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*Research Article*

# Chromosome Mapping of Repetitive Sequences in *Rachycentron canadum* (Perciformes: Rachycentridae): Implications for Karyotypic Evolution and Perspectives for Biotechnological Uses

**Uedson Pereira Jacobina,[1] Marcelo de Bello Cioffi,[2] Luiz Gustavo Rodrigues Souza,[3] Leonardo Luiz Calado,[1] Manoel Tavares,[4] João Manzella Jr.,[4] Luiz Antonio Carlos Bertollo,[2] and Wagner Franco Molina[1]**

[1] *Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN 59078-970, Brazil*
[2] *Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP 13565-905, Brazil*
[3] *Departamento de Botânica, Universidade Federal de Pernambuco, Recife, PE 50670-420, Brazil*
[4] *Aqualider, Laboratório de Piscicultura Marinha, Ipojuca, PE 55900-000, Brazil*

Correspondence should be addressed to Wagner Franco Molina, molinawf@yahoo.com.br

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The cobia, *Rachycentron canadum*, a species of marine fish, has been increasingly used in aquaculture worldwide. It is the only member of the family Rachycentridae (Perciformes) showing wide geographic distribution and phylogenetic patterns still not fully understood. In this study, the species was cytogenetically analyzed by different methodologies, including Ag-NOR and chromomycin $A_3$ ($CMA_3$)/DAPI staining, C-banding, early replication banding (RGB), and *in situ* fluorescent hybridization with probes for 18S and 5S ribosomal genes and for telomeric sequences $(TTAGGG)_n$. The results obtained allow a detailed chromosomal characterization of the Atlantic population. The chromosome diversification found in the karyotype of the cobia is apparently related to pericentric inversions, the main mechanism associated to the karyotypic evolution of Perciformes. The differential heterochromatin replication patterns found were in part associated to functional genes. Despite maintaining conservative chromosomal characteristics in relation to the basal pattern established for Perciformes, some chromosome pairs in the analyzed population exhibit markers that may be important for cytotaxonomic, population, and biodiversity studies as well as for monitoring the species in question.

## 1. Introduction

The cobia (*Rachycentron canadum*) is the only member of the family Rachycentridae (Perciformes) found in tropical seas worldwide. It is highly valued on the international fish market and is a promising species for marine aquaculture (Kaiser and Holt [1]; Liao et al. [2]; Benetti et al. [3]; Benetti et al. [4]). Among its favorable breeding traits are easy adaptation, prolificacy, rapid growth, and meat quality (Frank et al. [5]; Arnold et al. [6]). Although well-established breeding technology is available, there is little genetic information to guide biotechnological initiatives and/or genetic improvement of the species, especially with respect to cytogenetic aspects.

Cytogenetic analyses in fish have been used in natural environments to characterize cryptic species and/or populations (Bertollo et al. [7]; Jacobina et al. [8]; Cioffi et al. [9]) and identify polymorphisms (Mantovani et al. [10]; Molina and Galetti [11]), inventories of existing biodiversity (Galetti Jr. et al. [12]; Artoni et al. [13]), in addition to chromosomal evolution in large taxonomic groups (e.g., Bertollo et al. [7]; Molina [14]). The information obtained has contributed to a better understanding of genetic diversity, monitoring, and

conservation, as well as providing elements for a rational exploitation of fish stocks.

Current approaches, particularly involving the manipulation of chromosomal sets, have gone beyond the experimental field and are being increasingly used in aquaculture (Ocalewicz et al. [15]). From the technological viewpoint, the chromosomal data in fish have created conditions for implementing ploidy handling protocols aimed at increased growth and/or weight gain (Beardmore et al. [16]; Jankun et al. [17]), gynogenetic production (Devlin and Nagahama, [18]; Piferrer et al. [19]; Chen et al. [20]), establishment of monosex cultivations (Coimbra et al. [21]; Chen et al. [20]), and physical identification of quantitative trait loci (QTLs) (Ning et al. [22]) for several marine or fresh water species.

