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Are the chromosomal fusions that shaped the karyotype of *Tetranematichthys wallacei* (Siluriformes: Auchenipteridae) a shared feature among Ageneiosini species?

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The genus *Tetranematichthys* has only three species, and none of them have undergone cytogenetic analyses. Therefore, this study brings for the first time the analysis of *Tetranematichthys wallacei*, collected from the Igarapé Apaú, Guamá River basin, municipality of Castanhal, Pará State, Brazil. The diploid number found was 52 chromosomes (32m+18sm+2st, NF = 104), in both sexes, with predominantly terminal and some interstitial heterochromatin. Telomeric sequences were observed exclusively in terminal regions. The 18S rDNA sites were found on pair 17sm of all specimens and in only one of the homologous of pair 7 in three specimens. The 5S rDNA sites were found in pairs 8m and 10m. *Tetranematichthys wallacei* exhibits characteristics worthy of attention regarding its current phylogenetic position, including a probable diploid number reduction. Additionally, it shares with *Tympanopleura atronasus* the 18S rDNA allocated in the long arm of a large sm chromosome (first pair) but does not share with *Ageneiosus* the large first m pair with evidence of fusion, as observed in *Ageneiosus inermis*. The chromosomal data generated for *T. wallacei*, along with the data from the other two previously studied Ageneiosini taxa, reinforces proposals from morphology-based studies suggesting that the tribe represents the most distinct clade within the family.

Keywords: Chromosomal evolution, Driftwood catfish, Diploid number reduction, Interstitial telomeric sites, rDNA polymorphism.

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O gênero *Tetranematichthys* possui apenas três espécies, e nenhuma delas tinha sido submetida a análises citogenéticas. Assim, este estudo traz pela primeira vez a análise de *Tetranematichthys wallacei*, coletado no Igarapé Apaú, bacia do rio Guamá, cidade de Castanhal, estado do Pará, Brasil. O número diploide de 52 cromossomos (32m+18sm+2st, NF = 104) foi encontrado em ambos os sexos, com heterocromatina predominantemente terminal e algumas intersticiais. A sequência telomérica foi observada exclusivamente em regiões terminais. Os sítios de 18S rDNA foram encontrados no par 17sm de todos os exemplares e em apenas um dos homólogos do par 7 em três exemplares. Os sítios de 5S rDNA foram encontrados nos pares 8m e 10m. *Tetranematichthys wallacei* possui algumas características que dignas de quanto à sua posição filogenética atual, incluindo uma provável redução no número diploide. Além disso, *T. wallacei* compartilha com *Tympanopleura atronasus* o 18S rDNA alocado no braço longo de um grande cromossomo sm (primeiro par), mas não compartilha com *Ageneiosus* o primeiro par m grande com evidências de fusão, como observado em *Ageneiosus inermis*. Os dados cromossômicos gerados para *T. wallacei*, juntamente com os dados dos outros dois táxons de Ageneiosini estudados anteriormente, reforçam propostas de estudos baseados em morfologia que sugerem que a tribo representa o clado mais diferenciado dentro da família.

Palavras-chave: Bagres de troncos, Evolução cromossômica, Polimorfismo de DNAr, Redução do número diploide, Sítios teloméricos intersticiais.

INTRODUCTION

The Neotropical region extends from Mexico to Argentina and the Caribbean (Morrone, 2014), with an unparalleled representation in terms of fish biodiversity (Reis *et al*., 2016). The Auchenipteridae family comprises 128 valid species distributed across 25 genera (Fricke *et al*., 2024). *Ageneiosus* Lacepède, 1803 and *Tetranematichthys* Bleeker, 1858 belong to the tribe Ageneiosini within Auchenipterinae and have a closely linked taxonomic history, being considered sister groups (*e.g*., Bleeker, 1862; Miranda Ribeiro, 1911; Britski, 1972; Ferraris, 1988; Royero, 1999; Birindelli, 2014). These genera are so closely related that the first species of *Tetranematichthys* was initially described and classified within *Ageneiosus* (*Ageneiosus quadrifilis*; Walsh *et al*., 2015). Only a few years later, this species was reclassified as *Tetranematichthys quadrifilis* (Kner, 1858), leading to the establishment of the genus *Tetranematichthys* (Vari, Ferraris, 2006). Recently, Calegari *et al*. (2019) carried out research based on both morphological and molecular data in Auchenipteridae and concluded that *Tetranematichthys* and *Ageneiosus* share sufficient similarities, from both phylogenetic and historical perspectives, to be considered monophyletic sister clades.

Cytogenetic studies are increasingly valuable tools for exploring biodiversity in diverse fish groups (Cioffi, Bertollo, 2012; Ditcharoen *et al*., 2019). Classical characterization and banding techniques, combined with chromosomal mapping of repetitive DNA sequences, have become important to understand karyotypic

congruences and divergences (Bertollo *et al*., 2017). It can demonstrate characters that are usually not accessible by other research methods, contributing to the visualization of possible evolutionary paths in distinct groups of fish due to their specific chromosomal and genomic characteristics (Cioffi *et al*., 2018; Ditcharoen *et al*., 2019). One of the best well-known examples of the importance of cytogenetic analyses is the Wolf Fish *Hoplias malabaricus* (Bloch, 1794) (Characiformes, Erythrinidae). Although very similar morphologically, this is a species complex composed of seven major karyomorphs (A-G), which is possibly reproductively isolated and has been mainly diagnosed through cytogenetic methods (reviewed in Cioffi *et al*., 2018). Another noteworthy example is *Astyanax scabripinnis* (Jenyns, 1842), which was initially composed of six populations from different Brazilian watersheds. However, cytogenetic studies were pioneering in demonstrating a great hidden biodiversity within this taxon, which is currently recognized as a species complex with more than 30 species (reviewed in Cioffi *et al*., 2018). Other important examples can also be found in less-known Neotropical fish groups. For instance, within Auchenipteridae, a species complex is suggested for *Trachelyopterus galeatus* (Linnaeus, 1766) (Siluriformes: Auchenipteridae, Santos *et al*., 2021). Cytogenetic data was also crucial to identify hidden diversity within *Ancistrus* Kner, 1854 (Siluriformes, Loriicaridae) from the Paraná River basin (Prizon *et al*., 2017), and it was used to suggest the reallocation of genera in Hypostomini catfishes (Siluriformes, Anjos *et al*., 2019).

