Research Square

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# Recurrent fusions and structural rearrangements drive karyotype evolution in filefishes and triggerfishes (Tetraodontiformes)

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# Abstract

Tetraodontiformes, an ecologically and economically significant order of teleost fishes, exhibit extreme morphological specializations and extensive genomic and karyotypic reduction. The sister families Monacanthidae and Balistidae (filefishes and triggerfishes) possess reduced karyotypes whose underlying mechanisms remain poorly understood. To address this question, we performed a suite of cytogenetic analyses in *Cantherhines pullus* (Monacanthidae) and in *Melichthys niger* (two Atlantic insular populations), *Canthidermis maculata*, and *Canthidermis sufflamen* (Balistidae). The analyses included conventional staining, C-banding, Ag-NOR staining, base-specific fluorochrome staining, and fluorescence in situ hybridization (FISH) mapping of 18S and 5S rDNA and telomeric $(TTAGGG)_n$ sequences. In addition, the $(CA)_{15}$ and $(GA)_{15}$ microsatellites and the *Tol2* and *Rex3* transposable elements were mapped in *Canthidermis* species. All species shared 2n = 40 acrocentric chromosomes, except *Ca. maculata* (2n = 44; 4m+8sm+32a) and *Ca. sufflamen* (2n = 44; 2m+12sm+30a), with predominantly pericentromeric heterochromatin. Ag-NOR staining, $CMA_3$+/DAPI− banding, and rDNA mapping revealed single 18S and 5S rDNA loci, and the two populations of *M. niger* were cytogenetically identical. Microsatellites and transposable elements were dispersed along the chromosomes, with no association with specific cytogenetic features. Notably, besides terminal signals, FISH revealed interstitial telomeric sites (ITSs) on three acrocentric pairs of *C. pullus*, a hallmark of past tandem fusions. Together with Robertsonian fusions, these rarely reported sites suggest that sequential rounds of *tandem* and centric fusions independently drove karyotype reduction in Balistidae and Monacanthidae.


# Introduction

The Tetraodontiformes commonly known as puffers, porcupinefish, boxfish, filefish or triggerfish - represent a major branch in the teleost diversification (Nelson et al. 2016). This charismatic group occupies a wide range of marine and freshwater habitats worldwide and exhibits diverse morphological (Troyer et al. 2024), physiological (Liu et al. 2025), and genetic innovations. Among its adaptive traits, the body size stands out for its enormous variation (Troyer et al. 2022), ranging from approximately 20 mm in *Carinotetraodon travancoricus* (Tetraodontidae) (Devi et al. 2000) to over 3 m (Molidae), the largest known bony fish (Pope et al. 2010). They also display a remarkable arsenal of defensive adaptations, including body inflation, potent neurotoxins, dermal spines, and fused dermal plates (Tyler and Santini 2002).

From a genetic perspective, this diversity is also accompanied by a remarkable variation in the genome organization. Tetraodontiformes are notable for harboring the most compact vertebrate genomes (Clark and Elgar 2000; Crollius and Weissenbach 2005; Li et al. 2026). In the family Tetraodontidae, estimated genome sizes range from 340–400 Mb, approximately 8–9 times smaller than the human genome (Gao et al. 2016). This reduction has been primarily attributed to the loss of repetitive and non-coding DNA, a process associated with genome compaction in several teleost lineages (Neafsey and Palumbi 2003; Wang et al. 2024). Variation in genome size also occurs in other groups, such as Monacanthidae and

Balistidae (540–800 Mb), or Ostraciidae and Diodontidae (800–1100 Mb), indicating that independent genome expansions and contractions have occurred in each lineage (Venkatesh et al. 2000; Brainerd et al. 2001; Gregory 2025). Such variation is associated with chromosomal changes, which underscores the importance of cytogenetic approaches for understanding the evolutionary mechanisms in these groups.

Balistidae (triggerfishes) and Monacanthidae (filefishes) are sister lineages (Santini et al. 2013a, b), with origins in the Miocene and Oligocene, respectively (Yamanoue et al. 2009; Santini et al. 2013a, b; McCord and Westneat 2016). However, they have diverged markedly in their ecology, behavior, diversity, and body morphology. Triggerfishes comprise 39 carnivorous species with a circumtropical distribution, primarily on coral reefs and rocky shores. In turn, filefishes comprise 108 species (Fricke *et al*. 2026), largely restricted to the Indo-Pacific (with only a few Atlantic and Red Sea species; Santini et al. 2013b) and exhibit a wider range of body shapes, ecological habitats, and dietary patterns (Nelson et al. 2016). The adaptive divergence between these two closely related families raises important questions about their evolutionary histories, particularly the mechanisms underlying their karyotypic evolution (Supplementary Table 1). Balistids typically exhibit a reduced diploid number, ranging from 2n = 40–46 (e.g., Takai and Ojima 1987; Sá-Gabriel and Molina 2005), largely shaped by chromosomal fusions (Lima et al. 2011).

