Integrative Zoology

Comparative Satellitomics in Arowanas (Telostei, Osteoglossiformes) Sheds Light on the Evolution of Ancient Satellite DNAs

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Received: 13 November 2024 | **Revised:** 2 June 2025 | **Accepted:** 2 June 2025

Funding: This research was funded by the São Paulo Research Foundation (FAPESP) grant 2021/11169-2 (FHSS), 2022/00427-3 (GAT), and 2023/00955-2 (MBC); and the Brazilian National Council for Scientific and Technological Development (CNPq), grant number 302928/2021-9 (M.B.C.). This study was also financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES). Also, the publication of the paper was supported by DEAL (Deutsche Forschungsgemeinschaft = DFG, Germany).

Keywords: FISH | fishes | library hypothesis | repetitive DNAs | satellitome

ABSTRACT

A significant fraction of the genomes of most multicellular eukaryotes includes extensive arrays of tandemly repeated sequences, collectively referred to as satellite DNAs (satDNAs). However, the mechanisms responsible for generating and maintaining varying satDNA abundances across lineages and temporal scales are still unclear. This work focused on arowana fishes (Teleostei, Osteoglossiformes) as a model; their widespread intercontinental distribution and basal phylogenetic position within Teleostei make them a compelling model for evolutionary research, especially in the realm of satDNA molecular evolution. Through the integration of genomic and chromosomal data, we analyzed and compared the catalogs of satDNA families (i.e., satellitomes) of four out of the six extant arowana species, elucidating ancestral evolutionary trends and establishing their temporal history. Arowanas displayed a small number of satDNA families, ranging from 16 to 25 in *Osteoglossum bicirrhosum* and *Scleropages formosus*, respectively. Alongside the identification of some shared satDNAs, many considered species-specific, nonetheless possess a limited number of copies in other species. The minimal variation observed both within and across species highlights the long-term conservation of satDNAs during evolution, since specific ones (referred to as long-term conserved satDNAs) may have endured throughout a lengthy evolutionary period. Moreover, fluorescence *in situ* hybridization (FISH) investigations conducted with the most abundant satDNAs demonstrated unique hybridization patterns for homologous orthologous ones, signifying their dynamic genomic positioning. Besides, the similarities of satDNAs among species align with their phylogenetic relationships, showing the high dynamism of arowanas' satDNAs, with several evolutionary events driving their sequence diversity.

Fernando Henrique Santos de Souza and Gustavo Akira Toma contributed equally to this work

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1 | Introduction

Satellite DNAs (satDNAs) consist of arrays of repeated nucleotide sequence units organized in a head-to-tail configuration that constitute a great part of the Eukaryote genomes (Garrido-Ramos 2017; Lower et al. 2018; Šatović-Vukšić and Plohl 2023). Since their discovery in the early 1960s (Kit 1961; Sueoka 1961), understanding the general features and biological functions of satDNAs has been the primary focus of a great number of investigations (King and Cummings 1997; Robledillo et al. 2020; Shatskikh et al. 2020; Talbert and Henikoff 2022; Yamamoto and Miklos 1978). Previously regarded as useless and solely non-coding, satDNAs are nowadays seen as an important element of genomic architecture and centromere epigenetics (Bracewell et al. 2019; Cabral-de-Mello et al. 2021; Plohl et al. 2014; Talbert and Henikoff 2022). Novel evidence suggests that some satDNAs are transcriptionally active, participating in the modulation of gene expression (regulated through RNA interference-RNAi) and cellular functions (Ferreira et al. 2019; Lopes et al. 2024; Ninomiya et al. 2023; Shatskikh et al. 2020). Over the years, technological and methodological advances, such as the creation of in-depth computational pipelines, have brought a new perspective to satDNA investigations. Combined with newly developed nucleotide sequencing platforms, it is now possible to characterize a representative species catalog of satDNAs, even in non-model organisms, bringing forth the "Satellitome era" (Ruiz-Ruano et al. 2016). Thus, by using low-coverage short-read genome sequencing, one can characterize species satellitomes and investigate their wide patterns of conservation, homology, chromosome location, and evolutionary behavior (Camacho et al. 2022; de Oliveira et al. 2023; Peona et al. 2023; Toma et al. 2024; Utsunomia et al. 2019). These discoveries have led to the characterization of many satellitomes from various organisms and have helped to strengthen existing theories and shed new light on old ideas (see for example, Despot-Slade et al. 2022; Robledillo et al. 2020; Sales-Oliveira et al. 2024; Sena et al. 2020; Voleníková et al. 2023).

This work aims to provide a deeper insight into the processes shaping the evolution of satDNAs by investigating the arowanas (Osteoglossiformes). Due to their inter-continental distribution and basal phylogenetic position within Teleostei (Betancur-R et al. 2017; Dornburg and Near 2021; Near et al. 2012), they represent an interesting model for evolutionary studies, particularly molecular evolution (Capobianco and Friedman 2019; Hilton and Lavoué 2018; Lavoué 2016). With an estimated age of divergence of around 200 million years ago (MYA) (Capobianco and Friedman 2019), the bonytongue fishes, belonging to the order Osteoglossiformes, represent one of the oldest Teleost fish groups, with ~250 species that are found in various rivers and lakes across South America, Africa, Asia, and Oceania (Hilton and Lavoué 2018). The Osteoglossiformes comprise five families: Pantodontidae, Mormyridae, and Gymnarchidae are exclusively located in Africa; Notopteridae is present in both Africa and Asia, while Osteoglossidae is distributed across Asia, South America, Africa and Australia. Their current distribution pattern may have been influenced by instances of vicariance that happened after the Gondwana breakup (Kumazawa and Nishida 2000; Lavoué 2015).

Arowanas are classified within the monophyletic family Osteoglossidae, which is currently discontinuously distributed across South America, Southeast Asia, and Sahul (Figure 1). This family comprises six extant species categorized into two genera: *Scleropages* (*S. formosus* (Müller and Schlegel, 1840)*, S. jardinii* (Saville-Kent, 1982)*, S. leichardti* Günther, 1864, and *S. inscriptus* Roberts, 2012) and *Osteoglossum* (*O. bicirrhosum* (Cuvier, 1829) and *O. fererrai* Kanazawa, 1966) (Fricke et al. 2024; Hilton and Lavoué 2018). They represent one of the most beautiful freshwater fishes, thus being overfished in their natural habitats for aquarium trade, making some species (particularly the Asian arowana, *S. formosus)* listed as one of the most highly endangered fish species (Larson and Vidthayanon 2019). Additionally, by representing extant organisms with Gondwanic distribution, arowanas represent an excellent model for studying evolution and environmental adaptation.