However, out of the 25 currently valid genera in Auchenipteridae, chromosomal data are available for only eight genera: *Ageneiosus*, *Auchenipterus* Valenciennes, 1840, *Centromochlus* Kner, 1858, *Entomocorus* Eigenmann, 1917, *Glanidium* Lütken, 1874, *Trachelyopterus* Valenciennes, 1840, *Tatia* Miranda Ribeiro, 1911, and *Tympanopleura* Eigenmann, 1912 (Tab. 1). Notably, within the Ageneiosini tribe, cytogenetic investigations have been conducted only on *Ageneiosus* and *Tympanopleura*, with *Tetranematichthys* remaining unstudied. *Tetranematichthys* represents one of the earliest divergent lineages within Ageneiosini and is regarded as the sister group of *Ageneiosus* + *Tympanopleura* (Calegari *et al*., 2019). Therefore, the chromosomal characterization of *Tetranematichthys* species is essential for a more comprehensive understanding of the chromosomal evolutionary trajectory within Ageneiosini, including potential apomorphic and plesiomorphic conditions. *Tetranematichthys* currently comprises three species: *T. barthemi* Peixoto & Wosiacki, 2010, *T. quadrifilis*, *T. wallacei* Vari & Ferraris, 2006. This paper presents the first chromosomal analysis of a *Tetranematichthys* species: *Tetranematichthys wallacei*.

TABLE 1 | An overview of cytogenetic data in Auchenipteridae. 2n: diploid number; FN: fundamental number; SS: sex chromosome system; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; p: short arm; q: long arm; i: interstitial; t: terminal. CA: chromosome arm; PCA: position on the chromosome arm; CM: chromosome morphology; CITS: chromosome with interstitial telomere sequence; NI: ITS not investigated; ND: ITS not detected; Ref: References. AM: Amazonas; GO: Goiás; PA: Pará; PR: Paraná; MT: Mato Grosso; MG: Minas Gerais; MS: Mato Grosso do Sul; RN: Rio Grande do Norte. References: 1. Fenocchio, Bertollo (1992); 2. Lui *et al*. (2013a); 3. Present study; 4. Ravedutti, Júlio (2001); 5. Machado *et al*. (2021); 6. Santos *et al*. (2021); 7. Haerter *et al*. (2022); 8. Lui *et al*. (2021); 9. Lui *et al*. (2010); 10. Araújo, Molina (2013); 11. Fenocchio *et al*. (2008); 12. Lui *et al*. (2015); 13. Lui *et al*. (2013b); 14. Kowalski *et al*. (2020); 15. Kowalski *et al*. (2024); 16. Haerter *et al*. (2023); 17. Felicetti *et al*. (2023); 18. Felicetti *et al*. (2021); *species cited with a name different from that currently valid.

Species	Locality	2n	FN	Karyotype formula					AgNORs/18S rDNA				5S rDNA				CITS	Ref
				SS	m	sm	st	a	Pair	CA	PCA	CM	Pair	CA	PCA	CM		
Auchenipterinae																		
Ageneiosini																		
Ageneiosus inermis (*cited as *Ageneiosus brevifilis*)	Solimões River, Manaus (AM), Brazil	56	102	-	20	16	10	10		p	t	sm	-	-	-	-	NI	1*
	Araguaia River, Aragarças (GO), Brazil	56	108	-	32	16	4	4	20	p	t	sm	4	p		m	pair 1m, p	2
Tympanopleura atronasus (cited as *Ageneiosus atronasus*)	Solimões River, Manaus (AM), Brazil	56	100	-	16	16	12	12	-	q	i	sm	-	-	-	-	NI	1
Tetranematichthys wallacei	Guamá River (PA), Brazil	52	104	-	32	18	2	-	17	q	t	sm	8 10	p p	t t	m m	ND	3
Auchenipterini																		
Auchenipterus nuchalis	Araguaia River, Aragarças (GO), Brazil	58	110	-	22	16	14	6	14	p	t	sm	22	p	t	st	NI	5
Auchenipterus osteomystax (cited as *Auchenipterus nuchalis*)	Paraná River, Porto Rico (PR), Brazil	58	106	-	24	14	10	10	15	p	i	sm	-	-	-	-	NI	4
Entomocorus radiosus	Paraguai River, Poconé (MT), Brazil	58	106	-	22	12	14	10	21	p	t	st	12 13 14 15 16 18 19	p p p p p p p	t t t t t t t	sm sm sm sm sm st st	NI	5
Trachelyopterini																		
Trachelyopterus coriaceus	Araguaia, São Miguel do Araguaia (GO), Brazil	58	108	-	20	18	12	8	23	p	t	st	3 16	p q	t i	m sm	NI	6,7, 16
Trachelyopterus aff. *coriaceus* (*cited as *Trachelyopterus* sp.)	Bento Gomes, Poconé (MT), Brazil	58	108	-	22	20	8	8	22	p	t	st	16 18	p q	i t	sm sm	ND	8*, 16
Trachelyopterus galeatus (*cited as *Parauchenipterus galeatus*)	Amazonas River, Catalão Lake, Manaus (AM), Brazil	58	106	-	20	12	18	8	20	p	t	sm	14 17	p q	t t	sm sm	ND	7, 16 17, 18
	Paraná River, Paraná River basin (MS), Brazil	58	108	-	24	18	8	8	25	p	t	sm	16 17	p q	i i	sm sm	NI	9*
	Piumhi River, Paraná River basin (MG), Brazil	58	108	-	20	16	14	8	24	p	t	st	15 16	p q	i i	sm sm	NI	9*
	Lagoa da Prata, São Francisco River basin (MG), Brazil	58	108	-	22	16	12	8	23	p	t	st	16 17	p q	i i	sm sm	ND	9*
	Pium River, Paranamirim (RN), Brazil	58	108	-	24	16	10	8	-	p	-	sm	-	-	-	-	NI	10*
	Paraná River, Porto Rico (PR), Brazil	58	98		22	12	6	18	23	p	t	a	-	-	-	-	NI	4*
	Araguaia River, São Miguel do Araguaia (GO), Brazil	58	108	-	20	18	12	8	24	p	t	sm	3	p	t	m	NI	6*, 7, 16
	Miranda River, Paraguay River basin (PY), Brazil	58	108	-	24	12	14	8	24	p	t	st	14 17	p q	t i	sm sm	ND ND	17, 18
Trachelyopterus porosus	Catalão Lake, Amazonas River basin (AM), Brazil	58	106	-	22	16	10	10	23	p	-	st	3 4	p p	i t	m m	ND	7, 16, 17, 18
	Miranda River, Paraguay River basin (MS), Brazil	58	106	-	22	16	10	10	23	p	t	st	3 4	p p	i t	m m	ND	17