Although previous cytogenetic studies have proposed possible causes for the chromosome number reduction in filefishes and triggerfishes (Sá-Gabriel and Molina 2004 2005), detailed characterization remains limited (Lima et al. 2011; Sribenja et al. 2022; 2023), obscuring the mechanisms underlying their karyotypic evolution. To address this gap, we performed an integrated cytogenetic analysis of *Cantherhines pullus* and three balistid species: *Melichthys niger* (two oceanic populations), *Canthidermis maculata*, and *Canthidermis sufflamen*. Our approach combined classical banding techniques (Giemsa staining, C-banding, and Ag-NOR staining) with base-specific fluorochrome staining ($CMA_3$/DAPI) and FISH mapping of 18S rDNA, 5S rDNA, and telomeric $(TTAGGG)_n$ repeats. In addition, to explore additional dynamic karyotypic changes, we also mapped $(CA)_{15}$ and $(GA)_{15}$ microsatellite sequences and *Tol2* and *Rex3* transposable elements on the chromosomes of *Ca. maculata* and *Ca. sufflamen*. Our findings provide a comprehensive set of cytogenetic parameters for filefishes and triggerfishes, and the first direct evidence of *tandem* fusions as a mechanism driving karyotype evolution in these lineages.

## Materials and methods

# Specimen collection and mitotic chromosome preparation

Immature individuals of *Cantherhines pullus* (Ranzani, 1842) (n = 5) were collected off the coast of Salvador, Bahia, northeastern Brazil (12°58' S, 38°31' W). Samples of *Melichthys niger* (Bloch, 1786) (n = 8; 4 males and 4 females) were obtained from the central South Atlantic Ocean, near the Trindade and Martim Vaz Archipelago (n = 4; 3 males and 1 female; 20°30'13" S, 29°19'50" W) and the São Pedro and São Paulo Archipelago (n = 4; 1 male and 3 females; 00°55'15" N, 029°20'60" W). Specimens of *Canthidermis maculata* (Bloch, 1786) (n = 13; 8 males and 5 females) and *Canthidermis sufflamen*

(Mitchill 1815) (n = 7; 2 males and 5 females), were also collected in the São Pedro and São Paulo Archipelago region (Fig. 1).

## Chromosome banding and fluorochrome staining

Mitotic stimulation was induced by an intramuscular injection of a bacterial and fungal antigen complex, for 24–48 h before euthanasia (Molina et al. 2010). Specimens were then euthanized with clove oil, and fragments of the anterior kidney were dissected. Mitotic chromosomes were prepared from a cell suspension subjected to short-term culture (Gold et al. 1990). In addition, lymphocytes from 2 mL of blood, collected by cardiac puncture, were cultured in RPMI 1640 medium for 72 h at 37°C (Moorhead et al. 1960). Chromosomes were stained with 5% Giemsa solution (pH 6.8).

Heterochromatin and nucleolar organizer regions (NORs) were visualized using the C-banding (Sumner 1972) and Ag-NOR techniques (Howell and Black 1980), respectively. Chromosomes were also stained with the fluorochromes chromomycin $A_3$ ($CMA_3$) and 4',6-diamidino-2-phenylindole (DAPI), following Schweizer (1976). Metaphase spreads were analyzed using an Olympus™ BX51 epifluorescence microscope equipped with appropriate filters at 1000× magnification. Images were captured with an Olympus DP73 digital camera and processed with the Olympus cellSens software (Olympus Optical Co. Ltd.).

**Fluorescence in situ hybridization (FISH)**

## Probe preparation and labeling

The 5S rDNA (200 bp) and 18S rDNA (1400 bp) probes were obtained by PCR amplification from *Rachycentron canadum* (Teleostei, Perciformes) genomic DNA. The 5S rDNA probe was generated using primers A (5’-TAC CCG CGA TCT CGT CCG ATC-3’) and B (5’-CAG GGT GCT ATG CCG GTA AGC-3’) (Pendás et al. 1994), whereas the 18S rDNA probe was amplified using primers NS1 (5’-GTC ATA GTA TGC TTG TCT C-3’) and NS8 (5’-TCC GCA GGT TCA CCT ACG AG-3’) (White et al. 1990). The 18S rDNA and 5S rDNA probes were labeled by nick translation with digoxigenin-11-dUTP and biotin-14-dATP (Roche, Mannheim, Germany), respectively, according to the manufacturer's protocol. Telomeric $(TTAGGG)_n$ sequences were mapped using the PNA FISH Telomere Kit/FITC® (Dako) following the manufacturer's instructions.

Probes for the transposable elements *Rex3* and *Tol2* were generated by PCR amplification using genomic DNA extracted from *Canthidermis maculata*. The *Rex3* probe was amplified using primers Rex3-F (5'-CGG TGA TAA AGG GCA GCC GTC-3') and Rex3-R (5'-TGG CAG ACN GTG GTG GT-3') (Volff et al. 1999; 2000), whereas the *Tol2* probe was amplified with primers 4F (5'-ATA GCT GAA GCT GCT CTG ATC-3') and 4R (5'-CTC AAT ATG CTT CCT TAG G-3') (Kawakami and Shima 1999). These probes were labeled via nick translation (Roche, Mannheim, Germany). Additionally, the $(CA)_{15}$ and $(GA)_{15}$ microsatellite probes were prepared following the protocol of Kubat et al. (2008).