Previous cytogenetic studies included a set of cytogenetic and molecular analyses in all arowanas (except in *S. inscriptus*) evidencing variations in diploid numbers ($2n$) among the different species, that is, $2n = 44$ in *S. leichardti*, $2n = 48$ in *S. jardinii*, $2n = 50$ in *S. formosus*, $2n = 54$ in *O. ferreirai*, and $2n = 56$ in *O. bicirrhosum* (reviewed in Cioffi et al. 2019). However, genome-wide investigations in this group were never undertaken, particularly focusing on the process shaping the evolution of satDNAs. Here, by combining genomic and cytogenetic approaches, we isolated and compared the satellitomes of four of the six extant arowana species. The genomes of arowanas show ancient evolutionary patterns, enabling the examination and evaluation of the temporal history of satDNA families by employing this multispecies data collection.

2 | Material and Methods

2.1 | Sampling and Chromosome Suspensions

All analyzed individuals in this study are presented in Table 1. Chromosome suspensions were obtained using the caudal-fin regeneration method (Völker and Ráb 2015). Chromosomes on slides were stained with a 10% Giemsa solution (pH 6.8) for further visualization. Sampling was authorized by the Brazilian Environmental Agency ICMBIO/SISBIO (License 48628-14) and SISGEN (A96FF09). All experiments followed the ethical guidelines sanctioned by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number 7994170423).

2.2 | DNA Extraction and Genome Sequencing

The DNA was extracted using the phenol-chloroform-isoamyl alcohol method (Sambrook and Russell 2001), and we extracted genomic DNA (gDNA) of one female individual of each arowana species (Table 1). Low-coverage genome sequencing was performed on the BGISEQ-500 platform at BGI (BGI Shenzhen Corporation, Shenzhen, China) with PE reads (paired-end) of 150 bp length. All genomic raw reads were deposited in the Sequence Read Archive (SRA) under accession numbers SRR31136363 (*O. bicirrhosum*), SRR31136364 (*S. jardinii*), and SRR31136365 (*S. leichardti*).

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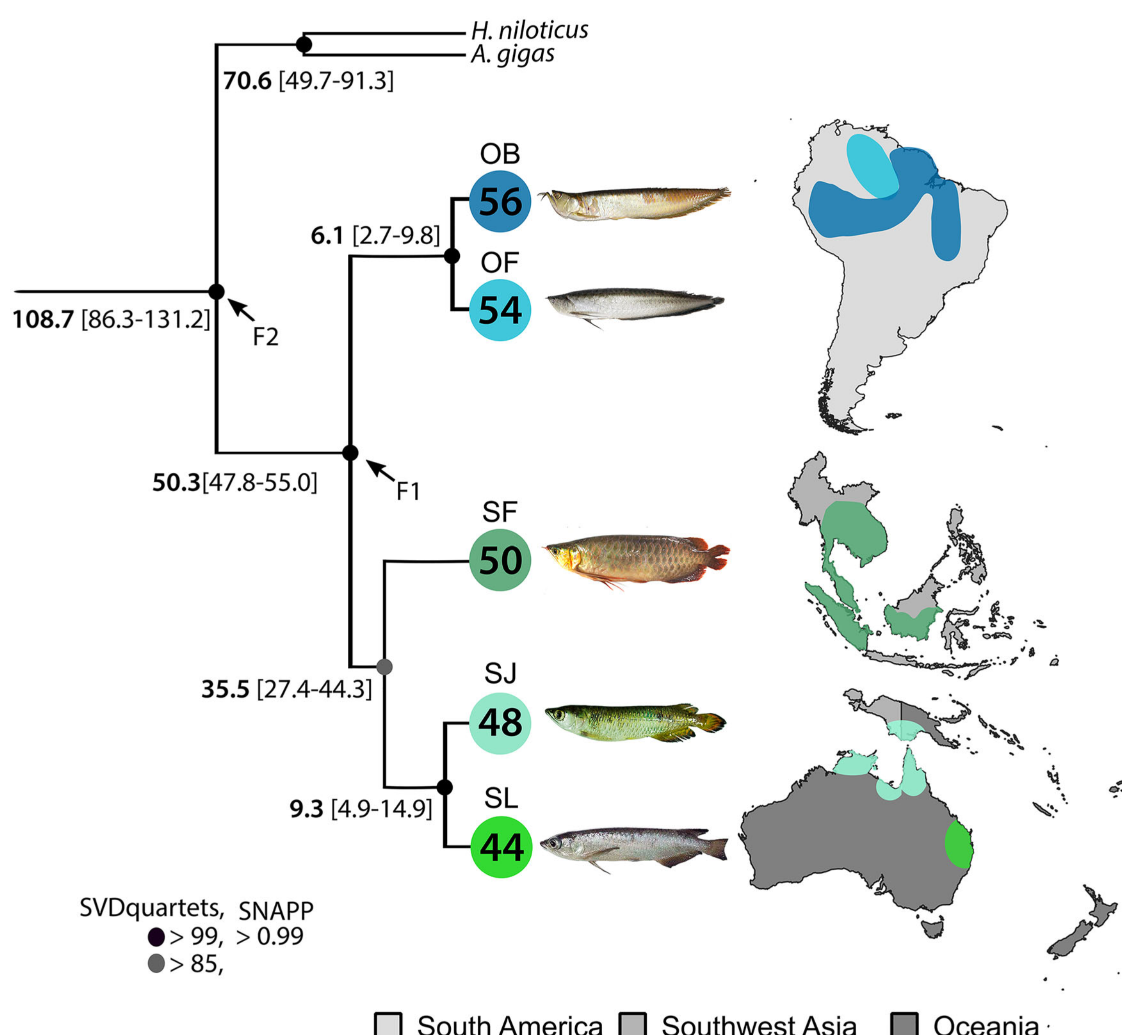

FIGURE 1 | Distribution and phylogenetic tree of Osteoglossidae species. Bold values indicate average divergence time, and values in brackets indicate 95% HPD intervals. Circles on each node indicate support for each tree estimation method. Arrows indicate calibration points. Tip circles represent each species, with the same color used for the distribution. The numbers inside the circles indicate the diploid chromosome number ($2n$) for each species. Shades of gray represent regions of the occurrence of each group (regions are not in proportion). OB = *Osteoglossum bicirrhosum*; OF = *Osteoglossum ferreirai*; SF = *Scleropages formosus;* SJ = *Scleropages jardinii*; SL = *Scleropages leichardti*. Figure adapted from Cioffi et al. (2019).

