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ORIGINAL ARTICLE

Chromosomal differentiation and speciation in sister-species of Grammatidae (Perciformes) from the Western Atlantic

Wagner Franco Molina · Gideão Wagner Werneck Felix da Costa · Marcelo de Bello Cioffi · Luiz Antonio Carlos Bertollo

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Abstract In the tropical Atlantic, the ichthyofauna between the coast of Brazil and the Caribbean regions, divided by the Amazon barrier, is very similar presenting several geminate species, including *Gramma brasiliensis*, endemic in Brazil, and its Caribbean counterpart *Gramma loreto*. Morphological and molecular studies have helped establish evolutionary patterns that sister-species of these two marine habitats are subjected to. However, their chromosomal characteristics are only beginning to be better characterized. Accordingly, a comparative cytogenetic analysis was carried out in *G. brasiliensis* and *G. loreto*, seeking evidence of cytotaxonomic markers implicated in the karyotypic diversification of these species and likely associated with speciation events. Heterochromatic regions and their affinity to fluorochromes GC- or AT-specific were identified, as well as the distribution of ribosomal DNA sites in chromosomes, either by silver nitrate impregnation (Ag-NORs) or dual-color FISH mapping with 18S and 5S rDNA probes. While displaying the same diploid number, $2n = 48$ chromosomes, considered basal for Perciformes, the two species differed in karyotype structure, showing karyotypic formulas and species-specific heterochromatin pattern. The cytological characters found support the differentiating status of these species, possibly achieved under the conditions of allopatry due to the Amazon/Orinoco barrier, showing chromosomal peculiarities in Grammatidae species when compared to other groups of Perciformes.

Keywords Marine fish · Chromosomal markers · Chromosomal evolution · Allopatric speciation · Marine barriers

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W. F. Molina (✉) · G. W. W. F. da Costa
Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte (UFRN), Natal, RN, Brazil
e-mail: molinawf@yahoo.com.br

M. de Bello Cioffi · L. A. C. Bertollo
Departamento de Genética e Evolução,
Universidade Federal de São Carlos, São Carlos, SP, Brazil

Introduction

The distribution patterns of marine biodiversity are complex, resulting from vicariance events and dispersion potential of species as well as local ecological and adaptive conditions. In the tropical Atlantic, the recognition of these patterns has been established incrementally (e.g., Rocha 2003; Floeter et al. 2008), including descriptions and revalidations of species endemic to the Brazilian coast (Rocha and Rosa 2001; Moura and Castro 2002; Moura and Lindeman 2007). Advances in reef fish research have identified species previously considered endemic to Brazil in the Southern Caribbean (Rocha et al. 2002). At the same time, species previously considered the same as to those of the Caribbean have been identified as new on the Brazilian coast (e.g., Rocha and Rosa 1999; Moura and Lindeman 2007). In many cases, molecular (e.g., Bowen et al. 2001; Rocha et al. 2002; Tornabene et al. 2010) and cytogenetic patterns (Nirchio et al. 2005; Rossi et al. 2005; Motta-Neto et al. 2011) have helped identify genetic diversity among species and populations of these marine provinces.

In the tropical Atlantic, strong environmental pressure and extraction occurring in coastal areas of Brazil and the Caribbean have endangered several small