Because of the growing economic importance and increasingly sophisticated cultivation techniques, recent offshore commercial breeding initiatives of *R. canadum* stocks from the Western Atlantic have been very successful (Benetti et al. [4]). In order to get new useful characters for comparative genomics at chromosomal levels and provide adequate conditions for future uses of genetic improvement and biotechnological innovations, a resolutive chromosomal characterization of this species is presented here for the first time, using conventional Giemsa staining, C-banding, position and frequency of nucleoli organizer regions (NORs), $CMA_3$ and DAPI fluorochrome staining, physical mapping of repetitive sequences using fluorescent *in situ* hybridization (FISH) with 18S and 5S rDNA probes and telomeric sequences $(TTAGGG)_n$, in addition to replication banding patterns by the incorporation of base analogue 5-BrdU in this species.

## 2. Material and Methods

Cytogenetic analyses were developed from a sample of 40 cobia fry, weighing around 35 grams, obtained from a commercial marine fish culture farm located in the state of Pernambuco, in Northeastern Brazil. The specimens were previously submitted to *in vivo* mitotic stimulation for 24 hours, using intramuscular and intraperitoneal inoculation of fungal and bacterial antigen complexes (Molina [23]), anesthetized with clove oil (eugenol) and sacrificed to remove the renal tissue. Metaphasic chromosomes were obtained from cell suspensions of anterior rim fragments, according to short-term *in vitro* methodology (Gold Jr. et al. [24]).

The cell suspension obtained was dropped onto clean slides and covered with a film of distilled water heated to 60°C. The chromosomes were stained with a solution of 5% Giemsa, and 30 metaphases of each individual were analyzed to determine the number of chromosomes. Males and females were identified by macroscopic or histologic examination of the gonads. The best metaphases were photographed under an Olympus BX50 epifluorescence microscope equipped with a DP70 digital image capture system.

*2.1. Chromosome Banding.* The heterochromatic regions and ribosomal sites were identified by techniques proposed by Sumner [25] and Howell and Black [26], respectively. The $CMA_3$/DAPI double-staining technique was employed for $CMA_3$ banding, using DAPI (4′,6-diamidino-2-phenylindole) as counterstain (Barros-e-Silva and Guerra, [27]). Slides aged for 3 days were stained with $CMA_3$ (0.1 mg/mL) for 60 min and restained with DAPI (1 $\mu$g/mL) for 30 min. Next, the slides were mounted in glycerol : McIlvaine buffer, pH 7.0 (1 : 1) and aged for three days before analysis under epifluorescence microscope equipped with appropriate filters.

*2.2. Replication Banding.* Replication bands were obtained in 10 specimens of *R. canadum* using *in vivo* incorporation of the base analog 5-Bromo-2-deoxyuridine (5-BrdU), following methodology developed by Giles et al. [28], with some modifications. A solution of 5-BrdU (5 mg/mL in 0.9% NaCl solution) was injected intraperitoneally into each specimen at a proportion of 1 mL/100 g of body weight, 6 h (early S phase) before animal sacrifice to obtain mitotic chromosomes. The FPG (Fluorochrome Photolysis Giemsa) staining method was used to reveal RBG (Replication bands by bromodeoxyuridine using Giemsa) bands. The slides with chromosomal preparations were washed in 2 × SSC saline solution. They were then stained with Hoescht 33258 solution (Sigma) (1 mg of Hoescht in 1 mL of methanol and 100 mL of 0.5 × SSC) for 40 min in a dark chamber, washed in distilled water, covered with a 2 × SSC film, and exposed to ultraviolet light (254 $\eta$m) at a distance of 10 cm, for 1 h. The slides were then incubated in 2 × SSC buffer for 90 minutes and stained with 6% Giemsa solution (pH 6.8) for 10 minutes.

*2.3. Chromosome Hybridization Probes.* Two tandem-arrayed DNA sequences isolated from the *Hoplias malabaricus* (Teleostei, Characiformes) genome were used. The first probe contained a 5S rDNA repeat copy and included 120 base pairs (bp) of the 5S rRNA encoding gene and 200 bp of the nontranscribed spacer (NTS) [29]. The second probe corresponded to a 1,400 bp segment of the 18S rRNA gene obtained via PCR from nuclear DNA (Cioffi et al. [30]). The 18S rDNA probe was labeled by nick translation with DIG-11-dUTP, according to the manufacturer's specifications (Roche) and the 5S rDNA probe was labeled with biotin-14-dATP by nick translation, also according to the manufacturer's specifications (Bionick Labelling System, Invitrogen).

