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Karyotypic characterization of *Symphurus tessellatus* and *Symphurus plagusia* (Pleuronectiformes, Cynoglossidae) from Brazilian coastal waters

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ABSTRACT. The family Cynoglossidae (tonguefishes) is a speciose group of Pleuronectiformes, encompassing about 20% of described species in this order, distributed among three genera (*Cynoglossus, Paraplagusia* and *Symphurus*). *Symphurus* is the only genus of tonguefish in the Western Atlantic, being characterized by species complexes and cryptic forms; consequently the actual species richness of this genus is likely to be underestimated. Comparative cytogenetic studies have proved to be useful to resolve taxonomic uncertainties in ichthyofauna. Therefore, we carried out the karyotypic characterization of *Symphurus tessellatus* and *Symphurus plagusia* from the Brazilian coast based on conventional analysis, C-banding, silver nitrate (Ag-NORs) and base-specific fluorochrome staining. The specimens of *S. tessellatus* presented 2n = 46, 20 m/sm+26st/a, differing from that previously reported (2n = 46, 22m/sm+24 st/a), with heterochromatic blocks in the centromeric region of most chromosomes, as usually described in this group. We also found interstitial segments on the long arms of the third chromosome; these were coincident to Ag-NORs and GC-rich sites.

In *S. plagusia*, the karyotype is composed of 32m/sm+14st/a (2n=46), with a single nucleolar organizer regions (NORs) system, which is the first cytogenetic data for this species. The unique karyotype formulae of these two species suggest that pericentric inversions played a major role in the chromosomal differentiation of tonguefishes, being useful for cytotaxonomy and diagnosis of evolutionary units in *Symphurus*.

Key words: Tonguefishes; *Symphurus*; Karyotype; Cytotaxonomy

INTRODUCTION

The genus *Symphurus* is widely distributed, being the only representative of the family Cynoglossidae from the Western Atlantic Ocean (Figueiredo and Menezes, 2000). A total of 81 species are recognized within *Symphurus* (Lee et al., 2017), with nine valid species for the Brazilian coast (Western South Atlantic): *S. diomedeanus*; *S. ginsburgi*; *S. jenynsi*; *S. kyaropterygium*; *S. marginatus*; *S. plagusia*; *S. tessellatus*; *S. trewavasae*; and *S. oculellus*. Nonetheless, the taxonomy of this group of tonguefishes remains controversial, including polymorphic taxa and species complexes. The imprecise morphological identification of *Symphurus* species is related to overlapping of meristic data in identification keys of Cynoglossidae (Figueiredo and Menezes, 2000) and the need of incorporation of oesteological, structural, and pigmentation data to their diagnosis (Munroe, 1991).

From this perspective, other data besides anatomical features should be incorporated to provide a proper delimitation of species or unique evolutionary lineages (Padial et al., 2010). The cytogenetic analyses, even though neglected in recent studies, are highly informative to interspecific comparisons, particularly in fishes (Cipriano et al., 2008; Kasahara, 2009; Argolo et al., 2018). Once chromosomal differences are able to impose reproductive isolation by gamete unbalance, unviability or sterility of hybrids (Allendorf and Luikart, 2007), chromosomal markers are useful to taxonomic inferences and to infer genome evolution of distinct groups (Cioffi et al., 2011).

However, most of marine fish species still lack basic karyotypic information. In Pleuronectiformes, cytogenetic reports are available for about 60 out of the 822 valid species (Azevedo et al., 2007), representing less than 10% of the recognized taxa from this order. Even though, this number has increased over the last years (Bitencourt et al., 2014; Bitencourt et al., 2017), the cytogenetic reports in this fish group remain underrepresented. In Cynoglossidae, cytogenetic data are restricted to less than 5% of species, including two representatives of the genus *Symphurus* (Le Grande, 1975; Azevedo et al., 2007).

Even though scarce, the available chromosomal data in Cynoglossidae (Table 1) indicate a remarkable genomic plasticity, with diploid numbers ranging from 34 to 46, as well as distinct sex chromosome systems such as ZZ/ZW in *Cynoglossus semilaevis* (2n= 42) (Jiang et al., 2014) and $X_1X_1X_2X_2/X_1X_2Y$ in *Symphurus plagiusa* (2n=45/46) (Le Grande, 1975). These data reinforce the role of chromosomal evolution during the speciation of tonguefishes, suggesting that cytogenetics might be useful to identify their representatives in comparative analyses.