benthic species (Leão and Dominguez 2000; Ferreira et al. 2005; Pandolfi et al. 2003). Cryptobenthic fish stand out for their trophic importance (Depczynski and Bellwood 2003). However, most of these species are not well studied for any biological parameters. Generally, they have a short larval period and some species, even in adulthood, appear to remain near the coast (Beldade et al. 2007). Highlighted here are fish from the Grammatidae family (Ferreira et al. 2005), a monophyletic group comprising 13 species with distribution restricted to the Western Atlantic (Gill and Mooi 1993). In this group, the genus *Gramma* is composed of only five species (Böhlke and Randall 1963; Starck and Colin 1978; Sazima et al. 1998; Victor and Randall 2010), inhabiting shallow waters, such as *G. loreto* and *G. brasiliensis*, or deeper regions such as *G. melacara*, *G. linki* and *G. dejongi* (Victor and Randall 2010). *Gramma loreto*, a small planktivorous species present in the Caribbean (Böhlke and Randall 1963), and *G. brasiliensis*, endemic to the Brazilian Western Atlantic (Sazima et al. 1998), have vibrant coloration patterns, with a distinctive purple and yellow bicolor body (Fig. 1). These species are increasingly exploited by the aquarist market, putting them at risk (Monteiro-Neto et al. 2003; Gasparini et al. 2005; Bruckner 2005). Indeed, the harvesting effect in some areas of northeast Brazil has reduced the population size of *G. brasiliensis*, given their low dispersal and recolonizing capacity. In addition, characteristics such as parental care and short larval stage may also increase the risk status of this species (Ferreira et al. 2005). Phylogenetically, the position of Grammatidae among Perciformes remains uncertain. They are considered to belong to the Percomorpha group with demersal eggs (Mooi 1990).

Although fauna similarities and endemism between the coast of Brazil and the Caribbean are recognized, chromosomal differences between species and populations of these regions have been neglected. In this study, two species of Grammatidae, *G. loreto* (Royal Gramma) and *G. brasiliensis* (Brazilian Royal Gramma), were compared cytogenetically with the use of conventional staining, C-banding, identification of nucleolar organizer regions by silver nitrate (Ag-NORs), staining with fluorochrome GC- and AT-specific and chromosome mapping by dual-color fluorescence in situ hybridization (dual-color FISH) with 18S and 5S rDNA probes. The results showed that these species constitute a suitable model for investigating chromosomal rearrangements in marine populations, revealing Grammatidae chromosomal singularities in relation to other Perciformes. Meanwhile, allowed to analyze the relationship between chromosomal rearrangements and speciation, an issue that is beginning to be more widely discussed (Molina 2007).

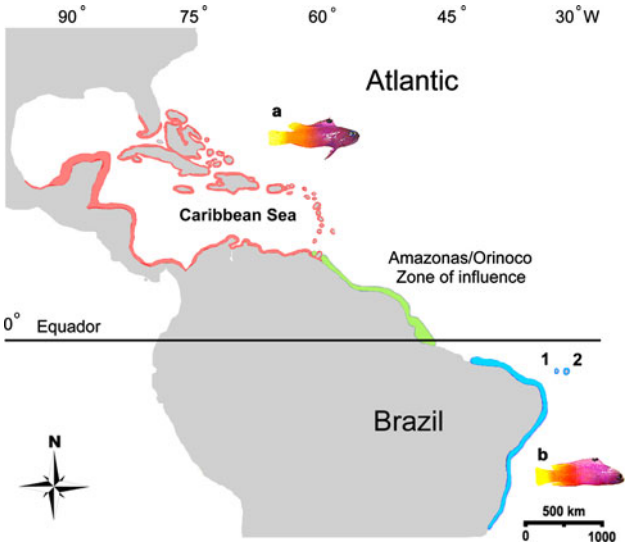

Fig. 1 Schematic presentation of the areas of occurrence of (*a*) *Gramma loreto* and (*b*) the sister-species *Gramma brasiliensis* along the Caribbean and Brazilian coast. Highlighted between these two areas is the zone of influence of discharges from the Orinoco and Amazon rivers into the Atlantic Ocean. The areas (*1*) and (*2*) correspond to Rocas Atoll and Fernando de Noronha Archipelago, respectively

Materials and methods

Specimens and chromosome preparations

Five *G. loreto* specimens (two males, two females, and one immature), obtained from ornamental fish importers, and ten specimens of *G. brasiliensis* (Sazima, Gasparini and Moura 1998) (five males, three females, and two immature), from the coast of Salvador (12°58′S/38°31′W), Bahia state, Northeast Brazil, were employed in chromosome analysis.

The specimens were subjected to mitotic stimulation using attenuated antigen compounds, for a period of 24 to 28 h, according to Molina (2001) and Molina et al. (2010). The animals were then anesthetized with clove oil (eugenol) and sacrificed for the removal of anterior kidney fragments and sexed by macroscopic and microscopic examination of the gonads. Chromosome preparations were obtained from cell suspensions of anterior kidney, through the in vitro interruption of the mitotic cycle, according to the method proposed by Gold et al. (1990).