The telomeric DNA sequence $(TTAGGG)_n$ was also used as a probe. This probe was generated by PCR (PCR DIG-Probe Synthesis Kit, Roche) in the absence of template using $(TTAGGG)_5$ and $(CCCTAA)_5$ as primers (Ijdo et al. [31]).

*2.4. Chromosome Hybridization and Analysis.* Fluorescent *in situ* hybridization (FISH) was performed on mitotic chromosome spreads (Pinkel et al., [32]). The metaphase chromosome slides were incubated with RNAse (40 $\mu$g/mL) for 1.5 h at 37°C. After denaturation of chromosomal DNA in 70% formamide, spreads were incubated in 2 × SSC for 4 min at 70°C. Hybridization mixtures containing 100 ng of denatured probe, 10 mg/mL dextran sulfate, 2 × SSC, and 50% formamide in a final volume of 30 $\mu$l were dropped onto the slides, and hybridization was performed overnight at 37°C in a moist chamber. Posthybridization washes were carried out at 37°C in 2 × SSC, 50% formamide for 15 min, followed by

a second wash in 2 × SSC for 15 min, and a final wash at room temperature in 4 × SSC for 15 min. Signal detection was performed using avidin-FITC (Sigma) for the 5S rDNA probe and anti-digoxigenin-rhodamine (Roche) for 18S rDNA and $(TTAGGG)_n$ probes. One-color FISH was performed to detect $(TTAGGG)_n$ repeats, while 5S and 18S rDNA were detected by double-FISH. The posthybridization washes were performed in a shaker (150 rpm). The chromosomes were counterstained with DAPI (1.2 $\mu$g/mL). FISH analysis was carried out with an epifluorescence microscope (Olympus BX50) and chromosomal plates were captured by the CoolSNAP system, Image Pro Plus, 4.1 (Media Cybernetics).

*2.5. Chromosome Measures and Idiogram.* Determination of chromosome types (Levan et al. [33]) and their measures were obtained using Image Tools 0.8.1 software, from five complete metaphases of *R. canadum*, where the locations of primary restrictions and telomeric regions of each chromosome were clearly defined. Total chromosome length (S), arm ratio (AR = long/short arm), in addition to size and position of heterochromatic blocks (numerical data not shown) were determined. An idiogram with chromosomal data for the species was created, using the Easy Idio program (Diniz and Xavier [34]), showing the position of 18S and 5s rDNA sites, late replication bands, and telomeric sequences.

## 3. Results

*R. canadum* has $2n = 48$ chromosomes, with a chromosomal formula composed of a submetacentric pair (sm), two subtelocentric pairs (st) and 21 acrocentric pairs (a), with a fundamental number (FN) of 54 (Figure 1(a)). Chromosome size ranged from 4.49 to 1.55 $\mu$m. The largest chromosome pair (2nd pair), subtelocentric, exhibited secondary constriction in the short arm (Figure 1(a)), which showed polymorphic behavior, causing substantial homologue size variation. This polymorphism was evidenced by both conventional staining and in chromosomes submitted to the other chromosome bandings.

C-positive heterochromatic blocks were observed in the pericentromeric regions of the 1st pair (sm), in the centromeric and telomeric position (reduced content) in most of the acrocentric chromosomes, in the terminal position on the long arm of the 3rd pair (st) and in the interstitial region of the long arm of some chromosome pairs. Secondary constriction, present in the 2nd subtelocentric pair, was heterochromatic ($CMA^+/DAPI^-$), corresponding to the location of the Ag-NOR sites (Figures 1(a) and 1(b)).

*In situ* hybridization with 18S and 5S rDNA probes characterized a nonsyntenic condition for these ribosomal subunits. The two 18S rDNA sites detected were located in the $C^+/CMA^+/DAPI^-$ region of the short arm of the 2nd chromosome pair. Conversely, four 5S rDNA sites were detected and mapped in the terminal position of the long arm of the 3rd pair and on the short arm of the 13th pair of chromosomes, both colocated with heterochromatic bands (Figure 1(d)).