## Hybridization and detection procedures

FISH procedures were performed according to Pinkel et al. (1986). Initially, chromosome preparations were treated with RNase A (20 µg/mL in 2×SSC) at 37°C for 1 hour, followed by pepsin digestion (0.005% in 10 mM HCl) at 37°C for 10 minutes. The slides were then fixed in 1% formaldehyde for 10 minutes and dehydrated through an ethanol series. Finally, chromosomal DNA was denatured in 70% formamide/2×SSC at 72°C for 5 minutes. The hybridization mixture consisted of 50% formamide, 2×SSC, 10% dextran sulfate, and the denatured probe (5 ng/µL). Following overnight hybridization at 37°C, post-hybridization washes were performed sequentially: in 15% formamide/0.2×SSC at 42°C for 20 minutes, in 0.1×SSC at 60°C for 15 minutes, and in 0.5% Tween-20/4×SSC at room temperature.

Hybridization signals were detected using streptavidin-FITC (Vector Laboratories, Burlingame, CA, USA) for the 5S rDNA probe and anti-digoxigenin-rhodamine (Roche, Mannheim, Germany) for the 18S rDNA probe. Chromosomes were counterstained with Vectashield mounting medium containing DAPI (1.5 µg/mL) (Vector Laboratories, Burlingame, CA, USA). FISH analysis and image acquisition followed the same procedures described for fluorochrome staining.

Chromosomes were classified by their arm ratio following Levan et al. (1964). The fundamental number (FN), defined as the total number of chromosome arms, was calculated by considering metacentric (m), submetacentric (sm), and subtelocentric (st) chromosomes as bi-armed, and acrocentric (a) chromosomes as uni-armed.

## Results

Both *C. pullus* and *M. niger* have 2n = 40 chromosomes, composed exclusively of acrocentric ones (FN = 40; Fig. 2), thus consistent with some previous cytogenetic reports (Sá-Gabriel and Molina 2005; Lima et al. 2011), and no detectable differentiation was found among *M. niger* individuals from the São Pedro and São Paulo and Trindade and Martim Vaz archipelagos. Both *Canthidermis maculata* and *Ca. sufflamen* have 2n = 44 chromosomes, but distinct karyotypic formulae: *Ca. maculata* has 4 metacentric, 8 submetacentric and 32 acrocentric chromosomes (FN = 56), whereas *Ca. sufflamen* has 2 metacentric, 12 submetacentric and 30 acrocentric chromosomes (FN = 58) (Fig. 2).

The heterochromatin is predominantly pericentromeric on all chromosomes, with some additional interstitial markings on some pairs (Fig. 2b). Single Ag-NOR/18S rDNA sites, coincident with $CMA_3^+/DAPI^-$ regions, are located interstitially on chromosome pair 5 in *C. pullus*, and pericentromerically on pair 2 in *M. niger* and on pair 1 in *Ca. maculata* and *Ca. sufflamen* (Fig. 2a-c).

A single 5S rDNA locus has a pericentromeric location on pair 10 of *C. pullus* and *M. niger*, and an interstitial location on the long arm of pair 8 of *Ca. maculata* and on the short arm of pair 7 of *Ca. sufflamen* (Fig. 2c). Telomeric $(TTAGGG)_n$ repeats are detected at the termini of all chromosomes, with some additional interstitial sites (ITSs) on pairs 1, 3, and 4 of *C. pullus* (Fig. 2d).

In *Ca. maculata* and *Ca. sufflamen*, the $(CA)_{15}$ and $(GA)_{15}$ microsatellites and the *Tol2* and *Rex3* transposable elements have a predominantly diffuse distribution along the chromosomes, although some accumulation also occurs in the centromeric and telomeric regions of a few chromosome pairs (Fig. 3).

# Discussion

The striking karyotypic diversity of Tetraodontiformes (2n = 28–52; FN = 36–96) (Arai 2011) includes a distinct trend toward a chromosomal reduction in the families Monacanthidae (2n = 33/34–40) and Balistidae (2n = 40–46) (Sá-Gabriel and Molina 2005). This reduction, shared since 50 Mya (McCord and Westneat 2016), is evident when their diploid numbers are compared with the putative ancestral percomorph karyotype (2n = 48) (Motta-Neto et al. 2019), which is retained in early-diverging tetraodontiform lineages, such as Triacanthidae (Rishi 1973; Choudhury et al. 1982). This reduction has occurred repeatedly in these lineages, suggesting that it is not a random process; rather, it is likely driven by convergent chromosomal mechanisms within the superfamily Balistoidea.