TABLE 1 | (Continued)

Species	Locality	2n	FN	Karyotype formula					AgNORs/18S rDNA				5S rDNA				CITS	Ref
				SS	m	sm	st	a	Pair	CA	PCA	CM	Pair	CA	PCA	CM		
Trachelyopterus striatulus (*cited as *Parauchenipterus striatulus*)	Doce River basin, Mariléia (MG), Brazil	58	106	-	18	20	10	10	23	p	t	st	10 13 15	p p q	i i i	sm sm sm	NI	6*, 16
Centromochlinae																		
Glanidiini																		
Glanidium ribeiroi	Iguaçu River, Reservoir Salto Caxias (PR), Brazil	58	112	-	28	16	10	4	17	p	i	sm	-	-	-	-	NI	4
	Iguaçu River, Reservoir Segredo (PR), Brazil	58	106	-	22	16	10	10	13	p	i	sm	-	-	-	-	NI	11
	Iguaçu River, Reservoir Salto Osório (PR), Brazil	58	106	-	22	16	10	10	13	p	i	sm	-	-	-	-	NI	11
	Iguaçu River, Capanema (PR), Brazil	58	110	-	22	20	10	6	14	p	i	sm	16	q	i	sm	ND	12
Tatia jaracatia	Iguaçu River, Capanema (PR), Brazil	58	116	-	20	26	12	-	28	p	t	st	4 18 19 29	p p q p	i t i t	m sm sm st	NI	13
Tatia neivai	Machado River, Denise (MT), Brazil	58	116	-	26	26	6	-	28	p	t	st	4 21 22	p p q	i t i	m sm sm	NI	13
Centromochlini																		
Centromochlus heckelii	Solimões River, Manaus (AM), Brazil	46	72	♂ ZZ	14	6	6	20	20 ZZ	p p	t p	a st	-				NI	14
				♀ ZW	15	6	5	20	20 ZW	p p	p t	a st/m						
Centromochlus schultzi	Xingu River basin, Altamira (PA), Brazil	58	108	-	26	16	8	8	24	p	i	st	4 24 27 28	p p p p	i i t t	m st a a	pair 1m, p pair 3m, c	15

MATERIAL AND METHODS

In this study, 11 individuals (3 females and 8 males) of *T. wallacei* were collected from the Igarapé Apaú, Guamá River basin, 01°23'20.5"S 47°59'07.4"W, in municipality of Castanhal, Pará State, Brazil (Fig. 1). The mitotic chromosomes were obtained from anterior kidney cells according to Bertollo *et al.* (2015). The animals were euthanized by an overdose of clove oil (Griffiths, 2000) and deposited at the ichthyology collection of the Universidade Tecnológica Federal do Paraná, Santa Helena (Voucher ID CISH 861). Chromosomal morphology was determined following the protocol outlined by Levan *et al.* (1964). The fundamental number (NF) was calculated considering metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes as having 2 arms, and acrocentric (a) as having only 1 arm. Nucleolus organizing regions (AgNORs) were visualized through silver nitrate impregnation (Howell, Black, 1980) and heterochromatin distribution was determined according to the C-band technique described by Sumner (1972), with modifications in staining step as proposed by Lui *et al.* (2012).

Fluorescent *in situ* hybridization (FISH) was performed according to Pinkel *et al.* (1986), with modifications suggested by Margarido, Moreira-Filho (2008). A stringency of 77% was applied for the 18S and 5S rDNA probes (200ng of each probe, 50% deionized formamide, 10% dextran sulfate, 2x SSC, at 37 °C overnight). Similarly, fluorescent *in situ* hybridizations with telomeric probes were initially performed using 77% of stringency. However, due to a probable diploid number reduction in this species,