Chromosome banding

Nucleolar organizer regions (NORs) were identified using the silver impregnation method (Ag-NORs), as described by Howell and Black (1980). C-banding according to

Sumner (1972), with minor modifications, was used to detect C-positive heterochromatin.

Sequential staining with the fluorochromes chromomycin A_3 (CMA_3) and 4′,6-diamidino-2-phenylindole (DAPI) was used to identify GC- and AT-rich chromosomal regions, respectively (Barros-Silva and Guerra 2009). Slides aged for 3 days were stained with CMA_3 (0.1/mg/ml) for 60 min and restained with DAPI (1 µg/ml) for 30 min. They were then mounted with antifading Vectashield and kept at 4°C for 5 days until analysis with an Olympus BX50 epifluorescence microscope, using appropriate excitation filters. Joint identification of positive fluorochromes CMA_3 and DAPI areas was obtained from the overlap of sequential images stained of the same metaphase, using Adobe Photoshop CS5 software.

Fluorescence in situ hybridization and karyotype analysis

Fluorescence in situ hybridization (FISH) was performed under high stringency conditions on mitotic chromosome spreads according to Pinkel et al. (1986). The 5S and 18S rDNA sequences were detected by dual-color FISH analysis. The two ribosomal sequences were isolated from the *Hoplias malabaricus* (Teleostei, Characiformes) genome. The 5S rDNA repeat copy included 120 base pairs (bp) of the 5S rRNA encoding gene and 200 bp of the non-transcribed spacer (NTS) (Martins et al. 2006). The 18S rDNA probe corresponded to a 1,400 bp segment of the 18S rRNA gene, obtained via PCR from nuclear DNA (Cioffi et al. 2009). The 5S and 18S rDNA sequences were cloned into the pGEM-T plasmid (Promega, Heidelberg, Germany) and propagated in DH5α *E. coli* competent cells (Invitrogen, San Diego, CA, USA). The 5S rDNA probe was labeled with biotin-14-dATP by nick translation according to manufacturer's recommendations (BioNick™Labeling System; Invitrogen, San Diego, CA, USA). The 18S rDNA was labeled by nick translation with DIG-11-dUTP, according to manufacturer's instructions (Roche, Mannheim, Germany).

Approximately thirty metaphases were analyzed for each specimen to determine diploid number and karyotype. The best metaphases were photographed with an Olympus BX50 epifluorescence microscope, equipped with a DP70 Olympus digital image capture system, used to determine karyotypes.

The chromosomes were grouped into metacentric (m), subtelocentric (st), and acrocentric (a) according to the position of the centromere (Levan et al. 1964) and arranged in descending order of size. Considering the difficulty of precise m and sm chromosome identification, they were grouped together in the karyotype.

Results

Both species have the same diploid number ($2n = 48$), with karyotypes 4 m + 6st + 38a in *G. loreto* and 6 m + 6st + 36a in *G. brasiliensis* (Fig. 2a, b). The smallest chromosome pair (pair no. 5 in *G. loreto* and pair no. 6 in *G. brasiliensis*) is significantly shorter than all other karyotype pairs. In this pair, it is difficult to identify the correct position of the centromere due to its small size (>1.5 µm). However, it was classified as subtelocentric, given data obtained in the majority of preparations examined. Thus, the two species differ in the number of m/sm chromosomes, which gives them different karyotypic formulas, as well as different fundamental number (FN). In fact, *G. loreto* is characterized by FN = 58 and *G. brasiliensis* by FN = 60, considering m/sm chromosomes, bi-armed st chromosomes, and one-armed acrocentric chromosomes.

Only one pair of Ag-NORs was detected, showing terminal location on the short arms of the largest subtelocentric pair (pair no. 3 in *G. loreto* and pair no. 4 in *G. brasiliensis*) (Fig. 2a, b, highlighted). Similarly, 18S rRNA genes were mapped in the same position and on the same chromosome pairs in both species (Fig. 2 g, h). In turn, 5S rRNA genes were mapped in both species at the terminal extremity of the chromosomes, located on the long arms of pairs 9 and 10 of *G. loreto* and *G. brasiliensis*, respectively, and the short arms of pairs 14 and 15 *G.* of *loreto* and *G. brasiliensis*, respectively (Fig. 2 g, h).

