a ZZ/ZW sex chromosome system (Ananias et al. 2007; Amaro et al. 2012; Silva et al. 2021), presents an intriguing model for investigating the origins and evolution of sex chromosomes, mainly due to the possible accumulation of repetitive sequences, leading to interspecific and intraspecific chromosomal differentiation.

Proceratophrys boiei females, specifically in specimens from southeastern Brazil, have a metacentric W chromosome showing heterochromatin in all chromosomal extension defined by the C-banding technique, e.g., C-positive blocks, being the W chromosome smaller than Z in females (Ananias et al. 2007; Amaro et al. 2012; Da Silva et al. 2020; Silva et al. 2021).

In contrast, individuals from southern Brazil do not display this heterochromatin-based differentiation. Therefore, these differences in heterochromatin composition, especially between different populations, reinforce the importance of investigating the influence of repetitive sequences on these evolutionary processes that are leading to notable chromosomal differentiation in *Proceratophrys boiei*.

Comprehensive genomic and cytogenetic analyzes performed on *Proceratophrys boiei* revealed its entire satDNA content (satellitoma), showing a high and abundant number of satellites repeats (João Da Silva et al. 2023). Therefore, taking into account the need for an interspecific comparative genomic analysis in *Proceratophrys boiei*, in this work we analyzed the presence, abundance and divergence of satDNAs and TEs sequences in females from different populations to reveal the composition and evolutionary history of these sequences, and it was possible to advance in comparative genomic studies involving a species of frog, specifically in understanding the repetitive composition of satDNAs and TEs, potentially contributing to the evolution of sex chromosomes observed for *P. boiei*.

Methods

The sequenced genomes of two female *P. boiei* were used for the analyzes in this work. The adult individuals in question, as well as the sequencing data, were part of previous work in which specimens were collected from two different populations, a: southeast

(Mogi das Cruzes, São Paulo, Brazil) and b: south (Tijucas do Sul, Paraná, Brazil), corresponding to areas of cytogenetic differentiation observed for the species (Da Silva et al. 2020; Silva et al. 2021; João da Silva et al. 2023).

For this work, it was utilized the *Proceratophrys boiei* satellitoma, previously described by João da Silva et al. (2023) and deposited in GenBank (NCBI) under accession numbers OP223503 - OP223728. The genome of *P. boiei* (south) was sequenced through Illumina® Hiseq™ 4000 sequencing (2 x 101 bp paired-end), yielded 16.342.588 paired-end reads and a G+C content of 42.84%. As for *P. boiei* (southeast) it was sequenced through Illumina® Hiseq™ 2000 sequencing (2 x 101 bp paired-end), yielded 19,382,286 paired-end reads and a G+C content of 42.84% (Da Silva et al. 2020; João da Silva et al. 2023). Reads from each sequence were merged using the Fastq-join FASTX-toolkit program (Gordon and Hannon, 2010) with default parameters and then processed and analyzed via FastQC (version 0.10.1, 2012).

To assess the abundance and average nucleotide divergence (Kimura-2-parameter, K2P) of satDNAs and TEs within the *P. boiei* genomes, it was utilized RepeatMasker (Smit et al. 2013) with the *rmblast* engine. All analyses via RepeatMasker were conducted remotely on the Kasahara server, hosted by the Insect Cytogenomics Group at Universidade Estadual Paulista - UNESP, Rio Claro, São Paulo, Brazil.

The data obtained from these analyses were used to construct graphs and repeat landscapes, illustrating the relative abundance (Y-axis) at 1% intervals of K2P distance from the consensus sequence (X-axis). This involved employing the calcDivergenceFromAlign.pl script from RepeatMasker utils. Subsequently, we generated individual and overall landscapes using Microsoft Excel 2016 and R programming (R Core Team, 2021 - version 4.1.2).

Specifically, TEs within the genomes were identified utilizing a homology-based approach employing RepeatMasker (Smit et al. 2013 - 2017). This method involved the amalgamation of Dfam consensus sequences (accession DF0000558), an external TE library of *Xenopus tropicalis* available at msRepDB (ID 8364, https://msrepdb.cbrc.kaust.edu.sa/pages/msRepDB/index.html), and Dfam version 3.6 (Storer et al. 2021) into a concatenated library. To estimate the abundance and divergence of transposable elements, we utilized RepeatMasker (RM) alongside a custom script (https://github.com/fjruizruano/satminer/blob/master/repeat_masker_run_big.py). This involved comparing TE consensus sequences with the entire collection of reads from both genomes. Likewise, for satDNAs, an external library was used to analyze the presence and homologies in both individuals of *P. boiei*. The library is available on Genbank NCBI, under accession numbers OP223503 - OP223728.

Results

The satellitome of *Proceratophrys boiei* was utilized for the analysis and identification of satDNA families in all populations (southeastern Brazil, state of São Paulo and south Brazil, estate of Paraná). It is known that this satellitome is composed of 226 families of satDNAs and all sequences were used as input in comparative analyses carried out in this work.

A subtractive landscape was constructed to compare the two genomes, SP and PR, with respect to the satellite in general (Figure 1). It is interesting to highlight that of the 226 families of satDNAs previously described for *P. boiei* (SP), only two (PboSat15-166 and PboSat92-93) were not found in *P. boiei* (PR), indicating the exclusivity of these sequences for the genome from SP populations (Figure 1). Despite the dynamic abundance and divergences, it is possible to reveal an elevated level of sharing of the

satellite among populations. The RepeatMasker analysis of *P. boiei* (PR) showed that 12.2% of the genome was composed of satDNA families (Figure 2; Supplementary material - Table 1), being 4.7% smaller in composition when compared to *P. boiei* (SP), which has a satellitome of 16.9% of families of satDNAs.

When comparing the SP/PR ratio, with numbers greater than 1 suggesting enrichment in the genome (SP), six satellites drew attention due to their high divergence when comparing the populations, PboSat03-25 (518,32), PboSat23-39 (67,76), PboSat29-68 (29,23), PboSat54-39 (10,92), PboSat97-41 (14,34) and PboSat219-26 (25,19) (Figure 3, Supplementary material - Table 1).

Species-specific transposon libraries were assembled through *de novo* methods, in conjunction with previously known TEs sourced from public databases. Analysis of partial repeat composition in *P. boiei* females, both with and without a differentiated W chromosome, exhibited comparably similar abundance scenarios across the populations studied (Figure 4). A total of 50 TEs superfamilies were identified in the two genomes, consisting of 24 DNA transposons and 26 retrotransposon superfamilies. The TE libraries constituted 3.05% of the analyzed genome of *P. boiei* (SP) with the W sex chromosome, in contrast to 4.35% of the genome proportion in *P. boiei* (PR) without the W sex chromosome (Figure 4).

Class II TEs (DNA transposons) were the most predominant group in both *P. boiei* populations, ranging approximately from 1.8% (SP) to 2.5% (PR), while retrotransposons encompassed 1.2% (SP) to 1.7% (PR) of genome proportion (Figure 4). The two predominant TE superfamilies in all analyzed populations were the cut-and-paste TcMariner and hAT. In the *P. boiei* (SP) population, DNA/TcMariner comprised 1.1%, whereas DNA/hAT constituted 0.2%. In *P. boiei* (PR) populations, DNA/TcMariner

represented 1.7%, and DNA/hAT constituted 0.1% (Figure 5). Both superfamilies constituted a substantial portion of DNA transposons in the genomes, collectively constituting more than half of the entire TE composition. Retrotransposons had weaker proliferation than DNA transposons wherein this class I of TEs were mainly represented by LTRs families such as LTR/Gypsy, the most predominant overall within the species, with 0.5% and 0.8% in the *P. boiei* genome, SP and PR populations, respectively (Figure 5).

Discussion

The dynamism of sequences of satDNAs and TEs in eukaryotic genomes can direct significant roles to these genomes, such as differentiation of sex and supernumerary chromosomes, genomic increase and organization, among others (Thakur et al. 2021). Recent advances in DNA sequencing and bioinformatics analyzes are enabling comparative genomic analyzes of repetitive DNAs in diverse groups of organisms revealing these structural and dynamic roles (Palacios-Gimenez et al. 2017; Serrano-Freitas et al. 2019; Utsunomia et al. 2019; Ferretti et al. 2020; Boštjancˇ ic et al. 2020; Crepaldi et al. 2021).