TABLE 1 | Species used in this study to analyze satDNAs, with the localization and number of individuals.

Species	Sampling site	Individuals (*N*)
Scleropages leichardti	Corroboree Billabong, Mary River, Australia	04♀/04♂
Scleropages jardinii	Fitzroy River, Australia	05♀/04♂
Osteoglossum bicirrhosum	Solimões River, Amazon, Brazil	10♀/12♂

2.3 | Characterization and BLAST Search of Arowana Satellitomes

We used the satMiner protocol (Ruiz-Ruano et al. 2016) (https://github.com/fjruizruano/satminer) that is based on consecutive rounds of clustering of Illumina reads by RepeatExplorer2 (Novák et al. 2013, 2020), which executes an integrated version of the Tandem Repeat Analyzer (TAREAN) tool (Novák et al. 2017), for automated identification of satellite DNA repeats based on the topology of their cluster graphs. First, to ensure the quality of the sequencing reads ($Q > 20$ for all nucleotides), we used the Trimmomatic software (version 3.0) (Bolger et al. 2014) to eliminate reads with low quality, following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN: 100 CROP. Then, to proceed with satDNA identification, each arowana satellitome was characterized independently by selecting a subset of 2 × 500 000 paired reads from each arowana sequencing library and using them as non-overlapping inputs to run RepeatExplorer2 (Novák et al. 2013, 2020). Then, cluster graphs with circular shapes were selected using TAREAN (Novák et al. 2017), which generates a consensus monomer sequence for each satDNA cluster. The putative satDNA detected in TAREAN

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was filtered from the trimmed reads, using the DeconSeq software (version 0.4.3) (Schmieder and Edwards 2011). Afterward, we performed an additional subset of 2 × 500 000 paired reads from each cleaned sequencing library and carried out multiple rounds of RepeatExplorer2/TAREAN and deconseq decontamination until no novel satDNAs were found. In the next step, we removed any non-satDNA repetitive DNA classes from our dataset. By conducting a homology search with the rm_homology.py tool and performing sequence alignments, we examined the similarity among the satellitomes isolated in the present study in conjunction with *S. formosus* catalog from Toma et al. (2023). Considering their level of sequence identity, we considered every collection of homologous sequences belonged to the same families when identity was >80% and to superfamilies when identity was >50%. All satDNA catalogs were deposited on the GenBank with access numbers PQ539088–PQ539109, PQ539110–PQ539132, and PQ539133–PQ539148 to *S. jardinii, S. leichardti*, and *O. bicirrhosum*, respectively.

We calculated the abundance of each satDNA (proportion of nucleotides related to the particular satDNA vs. total number of analyzed nucleotides) by selecting 2 × 5 000 000 paired reads from each Arowana genomic library and applying script repeat_masker_run_big.py in combination with RepeatMasker's "cross-match" tool (Smit et al. 2017). We applied the script calcDivergenceFromAlign.py in RepeatMasker to evaluate the genetic distances (Kimura-2-parameter) of each arowana satellitome. We also used this procedure to search for every satellite of each species in the rest of the species analyzed. For this, we aligned 2 × 5 000 000 randomly selected reads from each species to the consensus sequences in each species-specific satellitome database. To test whether the presence of mitochondrial DNA (mtDNA) sequences among raw reads could negatively affect satDNA abundance calculations, we estimated the abundance of mtDNA reads on the set of raw reads. For this, using the software NOVOPlasty, we characterized the mitochondrial genome for the four species under analysis and created a Bowtie index for each species' mitogenome. We then aligned the species' reads to their respective mitogenomes, obtaining that mtDNA sequences represented 0.11%, 0.22%, 0.40%, and 0.26% of the raw reads of *O. bicirrhosum*, *S. formosus*, *S. jardinii*, and *S. leichardti*, respectively. Therefore, this proportion of mtDNA in the general pool of reads of each species can be considered negligible when calculating the abundances of each satDNA per genome. In addition, to check that the satellitomes were free of mtDNA sequences, reads corresponding to the mitogenome were extracted and subsequently converted to FASTA format. A BLAST database was then created using the satellitomes of the species. Finally, we performed a BLAST analysis of the mitochondrial genome reads against these satellite DNA sequences, which did not yield any significant alignments between the inputs. Therefore, this proportion of mtDNA in the general pool of reads of each species can be considered negligible when calculating the abundances of each satDNA per genome.

To verify the structure of satDNAs in the raw reads, we used the satellitomes consensus of each species; so, we created an index of the satellites' DNAs and aligned the raw reads on each satDNAs as a reference, with Bowtie (–very-sensitive flag) to map the raw reads to these sequences. With the SAM file, we used samtools (samtools view -bS) (Li et al. 2009) to generate a sorted BAM file and analyzed the coverage of satellite DNA monomers larger than 1 kb in IGV version 2.17.2 (Robinson et al. 2011).

All satDNAs were named and numbered according to the suggestions of Ruiz-Ruano et al. (2016). Thus, *S. jardinii, S. leichardti*, and *O. bicirrhosum* satDNAs were named Sja, Sle, and Obi, respectively, and their numbers were organized according to their decreasing abundance in the genome. Finally, we conducted a +search of the recovered satDNAs against GenBank/NCBI and RepBase (CENSOR) to verify the sharing of any satDNA with other species. We also aimed to identify other repetitive DNA motifs (e.g., transposons, multigene families) that may take part in the evolution of these satellitomes. Additionally, to compare the set of satDNAs in Arowana species, we aligned the present study satDNAs with *S. formosus* satDNAs described by Toma et al. (2023) and analyzed the similarity among them.

2.4 | Primer Design, Polymerase Chain Reaction (PCR), and Fluorescence *In Situ* Hybridization (FISH)

Based on the total amount of satDNAs isolated in each species (Tables S1–S3; Table 2), we manually designed primers for 17 consensus monomers, using both Thermofisher multiple primer analyzer (https://www.thermofisher.com/br/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) and Biotools OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) to check for the hairpin formation and self-annealing. The monomers chosen for PCR and subsequent FISH were selected based on two criteria: (i) satDNAs that depicted 80% similarity or more and were shared between at least two arowana species (Table 3); and/or (ii) the two most abundant satDNAs in each catalog (e.g., satDNA01 and satDNA02).