Heterochromatic blocks were highlighted in the centromeres of practically all the chromosomes; however, they were more noticeable in *G. brasiliensis* than in *G. loreto* (Fig. 2c, d). GC-rich heterochromatin distribution (CMA_3^+/$DAPI^-$) with NOR sites was clearly characterized in both species. This correspondence was also observed for 5S rDNA sites, albeit less marked. In turn, most heterochromatic regions in other *G. loreto* and *G. brasiliensis* chromosomes also exhibited a GC-rich composition, responding positively to chromomycin A_3 staining (Fig. 2c–f).

Discussion

Chromosome conservation × differentiation and karyotype evolution

Characteristics, such as $2n = 48$ acrocentric chromosomes, unique NORs, reduced heterochromatic content, and restricted to centromeric/terminal regions, are likely basal for Perciformes (Brum and Galetti 1997; Molina 2007). Among the cytogenetically conserved groups in this order, such as Sciaenidae, some or all of these characteristics are present in more than 85% of the species analyzed (Accioly and Molina 2008). In this context,

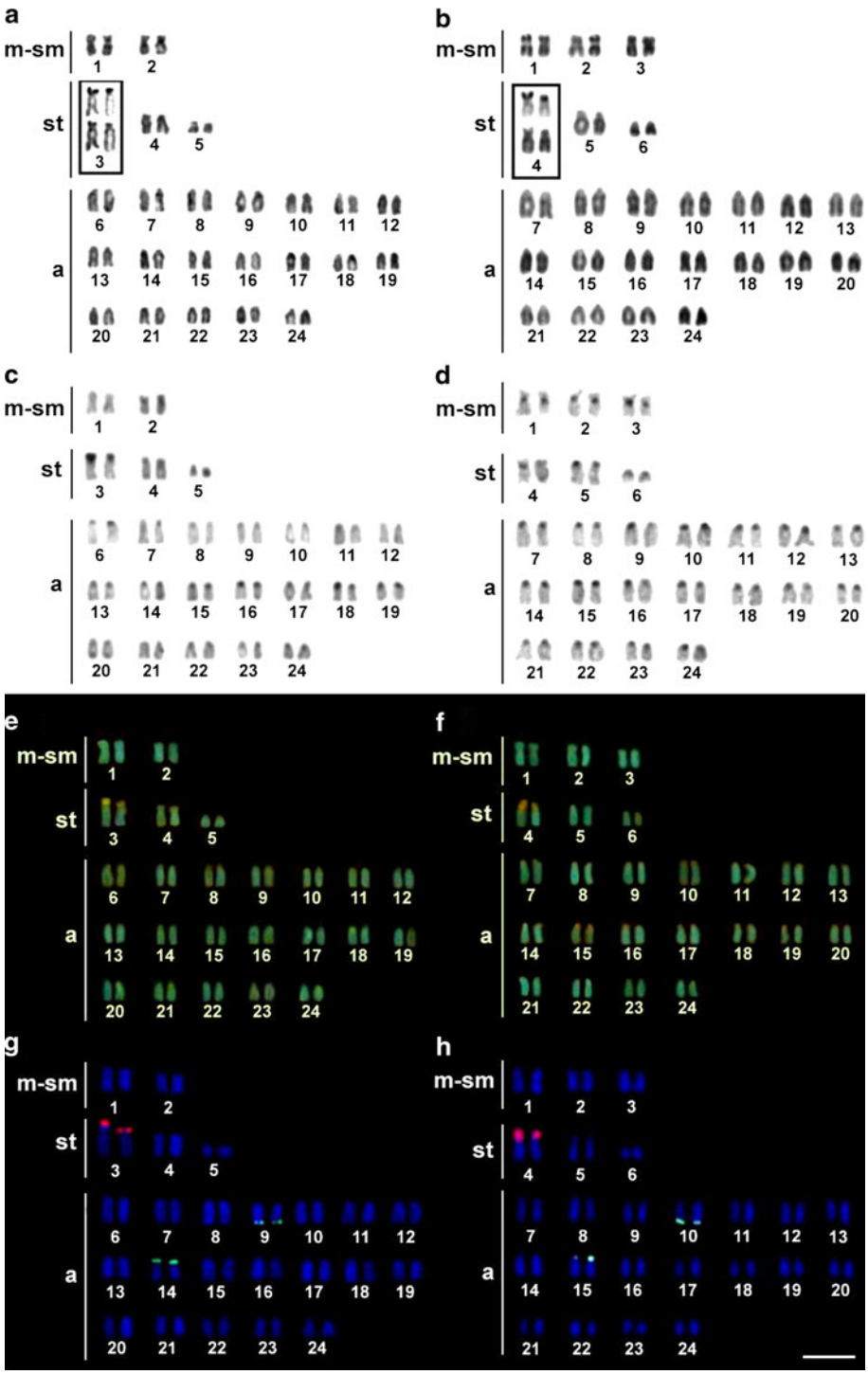

Fig. 2 Karyotype of *G. loreto* (**a**, **c**, **e**, **g**) and *G. brasiliensis* (**b**, **d**, **f**, **h**). Conventional Giemsa staining (**a**, **b**), highlighting Ag-NORs sites on the short arms of pairs 3 and 4 of *G. loreto* and *G. brasiliensis*, respectively; C-banding showing heterochromatin in centromeric chromosomal regions (**c**, **d**); Sequential staining with fluorochromes CMA_3/DAPI (**e**, **f**), showing the composition of most GC-rich heterochromatin blocks and location of ribosomal genes by dual-color FISH with rDNA 18S (pair 3 of *G. loreto* and pair 4 of *G. brasiliensis*) and 5S (pairs 9 and 14 of *G. loreto* and pairs 10 and 15 of *G. brasiliensis*) (**g**, **h**) probes. *Bar* = 5 μm

sister-species karyotypes of Grammatidae, *G. loreto* and *G. brasiliensis*, despite their basal chromosome number remaining unchanged ($2n = 48$), contrast in other aspects with the widely present conservatism of Perciformes. Whereas *G. loreto* exhibits a larger number of acrocentric chromosomes (FN = 58) and, therefore, more related to the karyotype considered basal for the group, *G. brasiliensis* shows a higher number of two-armed acrocentric chromosomes (FN = 60). Indeed, the most diagnosed cytotaxonomic