The comparison carried out in this work for revealed a composition of satDNAs and TEs that are quite abundant in the two populations analyzed, however, each genome has its particularities for certain sequences, with a different evolutionary history revealed by quantification analyzes. In *P. boiei*, analysis of repetitive content suggests that satDNAs and TEs are driving genomic evolution in the species, and possibly influencing the differentiation of the W sex chromosome (João Da Silva et al. 2023). However, as there are relevant interspecific cytogenetic differences in the species, and to shed new light on this subject, we followed the strategy of comparatively analyzing genomes from

different populations of *P. boiei*, to investigate the composition, sharing and influence of repetitive sequences in its differentiation of sex chromosome W. It is notable that *P. boiei* has a great diversity of satellites that make up its satellitoma, with more than 200 sequences (João Da Silva et al. 2023). In our comparative analyses, the southeastern population (SP) has some satellites with an abundance that is relatively exclusive to this population. The abundance of satellites PboSat03-25, PboSat23-39, PboSat29-68, PboSat54-39, PboSat97-41 and PboSat209-26 appears to be indicative of a relationship with a differentiation in genomic composition in relation to the southern population (PR).

Although usually less abundant in the genome than transposable elements, satDNAs may form longer arrays (tens of kilobases up to megabases) that often constitute blocks of heterochromatin, with specific structural and epigenetic properties (Garrido-Ramos 2017). Comparisons of satellite composition in genomes have highlighted the important role of these repetitive sequences in the differentiation of Y and W chromosomes, as well as in increasing genomic size and structural organization (Crepaldi et al., 2021; Ferretti et al., 2020; Kretschmer et al., 2022). For sex chromosomes, the accumulation of repeats leading to differentiation between heteromorphic chromosomes is a common phenomenon among eukaryotes (Charlesworth et al., 1994; Ezaz and Deakin, 2014). Our data indicates that despite significant cytogenetic differences, the two populations (SP and PR) have practically the same satDNAs, but different distributions, with specific abundance and divergence for each population, indicating a rapid evolution of tandem repeats in *Proceratophrys*.

The analysis of the *P. boiei* satellitome in other populations thus proved to be a useful tool for unraveling the sharing of sequences between different populations, which could be extended to other species of anuran amphibians. Using other techniques, for example *in-situ* Hybridization (FISH), this type of approach can define common origins

of sex and supernumerary B chromosomes, as observed by Silva et al. (2017) and Ferretti et al. (2020). Although FISH was not performed in this work, the *in-silico* data comparative indicate that this relationship can be reinforced using certain satellites to investigate their presence in sex chromosomes, not only of *Proceratophrys*, but that it can be expanded to other groups of anuran amphibians.

With regards to TEs, an in-depth analysis of these sequences could provide interesting insights into how selective constraints of genomic loci harboring these elements reshape the evolution of sex chromosomes (Srikulnath et al. 2022). Variations in the repetitive sequence content and structure among closely related species, including TEs, may serve as indicators of their evolutionary relatedness (Zhu et al. 2016; Ayres-Alves et al. 2017; Dodsworth et al. 2017).

Furthermore, the dynamic activity of TEs within a genome has the potential to induce mutations impacting gene expression and function (Raskina et al. 2004; Fransz et al. 2016; Bourque et al. 2018). That way, both retrotransposons and DNA transposons were predominant in all analyzed genomes in this work, although with a clear variation in abundance between *P. boiei* populations. However, variations observed in other groups of organisms, such as insects, indicate possible differentiation between sexes, related to the presence of heteromorphic sex chromosomes, and such variations observed in different populations of *P. boiei* may also be related to initial differentiation processes of sex chromosomes already observed for these populations.

TEs may play a significant role in sex chromosome differentiation by allowing W/Y chromosomes to reach a state of beneficial non-homology/non-recombination through TE insertions within a brief period (Charlesworth and Charlesworth, 2000; Hua-Van et al. 2005; Bachtrog, 2008). For *P. boiei*, this relationship between retrotransposons

and sex chromosome differentiation was initially reported (João Da Silva et al. 2023), however, a quantification of these sequences was necessary to deepen the knowledge of this relationship for sex chromosome differentiation. The southern population of *P. boiei* has a much greater number of TEs than the southeastern population. It is worth remembering that this population does not have a differentiated sex chromosome system, unlike the other population. Even though this relationship is not very clear, the great abundance of TEs in the southern population reveals that these sequences may be directly involved in the genomic evolution of this species, including in the differentiation of sex chromosomes.

It is known that new families of satellite DNA can arise from the independent duplication of different genomic sequences, such as intergenic spacers, or even those derived from other satellite DNAs (Garrido-Ramos, 2017). Furthermore, satDNA sequences can interact with transposable elements to create new repetitive DNA. Thus, it is suggested that transposable elements provide the mechanism by which satDNA repeats could propagate in the genome through arrays of dispersed short repeats (Macas et al. 2011; Bardella et al. 2014). It is a fact that the genome of *P. boiei* is rich in satDNAs and TEs, and speculation about the direct relationship between these two types of repetitive sequences, as well as the common origin of these sequences, still remains unknown and may be elucidated in future work.

In summary, this work represents the most comprehensive characterization of the repetitive elements in any species belonging to the genus *Proceratophrys*. In this study, we show that *P. boiei* harbors a wide variety of repetitive elements, including satDNAs and TEs, representing a major advance in understanding the genomic organization of amphibians. Our work provided strong support to the discussions regarding the *P. boiei* satellitome, in addition to providing initial data on TEs in the genome of this species.

With this, it was possible to infer that these repetitive sequences are present in the genome of the analyzed specimens, forming part of evolutionary dynamics that include the differentiation of sex chromosomes reported here. Our work reinforces the large collection of satDNA families described for *P. boiei* and highlights the need for robust analyzes of these sequences to determine new chromosomal markers, which can promote future research in the field of chromosomal evolution, especially of sexual chromosomes in anuran amphibians.

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Ethics statement

Not applicable.

Author contributions

MJS contributed to the work's conception, data acquisition, analysis and interpretation, and wrote the manuscript. PPM conceptualized, funded and coordinated the research. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of interest

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

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Data archiving

Not applicable.

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Figures

Figure 1 – Subtractive repetitive landscape (abundance vs divergence) for satDNAs identified in *Proceratophrys boiei*. The graphs show, for each color-coded element, the sequence divergence according to Kimura distance (x-axis) in relation to their copies in the genome (y-axis). Abundance values show the difference between the *P. boiei* (southeast) minus the *P. boiei* (south) genomes.

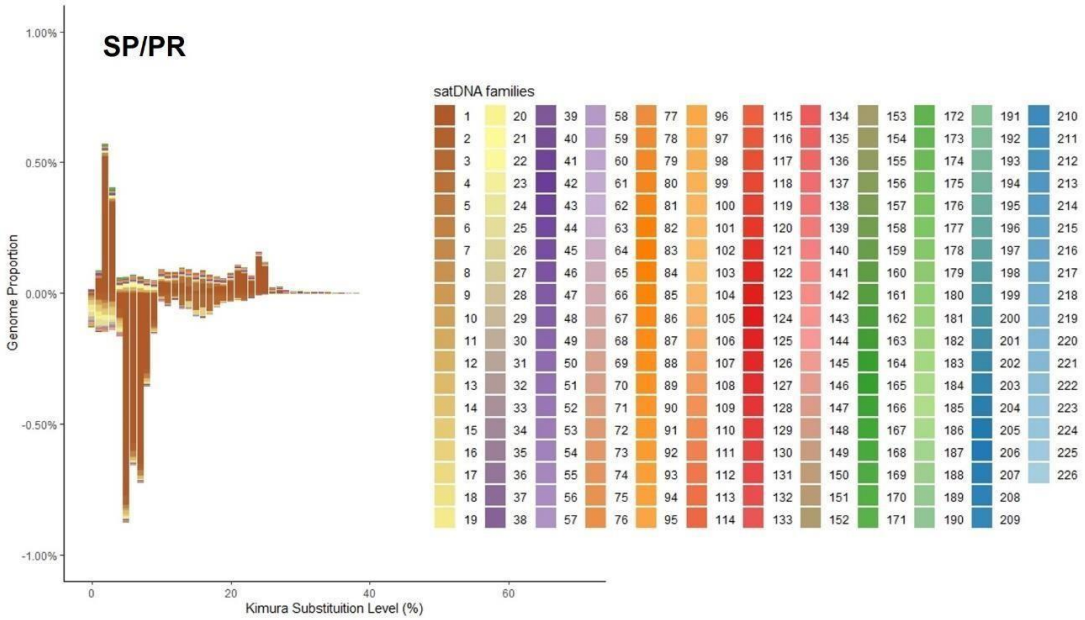


Figure 2 – Transposon, retrotransposon and satellite DNA composition in the *P. boiei* genomes based on analysis of different populations conducted with RepeatMasker.