The PCRs, performed in a T100 Bio-Rad Thermal Cycler (Bio-Rad Laboratories), followed the conditions from Kretschmer et al. (2022) with some modifications. Briefly, the amplification reaction consisted of an initial denaturation at 95°C for 7 min, followed by 34 cycles with denaturation at 95°C (45 s), annealing temperatures varying from 50°C to 62°C (60 s), extension at 72°C (60 s), and final extension at 72°C (7 min). All PCR products were quantified using a ThermoFisher NanoDrop spectrophotometer (ThermoFisher Scientific) and submitted to electrophoresis with a 1% or 2% agarose gel.

All 17 selected satDNAs were successfully amplified and the PCR products were labeled via nick-translation by incorporating the fluorophore Atto488-dUTP (green fluorescence) or Atto550-dUTP (red fluorescence) according to the instructions in the manufacturer's manual (Jena Bioscience, Jena, Germany). We conducted two separate FISH essays. For the first essay, we selected 13 monomers shared by arowana satellitomes as probes to identify their proper chromosomal location and check for possible chromosome rearrangements along the phylogeny. For the second essay, we utilized/selected the two most abundant satDNAs in each catalog to detect potential centromere-specific repetitive DNA motifs. All FISH procedures followed high-stringency conditions (Sassi et al. 2022): initial slide pretreatment

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steps with an RNAse (10 µg/mL in 2× SSC solution) for 1 h 30 min at 37°C and pepsin (50 µg/mL in 10 mM HCl) for 10 min, followed by denaturation in 70% formamide at 72°C for 3 min, 15 s, and hybridization in a 37°C moist chamber for at least 14 h. The final hybridization mixture was composed of 200 ng of the labeled probe, 50% formamide, 2× SSC, 10% SDS, 10% dextran sulfate, and Denhardt's buffer at pH 7.0 in a total volume of 20 µL. Finally, the chromosome slides were stained with Vectashield 4′,6-diamidino-2-phenylindole (DAPI) solution (Vector Laboratories, Burlingame, USA).

2.5 | Microscopy and Image Processing

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

3 | Results

3.1 | General Features of Arowanas Satellitome

After several iterations performed by Repeatexplorer2/TAREAN (*S. leichardti* = 3; *S. jardinii* = 3; *O. bicirrhosum* = 4), we identified 23, 22, and 16 satDNA families for *S. leichardti*, *S. jardinii*, and *O. bicirrhosum*, respectively. The main features of arowana's satellitomes are described in Table 2. All sets of satDNAs presented a great number of A+T-rich (>50%) monomers. A+T-rich domains made up 65% of *S. leichardti*'s (15 satDNAs), 72% of *S. jardinii*'s (16 satDNAs), and 62% of *O. bicirrhosum*'s (10 satDNAs) satDNA catalogs. The repeat unit lengths (RULs) varied from 28 bp (SjaSat19-28 and SleSat23-28) to 4037 bp (SleSat10-4037), with 49 RULs (80%) higher than 100 bp. Tables S1–S4 describe, individually for each species analyzed in this work (*S. leichardti*, *S. jardinii*, and *O. bicirrhosum*) and for the species *S. formosus* previously analyzed (Toma et al. 2023), the complete set of satDNAs composing their satellitomes as well as the characteristics of each satDNA (RUL, AT content, abundance, and divergence). Also, these tables include information about the superfamily to which each satDNA family belongs (see below). To confirm the correct structure of satDNAs formed by longer repetitive units (> 1 kb), we aligned raw reads to the TAREAN consensus satDNA sequences using bowtie2. All sequences showed high coverage across the entire monomer, confirming these as satDNA monomers and also confirming their lengths. In some cases, peaks in specific regions suggested the potential formation of new satDNAs originating from these sequences. Notwithstanding, SleSat10-4037 exhibits an abrupt drop in coverage around 2600 bp (spanning ~10 bp) that might indicate that this satellite of 4037 bp has arisen recently from the joining of two older independent satellites (one ~2600 bp and the other ~1500 bp), much more abundant than the newly composed satDNA. Therefore, most probably, the abundance is overestimated for SleSat10-4037 since the calculations should include the sum of the three satellites (the two older ones and the new one).

TABLE 2 | Main features of the arowana's satellitomes: *N* = number of satellite sequences; RUL = repeat unit length (bp); A + T = proportion of A + T nucleotides; Max = maximum; Min = minimal; Med = median.

Species	N	Max RUL	Min RUL	Med RUL	Max A+T	Min A+T	Med A+T	Reference
Scleropages leichardti	23	4037	28	680	66%	35%	53%	Present work
Scleropages jardinii	22	1682	28	398	68%	36%	55%	Present work
Scleropages formosus	25	4000	6	590	64%	36%	53%	Toma et al. (2023)
Osteoglossum bicirrhosum	16	1726	80	563	67%	37%	52%	Present work

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TABLE 3 | satDNA used for PCR and FISH experiment, with the similarity (%) and relationship of each arowana satDNA.

Scleropages leichardti	*Scleropages jardinii*	*Scleropages formosus*	*Osteoglossum bicirrhosum*	Similarity (%)
SleSat23-28	SjaSat19-28	—	—	100
—	—	SfoSat01-180	ObiSat09-180	100
SleSat15-183	SjaSat13-183	—	—	99.45
SleSat02-60	SjaSat07-60	—	—	98.89
SleSat21-1074	SjaSat21-1075	—	—	96.28
SleSat01-180	SjaSat05-180	—	—	92.22
SleSat03-840	SjaSat11-840	—	—	91.9
SleSat09-713	SjaSat22-713	—	—	90.48
SleSat08-687	SjaSat08-689	—	—	90.43
—	—	SfoSat03-198	ObiSat14-198	90
SleSat07-176	SjaSat09-177	—	—	88.15
SleSat17-597	SjaSat16-585	—	—	86.45
SleSat04-138	SjaSat06-146	—	—	80.82
—	SjaSat01-188	—	—	—
—	SjaSat02-676	—	—	—
—	—	—	ObiSat01-1726	—
—	—	—	ObiSat02-287	—

Among the analyzed satDNAs, we found 11 satDNA families shared between *S. leichardti* and *S. jardinii* and two shared between *S. formosus* and *O. bicirrhosum* (Table 3). Furthermore, the homology search revealed the presence of 12 superfamilies (SF1-SF12), of which only one (SF9) is exclusively composed of ObiSatDNAs (Table 4) SF2 and SF6 are the largest superfamilies: SF2 is composed of 11 families shared by *S. leichardti*, *S. jardinii*, and *S. formosus*, while SF6 is composed of 9 families shared by *S. leichardti*, *S. formosus*, and *O. bicirrhosum*. SF3, SF4, and SF7 are the only ones that have satDNA families shared by all analyzed species (Table 4).