marker for these species is found among the meta-submetacentric and acrocentric chromosomes. While 4 m-sm and 38a occur in *G. loreto*, 6 m-sm and 36a occur in *G. brasiliensis*, likely as a result of pericentric inversion, currently fixed in the homozygous condition of the latter species, changing one pair of acrocentric chromosomes into one pair of m-sm chromosomes.

It seems apparent that pericentric inversions correspond to the main chromosomal rearrangements found in Perciformes (Cano et al. 1982; Ozouf-Costaz et al. 1991; Galetti et al. 2000), as well as in other phylogenetically diverse marine groups, such as Tetraodontiformes (Sá-Gabriel and Molina 2005; Noleto et al. 2007; Martinez et al. 2010), Anguilliformes (Takai and Ojima 1985; Vasconcelos and Molina 2009), and Batrachoidiformes (Brum et al. 2002; Nirchio et al. 2002; Costa and Molina 2009), indicating their effective participation in the karyotypic evolution of these groups.

More recently, the role of inversions in the evolutionary process has been reassessed. After inversion occurs, it can be lost in the polymorphic state or, under the proper conditions, spread in the population until it is fixed. Even though few genes related to traits adapted to new environmental/climatic conditions and the speciation process were identified in the inversions, there are growing indications of their decisive role in these events. Inversions maintain areas of imbalance between alleles in *loci* within or influenced by these rearrangements, leading to an adaptive condition, primarily along environmental gradients (for a review see Hoffmann and Rieseberg 2008). This could occur, particularly in relation to possible historical expansion and adaptation to new environments more toward the south, for *G. brasiliensis*, compared to *G. loreto*. The different occurrence of pericentric inversions among coastal marine species and their likely derived insular forms have also been identified in other marine Perciformes, such as in species of the genus *Stegastes*. In this case, *S. sanctipauli* and *S. rocasensis*, inhabitants of ecologically diverse areas far from the mainland, had a larger number of detectable pericentric inversions when compared to land-based forms (Molina 2007), demonstrating the relevant role of these rearrangements in the evolutionary history of these groups.