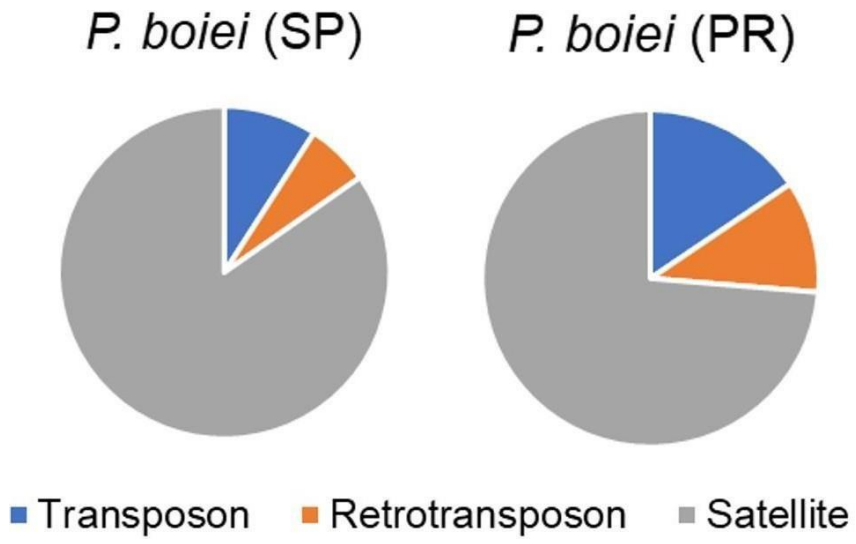


Figure 3 – Individual satDNA landscapes of *P. boiei* (SP) (blue) and *P. boiei* (PR) (orange) repeats for specific satDNAs families in the genomes.

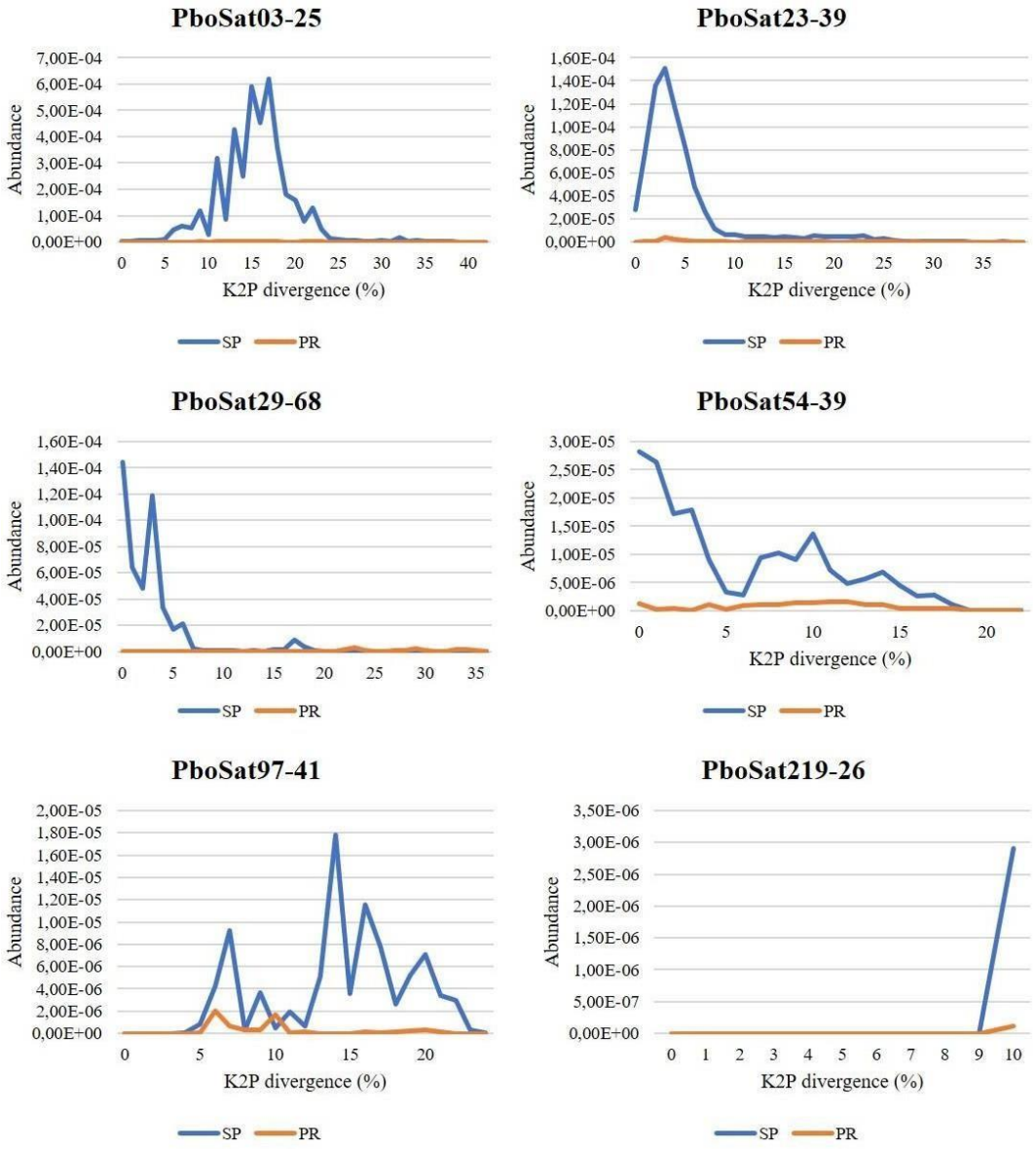


Figure 4 – Comparative repeat landscapes for TEs observed in *P. boiei* populations. In (a) and (b) *P. boiei* (SP), with transposons and retrotransposons, respectively. In (c) and (d), *P. boiei* (PR). The graphs represent the Kimura distance-based divergence from each element to their consensus sequences (x axis).