The karyotypic reduction in Monacanthidae and Balistidae can be interpreted as a byproduct of karyotypic orthoselection (Molina 2007), a process in which specific chromosomal rearrangements become fixed in a recurrent manner, potentially through mechanisms such as meiotic drive (Molina et al. 2014a; b). This pattern implies that similar rearrangements have been fixed independently across lineages, possibly owing to meiotic advantages or intrinsic structural constraints, reinforcing a scenario of parallel karyotype evolution. In this framework, recurrent fusions and inversions likely represent non-random trajectories that contribute to stabilizing reduced diploid numbers, rather than merely stochastic events. This view is further supported by the largely conserved cytogenetic architecture of the studied species, including the predominance of one-armed chromosomes and the conserved organization of some other important chromosomal characteristics: a single chromosome pair bearing Ag-NORs, 18S rDNA, and GC-rich sites; one to two pairs carrying 5S rDNA clusters; and constitutive heterochromatin mainly in pericentromeric regions (Lima et al. 2011; Sribenja et al. 2022; 2023). Together, these features suggest that shared genomic constraints may underlie the directional nature of karyotype evolution in these groups.

The moderate to low amount of heterochromatin in most chromosome pairs of these species appears to be related to losses of repetitive DNA during the karyotype evolution (Petrov 2001), a characteristic condition of several teleosts (Galetti *et al*. 2000), and particularly of some tetraodontiform groups (Grützner *et al*. 1999; Mandrioli 2000; Fischer *et al*. 2004). Compact genomes can result from the cumulative effect of deletions (Li et al. 2026), in stark contrast to the genome expansions of other groups due to *tandem* duplications, insertions, polyploidy, transposable element activity, or the accumulation of repetitive sequences (Venkatesh et al. 2000).

Interstitial telomeric sequences (ITSs) have been identified in several fish species and are frequently interpreted as historical remnants of chromosomal fusions (Ocalewicz 2013). These ectopic cytogenetic

markers have revealed key chromosomal rearrangements, including cryptic *tandem* fusions (Amaro et al. 1996) and centric fusions in diverse fish groups (Reed and Phillips 1995; Cioffi et al. 2010; 2011). Beyond their well-documented role in general karyotypic rearrangements, as exemplified in salmonids (Phillips and Ráb 2001; Pomianowski et al. 2012), chromosomal fusions also contribute to more specific evolutionary pathways. In Monacanthidae, for example, they are linked to the establishment of multiple sex chromosome systems with male heterogamety (Murofushi et al. 1980; Sá-Gabriel and Molina 2004; Nirchio and Oliveira 2007). This rare condition among fishes (Devlin and Nagahama 2002) may promote reproductive isolation (Kitano and Peichel 2012) and greater phenotypic divergence (McCord and Westneat 2016), thereby potentially driving independent evolutionary trajectories among lineages.

In Monacanthidae and Balistidae, the occurrence of ITSs might be anticipated given the multiple sex chromosome systems and the consistent reduction in diploid number (Lima et al. 2011; Sribenja et al. 2022; 2023; Supplementary Table 1). However, such sequences had not been previously reported in these families. So, against this cytogenetic background, we are now clearly demonstrating their occurrence in *C. pullus*. These ectopic signals provide direct evidence for at least three cryptic *tandem* fusion events in this species, underscoring the role of these rearrangements as an active mechanism of karyotypic differentiation. However, the absence of ITSs in the balistids analyzed does not necessarily imply the absence of past fusions in these species. This scenario is well documented in other reef fish families, as in Acanthuridae and Pomacentridae, where the degeneration or non-incorporation of ITSs during fusion events was reported (Molina and Galetti 2002; Fernandes et al. 2015; Getlekha et al. 2016), potentially obscuring their detection.

The transposable elements *Tol2* and *Rex3* showed a dispersed distribution across the chromosomes of *Canthidermis* species, with no specific association with the most variable chromosome types in the karyotypes. Such elements may cause mutations and alter gene expression or structure (Kapitonov and Jurka 2007), promoting structural and functional genomic changes (Böhne et al. 2008), including increases or decreases in genome size (Feschotte and Pritham 2007). The absence of differential accumulation on specific heterochromatic regions or on the microsatellites analyzed in *Canthidermis* contrasts with the distribution patterns reported for other fish groups, in which clear co-localization with heterochromatin-rich regions has been documented (Ozouf-Costaz et al. 2004; Gross et al. 2009; Valente et al. 2011). This predominantly diffuse distribution suggests that transposable elements play only a secondary role in the chromosomal restructuring of these species, unlike other teleost groups, where such elements act as major drivers of genomic reorganization (Venkatesh et al. 2000).

The rDNA regions are notable for their evolutionary dynamism in frequency, location, and organization (Gornung 2013). Indeed, Ag-NOR/GC-rich/18S rDNA and 5S rDNA clusters have high recombination rates (Redi et al. 1990), frequently acting as rearrangement hotspots (Sola et al. 2003). However, numerical variation of rDNA sites is markedly reduced in Monacanthidae and Balistidae (Lima et al. 2011; this study), a pattern also reported for other tetraodontiform families, including Diodontidae, Ostraciidae, and Tetraodontidae (Noleto et al. 2007; Martinez et al. 2011; Vicari et al. 2012). In all species analyzed, the

5S rDNA sites were located on chromosomes distinct from those bearing the 18S rDNA sites, suggesting an ancestral condition conserved across different fish groups (Motta-Neto et al. 2011).