Therefore, the goal of the present study was to cytogenetically characterize populations of *S. tessellatus* and *S. plagusia* from northeastern and southeastern Brazilian coastal waters and evaluate the efficiency of chromosomal markers in cytotaxonomy of tonguefishes.

MATERIAL AND METHODS

Eight specimens of *Symphurus* were collected along the northeastern and southeastern coast of Brazil according to the license provided by Instituto Chico Mendes de Conservação da Biodiversidade - ICMBIO (SISBIO 31360-1). The individuals were identified as *Symphurus tesselatus* and *S. plagusia* (Figure 1) by experts and deposited in the fish collection from Universidade Federal da Paraíba (voucher UFPB 9851).

Figure 1. Map showing the collection sites (indicated by numbers) of *S. tesselatus* (red circle) and *S. plagusia* (black circle) in northeastern and southeastern coast of Brazil.

After euthanasia in iced water (Blessing et al., 2010), kidney fragments were removed to obtain mitotic chromosomes as proposed by Netto et al. (2007). All procedures were approved by the Committee of Ethics in Animal Utilization of the Universidade Estadual do Sudoeste da Bahia - UESB (CEUA/UESB n° 71/2014).

The chromosomal morphology was established based on arm ratios (Levan et al., 1964) and classified into metacentric/submetacentric (m/sm) and subtelocentric/acrocentric (st/a). The heterochromatin was visualized by C-banding according to Sumner (1972), with slight modifications. Active nucleolar organizer regions (NORs) were identified by silver nitrate staining (Ag-NOR) (Howell and Black, 1980) and base-specific fluorochrome staining was used to detected GC- and AT-rich sites using chromomycin A_3 (CMA_3), distamycin (DA) and 4,6-diamidino-2-phenylindole (DAPI), respectively (Schweizer, 1980).

RESULTS

All specimens of *S. tessellatus* and *S. plagusia* shared a modal diploid number of 2n = 46, but they differed in relation to their karyotype formulae, being equal to 20 m/sm+26st/a (Figure 2 A) and 32m/sm+14 st/a (Figure 2 B), respectively. Heteromorphic sex chromosomes were not evidenced.