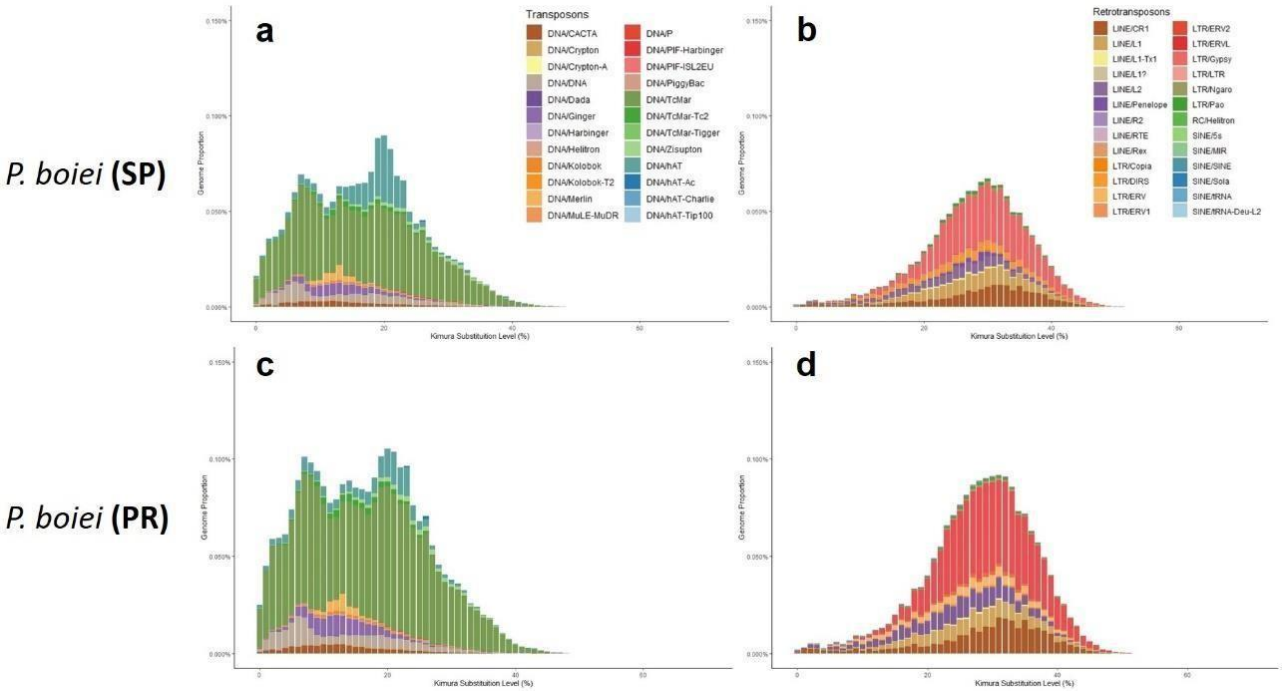
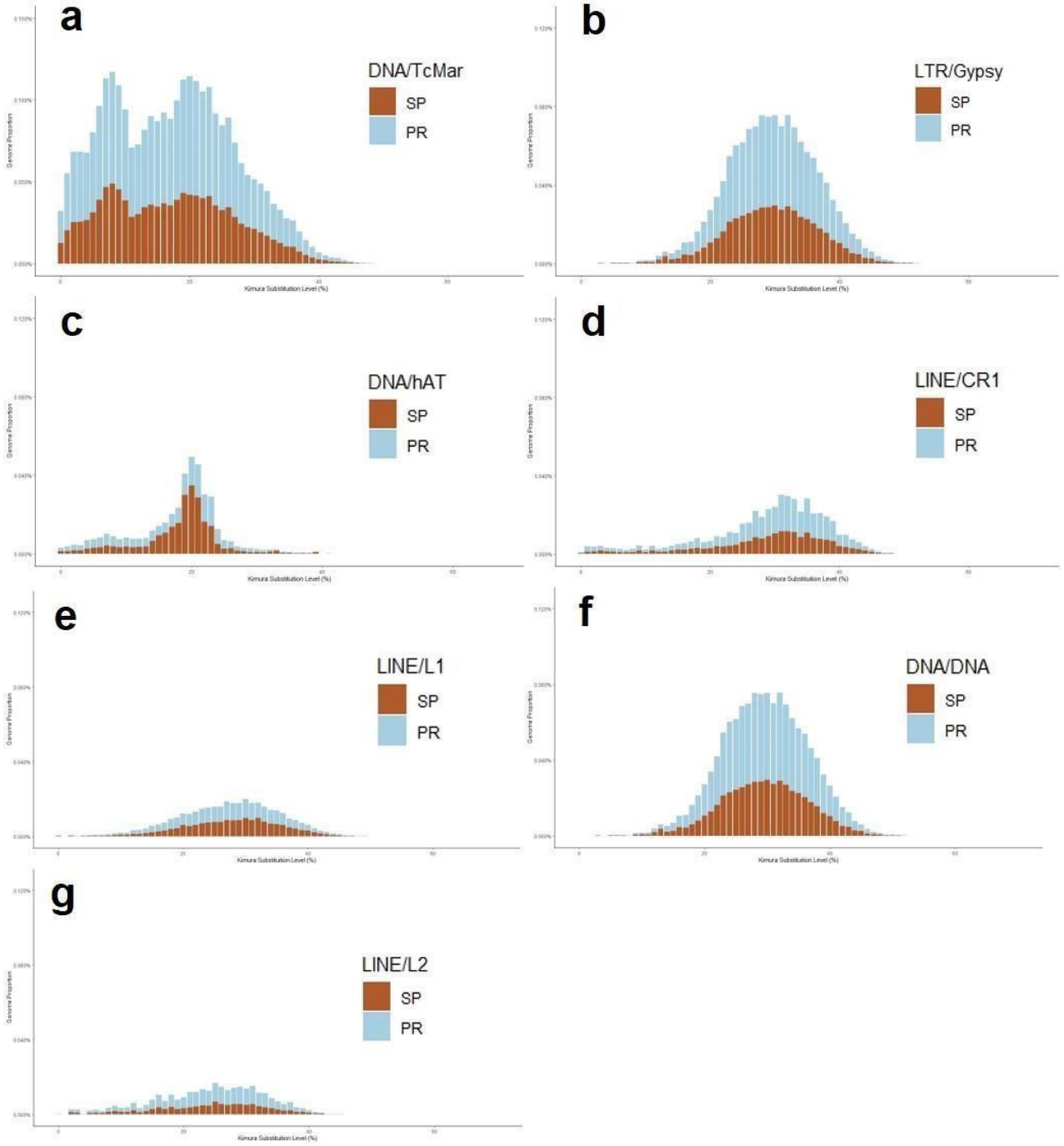
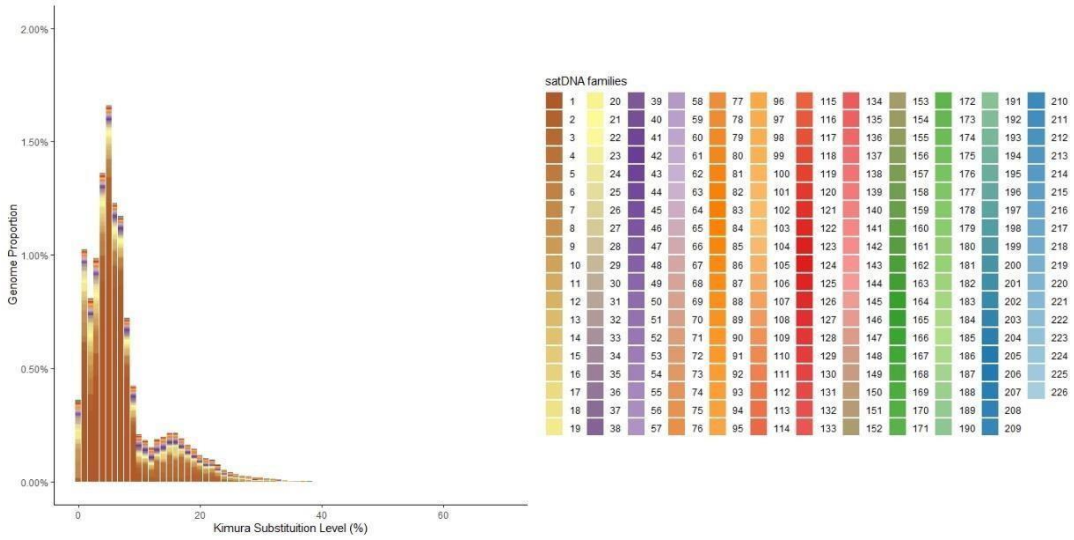


Figure 5 – Repeat landscapes for the most abundant TEs observed in *Proceratophrys boiei* populations.