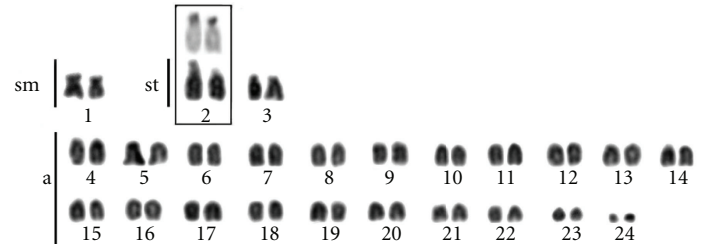
FISH with telomeric probes $(TTAGGG)n$ showed no ectopic signals, other than those present in the terminal region of the chromosome complement (Figure 1(e)). The wide range in telomeric signal intensity observed between chromosome pairs was markedly higher in some pairs than in others (Figure 1(e)).

The replication band pattern enabled a better identification of chromosome pairs (Figures 1(c) and 2). Early replication bands coincided with the euchromatic chromosome regions. The heterochromatin blocks identified by C-banding showed a synchronous pattern of late replication. Interestingly, the heterochromatinized 18S and 5S rDNA sites exhibited a typical initial replication pattern.

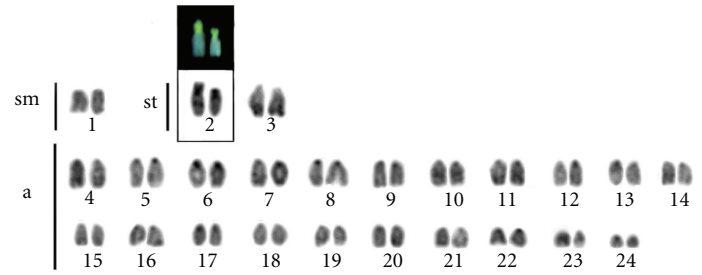
## 4. Discussion

The karyotype patterns of *R. canadum* are similar to those considered basal and conserved in most Perciformes fish. These symplesiomorphies are characterized by a diploid number of chromosomes equal to 48, karyotypes composed mainly of acrocentric chromosomes, simple NORs, and a reduced amount of heterochromatin (Molina [14]). Indeed, this relatively heterochromatin-poor karyotypic pattern, preferentially located in the pericentromeric region of acrocentric chromosomes, has been identified in several groups of marine Perciformes (e.g., Molina and Galetti Jr., [11]; Molina and Bacurau [35]; De Araújo et al. [36]). However, the presence of six bi-armed chromosomes (pairs 1, 2, and 3) in the karyotype of *R. canadum* reflects the more pronounced diversification in the karyotypic macrostructure of this species, indicating the occurrence of pericentric inversions associated to chromosomal evolution. Pericentric inversions are the prevalent evolutionary events in some groups of marine fish, such as Pomacentridae, Carangidae, and Apogonidae (Molina and Galetti Jr. [37]; Rodrigues et al. [38]; De Araújo et al. [36]) and have been reported as being the main mechanism of chromosomal diversification in Perciformes (Galetti Jr. et al. [39]).

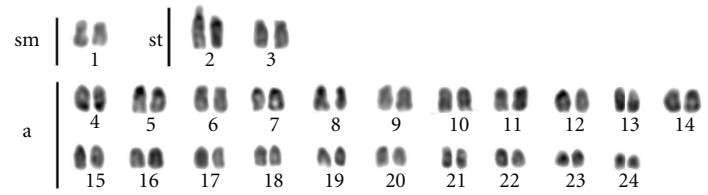
Replication bands in fish chromosomes are still restricted to a reduced number of species. Although the R band patterns produced help achieve better homologue pairing, the symmetrical karyotype and relatively small size of *R. canadum* chromosomes enable more precise chromosomal individualization, as observed in a number of other fish species. The replication patterns obtained revealed the presence of three functional groups of chromatin in the karyotype of this species. The first corresponds to euchromatic regions, exhibiting the characteristic pattern of initial replication. The second group corresponds to heterochromatic regions composed of ribosomal gene repeats, with initial replication and where the genic clusters of 18S rDNA showed characteristically strong fluorescence with GC-specific fluorochromes. A similar condition was identified earlier in three species of the genus *Leporinus* (Anostomidae), in which intensely GC-rich positive mitramycin bands ($MM^+$) also showed an initial replication pattern, while medium- or low-mitramycin fluorescence bands exhibited late replication (Molina and Galetti Jr. [40]). Finally, the third group of chromatin from *R. canadum* corresponds to repetitive DNAs