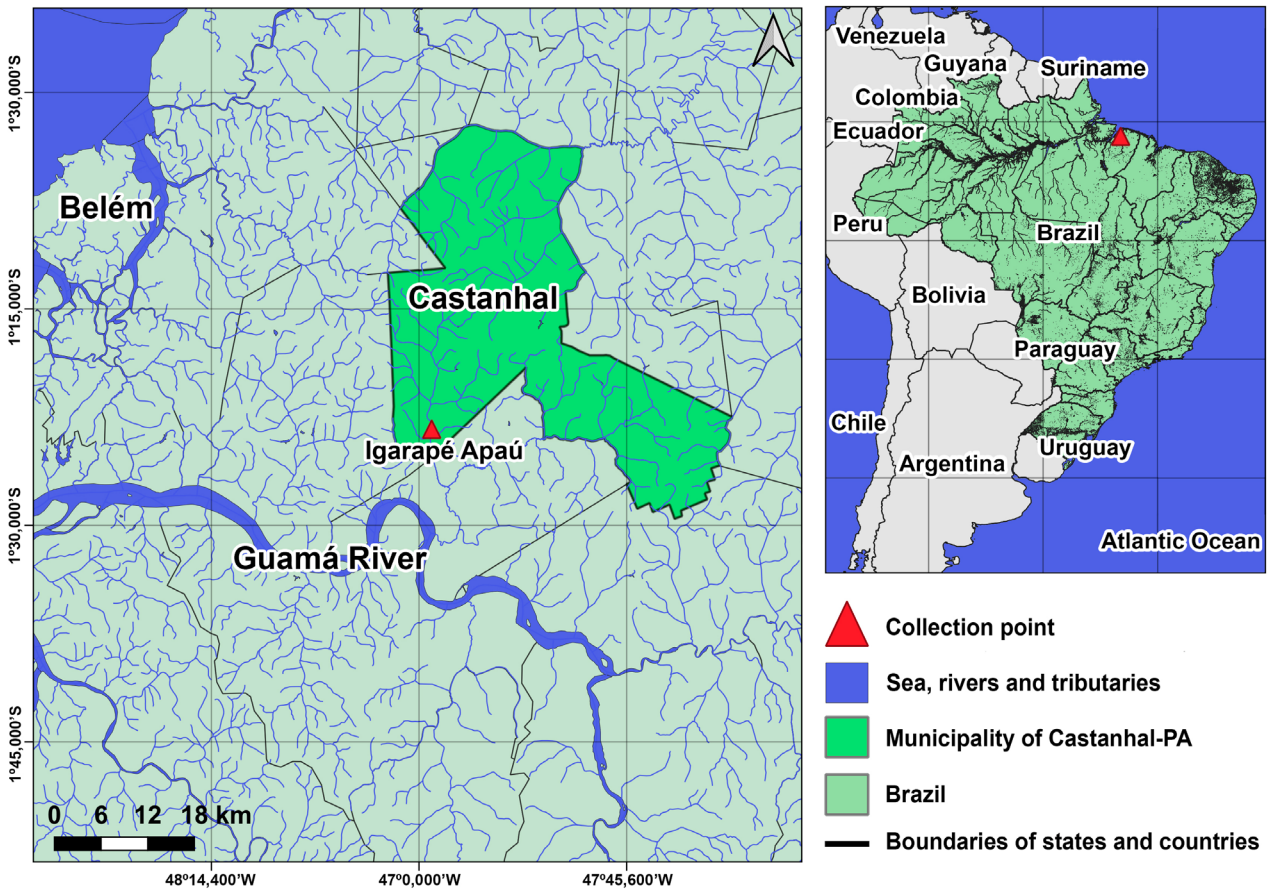

FIGURE 1 | Map showing the sampling location of *Tetranematichthys wallacei* from the Guamá River basin. Geographical data source: Instituto Brasileiro de Geografia e Estatística (IBGE). Datum: SIRGAS 2000.

FISHs were also carried out at 62% stringency to identify potential degenerated ITSs. The 5S rDNA and 18S rDNA probes were obtained from minipreps of *Megaleporinus elongatus* (Valenciennes, 1850) (Martins, Galetti Jr., 1999) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti, 2004), respectively. They were labeled with Digoxigenin-11-dUTP (Dig-Nick-Translation Mix, Roche, according to the manufacturer's instructions) and detected using Anti-Digoxigenin-Rhodamine or labeled with Biotin-16-dUTP (Bio-Nick-Translation Mix, Roche, according to the manufacturer's instructions) and detected with Streptavidin-FITC (Roche).

The telomeric probes were generated and labeled by PCR reaction using the primers described by Ijdo *et al.* (1991) with the following specifications: 1x buffer, 25 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, 0.5 U of Taq DNA polymerase (Roche) and 0.025 mM Tetramethyl-Rhodamine-5-dUTP (Roche). The PCR conditions were: 95°C (1 min), 10 cycles of 95°C (1 min), 55°C (30 s) and 72°C (1 min); of 30 cycles of 95°C (1 min), 60°C (30 s) 72°C (30 s), and final extension at 72°C (1 min). Digital images were captured using the DP Controller 3.2.1.276 software with an Olympus DP71 digital camera connected to a BX61 epifluorescence microscope (Olympus America Inc., Center Valley, PA, United States of America).

RESULTS

The diploid number observed for *T. wallacei* was 2n = 52 chromosomes (32m + 18sm + 2st, NF = 104), with no differences between the sexes (Fig. 2A). Most heterochromatic blocks appeared pale and were located at the terminal regions; however, some pericentromeric blocks were also observed in pairs 2m, 6m, and 17sm, and centromeric blocks were evident in pairs 1m, 4m, 5m, 18sm, and 20sm (Fig. 2B). Fluorescent in situ hybridization (FISH) with 18S rDNA probes revealed markings at the terminal positions on the long arm of pair 17sm in all analyzed specimens (Fig. 3A). Additionally, three individuals (two males and one female) exhibited an additional 18S rDNA site on a single chromosome of pair 7m, located at the terminal position of its short arm (Fig. 3A, in box). No specimen in our sample exhibited two pairs completely marked (four chromosomes) with the 18S rDNA probes. The AgNORs were coincident with the 18S rDNA sites on pair 17 (Fig. 2A, in box). FISHs with 5S rDNA probes showed this cistron is located at the proximal position of the short arm at the pairs 8m and 10m (Fig. 3A). FISH with the telomeric sequence $(TTAGGG)_n$ revealed markings exclusively at the terminal regions, with no presence of interstitial telomeric sites (ITS) (Fig. 3B).