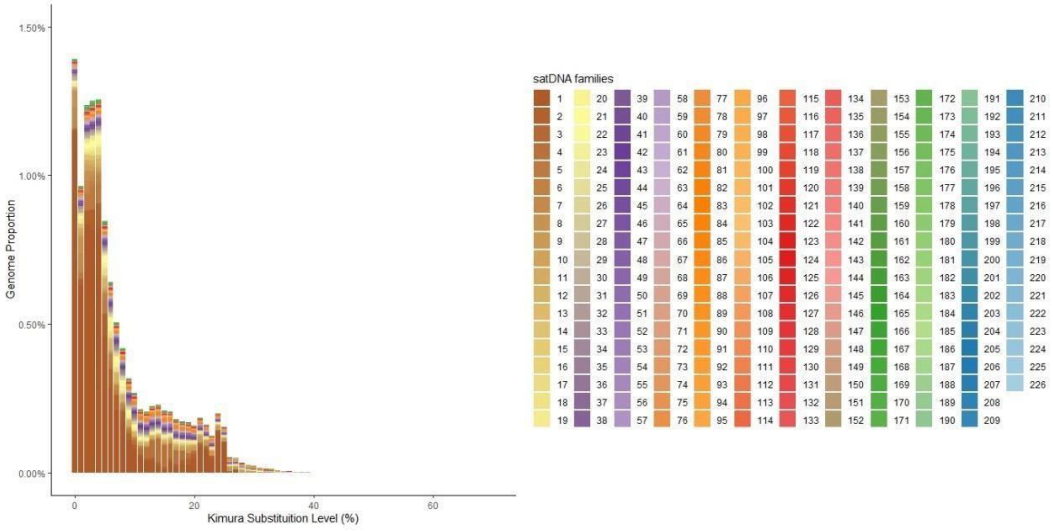


Supplementary material

Supplementary Figure S1. Repeat landscape (abundance vs divergence) for satDNAs identified in *Proceratophrys boiei* (from SP region). The graphs show, for each color-coded element, the sequence divergence according to Kimura distance (x-axis) in relation to their copies in the genome (y-axis). Each color represents a satDNA family.



Supplementary Figure S2. Repeat landscape (abundance vs divergence) for satDNAs identified in *Proceratophrys boiei* (from SP region). The graphs show, for each color-coded element, the sequence divergence according to Kimura distance (x-axis) in relation to their copies in the genome (y-axis). Each color represents a satDNA family



Supplementary Table S1 – General characteristics of satDNA families analyzed from genomes of *Proceratophrys boiei*.

Satellite DNA	Length	Abundance		Divergence		SP/PR
		SP	PR	SP	PR	
PboSat01-176	176	10,4973	6,3674	3,10	5,31	1,65
PboSat02-192	192	0,9736	0,6340	8,61	8,34	1,54
PboSat03-25	25	0,4102	0,0008	15,98	16,11	518,32
PboSat04-60	60	0,3294	0,4784	15,42	14,82	0,69
PboSat05-36	36	0,3123	0,3170	4,0	4,33	0,99
PboSat06-123	123	0,2463	0,1661	7,12	6,73	1,48
PboSat07-121	121	0,1961	0,2076	2,05	2,39	0,94
PboSat08-92	92	0,1743	0,2051	2,85	3,11	0,85
PboSat09-150	150	0,1635	0,2005	21,42	21,12	0,82
PboSat10-38	38	0,1557	0,0743	2,76	2,92	2,10
PboSat11-72	72	0,1427	0,1507	9,46	10,62	0,95
PboSat12-52	52	0,1313	0,2015	7,56	7,71	0,65
PboSat13-181	181	0,1162	0,0251	8,95	14,60	4,63
PboSat14-41	41	0,1155	0,1343	6,03	9,66	0,86
PboSat15-166	166	0,1118	0,0000	3,26	-	-
PboSat16-25	25	0,1063	0,0159	15,68	16,73	6,70
PboSat17-90	90	0,1057	0,0856	5,71	5,98	1,23
PboSat18-121	121	0,0971	0,1102	11,98	12,53	0,88
PboSat19-178	178	0,0931	0,0573	1,90	3,37	1,63
PboSat20-52	52	0,0855	0,1195	7,60	7,72	0,72
PboSat21-44	44	0,0853	0,1589	6,92	6,87	0,54
PboSat22-90	90	0,0827	0,0814	9,12	10,63	1,02
PboSat23-39	39	0,0753	0,0011	4,86	7,02	67,76
PboSat24-67	67	0,0746	0,0763	6,23	7,31	0,98
PboSat25-110	110	0,0733	0,0259	2,46	14,42	2,83
PboSat26-115	115	0,0680	0,0761	8,28	8,32	0,89
PboSat27-24	24	0,0507	0,0673	26,88	26,71	0,75
PboSat28-62	62	0,0499	0,0483	8,66	9,80	1,03
PboSat29-68	68	0,0473	0,0016	2,74	28,42	29,23
PboSat30-91	91	0,0464	0,0499	2,25	2,29	0,93
PboSat31-33	33	0,0419	0,0456	11,45	12,31	0,92
PboSat32-43	43	0,0407	0,0511	4,63	5,05	0,80
PboSat33-986	986	0,0390	0,0185	1,93	1,29	2,11
PboSat34-43	43	0,0374	0,0531	9,13	9,41	0,71
PboSat35-30	30	0,0358	0,0456	12,05	10,09	0,79
PboSat36-39	39	0,0330	0,0303	10,47	10,61	1,09
PboSat37-31	31	0,0319	0,0373	13,78	14,38	0,86
PboSat38-96	96	0,0311	0,0230	11,99	13,56	1,35
PboSat39-64	64	0,0311	0,0729	8,34	7,98	0,43
PboSat40-40	40	0,0296	0,0315	11,90	11,39	0,94
PboSat41-84	84	0,0292	0,0387	20,13	20,00	0,75
PboSat42-51	51	0,0282	0,0383	11,04	11,33	0,74
PboSat43-33	33	0,0235	0,0107	15,13	14,64	2,20
PboSat44-31	31	0,0225	0,0222	10,01	10,08	1,01
PboSat45-110	110	0,0214	0,0247	10,91	11,03	0,87
PboSat46-106	106	0,0202	0,0234	6,96	7,14	0,86
PboSat47-91	91	0,0199	0,0240	1,35	1,82	0,83
PboSat48-93	93	0,0196	0,0351	5,29	4,86	0,56
PboSat49-37	37	0,0195	0,0250	21,20	21,83	0,78