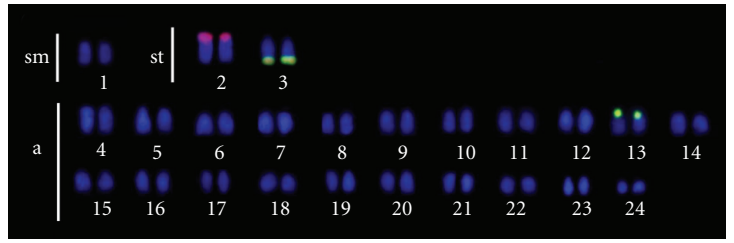

(a)



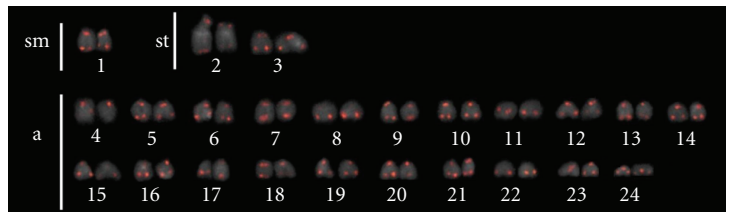

(b)




(c)




(d)




(e)

Figure 1: Karyotypes of *Rachycentron canadum*. Conventional staining (a) highlighting the Ag-NOR sites in chromosome 2; (b) C-banding, highlighting the NORs as $CMA_3^+/DAPI^-$ heterochromatic regions; (c) replication bands, showing 18S (pair 2) and 5S (pairs 3 and 13) rDNA sites with early replication; (d) double-FISH with 18S (pink) and 5S (green) rDNA probes, showing the location of 18S rDNA sites in pair 2 and of 5S rDNA sites in pairs 3 and 13; (e) FISH with $(TTAGGG)_n$ sequences showing the location of telomeric sites in the chromosomes (orange). Bar = 5 $\mu$m.

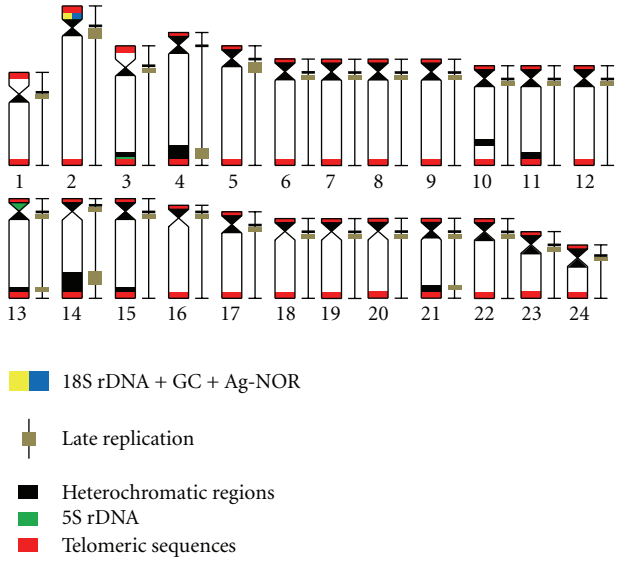



Figure 2: Idiogram of the chromosome complement of *Rachycentron canadum*, exhibiting cytogenetic mapping of ribosomal sequences, Ag-NORs, heterochromatic regions, and chromosome replication bands. Bar = 5 $\mu$m.

with late replication, exhibiting no positive response to AT or GC-specific fluorochromes.

In general, replication bands indicate less heterogeneity in heterochromatins from *R. canadum*, compared to other species (Molina and Galetti Jr. [40]). This apparent composition homogeneity in the heterochromatic portions of the *R. canadum* genome may reflect the heterochromatic segments conserved in the karyotype. Such a situation seems to be particularly disseminated in Perciformes, suggesting the occurrence of similar chromosomal diversification mechanisms in phylogenetically similar groups, characterizing karyotypic orthoselection processes (Molina [14]). Despite the controversies regarding the different compositions and functionalities of heterochromatins and their evolutionary role (Allshire [41]; Huisinga et al. [42]; Djupedal and Ekwall, [43]), analysis of their location, distribution, and composition has been essential in the chromosomal characterization of many fish groups and often effective as cytotaxonomic markers (Moreira-Filho and Carlos Bertollo [44]; Sola et al. [45]).