To search for the presence of species-specific satDNAs in the rest of the species, we aligned 2 × 5 000 000 randomly selected reads from each species to the consensus sequences in the species-specific satDNA database, using RepeatMasker (Smit et al. 2017) as indicated in the Materials and Methods section (Table S5). According to the information in this table, many satellites that have been determined to be unique to a species nonetheless have a small number of copies in other species. For example, the case of *O. bicirrhosum* reveals that 11 of the 16 detected satellites exhibit low-copy counterparts across all other species, while an additional three satellites also have low-copy counterparts in *S. formosus*. All 25 satellites of *S. formosus* possess relict copies in *O. bicirrhosum*. Nevertheless, no copies of seven of them remain in any of the other two *Scleropages* species. *S. leichardti* and *S. jardinii* possess identical satellites; nevertheless, each species contains a distinct array of satellites that are amplified within its genome, exhibiting low copy numbers in the counterpart species. Traces of the majority of these satellites persist in *S. formosus* and *O. bicirrhosum* (Table S5). These data have been illustrated for some satellites in Figure 2, which contains a graphical representation of data from Table S5 retrieved for the satellites in Table 3 (those shared between species).

Several satDNA families included sequences matching portions of transposable elements (TEs), including Penelope, NonLTR/SINE/SINE2, LTR/Gypsy, among others (Table S6).

3.2 | FISH Patterns of Arowana satDNAs

FISH experiments were performed using the most abundant satDNAs present in each satDNA catalog (Tables S1–S4) and the ones with >80% similarity among the analyzed arowana species (Table 3) as probes. Thus, for interspecific FISH with satDNA families, we chose only to use metaphase plates of the arowanas that shared that specific satellite (e.g., if a monomer variant was found shared between two species, only metaphase plates of those two species were used). For the two most abundant satDNAs present in each species, we performed only intraspecific FISH, and the findings indicated their presence in the centromere of the many chromosomes (Figures 3 and 4). Thus, our intraspecific FISH with the four most abundant satDNAs (SjaSat01-188; SjaSat02-676; ObiSat01-1726, and ObiSat02-287) evidenced centromeric/pericentromeric signals in several chromosomes. SjaSat01-188 and SjaSat02-676 displayed co-localized centromeric/pericentromeric signals in 11 chromosome pairs, while ObiSat01-1726 and ObiSat02-287 also presented syntenic clusters in almost all chromosomes (Figure 3). On the other hand, among the 13 satDNA families shared by the arowanas genomes, five of them (SleSat04-138/Sja06-146; SleSat07-176/SjaSat09-177;

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TABLE 4 | The different satellite superfamilies (SF1-SF12) that compose the satellitomes of Arowana species.

Superfamily	Species			
	Scleropages leicharditii	***Scleropages jardinii***	***Scleropages formosus***	***Osteoglossum bicirrosum***
	SleSat01-180	SjaSat05-180		ObiSat01-1726
SF1	SleSat07-176	SjaSat09-177		
	SleSat08-687	SjaSat08-689		
SF2	SleSat02-60	SjaSat07-60	SfoSat05-107	
	SleSat05-752	SjaSat02-676	SfoSat10-239	
		SjaSat04-120	SfoSat12-337	
		SjaSat03-388		
		SjaSat10-238		
		SjaSat15-447		
SF3	SleSat14-473	SjaSat12-1682	SfoSat11-1682	ObiSat16-226
SF4	SleSat15-183	SjaSat13-183	SfoSat15-261	ObiSat15-129
SF5	SleSat17-597	SjaSat16-585	SfoSat17-699	
SF6	SleSat06-1726		SfoSat18-767	ObiSat07-715
	SleSat10-4037		SfoSat23-291	ObiSat11-653
	SleSat19-849			ObiSat12-1002
				ObiSat13-779
SF7	SleSat11-42	SjaSat17-36	SfoSat01-180	ObiSat09-180
SF8	SleSat13-992		SfoSat19-993	
SF9				ObiSat02-287
				ObiSat03285
SF10	SleSat22-34	SjaSat14-69		
SF11	SleSat09-713	SjaSat22-713	SfoSat20-677	
SF12			SfoSat02-39	ObiSat10-80

SleSat17-597/SjaSat16-585; SjaSat21-1075/SleSat21-1074; and SjaSat19-28/SleSat23-28) did not produce any detectable hybridization signals after FISH assays (Figure S2), while the remaining ones depicted three different patterns. The first, presented by SleSat08-687/SjaSat08-689; SleSat03-840/SjaSat11-840; SleSat15-183/SjaSat130-183; and SleSat09-713/SjaSat22-713, showed a similar hybridization pattern between *S. leichardti* and *S. jardinii* with centromeric/pericentromeric clusters in only one pair of chromosomes (Figure 4C–F). The second pattern, however, found in SleSat01-180/SjaSat05-180 and SleSat02-60/SjaSat07-60, demonstrated conspicuous changes in chromosome location and site number between *S. leichardti* and *S. jardinii*. In the case of SleSat01-180/SjaSat05-180, we found centromeric/pericentromeric clusters in four and two chromosome pairs of *S. leichardti* and *S. jardinii*, respectively, while for SjaSat07-60/SleSat02-60, we found signals in 12 chromosome pairs in *S. leichardti* and only one pair in *S. jardinii* (Figure 4A,B). The third pattern involved two pairs of satDNAs with 100% (SfoSat01-180/ObiSat09-180) and 90% (SfoSat03-198/ObiSat14-198) of similarities present between *O. bicirrhosum* and *S. formosus*, which only produced detectable hybridization signals in the latter (Figure S3).