PboSat50-30	30	0,0194	0,0752	14,89	18,32	0,26
PboSat51-47	47	0,0193	0,0172	8,52	8,68	1,12
PboSat52-44	44	0,0184	0,0215	9,29	10,44	0,86
PboSat53-32	32	0,0184	0,0248	19,91	20,44	0,74
PboSat54-39	39	0,0183	0,0017	5,94	9,74	10,92
PboSat55-28	28	0,0181	0,0180	11,02	12,17	1,01
PboSat56-54	54	0,0180	0,0207	8,55	10,21	0,87
PboSat57-88	88	0,0171	0,0267	11,67	12,74	0,64
PboSat58-75	75	0,0170	0,0077	16,29	18,30	2,22
PboSat59-50	50	0,0160	0,0026	10,70	24,83	6,29
PboSat60-78	78	0,0157	0,0187	4,97	5,31	0,84
PboSat61-74	74	0,0154	0,0145	4,17	5,05	1,07
PboSat62-47	47	0,0154	0,0161	7,40	8,08	0,96
PboSat63-28	28	0,0152	0,0132	11,1	10,88	1,15
PboSat64-29	29	0,0151	0,0135	9,50	9,68	1,12
PboSat65-45	45	0,0150	0,0164	12,71	13,60	0,92
PboSat66-31	31	0,0145	0,0218	21,0	21,81	0,66
PboSat67-44	44	0,0143	0,0101	3,95	5,02	1,42
PboSat68-30	30	0,0142	0,0203	18,97	18,75	0,70
PboSat69-62	62	0,0138	0,0147	6,51	7,48	0,94
PboSat70-88	88	0,0138	0,0116	3,56	4,40	1,19
PboSat71-46	46	0,0137	0,0129	3,56	3,39	1,06
PboSat72-67	67	0,0133	0,0182	24,75	25,53	0,73
PboSat73-34	34	0,0132	0,0146	10,65	11,29	0,90
PboSat74-88	88	0,0131	0,0126	13,21	13,31	1,05
PboSat75-43	43	0,0127	0,0140	17,12	17,26	0,91
PboSat76-61	61	0,0126	0,0189	17,28	17,32	0,67
PboSat77-33	33	0,0125	0,0096	10,82	12,45	1,31
PboSat78-29	29	0,0124	0,0137	11,33	11,81	0,91
PboSat79-69	69	0,0124	0,0155	13,71	14,52	0,80
PboSat80-77	77	0,0122	0,0166	7,52	7,94	0,74
PboSat81-33	33	0,0122	0,0118	11,50	12,70	1,04
PboSat82-34	34	0,0116	0,0151	8,84	8,54	0,77
PboSat83-78	78	0,0115	0,0131	23,64	23,47	0,88
PboSat84-75	75	0,0113	0,0181	13,57	12,92	0,63
PboSat85-24	24	0,0110	0,0135	28,02	29,35	0,82
PboSat86-25	25	0,0110	0,0718	20,35	15,29	0,15
PboSat87-33	33	0,0110	0,0108	10,16	11,70	1,02
PboSat88-45	45	0,0110	0,0132	14,77	15,07	0,84
PboSat89-38	38	0,0104	0,0101	14,67	13,11	1,03
PboSat90-31	31	0,0103	0,0111	19,88	20,25	0,93
PboSat91-60	60	0,0101	0,0137	11,00	11,07	0,74
PboSat92-93	93	0,0098	0,0000	0,27	-	-
PboSat93-60	60	0,0094	0,0120	11,49	11,96	0,79
PboSat94-73	73	0,0094	0,0122	18,46	18,24	0,77
PboSat95-44	44	0,0090	0,0094	6,52	7,14	0,96
PboSat96-30	30	0,0090	0,0106	15,52	15,88	0,85
PboSat97-41	41	0,0088	0,0006	14,89	10,38	14,34
PboSat98-30	30	0,0088	0,0097	12,78	13,06	0,91
PboSat99-131	131	0,0086	0,0052	4,46	4,63	1,66
PboSat100-36	36	0,0086	0,0094	21,58	21,94	0,92
PboSat101-351	351	0,0085	0,0103	13,96	13,44	0,83
PboSat102-37	37	0,0084	0,0097	15,66	14,88	0,87
PboSat103-97	97	0,0084	0,0064	10,85	12,07	1,33
PboSat104-84	84	0,0083	0,0096	16,02	16,32	0,87
PboSat105-141	141	0,0082	0,0081	14,66	14,89	1,01
PboSat106-405	405	0,0081	0,0040	5,59	7,81	2,02
PboSat107-34	34	0,0078	0,0111	6,54	6,35	0,71

PboSat108-435	435	0,0078	0,0062	3,76	4,74	1,26
PboSat109-88	88	0,0078	0,0090	15,11	16,10	0,87
PboSat110-742	742	0,0076	0,0128	1,34	1,95	0,60
PboSat111-25	25	0,0076	0,0141	14,07	13,12	0,54
PboSat112-31	31	0,0075	0,0064	10,62	15,32	1,18
PboSat113-27	27	0,0074	0,0069	4,42	4,59	1,08
PboSat114-27	27	0,0073	0,0078	14,26	15,65	0,94
PboSat115-20	20	0,0073	0,0051	8,96	8,89	1,44
PboSat116-44	44	0,0073	0,0089	4,44	4,20	0,83
PboSat117-543	543	0,0072	0,0093	6,13	6,73	0,77
PboSat118-73	73	0,0071	0,0084	4,08	5,54	0,85
PboSat119-58	58	0,0067	0,0147	9,26	8,91	0,46
PboSat120-21	21	0,0066	0,0043	7,28	7,06	1,56
PboSat121-31	31	0,0066	0,0054	6,30	5,20	1,22
PboSat122-78	78	0,0064	0,0056	21,73	24,56	1,15
PboSat123-45	45	0,0063	0,0071	8,01	8,60	0,90
PboSat124-62	62	0,0063	0,0094	16,39	16,73	0,67
PboSat125-47	47	0,0062	0,0044	7,20	7,49	1,42
PboSat126-52	52	0,0062	0,0055	17,88	19,70	1,13
PboSat127-44	44	0,0061	0,0060	12,24	11,76	1,03
PboSat128-68	68	0,0060	0,0057	8,61	10,58	1,05
PboSat129-44	44	0,0059	0,0099	8,66	8,38	0,60
PboSat130-34	34	0,0059	0,0145	21,52	22,78	0,41
PboSat131-41	41	0,0059	0,0079	9,28	11,41	0,75
PboSat132-31	31	0,0056	0,0041	14,35	16,14	1,37
PboSat133-78	78	0,0054	0,0068	6,43	7,07	0,80
PboSat134-39	39	0,0053	0,0067	15,43	17,00	0,81
PboSat135-68	68	0,0053	0,0068	17,84	18,27	0,78
PboSat136-59	59	0,0053	0,0045	4,03	4,54	1,18
PboSat137-34	34	0,0051	0,0062	11,66	11,88	0,83
PboSat138-29	29	0,0047	0,0051	11,69	10,83	0,93
PboSat139-65	65	0,0047	0,0051	21,78	21,65	0,92
PboSat140-36	36	0,0044	0,0043	7,44	9,25	1,03
PboSat141-39	39	0,0043	0,0100	11,02	12,40	0,44
PboSat142-60	60	0,0043	0,0056	10,80	11,39	0,77
PboSat143-38	38	0,0041	0,0043	4,96	5,50	0,96
PboSat144-98	98	0,0041	0,0066	8,59	7,81	0,63
PboSat145-33	33	0,0040	0,0022	10,51	13,53	1,85
PboSat146-27	27	0,0039	0,0042	5,58	6,10	0,93
PboSat147-21	21	0,0038	0,0024	7,88	12,00	1,59
PboSat148-22	22	0,0038	0,0022	3,85	3,63	1,74
PboSat149-25	25	0,0038	0,0038	3,64	3,52	0,99
PboSat150-51	51	0,0038	0,0039	12,98	12,17	0,97
PboSat151-52	52	0,0037	0,0028	5,78	6,53	1,33
PboSat152-31	31	0,0036	0,0029	17,68	19,28	1,27
PboSat153-75	75	0,0035	0,0040	4,01	4,10	0,90
PboSat154-25	25	0,0035	0,0039	8,31	9,22	0,92
PboSat155-20	20	0,0035	0,0044	11,04	8,58	0,82
PboSat156-40	40	0,0035	0,0035	14,43	14,45	1,01
PboSat157-30	30	0,0035	0,0039	16,48	18,32	0,89
PboSat158-41	41	0,0034	0,0040	13,22	13,15	0,86
PboSat159-36	36	0,0033	0,0039	19,21	19,39	0,85
PboSat160-58	58	0,0032	0,0030	13,74	15,44	1,08
PboSat161-32	32	0,0032	0,0044	11,82	11,37	0,74
PboSat162-87	87	0,0032	0,0037	3,34	4,25	0,89
PboSat163-39	39	0,0031	0,0043	8,76	9,11	0,72
PboSat164-30	30	0,0030	0,0090	14,15	16,10	0,34
PboSat165-452	452	0,0029	0,0033	0,35	1,13	0,89