Martins and Galetti Jr. [46] proposed that the location of 5S and 18S rDNA sites in different chromosomes, as observed for most vertebrates, could allow these loci to evolve independently, since their divergent functional dynamics requires a physical distance. This divergent location of 18S and 5S rDNA loci seems to be the most common situation observed in fish, as well as in other vertebrates (Lucchini et al. [47]; Suzuki et al. [48]). However, although present in smaller numbers, syntenic or even equilocal arrangements of these rDNA families have also been observed (Fujiwara et al. [49]), possibly even characterizing a frequent condition in some groups, such as Channichthyidae (Mazzei et al. [50]).

The occurrence of evolutionary dynamics involved in these genes in some species has been confirmed by a greater variability in the distribution of 45S rDNA sites in the chromosomes (Mantovani et al. [51]). Similarly, heterochromatins associated to ribosomal genes play a different evolutionary role in *R. canadum*, involving composition, position, and/or functional aspects, compared to other merely heterochromatic regions of the karyotype. Whereas the first contained high GC levels and replication at the onset of the S phase, the remaining heterochromatic portions showed late replication and $CMA_3$/DAPI staining neutrality. However, examples of nonheterochromatinized ribosomal sites are also present in different fish groups (e.g., Fujiwara et al. [49]; Ráb et al. [52]; Rábová et al. [53]).

Telomeric $(TTAGGG)_n$ sequences are present in the telomeres of vertebrate chromosomes, and their analysis allows one to establish the presence of chromosomal rearrangements, such as Robertsonian fusions or inversions, which are involved in chromosomal evolution (Meyne et al. [54]). These sequences have been used to identify chromosomal rearrangements in fish (Saitoh et al. [55]), such as some species of Salmoniformes, *Salvelinus namaycush,* S. *fontinalis,* S. *alpinus,* and *Oncorhynchus* spp. (Reed and Phillips [56]; Phillips and Reed [57]); Characiformes, *Hoplias malabaricus* (Cioffi and Bertollo [58]), and *Erythrinus erythrinus* (Cioffi et al. [59]); Anguilliformes and Perciformes (Salvadori et al. [60]; Gornung et al. [61]; Caputo et al. [62]). FISH with the telomeric probe $(TTAGGG)_n$ revealed hybridization signals in the telomeric region of the chromosomes. Hybridization signals exhibited intensity variations in some chromosome pairs, suggesting the occurrence of telomeric repeat amplification or their dispersion into telomeric heterochromatic regions. Interstitial telomeric sites (ITS) were not detected, which indicates that Robertsonian fusions or chromosomal translocations were likely not involved in the karyotypic evolution of *R. canadum*. However, we cannot rule out this possibility, since it is known that loss of telomeric sequences may occur after such rearrangements (Slijepcevic [63]; Nanda et al. [64], De Almeida-Toledo et al. [65]). Maintaining the basal diploid number for Perciformes, as well as the relative conservatism of the karyotype, in fact do not appear to indicate that recent chromosomal rearrangements have occurred in the chromosomal evolution of cobia fish.

The phylogenetic relationships of the family Rachycentridae, based on morphological characters, suggest its proximity with Nematistiidae, Echeneidae, Carangidae, and Coryphaenidae (Johnson [66]; Springer and Smith-Vaniz [67]). Indeed, the evolutionary proximity between families has been corroborated by cytogenetic data available for a number of species belonging to these groups (Caputo et al. [68]; Sola et al. [45]; Rodrigues et al. [38]; Chai et al. [69]; present study), complemented by the investigation of mitochondrial sequences (Cit B) (Reed et al. [70]) and larval morphology (Ditty and Shaw [71]), which point to Coryphaenidae as a sister group of *R. canadum*.

*R. canadum* has emerged as an important model for marine fish culture. Nevertheless, there are few genetic studies on this species (Garber et al. [72]; Liu et al. [73]). Given that it belongs to a monotypic family with vast worldwide distribution in tropical and subtropical areas, its populations may be subject to marked genetic structuring, like others species with similar biogeographic characteristics. The

presence of some chromosome pairs, such as the first three (sm, st, st, resp.) of the karyotype is particularly indicated as cytotaxonomic markers in the cobia population studied, considering its morphology, size, presence of heterochromatin, and 18S and 5S rDNA sites. In this respect, such chromosomes may contain important characters for interpopulation approaches, biodiversity characterization and monitoring, in addition to contributing to future biotechnological assays.

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