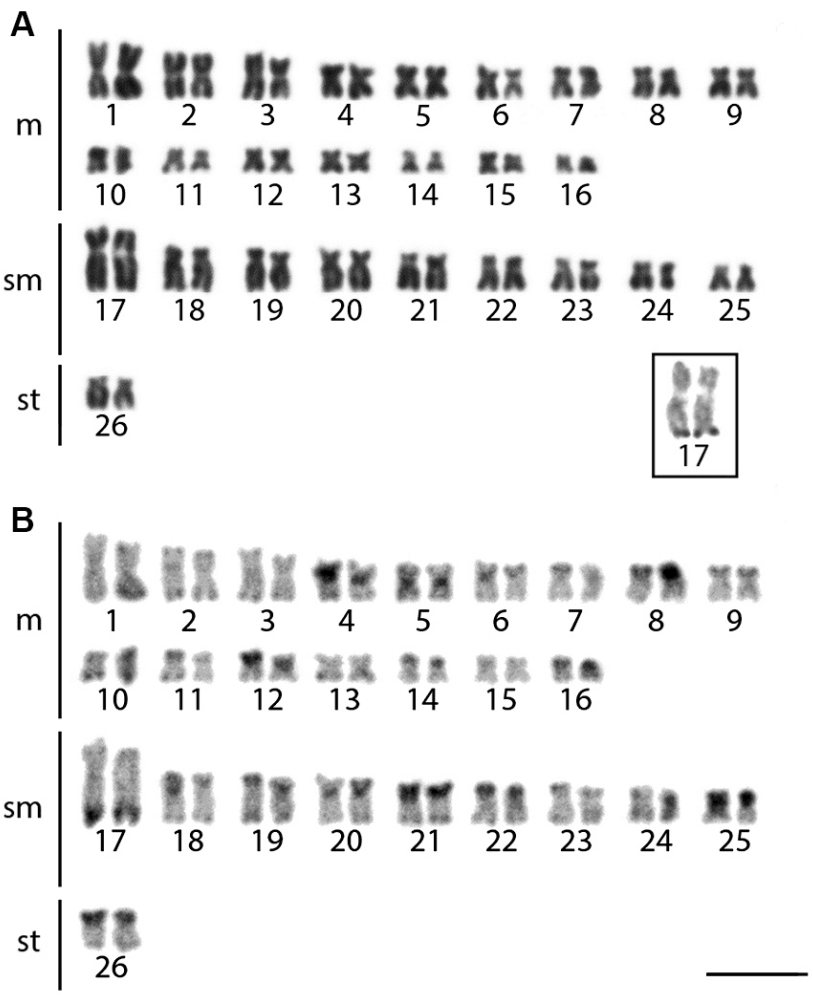

FIGURE 2 | **A.** Karyotype of *Tetranematichthys wallacei* Stained with Giemsa; and **B.** Karyotype of *T. wallacei* sequentially submitted to C-banding. AgNORs presented in the box. Scale bar = 10 µm.

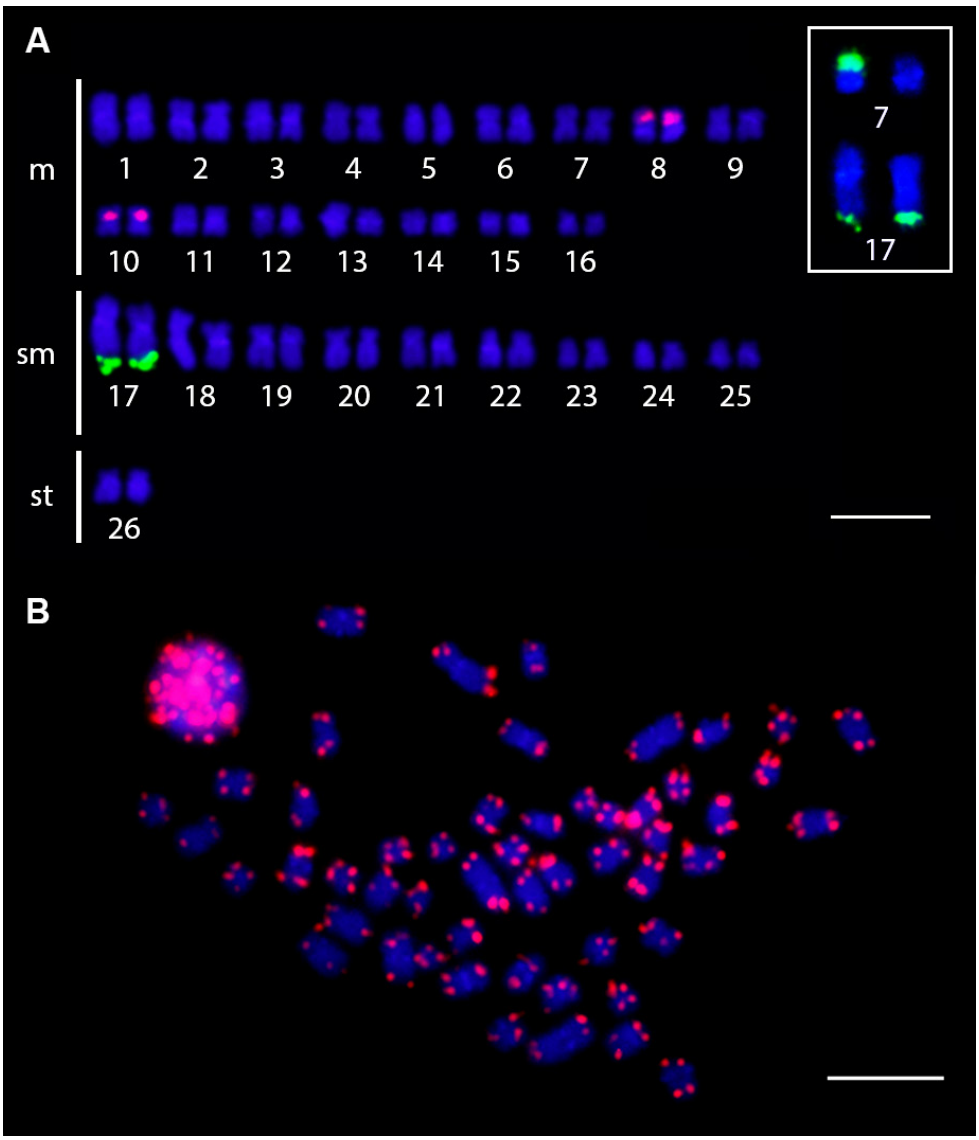

FIGURE 3 | **A.** Karyotype of *Tetranematichthys wallacei* hybridized with 18S rDNA (green signal in pair 17) and 5S rDNA (red signal in pairs 8 and 10) probes, counterstained with DAPI. The 18S rDNA polymorphism, represented by an additional chromosome carrying the 18S rDNA sites (pair 7) are presented in the box. **B.** Methaphase plate of *T. wallacei* hybridized with telomeric probes under 77% of stringency. Scale bars = 10 µm.