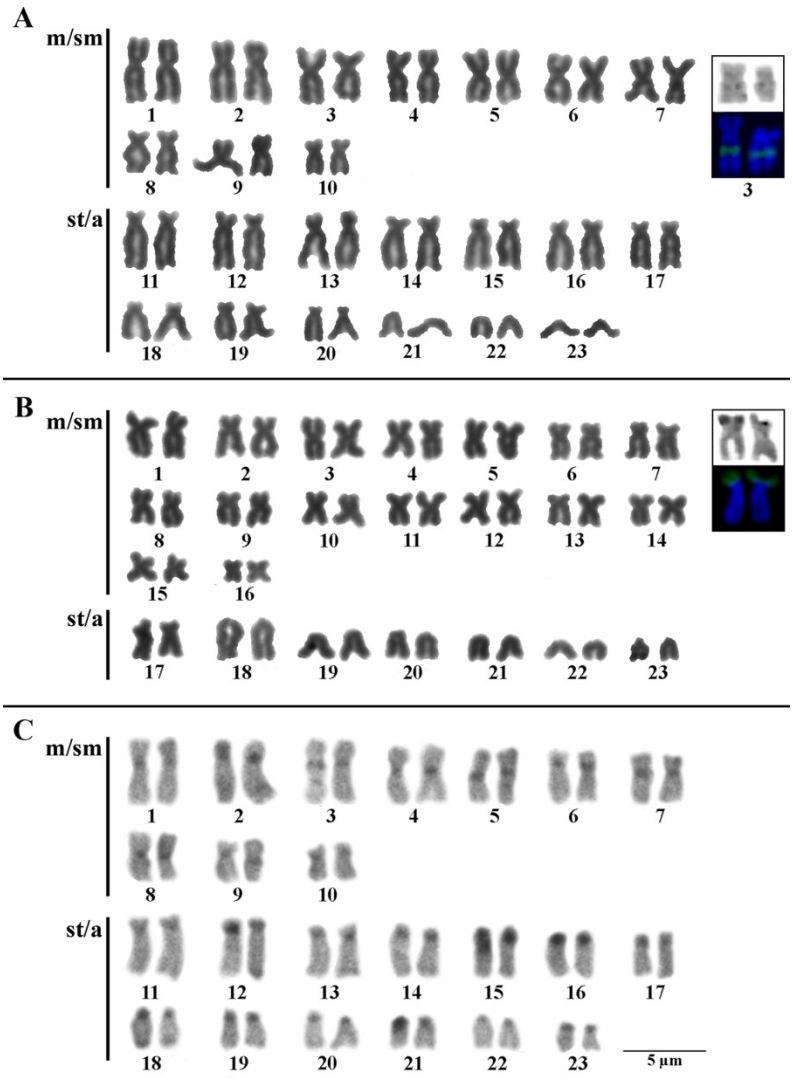

Figure 2. Conventionally stained (A, B) and C-banded (C) karyotypes of *S. tesselatus* (A, C) and *S. plagusia* (B). In detail, the NOR-bearing pairs after silver nitrate staining (Ag-NOR) and base-specific fluorochrome staining revealing GC-rich sites (CMA3+).

A single Ag-NOR system was observed in both species, located at interstitial region of long arms of the third m/sm pair in *S. tesselatus* (Figure 2 A, detail) and at terminal

region on short arms of a sm pair in *S. plagusia* (Figure 2 B, detail), In both species, the NORs were interspersed with GC-rich sites (CMA_3^+/$DAPI^-$) (Figure 2 A and B, detail).

In *S. tesselatus*, heterochromatic blocks were visualized at centromeric region of most chromosomes and interstitially in one pair, being coincident with Ag-NORs (Figure 2 C). Unfortunately, C-banding was not successful in *S. plagusia*.

DISCUSSION

The diploid number in representatives of Pleuronectiformes ranges from 28 to 48, the later being reported in 40% of analyzed species. The presence of 48 chromosomes is regarded as a plesiomorphic feature of Pleuronectiformes and their karyotype diversification seems to have evolved from two main chromosomal rearrangements: centric fusions leading to reduction of 2n values and pericentric inversions that have increased the number of chromosomal arms (FN) (Azevedo et al., 2007; Bitencourt et al., 2014).

In addition, most of Pleuronectiformes are characterized by a single NOR-bearing pair, usually located at terminal position of biarmed chromosomes or at pericentromeric region of subtelo/acrocentric chromosomes, with few cases of multiple NORs (Azevedo et al., 2007). Invariably, NORs are interspersed with GC-rich heterochromatic blocks (Azevedo et al., 2005; Fujiwara et al., 2007; Ocalewicz et al., 2008; Bitencourt et al., 2014; Bitencourt et al., 2017). These data suggest that the changes in karyotype macrostructure are not followed by significant microstructural variations.

In Cynoglossidae (Table 1), karyotype reports are restricted to two species of S*ymphurus: S. tessellatus* (Azevedo et al., 2007) and *S. plagiusa* (LeGrande, 1975). Both species share a chromosomal number close to the plesiomorphic condition (2n=48a), being equal to 2n=45/46 in *Symphurus plagiusa* and 2n=46 for *S. tessellatus*. Accordingly, a single event of centric fusion accompanied by multiple pericentric inversions could account for the karyotype formulae described in *Symphurus* since they present several m/sm pairs (Azevedo et al., 2007). In the case of *S. plagiusa*, a multiple sex chromosome system has been reported (LeGrande, 1975) represent a peculiar situation rarely observed in Pleuronectiformes (Bitencourt et al., 2017).

Here, we increased the cytogenetic data for Cynoglossidae by providing the first report of the karyotype structure of *S. plagusia*. Similar to previous reports in other species, *S. plagusia* presented 2n values lower than 48 (2n=46) and several biarmed chromosomes (32m/sm+14st/a; FN=78). The high number of meta/submetacentric in this species suggests that several pericentric inversions have taken place during diversification and speciation.

On the other hand, the specimens of *Symphurus tessellatus* presented the same diploid number previously reported for this species - 2n=46 – but they differed in relation to the karyotype formulae (Azevedo et al., 2007). While the populations herein analyzed presented 20m/sm+26st/a (FN=66), the population from São Paulo (Southeastern Brazil) was characterized by 22m/sm+24st/a (FN=68) (Azevedo et al., 2007). These differentiated karyotype formulae indicate that both populations diverged in the number of pericentric inversions. Nonetheless, these subtle differences between close chromosomal categories such as submetacentric and subtelocentric might be artifactual as a result of chromosomal condensation or the criteria established by each author. Moreover, the early development stages (eggs and larvae) of *Symphurus* are pelagic (Aceves-Medina et al., 2006) and some reports indicate that the metamorphosis of species in this genus might take one year to be

complete (Munroe and Krabbenhoft, 2010; Tunnicliffe et al., 2013). Therefore, this long period of pelagic stages might favor dispersal over long distances such as Northeastern and Southeastern coast of Brazil and hinder the fixation of interpopulation cytogenetic differences.