PboSat166-25	25	0,0028	0,0036	8,71	8,34	0,80
PboSat167-25	25	0,0028	0,0115	10,42	11,14	0,25
PboSat168-77	77	0,0028	0,0006	3,17	7,35	4,99
PboSat169-30	30	0,0027	0,0015	11,55	14,41	1,84
PboSat170-29	29	0,0026	0,0016	8,18	15,95	1,63
PboSat171-39	39	0,0026	0,0633	4,09	5,40	0,04
PboSat172-31	31	0,0026	0,0049	17,81	18,74	0,53
PboSat173-29	29	0,0024	0,0246	15,59	15,24	0,10
PboSat174-82	82	0,0024	0,0025	8,33	13,01	0,97
PboSat175-49	49	0,0024	0,0030	19,96	19,96	0,81
PboSat176-45	45	0,0021	0,0034	21,03	21,77	0,64
PboSat177-75	75	0,0020	0,0015	4,78	5,80	1,35
PboSat178-69	69	0,0019	0,0020	3,18	3,36	1,00
PboSat179-23	23	0,0019	0,0007	11,26	11,17	2,77
PboSat180-42	42	0,0019	0,0020	3,14	2,87	0,98
PboSat181-33	33	0,0017	0,0025	19,85	19,43	0,70
PboSat182-32	32	0,0016	0,0030	15,92	15,52	0,55
PboSat183-97	97	0,0016	0,0007	9,91	15,21	2,27
PboSat184-39	39	0,0016	0,0023	5,58	5,53	0,70
PboSat185-31	31	0,0015	0,0021	14,08	13,52	0,74
PboSat186-30	30	0,0015	0,0024	5,63	5,28	0,62
PboSat187-64	64	0,0014	0,0016	18,90	19,70	0,93
PboSat188-26	26	0,0014	0,0012	12,82	12,93	1,19
PboSat189-20	20	0,0013	0,0018	5,42	5,08	0,76
PboSat190-80	80	0,0013	0,0027	2,91	2,03	0,51
PboSat191-51	51	0,0013	0,0020	11,91	11,87	0,65
PboSat192-30	30	0,0012	0,0012	14,46	16,07	1,03
PboSat193-33	33	0,0011	0,0008	8,05	9,84	1,48
PboSat194-59	59	0,0011	0,0009	5,92	8,89	1,30
PboSat195-32	32	0,0011	0,0008	14,58	20,89	1,52
PboSat196-77	77	0,0010	0,0011	11,67	11,56	0,97
PboSat197-31	31	0,0009	0,0011	17,66	18,10	0,87
PboSat198-44	44	0,0009	0,0015	3,16	1,35	0,65
PboSat199-80	80	0,0009	0,0015	5,22	5,44	0,61
PboSat200-45	45	0,0009	0,0012	24,01	22,23	0,77
PboSat201-75	75	0,0008	0,0007	0,44	0,43	1,32
PboSat202-35	35	0,0008	0,0007	10,92	10,54	1,17
PboSat203-52	52	0,0007	0,0008	4,98	5,41	0,99
PboSat204-36	36	0,0007	0,0010	18,91	18,78	0,79
PboSat205-27	27	0,0007	0,0002	6,57	7,40	4,31
PboSat206-69	69	0,0007	0,0009	19,67	19,89	0,82
PboSat207-23	23	0,0007	0,0007	9,92	11,54	1,06
PboSat208-33	33	0,0006	0,0006	7,23	8,57	1,11
PboSat209-51	51	0,0006	0,0010	19,16	19,18	0,66
PboSat210-30	30	0,0006	0,0007	10,66	12,01	0,91
PboSat211-50	50	0,0006	0,0008	20,68	19,13	0,81
PboSat212-31	31	0,0005	0,0006	16,74	19,98	0,93
PboSat213-80	80	0,0004	0,0004	5,08	5,77	1,15
PboSat214-54	54	0,0004	0,0003	7,98	8,67	1,46
PboSat215-25	25	0,0003	0,0004	7,76	7,99	0,89
PboSat216-43	43	0,0003	0,0003	20,11	21,33	1,20
PboSat217-26	26	0,0003	0,0002	16,36	16,89	1,41
PboSat218-25	25	0,0003	0,0003	13,82	12,43	0,92
PboSat219-26	26	0,0002	0,0000	10,74	10,74	25,19
PboSat220-31	31	0,0002	0,0004	19,60	20,11	0,57
PboSat221-28	28	0,0001	0,0002	17,88	27,42	0,68
PboSat222-29	29	0,0001	0,0003	12,51	12,95	0,57
PboSat223-28	28	0,00009	0,0001	16,16	14,55	0,70

PboSat224-38	38	0,00009	0,0000	21,89	21,43	9,47
PboSat225-31	31	0,00008	0,000067	16,76	20,54	1,20
PboSat226-20	20	0,00002	0,000021	18,40	23,72	0,97
Average	**98**	**16,9**	**12,2**	**10,75**	**11,94**	**4,05**

5 CONSIDERAÇÕES FINAIS

Esse trabalho deu continuidade a pesquisas iniciadas no Laboratório de Citogenética Animal, localizado no Instituto de Biociências, UNESP Rio Claro. No primeiro capítulo, foram apresentados os resultados da pesquisa que investigou o satelitoma completo do sapo brasileiro *Proceratophrys boiei*, uma espécie com características cromossômicas atípicas. Utilizando abordagens genômicas, bioinformáticas e citogenéticas, foram identificadas inicialmente 226 famílias de DNA satélite, destacando-se a família PboSat01-176 como a mais proeminente, compreendendo 10,49% do genoma dessa espécie. Com as análises de FISH, os satélites mais abundantes foram localizados em regiões pericentroméricas, sugerindo um papel na formação e manutenção da heterocromatina centromérica.

Surpreendentemente, não foram encontrados satDNAs específicos para o cromossomo sexual W de *P. boiei*, indicando uma possível fase inicial de diferenciação, no qual satélites ainda são compartilhados por ambos os sexos e cromossomos homólogos. No entanto, a diversidade de satDNAs sugere eventos recentes de amplificação e diversificação genômica, reforçando o dinamismo reportado para esse tipo de sequência repetitiva. Em resumo, no capítulo 1, a pesquisa contribui para a compreensão da evolução genômica e da dinâmica dos satDNAs em uma espécie de sapo com características cromossômicas distintas.

No segundo Capítulo, foi abordado o estudo do satélite PboSat01-176 de forma comparativa entre espécies de *Proceratophrys*, no intuito de investigar a presença, localização e padrões evolutivos deste satélite em uma abordagem interespecífica. Inicialmente identificado apenas para *P. boiei*, PboSat01-176 chamou a atenção por ser um componente genômico altamente abundante, representando 93,62% do conteúdo total de satDNA na espécie. O PboSat01-176 exibiu variações significativas em termos de abundância e divergência em diferentes espécies de *Proceratophrys*.

A abundância mais elevada em *P. boiei*, indica uma amplificação mais recente em comparação com outras espécies analisadas, com padrões distintos de amplificação e divergência, permitindo sugerir dinâmicas evolutivas específicas em cada espécie. Interessantemente, a FISH revelou que o PboSat01-176 está predominantemente localizado nas regiões peri e centroméricas de todos os pares cromossômicos em diferentes espécies de *Proceratophrys*, sugerindo seu envolvimento em processos

importantes nessas áreas cromossômicas, emergindo como um candidato promissor para um marcador centromérico de cromossomos, devido à sua localização genômica precisa, alta abundância, conteúdo elevado de A+T e tamanho monomérico. Portanto, o capítulo destaca o satélite PboSat01-176 como uma ferramenta valiosa para estudos genômicos e evolutivos em *Proceratophrys*, oferecendo *insights* significativos sobre a dinâmica do satDNA e sua contribuição para a evolução do genoma em um gênero de sapos, em que futuras pesquisas poderão explorar a aplicabilidade do PboSat01-176 em outras espécies de anfíbios anuros, ampliando a compreensão da organização genômica repetitiva e da evolução cromossômica nesse grupo.

Por fim, no terceiro capítulo, a pesquisa realizada teve como objetivo analisar a composição e a história evolutiva das sequências repetitivas de DNA em *P. boiei*, levando em consideração dois tipos de repetições: satDNAs e os elementos transponíveis (TEs), que incluem DNA transposons e retrotransposons. O acúmulo dessas sequências repetitivas é comum em cromossomos sexuais recém-formados, como os cromossomos W/Y, influenciando na não-homologia e não-recombinação. Como há diferenças cromossômicas relevantes para diferentes populações de *P. boiei*, essa investigação foi importante para definir o nível de compartilhamento de sequências e a possível influência para a diferenciação de cromossomos sexuais na espécie.