DISCUSSION

Doradidae and Auchenipteridae are the only families within the superfamily Doradoidea, a monophyletic clade supported by several morphological and molecular data (Birindelli, 2014; Calegari *et al*., 2019). The cytogenetic studies on most Auchenipteridae species have revealed a diploid number (2n) of 58 chromosomes (Tab. 1). This 2n is also prevalent in the sister group Doradidae (Takagui *et al*., 2021), which may suggest that it represents the plesiomorphic condition for both families, as indicated by other studies (Baumgärtner *et al*., 2016; Kowalski *et al*., 2020; Machado *et al*., 2021). However, the reconstruction

of the basal diploid number (2n) in Doradidae provided limited support for the 58 chromosomes as the plesiomorphic condition, as it was equally parsimonious as the 2n = 56 (Takagui *et al*., 2021). The low support values in this case may be attributed to the small number of species karyotyped so far, a challenge that also affects Auchenipteridae. This makes the karyotypic description of new species, such as *T. wallacei*, even more important to reconstruct the ancestral 2n for Auchenipteridae and Doradoidea.

Although Takagui *et al*. (2021) had focused only on Doradidae, such data also introduces uncertainty about the 2n = 58 being the plesiomorphic condition in Auchenipteridae. Thus, also considering the diploid numbers of 56 and 58 chromosomes as potential plesiomorphic conditions for Auchenipteridae, the 2n = 52 chromosomes in *T. wallacei* indicates a diploid number reduction. In addition, the diploid number reduction in other members of *Ageneiosini*, including *Ageneiosus inermis* (Linnaeus, 1766) (cited as *Ageneiosus brevifilis*, Fenocchio, Bertollo, 1992; Lui *et al*., 2013a) and *Tympanopleura atronasus* (Eigenmann & Eigenmann, 1888) (Fenocchio, Bertollo, 1992) with 56 chromosomes, indicates that the reduction of the 2n can be a shared feature among the species of this tribe.

While most Auchenipteridae species exhibit a 2n = 58 (Tab. 1), various diploid numbers have been observed within the family. For instance, *A. inermis* (cited as *A. brevifilis*, Fenocchio, Bertollo, 1992; Lui *et al*., 2013a) and *T. atronasus* (Fenocchio, Bertollo, 1992) with 56 chromosomes, and *Centromochlus heckelii* (De Filippi, 1853) (Kowalski *et al*., 2020) with 46 chromosomes. Considering that *A. inermis*, *T. atronasus* and *T. wallacei* belong to Auchenipterinae, and *C. heckelii* to Centromochlinae, which is strongly supported by an extensive database and previous research (*e.g*., Ferraris, 2007; Birindelli, 2014; Calegari *et al*., 2019), it is evident that these lower diploid numbers (56 and 52 in Auchenipterinae-Ageneiosini *versus* 46 in Centromochlinae) result from independent evolutionary processes. Although it might be intuitive to attribute these lower diploid numbers in Auchenipteridae (56, 52 and 46) to chromosomal fusion events, as suggested by some studies (Lui *et al*., 2013a; Kowalski *et al*., 2020), ITS (Interstitial Telomeric Sequence) sites have been detected only in *A. inermis* from the Araguaia River basin (Lui *et al*., 2013a).

Telomeres consist in noncoding TTAGGG repeats and associated proteins that protect chromosome ends from degradation, aberrant recombination, and end-to-end fusion (Hartmann *et al*., 2004). The absence or inactivation of this structure/sequence allows the chromosomal end fusion. In some groups of vertebrates these chromosomal fusions occur without several loss of telomeric sequence, resulting in interstitial telomeric sites (Meyne *et al*., 1989, 1990; Slijepcevic, 1998), whose detection by FISH is considered a strong indicator of a fusion point by some researchers (*e.g*., Rosa *et al*., 2012; Lui *et al*., 2013a; Deon *et al*., 2020). *Tetranematichthys wallacei* have not presented ITS under 77% (Fig. 3B) or 62% of stringency (data not shown, same result as 77%). However, it is worth noting that there are species with no ITS even though fusion events were suggested to explain a 2n reduction, as in the case of *Heptapterus hollandi* (Haseman, 1911), which presented the lowest diploid number at that time in Heptapteridae (Margarido, Moreira-Filho, 2008). Therefore, the absence of ITSs in *T. wallacei* does not necessarily mean that chromosomal fusions have not occurred.

In Auchenipteridae, heterochromatin is typically pale and located at the terminal regions of the chromosomes (*e.g*., Lui *et al*., 2010, 2013a,b, 2015; Kowalski *et al*.,

2020; Machado *et al*., 2021; Santos *et al*., 2021). However, heterochromatic blocks in centromeric, pericentromeric or interstitial position have been reported in some species (*e.g*., Lui *et al*., 2013a,b; Kowalski *et al*., 2020; Machado *et al*., 2021). In addition to the terminal heterochromatin commonly found in Auchenipteridae, *T. wallacei* also exhibited chromosomes with centromeric and pericentromeric heterochromatic blocks (Fig. 2B). In other Ageneiosini species, only the pair 1m of *A. inermis* from the Araguaia River basin has shown pericentromeric heterochromatin, which coincided with the ITS found in this population (Lui *et al*., 2013a).

In Ageneiosini, C-banding techniques were performed on three species: *A. inermis* from the Araguaia River (Lui *et al*., 2013a) and the Solimões River (Fenocchio, Bertollo, 1992), and *T. atronasus* (Fenocchio, Bertollo, 1992). However, the organization of the chromosomes into a karyotype was only performed in *A. inermis* from the Araguaia River (Lui *et al*., 2013a). In the other species, only the metaphases were presented, and in *T. atronasus*, the C-banding was presented only for the pair carrying the NORs (Fenocchio, Bertollo, 1992). This scarcity of non-terminal heterochromatins in *Ageneiosus* (except for the first m chromosome pair) and their absence in *Tympanopleura* species analyzed so far, allows us to suggest that the non-terminal blocks detected in *T. wallacei* might correspond to chromosomal fusion sites. There are several reports of species from different groups of fish presenting co-located ITS and heterochromatin, such as *Trachydoras paraguayensis* (Eigenmann & Ward, 1907) (Baumgärtner *et al*., 2016), *Harttia* sp. 2 (Deon *et al*., 2020), *Rineloricaria lima* (Kner, 1853) (Rosa *et al*., 2012) and *Corydoras lacrimostigmata* Tencatt, Britto & Pavanelli, 2014 (Barbosa *et al*., 2017).