4 | Discussion

This study aimed to gain a deeper understanding of the mechanisms that influence the evolution of satDNAs by examining a primitive group of Teleost fishes. SatDNA sequences are recognized for their rapid evolution in eukaryotes (Plohl et al. 2010); nonetheless, several species exhibit sequences that remain conserved across extensive evolutionary periods (millions of years) (Palacios-Gimenez et al. 2020; Sales-Oliveira et al. 2024; Tunjić-Cvitanić et al. 2021). Examples have been documented in several taxa, including snakes (Lisachov et al. 2023), monkeys (Ahmad et al. 2020), and fishes (Goes et al. 2022; Robles et al. 2004; dos Santos et al. 2021; Silva et al. 2017; Utsunomia et al. 2019). In this study, we have described three new Osteoglossiformes satDNA sets and analyzed their evolutionary characteristics together with previous *S. formosus* satellitome data (Toma et al. 2023). We demonstrated that certain satellites, which are referred to as long-term conserved satDNAs, have persisted across an extensive evolutionary time frame. *S. formosus* exhibited the highest number of satDNAs (25), while *O. bicirrhosum* had the smallest one (16) (Table 2). The generally elevated number of SatDNAs appears to be a regular trend in numerous fish

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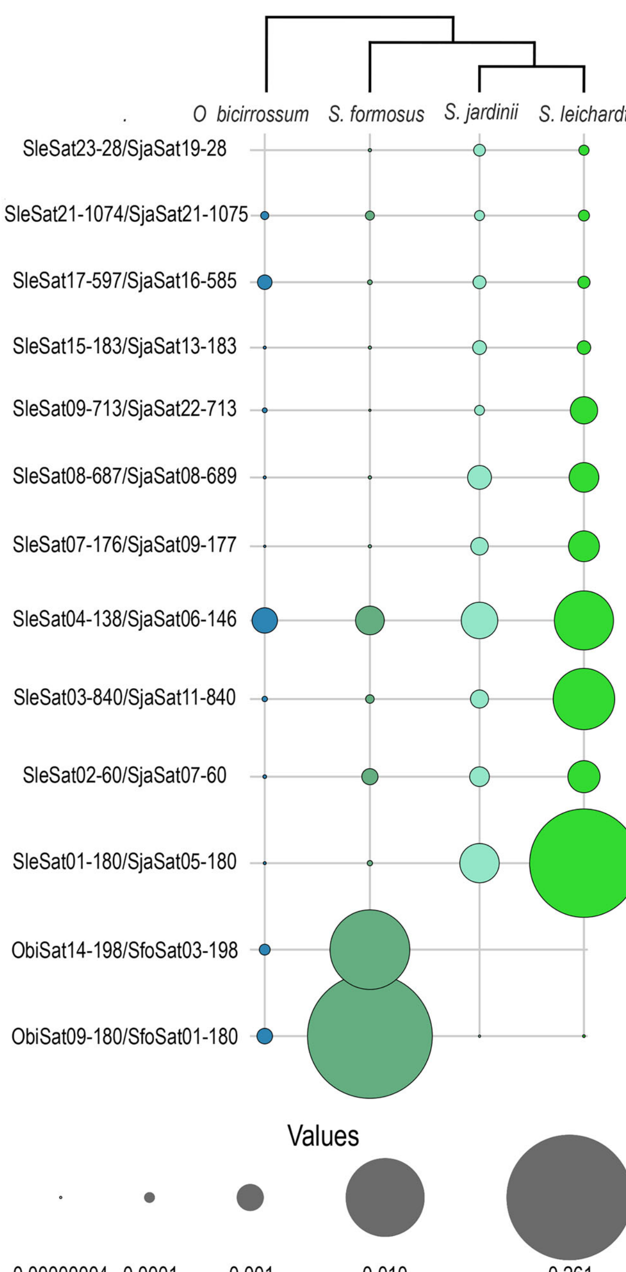

FIGURE 2 | Quantification of the shared satDNA families between arowanas species. Within each satDNA cluster, the size of the circle represents the relative satDNA abundance. The information was retrieved from Table 3 and Table S5.

satellitomes (Silva et al. 2017; Kretschmer et al. 2022; Goes et al. 2022; dos Santos et al. 2025). Extreme examples of highly diverse catalogs are observed in certain fish species, exemplified by the *Megaleporinus* genus, which possesses over 100 satDNAs (Crepaldi et al. 2021; Utsunomia et al. 2019). By analyzing all satDNAs across the four species and matching the sequences with one another, we could uncover their similarities (Table 3). Homologous satDNAs (i.e., satDNAs with > 80% similarity) were absent in four species, occurring just between *S. leichardti* and *S. jardinii*, as well as between *S. formosus* and *O. bicirrhosum* (Table 3). Upon increasing the similarity threshold to 50%, related sequences were identified across all studied species (Table 4).

The similarity and divergence of the set of satDNA found in this study are congruent with previous phylogeographic studies (Figure 1; Cioffi et al. 2019; Capobianco and Friedman 2024). Shared satellite families between *S. leichardti* and *S. jardinii* are expected, as these species diverged recently (between 4.9 and 14.9 MYA) (Cioffi et al. 2019), and the evolutionary rate of satellite families is highly time-dependent under no biological constraints (Pérez-Gutiérrez et al. 2012). Although there are different superfamilies consisting of several satellites of the three *Scleropages* species, suggesting a common origin for those of the same superfamily, the absence of shared satellites between *S. formosus* and the two other *Scleropages* species is puzzling. This result suggests that there is a considerable amount of genetic differentiation between the satellitomes from *S. leichardti* and *S. jardinii's* when compared with *S. formosus*. This is plausible considering that *Scleropages* diversification occurred at approximately 35 MYA through marine dispersal events (Cioffi et al. 2019; Lavoué 2015). However, we cannot rule out that these findings are due to limitations in bioinformatics tools. Our screening shows that there are traces of most of the satellites of *S. jardinii* and *S. leichardti* in the genome of *S. formosus* and vice versa (Table S5). This aligns with Salser et al. (1976) hypothesis, which posits that from a collection of satDNA families in the genome of an ancestral species, each satellite family will experience differential amplification and/or contraction in each descendent species. This leads to species-specific profiles that become increasingly distinct with greater divergence time between the comparing species, where a residual copy number of repeats in one species may persist from a satDNA family that has been significantly amplified in other species, and vice versa (Garrido-Ramos 2017; Ruiz-Ruano et al. 2016; Salser et al. 1976).