In general, reduced heterochromatic/centromeric/telomeric content corresponds to the widely dispersed pattern among Perciformes (Molina 2007). A few exceptions are known, such as a species of *Centropyge* (Pomacanthidae) on the coast of Brazil, which exhibits conspicuous heterochromatic blocks on the short arm of a number of subtelocentric chromosomes (Affonso and Galetti 2005). C-positive heterochromatin in *G. loreto* and *G. brasiliensis* maintains the preferential pericentromeric pattern, but distributed to practically all chromosomes of the karyotype, in addition to the short arms of NOR-bearing subtelocentric chromosomes. However, there is predominance of more conspicuous heterochromatic blocks in *G. brasiliensis*, underscoring a different heterochromatinization process when compared to *G. loreto.* In this sense, *G. brasiliensis* once again stands out as a relatively more differentiating karyotype, reinforcing its condition, derived from the genus *Gramma*. On the other hand, the occurrence of GC-rich heterochromatin dispersed in different chromosomes of the karyotype in both species, in addition to ribosomal sites, contrasts with the pattern usually observed in Perciformes, where a neutral response to AT- or GC-specific fluorochromes is observed (except the major rDNA sites), indicating a probably apomorphy of the family Grammatidae.

However, in addition to these differentiating characters between *G. loreto* and *G. brasiliensis,* other chromosomal markers were conserved in both species, such as the number and location of Ag-NORs and in turn 18S rDNA and 5S rDNA sites. There is a clear association between chromosomes at these sites in the two species, suggesting homeology between them. Thus, while some of the chromosomal characteristics of *G. loreto* and *G. brasiliensis* underwent differential processes, others remained stable and conserved throughout their evolutionary history. Similar observations were also made for pairs 5 and 6 of *G. loreto* and *G. brasiliensis*, respectively, which can be considered microchromosomes because of their reduced size (1.5 μm), markedly different from the other pairs of the karyotype. Microchromosomes have often been found in different groups of vertebrates (King 1990), primitive fish (Rock et al. 1996), and various marine Perciformes (Takai and Ojima 1987). In both *Gramma* species, these chromosomes were constant in all metaphases analyzed, thereby characterizing them as regular components of their chromosomal lots and excluding them as possible B or supranumerary chromosomes, which can vary between specimens or even among the cells of an individual (Carvalho et al. 2008). This karyotypic trait is shared by *G. loreto* and *G. brasiliensis*, allowing us to infer about the ancestry of their presence, likely prior to the speciation process that differentiated these species, thereby constituting a phylogenetic marker for these forms.

Chromosomal differentiation and speciation

Fish fauna comparisons between Atlantic regions have pointed to the Caribbean as a center of diversity and active exporter of a range of reef fish. Mitochondrial sequence analyses in Caribbean populations of the pomacentrid *Chromis multilineata* support this idea, indicating that they are older than in other Atlantic regions, including Brazil (Rocha 2003). Historical environmental variations caused by glacial events (e.g., Gysels et al. 2004), as well as the emergence of the Amazon/Orinoco river barrier (Rocha 2003, Rocha et al. 2008), had a potential influence on low-vagility cryptobenthic species inhabiting shallow waters, leading to population restrictions and the fixation of genetic divergences, such as the chromosomal modifications observed in the *Gramma* species analyzed here. Indeed, the role of glacial events in fractioning and restricting populations is considered relevant for the chromosomal diversification and transitory polymorphisms present in marine

species of the genus *Chromis* (Molina and Galetti 2002). Moreover, the barrier formed by the discharge of the Amazon/Orinoco Rivers (≈10 m.a) has also played a crucial role in the evolution of reef fish in the tropical Atlantic (Briggs 1974; Robertson et al. 2006). This barrier is considered the primary cause of sister-species formed in the different families of reef fish (Floeter and Gasparini 2001; Rocha 2003), promoting an effective or sufficient impediment to gene flow between the Caribbean and Brazilian coast, mainly in shallow water species.