Regarding the proposal by Lui *et al*. (2013a) that the chromosomal fusion detected in *A. inermis* could be a basal event among the *Ageneiosus*, our data make it possible to expand this hypothesis. At the time of the publication by Lui *et al*. (2013a), only *Ageneiosus* and *Tetranematichthys* had been included in Ageneiosini. However, Walsh *et al*. (2015) later revalidated *Tympanopleura*, which was composed by a fraction of the species previously allocated in *Ageneiosus*. According to this new scenario, it is possible to assume that the fusion event reported by Lui *et al*. (2013a) can constitute a basal event before the cladogenesis of *Ageneiosus* and *Tympanopleura*.

The first m chromosome pair, visibly larger in size than the others, seems to be shared between the Ageneiosini species (Lui *et al*., 2013; Fenocchio, Bertollo, 1992). This same chromosome pair was suggested to have originated through a fusion event in *A. inermis* due to the presence of an ITS at the proximal region of the short arm (Lui *et al*., 2013a). While *Tetranematichthys* is considered sister group of *Ageneiosus* (Birindelli, 2014; Calegari *et al*., 2019), it was not possible to distinguish any chromosomal pair that could represent this chromosome pair in *T. wallacei* (Figs. 2–4). Two hypothesis can be proposed to explain this chromosomal arrangement: (1) the basal fusion proposed by Lui *et al*. (2013a) occurred before the diversification of the clade that gave rise to the species of *Ageneiosus* and *Tympanopleura*, and consequently, after the cladogenesis that originated *Tetranematichthys*; or (2) the fusion occurred at the clade base of all Ageneiosini and had underwent subsequent rearrangements in the *Tetranematichthys* lineage, reducing its size compared to its probable counterpart chromosome, the large chromosome pair present in *Ageneiosus* and *Tympanopleura* species.

In Ageneiosini, despite the limited cytogenetic data, the first m chromosome pair is not the only chromosomal pair that stands out due to its size. The first submetacentric

pair detected in *T. atronasus* and *T. wallacei* is notably similar as well, suggesting that *T. atronasus* and *T. wallacei* also share a common characteristic, which has not been identified in *Ageneiosus* species yet (Fig. 4). Considering the phylogenetic proposals for the group, it seems that *Ageneiosus* might have lost this character during its karyotypic evolution. On the other hand, and considering this first sm pair shared between *Tetranematichthys* and *Tympanopleura*, the following question may emerge: could the phylogenetic relationships among the three Ageneiosini genera be different? This issue becomes even more interesting if we remember that this sm chromosome pair is the 18S rDNA carrier in *T. atronasus* and *T. wallacei*.

Several characteristics regarding the distribution of 18S rDNA sites in Auchenipteridae are worth highlighting: (1) in most species the 18S rDNA has been found at terminal position of st or a chromosome pairs, and less frequently at sm pairs and interstitial positions (Tab. 1); (2) except for *T. atronasus* and *T. wallacei*, all the species exhibit the 18S rDNA at the short arm (Tab. 1); (3) except for *T. atronasus* and *T. wallacei*, all the species exhibit 18S rDNA at chromosome pairs with medium or small size compared to the other chromosomes into its respective karyotype (Tab. 1; Figs. 2, 4). The unique characteristic of the first sm chromosome pair bearing the 18S rDNA on the long arm further distinguishes *T. atronasus* and *T. wallacei* from other karyotyped Auchenipteridae species. The noticeably larger size of this chromosome pair also contributes to this distinction. This raises the possibility of a homoplastic character, particularly in light of the most recent phylogenetic proposal for the Ageneiosini, which consider

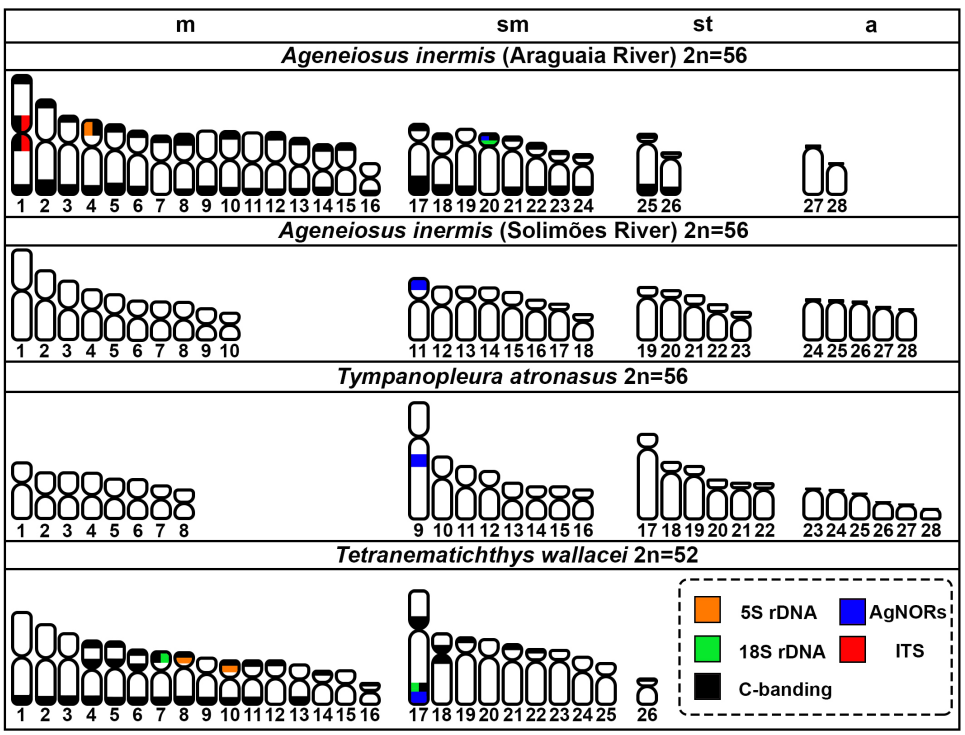

FIGURE 4 | Idiogram representing the karyotypes of *Ageneiosus inermis* (adapted from Fenocchio, Bertollo, 1992; Lui *et al.*, 2013a), *Tympanopleura atronasus* (adapted from Fenocchio, Bertollo, 1992) and *Tetranematichthys wallacei* (present study).