Likewise, the presence of shared satellites between *O. bicirrhosum* and *S. formosus* is not completely unexpected (Table 2). These satDNA families could have been present on the *Osteoglossum* and *Scleropages*' common ancestor, accumulating several sequence differences, or even being lost on the recently diverged *Scleropages* species. Following the library hypothesis, species with lower divergence times are thought to share more sequences than phylogenetically distant species, as shown by several studies (de Lima and Ruiz-Ruano 2022; Peona et al. 2023; Da Silva et al. 2023). Despite any satDNA family being found across all studied arowana species, some sequences classified as superfamilies were shared among all *Scleropages* species. The superfamily classification encompasses sequences with 50%–80% similarity among them (Ruiz-Ruano et al. 2016); these sequences can derive from a common ancestor but diverge due to the accumulation of mutations and other processes associated with satDNA evolution (Mora et al. 2024; Ruiz-Ruano et al. 2016). The divergence time among arowana species, together with the elevated mutation rate of satDNAs, may lead to significant satDNA divergence within this group. Consequently, our investigation reveals conserved satDNAs, cluster amplification, reduction, de novo emergence, and remnants of a likely ancestral sequence (i.e., superfamilies; Tables 3 and 4 and Figure 2; Tables S1–S5). We suggest that most of the satellites analyzed here predate these species and were present in the genome of their common ancestor (Table S5). However, 13 satDNA families have been completely lost in the *S. leichardti/S. jardinii* lineage, in which two new satellites (SjaSat01-188 and SjaSat15-447) would have appeared *de novo* (Table S5). Five satellites (SjaSat03-388, SjaSat04-120,

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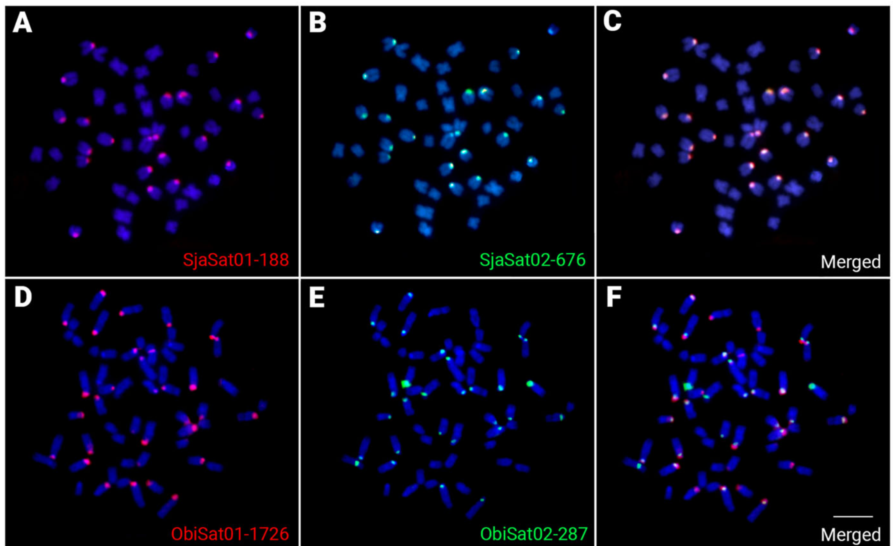

FIGURE 3 | Metaphases of *Scleropages jardinii* (A–C) and *Osteoglossum bicirrhosum* (D–F) showing the chromosome localization of the two most abundant SatDNAs in each species' genome. Bars = 5 μm.

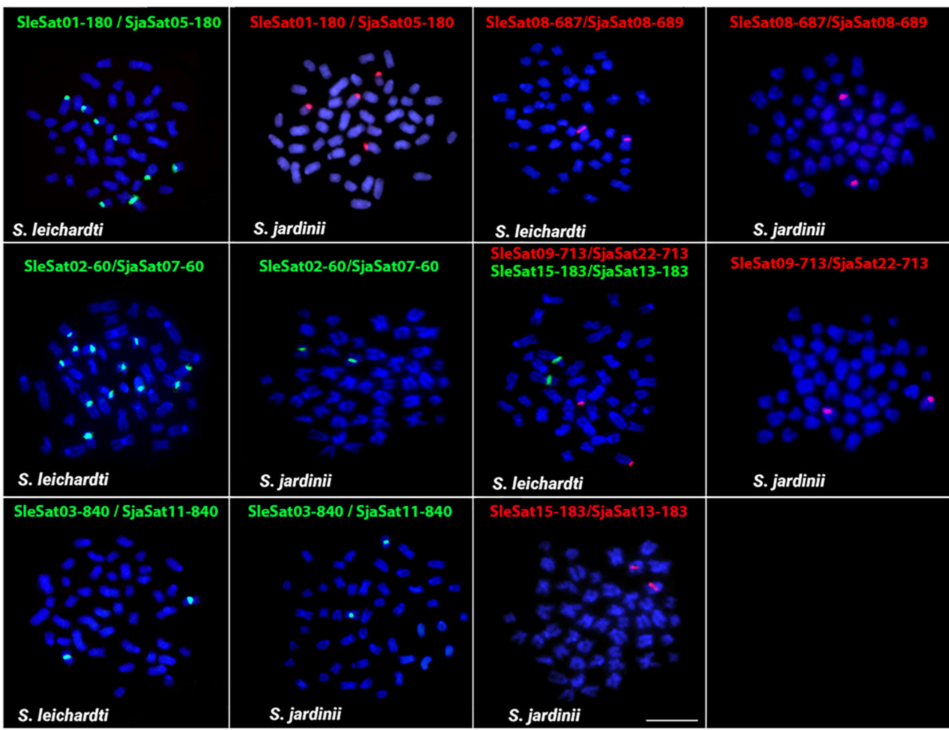

FIGURE 4 | Metaphase plates of *Scleropages leichardti* and *Scleropages jardinii* showing the localization of satDNAs of the same family. Bars = 5 μm.

SjaSat10-238, SjaSat16-585, and SleSat23-28/SjaSat19-28) appear to have arisen de novo in *Scleropages*, given there is no evidence of their presence in *Osteoglossum* (Table S5). Notably, the satellites SjaSat14-69, SleSat20-37, and SleSat22-34, which are shared by both examined genera, would have been lost in *S. formosus* (Table S5). This data not only corroborate the library theory (Salser et al. 1976) but also illustrate the evolution of each family within the satellitomes of Osteoglossidae and validates prior phylogenetic findings for this group (Cioffi et al. 2019).