In Monacanthidae and Balistidae, the distribution of rDNA sites suggests an evolutionarily stable condition, although some arrays may have participated in karyotypic changes. This assertion is supported by the absence of interpopulation polymorphisms across geographically distant populations of *M. niger*, as well as by simple and conserved rDNA sites overall. However, the trend of rDNA regions in participating of fusion or inversion events is consistent with the karyotypic reduction and marked diversification observed in *Canthidermis* species. In fact, ribosomal sites can facilitate some events, such as centric fusions, without detectable ITS traces, as documented in Pomacentridae (Molina and Galetti 2002; Getlekha et al. 2016) and Loricariidae (Rosa et al. 2012; Glugoski et al. 2020). Thus, the pericentromeric 18S rDNA loci in the large metacentric chromosome of *Canthidermis* species may be indicative of a centric fusion. In addition, the interstitial 5S rDNA loci on pair 5 of *Ca. maculata* potentially points to internal chromosomal reorganization resulting from *tandem* fusions.

Overall, the available evidence is consistent with a model in which an ancestral karyotype (2n = 48) underwent progressive reduction through centric and *tandem* fusions, followed by structural refinements via pericentric inversions. This process, coupled with the relative stability of rDNA loci and the limited structural role of transposable elements, may define a distinctive trajetory of karyotype evolution in Monacanthidae and Balistidae.

# Conclusions

Genomic minimization is a recognized in Tetraodontiformes, and, in this context, the sister families Monacanthidae and Balistidae offer a compelling case. Despite their ecological and morphological divergence, they share a trend toward karyotypic reduction. Centric fusions appear to play a main role in this process, leading to reduced karyotypes and the occurrence of multiple sex chromosomes in filefishes, and a congruent pattern of pericentric inversions (or centromere repositioning) favors bi-armed chromosomes in triggerfishes. These findings implicate *tandem* fusions in the karyotypic evolution of filefishes. Taken together, centric and *tandem* fusions, indicate exclusive and recurrent pathways that drive karyotypic evolution within Balistoidea.

# Declarations

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### Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

### Consent for Publication

Not applicable.

### Consent to Participate

Not applicable.

### Ethics Approval

All procedures involving animals were conducted in accordance with national and international guidelines for the care and use of laboratory and wild animals and were approved by the Ethics Committee on Animal Use of the Federal University of Rio Grande do Norte (CEUA/UFRN) (Proc. # 41/2015). Specimen collections were authorized by the Ministry of the Environment through the Biodiversity Information and Authorization System (SISBIO/ICMBio).

### Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and cytogenetic experiments were performed by Juliana G. Bezerra, Glaicon S. Santos, and Gideão W. W. F. Costa. Data analysis and cytogenetic interpretation were carried out by Juliana G. Bezerra, Glaicon S. Santos, and Wagner F. Molina. The first draft of the manuscript was written by Juliana G. Bezerra, Glaicon S. Santos, and Wagner F. Molina, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

### Competing Interests

The authors declare that they have no competing financial or non-financial interests directly or indirectly related to the work submitted for publication.