Tetranematichthys as the sister group of *Ageneiosus* + *Tympanopleura*. Future research on other Ageneiosini species will be crucial to expand this discussion. Currently, only three species out of the 21 in the tribe (Fricke *et al*., 2024) have been cytogenetically studied, with *T. wallacei* being the most recent addition.

In *T. atronasus* and *T. wallacei*, the 18S rDNA has been observed on the long arm of the first submetacentric pair, but their positions differ, being proximal and terminal, respectively (Tab. 1; Fig. 4). Among Auchenipteridae, only *Glanidium ribeiroi* Haseman, 1911 (Ravedutti, Júlio, 2001; Fenocchio *et al*., 2008; Lui *et al*., 2015) and *Auchenipterus osteomystax* Miranda Ribeiro, 1918 (cit. *Auchenipterus nuchalis*, Ravedutti, Júlio, 2001) exhibited NORs located in interstitial chromosomal regions (Tab. 1). Usually, the occurrence of interstitial NORs for some species within groups with a history of terminal position, such as Auchenipteridae and Doradidae, have been attributed to pericentric and/or paracentric inversion occurrence (Ravedutti, Júlio, 2001; Eler *et al*., 2007; Milhomem *et al*., 2008; Takagui *et al*., 2022). Therefore, the variation in the position 18S rDNA in this sm chromosome pair shared between *T. wallacei* and *T. atronasus* might also be attributed to a paracentric inversion.

In addition to pair 17sm, three specimens (two males and one female) showed 18S rDNA probe signals at the terminal position of the short arm of a single chromosome from pair 7m, indicating an intrapopulation chromosomal polymorphism. Another noteworthy observation is that *T. wallacei* and *C. heckelii* are the only Auchenipteridae species with multiple 18S rDNA sites, and this marks the first report of a polymorphism involving the 18S rDNA in the family. Since there is no similar description in Auchenipteridae, it is worth noting the observation in *Anadoras grypus* (Cope, 1872), from the sister group Doradidae, which also had up to three chromosomes bearing the 18S rDNA and similar variations in the position of the rDNA sequence as those found in *T. wallacei* (Takagui *et al*., 2022). Among the different possible chromosomal rearrangements, the remnant of a non-reciprocal translocation event is pointed as the hypothesis to explain the origin of the polymorphism found in *Anadoras grypus* (Takagui *et al*., 2022), and from a second pair bearing 18S rDNA in *C. heckelii* (Kowalski *et al*., 2020). Likewise, a transposition event could be responsible for the dissemination of this sequence in *T. wallacei*.

In contrast to the 18S rDNA, which shows a more varied distribution, the 5S rDNA has been identified in various chromosomes and positions across Auchenipteridae (Machado *et al*., 2021; Santos *et al*., 2021; Haerter *et al*., 2022). In Ageneiosini, only one population had been analyzed with this marker, *A. inermis* (Lui *et al*., 2013a). When compared to *T. wallacei*, it is notable that the 5S rDNA was found on chromosomes with the same morphology (metacentric) and on short arms of both species. Given the similarity in chromosomal morphology and the location of the 5S rDNA site, one of the chromosomal pairs found in *T. wallacei* may correspond to pair 4m in *A. inermis* (Lui *et al*., 2013a). The 5S rDNA exhibits high diversity in Auchenipteridae, presenting both simple and multiple sites (Tab. 1), a barrier to the establishment of a plesiomorphic condition. Hence, two hypothetical scenarios can be considered for the chromosomal evolution of this marker in Ageneiosini: (1) if the plesiomorphic condition entails only one chromosome carrying the 5S rDNA sites, the second pair of *T. wallacei* may have been originated from a transposition event; or (2) if the ancestral condition involves multiple sites, *A. inermis* may have lost 5S rDNA sites during its evolutionary trajectory.

In addition to the potential transposition events involving ribosomal sequences in *T. wallacei*, it is important to consider the organization of mitotic chromosomes during interphase. Chromosomes are arranged into specific chromosomal territories to maintain their individuality throughout the cell cycle, positioning adjacent to each metaphase phase, a concept known as the "Rabl Model" (Cremer *et al*., 1982, 2018; Cremer, Cremer, 2010). This arrangement facilitates spatial organization in a paired manner, enhancing the proximity and/or contact between chromosomes, thereby enabling the potential transfer of genetic material in the same position between adjacent chromosomes (Cremer *et al*., 1982, 2010, 2018), including rDNAs, as previously suggested for other Auchenipteridae species (*e.g*., Kowalski *et al*., 2020; Machado *et al*., 2021).

Tetranematichthys wallacei exhibits some characteristics that stand out: (1) a diploid number reduction unprecedented for Auchenipteridae; (2) it shares with *T. atronasus* the 18S rDNA in the long arm of a submetacentric chromosome, which have not been reported in Auchenipteridae yet; (3) first submetacentric pair possibly shared with *T. atronasus* and, thus far, exclusive to Ageneiosini. Some studies (Regan, 1911; Miranda Ribeiro, 1911; Eigenmann, 1925; Fowler, 1951; Greenwood *et al*., 1966; among others) suggested the members of Ageneiosini should be allocated into an exclusive clade, the Ageneiosidae. However, with the advancement of analyses involving new tools and more samples, this classification proved to be unsustainable, and recent data based on morphological and molecular characters by Calegari *et al*. (2019) definitively ruled out this possibility. Although there is no doubt about the species in this group being part of Ageneiosini, it is important to highlight that this tribe seems to be markedly different from other Auchenipteridae.

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Cleisson de Cristo Casarotto: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review and editing.
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ETHICAL STATEMENT

Specimens were collected under permission license of Sistema de Autorização e Informação em Biodiversidade (SISBIO #49379). The fish were euthanized by an overdose of clove oil and following the guidelines of the Ethics Committee on Animal Experimentation and Practical Classes at Unioeste (Protocol 13/09 – CEEAAP/Unioeste).

COMPETING INTERESTS

The author declares no competing interests.

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