Nearly all Grammatidae species are found in the Caribbean, suggesting that this region, similarly to what is observed in other groups (Rocha et al. 2008), is also the diversification center of this family. On the other hand, *G. brasiliensis*, a species endemic to the Brazilian coast, likely represents a more recent derived form than *G. loreto*. This hypothesis is supported by the more conservative karyotypic characteristics of *G. loreto*, when compared to *G. brasiliensis*. Indeed, cytogenetic data indicate that *G. brasiliensis* exhibits a more derived karyotype, considering its karyotypic formula and the more conspicuous heterochromatinization of its chromosomes.

Sympatric and parapatric speciation in marine fish have been increasingly reported (Rocha 2003; Rocha and Bowen 2008), demonstrating that they are more frequent events than were previously believed. One possible example of sympatric speciation was reported in Grammatidae. The species *G. dejongi*, inhabiting the deep waters off Cuba, occurs within the distribution area and in sympatry with *G. loreto*. The two species share morphological characteristics and have similar mtDNA barcodes (Victor and Randall 2010), suggesting a valid species with recent sympatric speciation. On the other hand, the areas of influence belonging to the Amazon/Orinoco Rivers demonstrate marked ecological effects on *G. loreto,* and its germinate species, *G. brasiliensis*, promoting a conspicuous environmental exclusion zone between them, as well as between any other Grammatidae species. This barrier probably led to reproductive isolation between populations of *G. loreto* for enough time to allow differentiation and allopatric speciation between them. The premise of allopatric speciation is more plausible for shallow water species and specialized habits, such as *G. loreto*, where the Amazon/Orinoco barrier is a physical, physiological, ecological, and possibly insurmountable obstacle, even during glaciations events. Two aspects support this hypothesis. The first is phylogenetic (phylogeographic), evidenced by the absence of Grammatidae species along the Brazilian coast, as well as in the zone affected by the Amazon/Orinoco Rivers, suggesting impediment to subsequent dispersion and colonization by other Caribbean species in Brazil. The second is cytogenetic, represented by the conspicuous karyotypic differentiations currently fixed in *G. brasiliensis*, which is much more likely to occur in the conditions of geographic isolation than in sympatric or even parapatric conditions.

The allopatric effects on the karyotype of these species indicate forms with large steps of divergence and potential degree of postzygotic reproductive isolation. It is known that chromosome rearrangements, such as inversions, can represent effective postzygotic reproductive isolation mechanisms (Noor et al. 2001; Brown et al. 2004). In this sense, reproductive behavior analysis of *G. loreto* and *G. brasiliensis* under sympatric conditions in nature would be highly elucidative, although such a possibility seems to be very remote, considering the biogeographic isolation barrier between them and the difficult transposition for any of these species.

Final considerations

The karyotypic pattern of sister-species *G. loreto* and *G. brasiliensis*, in addition to expected similarities, also shows a specific chromosomal diagnosis. Data strongly suggest that *G. brasiliensis* is a more recent species, derived from *G. loreto* through a process of allopatric speciation afforded by biogeography isolation from the Amazon/Orinoco barrier.

In this case, chromosomal differences between these two *Gramma* species reflect about ten million years of evolutionary differentiation. Very little is known about the chromosomal alterations involved in the speciation process in a marine environment. Although prezygotic barriers can be considered isolation mechanisms between species of Perciformes (Kocher 2004), postzygotic chromosomal barriers may have been established between the two *Gramma* species analyzed, hindering or even preventing possible hybridizations and introgression between them. Thus, cytogenetic studies in different regions of the Atlantic might provide informative data for diagnosing interspecific and/or interpopulation differentiation, especially in the investigation of chromosomal events that may have promoted the speciation process in this environment.

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