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# Figures

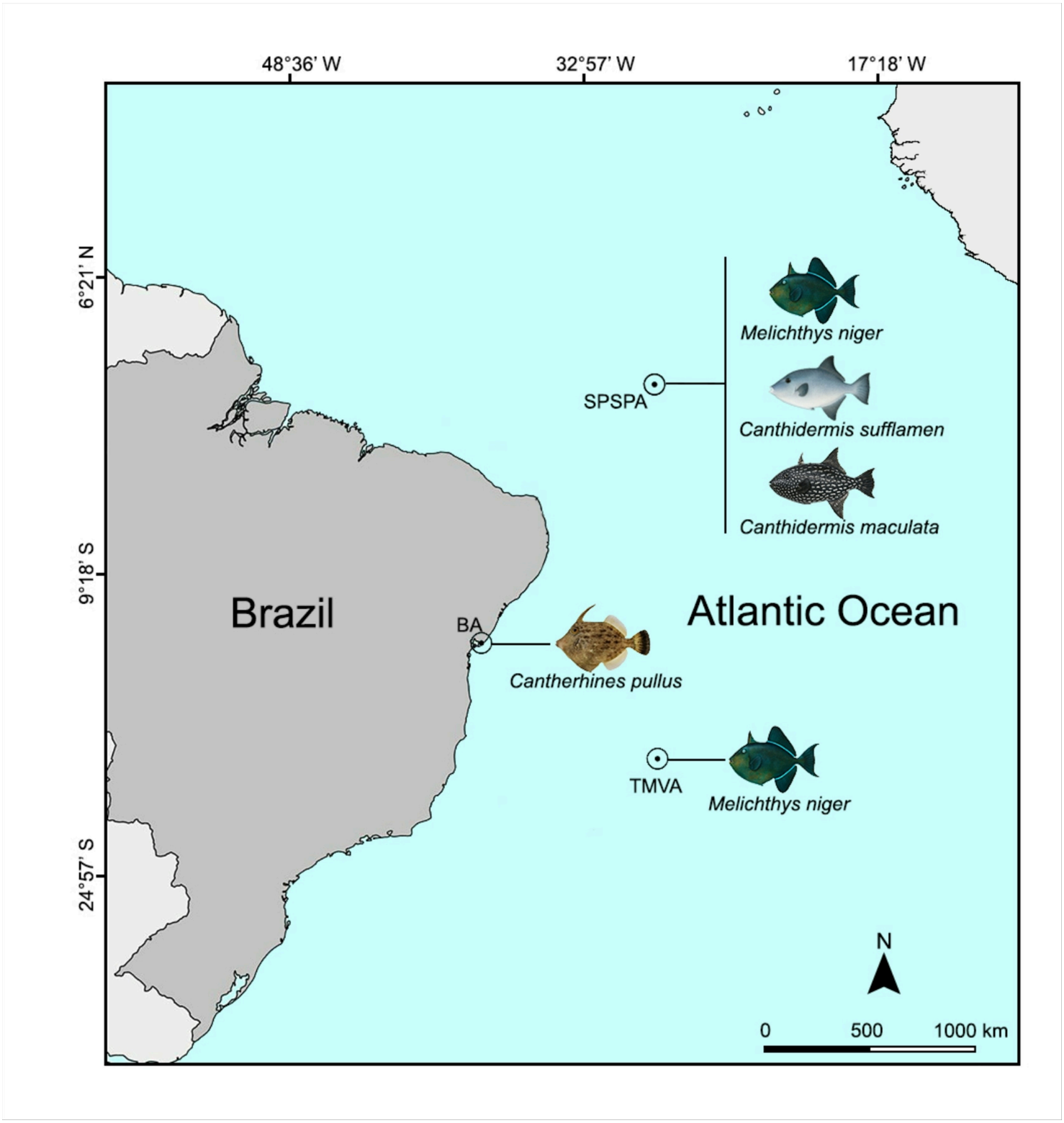
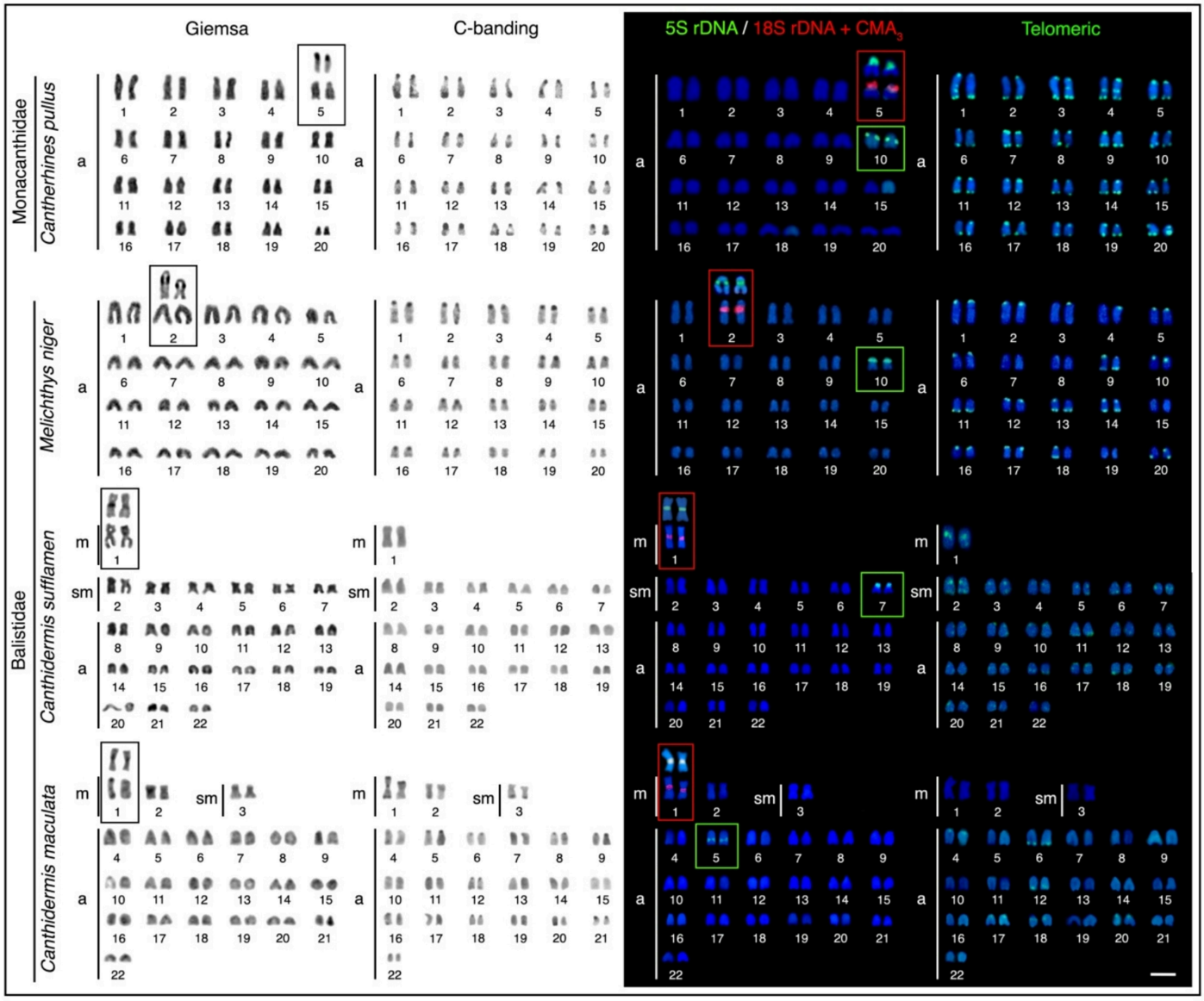