Despite their similarities, certain satDNAs are not sufficiently clustered to produce positive hybridization signals after FISH across all species, thus displaying species-specific patterns. The analyses conducted by Cioffi et al. (2019) also indicated that there is a limited amount of repetitive DNA shared between *Osteoglossum* and *Scleropages*. However, many repetitive sequences are shared among *Osteoglossum* species, primarily located in the centromeric and terminal regions of certain chromosomes (Souza et al. 2019). The limited degree of repeated DNA

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similarity observed between *Osteoglossum* and *Scleropages* may be attributed to the significant disparity in satellite DNA content between the two genera. Nevertheless, the similar composition of satDNA found between *S. jardinii* and *S. leichardti* is explained due to their closer phylogenetic relationship (Figure 1). As expected, both *Scleropages* species shared a greater number of satDNAs that mostly demonstrated the same pattern of chromosomal organization (Figure 3). Moreover, two sets of satDNAs demonstrate 100% similarity (SfoSat01-180/ObiSat09-180), and 90% similarity (SfoSat03-198/ObiSat14-198) between *O. bicirrhosum* and *S. formosus* produced detectable hybridization signals exclusively in the latter, indicating a limited clustering of these sequences in *O. bicirrhosum*, consistent with the small number of such sequences identified in this species (Tables S3 and S5). Thus, low-copy equivalents of dominant satDNAs of one species remained in related species' genomes and vice versa, following previous assumptions (Salser et al. 1976). The comparisons of high-copy and low-copy sequence variants of these satellites in each species show high interspecific sequence conservation and the complete lack of any species-diagnostic mutations, as found in *Palorus* (Mestrovic et al. 1998) or in Cardueae (del Bosque et al. 2014, 2013) and the variation in satellite profiles is found by changes in copy number (Plohl et al. 2010). The consensus sequences of SfoSat01-180/ObiSat09-180 and SfoSat03-198/ObiSat14-198 showed higher interspecific similarity than expected according to the divergence time between both species (*S. formosus* and *O. bicirrhosum*). Indeed, there are no distinctions between the consensus sequences of SfoSat01-180 and ObiSat09-180. This phenomenon may be elucidated by evolutionary convergence since the consensus sequence is contingent upon the relative frequency of the four potential nucleotides in each species (Camacho et al. 2022).

TEs constitute a category of repetitive DNA that is highly prevalent in the genomes of various species (López-Flores and Garrido-Ramos 2012; Meštrović et al. 2015; Wells and Feschotte 2020). This class is related to various functions, including genome control and karyotype remodeling (Scarpato et al. 2015). TEs exhibit a robust association with satDNAs, influencing their evolution and aiding in their diversification, dispersion, and de novo emergence (Garrido-Ramos 2017; Hayward and Gilbert 2022; Kejnovsky et al. 2006; Šatović-Vukšić and Plohl 2023; Tunjić-Cvitanić et al. 2024). Here, despite not being the main focus of the present work, we could identify that TEs likely played a significant role in the evolution of arowana's satellite DNAs (satDNAs), as the similarity between satDNAs and TEs may have facilitated the evolutionary development of these satellites (Table S6).

Due to the high rate of changes that encompass the centromeric/pericentromeric region, some species can present species-specific or even chromosome-specific satDNAs (Robledillo et al. 2020). The latter is true for most of our results (Figure 2), except for *S. formosus*, in which SfoSat01-180 was detected in all centromeres (Toma et al. 2023). Chromosome-specific satDNA may be the result of high centromere turnover rates, being previously associated with the centromere rearrangements found in *Drosophila obscura* (Bracewell et al. 2019). The abundance of satellite DNA (satDNA) in vertebrate genomes has been linked to chromosomal instabilities, potentially leading to events such as chromosomal rearrangements that result in increased karyotypic divergence (Paço et al. 2015). Therefore, it would be reasonable to suggest a possible correlation between satellite DNA (satDNA) and the variation in chromosome structure (karyotypic diversification) in Osteoglossinae. The ancestral karyotype for Osteoglossidae is likely $2n = 56$, as shown by *Arapaima*, *Heterotis*, and *O. bicirrhossum*. One hypothesis about the evolutionary history of Osteoglossinae indicates that fusion events occurred, leading to a decreased chromosome count in *Scleropages* (Figure 1; Cioffi et al. 2019). However, our findings do not reveal any correlation between the extensively restructured *Scleropages* karyotypes and an elevated amount of satDNAs, notwithstanding the variation in the number of signals detected in *Scleropages* species (Figure 2A,B).

Comprehending the entire repetitive DNA content and its role in the evolution is an unresolved issue, mostly due to the challenges in assembling contigs of repetitive regions, as most genome assemblies lack a comprehensive representation of repetitive DNA (Kapusta and Suh 2017; Mora et al. 2024). In past years, traditional cytogenetic methods were the primary tools for examining repetitive DNA content in chromosomes; however, advancements in sequencing techniques and bioinformatics have facilitated the emergence of a new research domain known as cytogenomics. This field study offers an in-depth examination of these repeated areas, namely in satellite DNAs. Nonetheless, the sheer number of fish species is minimal in relation to the remarkable variety of this group.

The current study elucidates the satellitome of most Arowana species, enhancing the understanding of satDNA evolution in an ancient teleost group. Our findings indicate that specific satellites, referred to as long-term conserved satDNAs, may have persisted across an extensive evolutionary time frame. We demonstrated that Arowanas' satDNA has significant dynamism, including numerous evolutionary events contributing to the diversification of these sequences. The similarities of satDNA may suggest the phylogenetic structure within this group, wherein closely related species display a higher quantity of satDNA relative to phylogenetically distant ones. Subsequent studies, including additional ancestral Teleost species, may enhance our comprehension of the evolution of ancient satellite DNA.

Acknowledgments

This research was funded by the São Paulo Research Foundation (FAPESP) grant 2021/11169-2 (F.H.S.S.), 2022/00427-3 (G.A.T.), and 2023/00955-2 (M.B.C.); and the Brazilian National Council for Scientific and Technological Development (CNPq), grant number 302928/2021-9 (M.B.C.). This study was also financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES) (PROBRAL 88887.805967/2023-00). Also, the publication of the paper was supported by DEAL (Deutsche Forschungsgemeinschaft = DFG, Germany). All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1 General features of *Scleropages leichardti* satellitome **Table S2** General features of *Scleropages jardinii* satellitome **Table S3** General features of *Osteoglossum bicirrhosum* satellitome **Table S4** General features of *Scleropages formosus* satellitome **Table S5** Abundance and divergence of Arowanas´satDNA in the genome of each analyzed species **Table S6** Shared satDNA families between Arowanas species associated with the other repetitive sequences in CENSOR-Giri **Figure S1** Workflow of the analysis carried out in the present paper. First, we perform the DNA extraction through phenol-chloroform-isoamyl alcohol, followed by BGISEQ-500 pair-end sequencing, and subsequent library filtering and trimming steps on Trimmomatic. **Figure S2** Metaphase plates of *Scleropages leichardti* and *Scleropages jardinii* showing the negative FISH of satDNAs with similarity greater than 80%. Bars = 5 μm. **Figure S3** Metaphase plates of *Scleropages formosus* and *Osteoglossum bicirrhosum* showing the localization of satDNAs with similarity greater than 80%. Bars = 5 μm. **Supplementary Figures and Tables**: inz213008-sup-0002-SuppMat.docx

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