Table 1. Cytogenetic data for Cynoglossidae: diploid number (2n), fundamental number (FN) and banding techniques.

Species	Locality	2n	FN	Karyotype	C-banding	Ag-NOR	Reference
Cynoglossus semilaevis	Yantai, China	42	42	42a	Interstitial	2nd pair (telomeric region; C^+)	Jiang et al. (2014), Di et al. (2006) and Zhou et al. (2005)
Cynoglossus interruptus	Yanai, Japan	34	34	34a	-	-	Sakamoto and Nishikawa (1980)
Cynoglossus puncticeps	Bengal Bay, India	♀39	40	1 m + 38 a	-	-	Patro and Prasad (1981)
Paraplagusia bilineata	Bengal Bay, India	38	44	8 m/sm + 32 a	-	-	Patro and Prasad (1981)
Paraplagusia japonica	Futami, Japan	38	46	6 m + 32st/a	-	-	Sakamoto and Nishikawa (1980)
Symphurus plagiusa	Louisiana, USA	♂45	68	♂23 m/sm + 22st/a	-	-	Le Grande (1975)
		♀46	70	♀24 m/sm + 22st/a			
Symphurus plagusia	Paraíba, Brazil	46	78	32 m/sm + 14 st/a	-	st/a; CMA_3^+	Present study
Symphurus tessellatus	São Paulo, Brazil	46	68	22 m/sm + 24 st/a	Mostly centromeric	3rd m/sm pair (i/la)/; bc^-; CMA_3^+	Azevedo et al. (2007)
	Rio de Janeiro/Bahia, Brazil	46	66	20 m/sm + 26st/a	Mostly centromeric	3rd m/sm (i/la); bc^+; CMA_3^+	Present study

st/a: subtelocentric/acrocentric; m/sm: metacentric/submetacentric; T: terminal; i: interstitial; as: short arms; la: long arms; bc: C-bands; CMA_3: Chromomycin A_3

In this sense, the inclusion of additional chromosomal markers based on banding techniques should be useful to refine the cytogenetic analysis in *Symphurus*, such as those to reveal the distribution of heterochromatin and repetitive DNA clusters. In the present study, the specimens of *Symphurus tessellatus* were characterized by C-bands at pericentromeric region and at interstitial position of few pairs, as commonly reported in Cynoglossidae so far (Azevedo et al., 2007). Nonetheless, representatives of the families Bothidae (Vitturi et al., 1993; Argolo et al., 2018) and Achiridae (Bitencourt et al., 2014) usually present conspicuous heterochromatin segments, what seems to represent a trend for some lineages of Pleuronectiformes despite the limitations of these data for cytotaxonomic inferences.

In addition, the single NOR system associated with GC-rich sites, as presently described (Figure 2) has been widely reported in fishes (deAlmeida-Toledo et al., 1996), reptilians and amphibians (Kasahara, 2009). However, the presence of interstitial Ag-NORs

might be interpreted as a derived feature in Pleuronectiformes (Vitturi et al., 1993; Azevedo et al., 2007), usually derived from pericentric inversions.

Again, the NOR location reported in *Symphurus tesselatus* from this study is similar to that reported in *S. tessellatus* by Azevedo et al. (2007) but they differed in relation to the presence (this study) or lack (Azevedo et al., 2007) of C-bands associated with NORs, being the former a common situation in Pleuronectiformes (Kikuno et al., 1986; Pardo, 2001; Azevedo et al., 2007; Fujiwara et al., 2007). Therefore, the C-banding might be a potential chromosomal marker to differentiate species or populations of *Symphurus*, what remains to be investigated.

In *S. plagusia*, a distinctive distribution of NORs was observed, being located at terminal region on short arms of a metacentric pair, thus differing from previous reports in this genus. These data suggest that cytogenetics is efficient to validate species of *Symphurus*, a group recognized by their confusing taxonomy. Based on karyotype macrostructure and conventional banding analyses we were able to recognize each taxon, being potentially useful to discriminate cryptic forms. Nonetheless, the cytogenetic data in Cynoglossidae are too scarce and further information are required to refine the role of chromosomal changes in the diversification of *Symphurus* species.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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