**Figure 1**

Collection sites of *Cantherhines pullus*, *Melichthys niger*, *Canthidermis sufflamen* and *Canthidermis maculata* in the Atlantic Ocean. BA: Bahia (Brazil); SPSPA: São Pedro and São Paulo Archipelago; TMVA: Trindade and Martim Vaz Archipelago.




## Figure 2

Comparative cytogenetic data of *Cantherhines pullus* (Monacanthidae), *Melichthys niger*, *Canthidermis sufflamen*, and *Canthidermis maculata* (Balistidae). Karyotypes are arranged according to Giemsa-stained chromosomes (left), C-banded chromosomes (middle), and FISH-mapped chromosomes (right) showing 5S rDNA (green), 18S rDNA (red), $CMA_3$ signals (highlighted by red boxes) and telomeric $(TTAGGG)_n$ sequences (green). Regions of interest are highlighted with colored boxes. Scale bar = 5 μm.

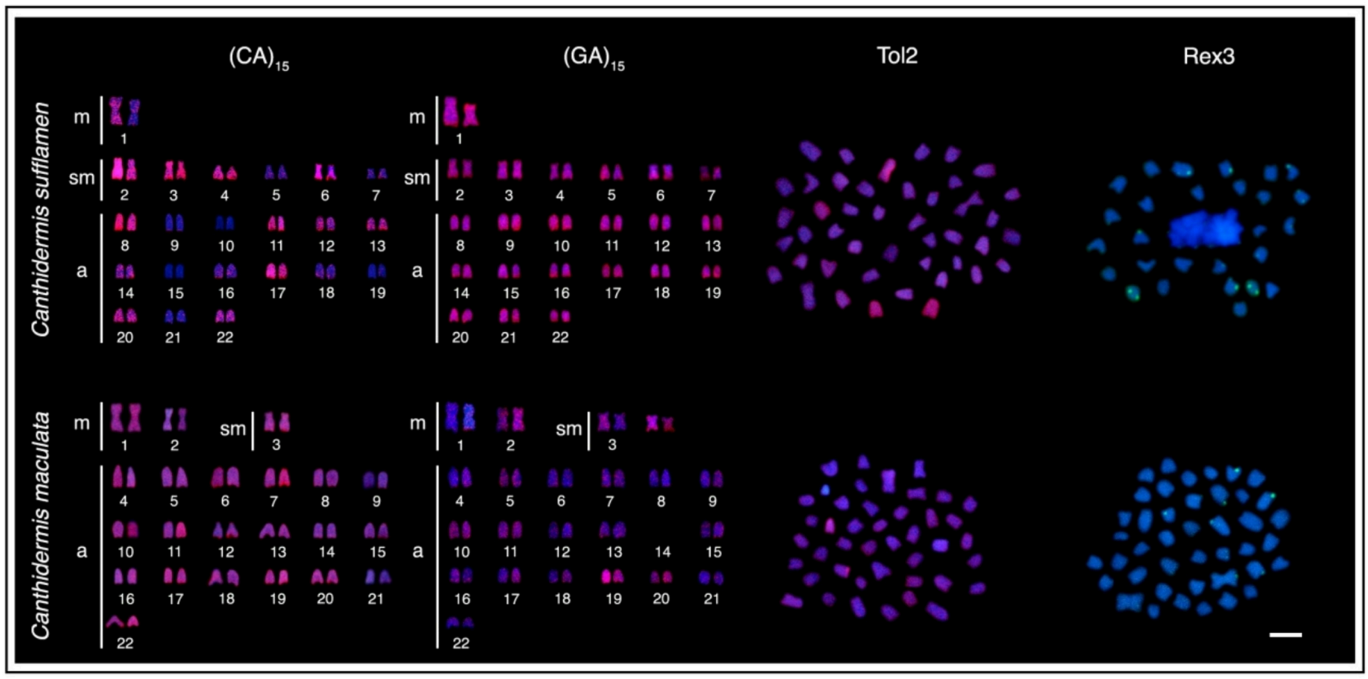



**Figure 3**

Fluorescence *in situ* hybridization (FISH) mapping of repetitive DNA and transposable elements in *Canthidermis sufflamen* and *Canthidermis maculata*. Organized karyotypes (left) show the chromosomal distribution of $(CA)_{15}$ and $(GA)_{15}$ microsatellites. Metaphase spreads (right) display the mapping of *Tol2* and *Rex3* transposable elements. Red signals indicate probe hybridization, and chromosomes are counterstained with DAPI (blue). Scale bar = 5 μm.