A população de *P. boiei* da região sudeste do Brasil (SP) apresenta heterocromatina no cromossomo W, enquanto a população do sul (PR) não exibe essa diferenciação. Isso ressalta a importância de investigar a influência das sequências repetitivas nesses processos evolutivos. A pesquisa utilizou genomas sequenciados de fêmeas de duas populações distintas para analisar o conteúdo de satDNAs e TEs. Os resultados revelaram um satellitoma compartilhado entre os genomas analisados, com uma porcentagem menor na população sul (PR) em comparação com a do sudeste (SP). Algumas sequências de satDNA mostraram divergência significativa entre as populações, indicando uma rápida evolução dessas repetições. Quanto aos TEs, foram identificadas 50 superfamílias, com os DNA transposons sendo mais predominantes. A população do sul (PR), sem cromossomo sexual diferenciado, apresentou uma maior abundância de TEs em comparação com a população do sudeste (SP).

A pesquisa destacou a dinâmica evolutiva das sequências repetitivas em *P. boiei*, sugerindo que satDNAs e TEs desempenham papéis importantes na evolução genômica,

incluindo a diferenciação dos cromossomos sexuais, fornecendo *insights* valiosos para futuras pesquisas sobre a evolução cromossômica. É notável que pesquisas envolvendo citogenética e citogenômica em sapos é fascinante, com potencial para desvendar e contribuir para o entendimento da evolução cromossômica em diversas espécies de anfíbios anuros. Particularmente, em *Proceratophrys*, os estudos realizados nesta tese demonstram esse potencial de abordagens integrativas em um grupo de organismos subexplorado, com uma gama de possibilidades a serem realizadas e descobertas a partir da diversidade de espécies de anfíbios brasileiros.

6 PRÓXIMAS ETAPAS

a) Testar e comparar a expressão diferencial de DNA satélites entre diferentes tecidos, sexos e espécies de *Proceratophrys*, a partir de transcriptomas, com o objetivo de entender os possíveis papéis funcionais destas sequências na regulação gênica, modulação da cromatina e como componentes funcionais de importantes estruturas como telômeros, centrômeros e cromossomos sexuais;
b) A partir de transcriptomas de espécies de *Proceratophrys*, prospectar genes codificadores, sobretudo relacionados com a determinação sexual;
c) Fazer FISH, mapeamento físico de satDNAs específicos em ambos os sexos e espécies de *Proceratophrys*, investigando os padrões de evolução e influência dessas sequências na organização genômica;
d) Investigar e mapear proteínas centroméricas CENP-A, entre outras para estabelecer os limites centroméricos e associar essas proteínas aos satDNAs centroméricos;
e) Coletar e descrever o cariótipo e bandamentos gerais em outras espécies de *Proceratophrys*, no intuito de entender os cromossomos e organização genômica repetitiva através do sequenciamento genômico;
f) Sequenciar espécies de *Proceratophrys* com tecnologias de sequenciamento *long reads* e caracterizar todo o repeatoma, incluindo satellitoma e mobiloma em todas elas, usando genômica, bioinformática e citogenética clássica;
g) Confeccionar árvore de abrangência mínima mostrando as relações entre os diferentes haplótipos de sequências do satélite PboSat01-176 e outros satélites específicos do gDNA de espécies de *Proceratophrys*.

7 BREVE HISTÓRICO PROFISSIONAL

A graduação em Biologia veio no ano de 2016, em que obtive o título de licenciado em Ciências Biológicas pela Universidade Federal do Piauí (UFPI), *Campus* Senador Helvídio Nunes de Barros, em Picos, Piauí. Embora fosse um curso voltado para a Licenciatura, durante a graduação desenvolvi pesquisas científicas voltadas para Citogenética de lagartos no Instituto Federal do Piauí (IFPI), sob a orientação dos professores Dr. Edson Lourenço da Silva e Dra. Tamaris Gimenez Pinheiro e que resultaram no meu trabalho de conclusão de curso.

Obtive o título de Mestre em Ciências Biológicas (Biologia Celular e Molecular) em 2019, pela Universidade Estadual Paulista Júlio de Mesquita Filho, *Campus* de Rio Claro (SP), com dissertação entitulada "Estudo da organização estrutural de elementos repetitivos isolados do genoma de espécies de *Proceratophrys* (AMPHIBIA, ANURA, ODONTOPHRYNIDAE)", sob a orientação da professora Dra Patricia Pasquali Parise-Maltempi. Em abril desse mesmo ano, ingressei no Programa de Pós-graduação em Ciências Biológicas (Biologia Celular, Molecular e Microbiologia) para continuar os trabalhos inciados no mestrado. Todas as atividades referentes ao curso de doutorado foram realizadas no Laboratório de Citogenética Animal, Departamento de Biologia Geral e Aplicada, no Instituto de Biociências. A orientação da Prof. Dra Patricia Pasquali Parise-Maltempi permanceu durante todo o doutorado, que durou cinco anos, entre atividades de pesquisa, treinamento de docência, disciplinas e tantas outras realizadas. Concomitante com as atividades do doutorado, em outubro de 2020 ingressei no curso de Pós-graduação *Lato Sensu* em Processos didáticos-pedagógicos para cursos na modalidade a distância, pela Universidade Virtual do Estado de São Paulo (UNIVESP), através de parceria com a UNESP, no qual obtive o título de Especialista na área.

01 de abril 2019, início das atividades do doutorado;

11 de março de 2020, a COVID-19 foi caracterizada pela OMS como uma pandemia;

10 de fevereiro de 2021, retorno parcial aos laboratórios da UNESP.

31 de agosto de 2022, incêndio destrói laboratórios do Instituto de Biociências da Unesp, em Rio Claro, incluindo o Laboratório de Citogenética Animal.

Além dos resultados apresentados nesta tese, durante o período de doutoramento,

participei de diversas atividades, como estágios de docência, seminários, congressos com apresentação de trabalhos, simpósios, encontros, ministramento de cursos e aulas, etc. Além disso, algumas atividades merecem destaque, como segue:

a) De 2020 a 2022, participação no grupo de extensão UnATI - Universidade Aberta à Terceira Idade;

b) Em 2018, participação no grupo de extensão Primeiros Passos na Ciência;

c) Participação como autor/co-autor em 15 trabalhos de apresentações em congressos;

d) Participação em 20 eventos regionais, nacionais ou internacionais;

e) Coorientação em sete trabalhos de conclusão de curso de graduação, 10 iniciações científicas e um trabalho de estágio obrigatório;

f) Participação como membro titular na banca de cinco trabalhos de conclusão de curso.

Durante o período de doutoramento, além dos artigos mencionados nesta tese, outros trabalhos foram publicados, seja como primeiro autor ou como colaborador em trabalhos do grupo de pesquisa. Esses trabalhos foram resultantes de análises e parcerias feitas durante a graduação e o mestrado.

a) **Da Silva, Marcelo J.**; De Araújo Vieira, Ana P.; Galvão Cipriano, Flávia M.; Dos Santos Cândido, Maria R.; De Oliveira, Edivaldo H. C. ; Gimenez Pinheiro, Tamaris; Da Silva, Edson L. The Karyotype of *Salvator merianae* (Squamata, Teiidae): Analyses by Classical and Molecular Cytogenetic Techniques. **Cytogenetic and Genome Research**, v. 160, p. 94-99, 2020.

b) **Da Silva, Marcelo J.**; Fogarin Destro, Raquel; Gazoni, Thiago; Narimatsu, Hideki; Pereira Dos Santos, Paulo S.; Haddad, Célio F. B.; Parise-Maltempi, Patricia P. Great Abundance of Satellite DNA in *Proceratophrys* (Anura, Odontophrynidae) Revealed by Genome Sequencing. **Cytogenetic and Genome Research**, v. 160, p. 141-147, 2020.

c) **Silva, M. J.**; Cipriano, F. M. G.; Vieira, A. P. A.; Candido, M. R. S.; Pinheiro, T. G.; Silva, E. L. Cytogenetic characterization of *Ameivula ocellifera* (Spix, 1825) (Squamata, Teiidae) from the Brazilian Northeast. **Bioscience Journal**, v. 36, p. 1018-1023, 2020.

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