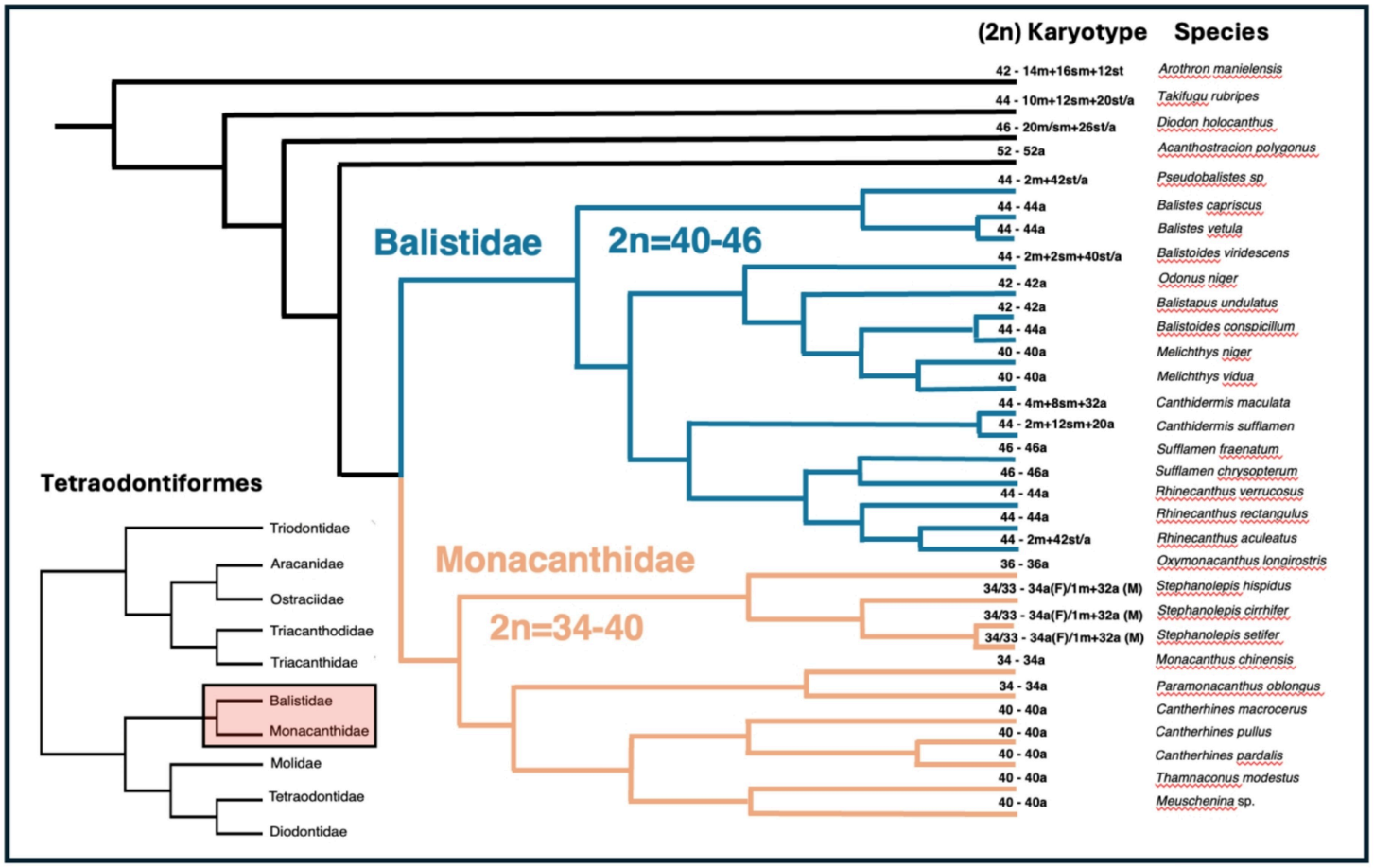



**Figure 4**

Phylogeny of the order Tetraodontiformes illustrating karyotypic evolution within the Balistidae and Monacanthidae. The phylogenetic framework was adapted from Santini et al. (2013b), with the internal topology of the Balistoidea (Balistidae + Monacanthidae) following McCord and Westneat (2016). Karyotype data, including diploid chromosome numbers and chromosomal morphology, were primarily sourced from Arai (2011) (Supplementary Table 1). Branches and terminal taxa are color-coded to indicate the distribution of the karyotypic characteristics across the two families, highlighting the diversification of chromosomal forms within lineages.

## Supplementary Files

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- SupplementaryTable1.docx