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ORIGINAL ARTICLE

Atlantic moonfishes: independent pathways of karyotypic and morphological differentiation

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Abstract Fish of the genus *Selene*, known as lookdowns or moonfish, are one of the most morphologically derived groups of the family Carangidae, whose phylogenetic relationships are still largely unknown. In this study, we discuss karyoevolutionary aspects of three representatives of this genus from the Western Atlantic: *Selene brownii* ($2n = 48$; FN = 48), *Selene setapinnis* ($2n = 46$; FN = 48), and *Selene vomer* ($2n = 48$; FN = 50). Their body patterns were also investigated and compared to one another and in relation to two other species of different genera. Two mechanisms of karyotypic evolution seem to have acted in the diversification of this genus, namely pericentric inversions and centric fusions. Mapping of rDNA sequences showed that chromosome pairs bearing 5S rDNA sites are similar, whereas those bearing 18 rDNA sites are morphologically distinct while apparently also exhibiting interspecies synteny. Although the nucleolar organizer-bearing chromosomes are extremely efficient cytotaxonomic markers among *Selene* species, others cytogenetic patterns of these species are relatively conserved. Hybridization with telomeric probes $(TTAGGG)_n$ did not exhibit interstitial telomeric sites (ITS), especially in *S. setapinnis*, where, along with a reduction in diploid number, a large metacentric pair derived from centric fusion is present. Data obtained by geometric morphometrics enable a clear morphological distinction among the three species, as well as in relation to two other species of the genus *Caranx* and *Oligoplites*. Data obtained suggest that morphologic evolution in *Selene* species was primarily dissociated from visible changes that occurred at the chromosomal level.

Keywords *Selene* · Pericentric inversions · Centric fusion · Morphological divergence

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Introduction

Fish of the genus *Selene*, known as lookdowns or moonfish, belong to one of the most morphologically derived groups compared to the 32 genera that make up the family Carangidae. Indeed, they exhibit a tall round body, laterally compressed, with a unique steep head (Smith-Vaniz 1979). This genus is composed of only the following seven species: *Selene brownii* (Cuvier 1816), *Selene setapinnis* (Mitchill 1815), *Selene vomer* (Linnaeus 1758), in the Western Atlantic, *Selene dorsalis* (Gill, 1862), in the Eastern Atlantic, and *Selene peruviana* (Guichenot 1866), *Selene brevoorti* (Gill 1863), and *Selene orstedii* (Lütken, 1880), in the Eastern Pacific.

Phylogenetic relationships among *Selene* species are scarcely known, where there is no uniquely derived character identified for this genus putting your taxonomic composition in family Carangidae still in question (Smith-Vaniz 1984).

From a morphological standpoint, the absence of an exclusive diagnostic character for *Selene* makes its taxonomic composition questionable, for example, validation of the species *S. setapinnis* and *S. brownii*, whose differentiation characters significantly overlap (Smith-Vaniz 1979). Analyses based on cytochrome b gene sequencing have as yet not been able to explain relationships among its species (Reed et al. 2001). Questions remain concerning the real phylogenetic positioning and monophyletism of this genus, although the differentiation of marine species in many groups is not seem to have been followed by significant karyotype diversification (Brum and Galetti 1997).

Rearrangements in the chromosome complement are often associated with speciative processes, favoring their use in phylogenetic and evolutionary analyses (King 1993). Scant cytogenetic information is available for Atlantic species of Carangidae (Rodrigues et al. 2007) in contrast to Mediterranean species, which are the focus of most cytogenetic studies (Caputo et al. 1996; Sola et al. 1997; Chai et al. 2009).

In order to compare evolutionary aspects of the karyotype and establish a possible relationship with its diversified body shapes, the species *S. brownii*, *S. setapinnis* and *S. vomer*, occurring in the Western Atlantic, were analyzed cytogenetically by conventional staining, Ag-NORs, C-banding, chromomycin A_3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) staining, and mapping of 18S, 5S rDNA and telomeric sequences, as well as by geometric morphometrics. Both methodologies were able to discriminate species, showing their chromosomal evolution and validating the sibling species *S. brownii* and *S. setapinnis*.

Materials and methods

Cytogenetic analyses were conducted with the Caribbean moonfish *Selene brownii* ($n = 8$), Atlantic moonfish *S. setapinnis* ($n = 7$), and the Atlantic lookdown *S. vomer* ($n = 7$) from the coast of Rio Grande do Norte State (05°05′26″S/36°16′31″W), Northeastern Brazil. Before chromosomal preparations, specimens were submitted to in vivo mitotic stimulation for 24 h, by intramuscular and intraperitoneal inoculation of bacterial and fungal antigen complexes (Molina et al. 2010). Specimens were anesthetized with clove oil (Griffiths 2000) and then killed. Metaphase chromosomes were obtained from cell suspensions of the anterior kidney, using short-term in vitro methodology (Gold et al. 1990).

Chromosome banding

Heterochromatic regions and ribosomal sites were identified with techniques described by Sumner (1972) and Howell and Black (1980), respectively. CMA_3/DAPI double staining was employed, using DAPI as counterstain (Barros-e-Silva and Guerra 2010). Briefly, slides aged for 3 days were stained with CMA_3 (0.1 mg/ml) for 60 min and retained with DAPI (1 μg/ml) for 30 min. Next, the slides were mounted in glycerol:McIlvaine buffer pH 7.0 (1:1) and aged for 3 days before being analyzed with an epifluorescence microscope under appropriate filters.

Cytogenetic mapping

Three probes were used: an 18S rDNA probe obtained from the nuclear DNA of *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti 2004), a 5S rDNA probe isolated from the genomic DNA of *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti 1999), and a probe for the general vertebrate telomeric sequence $(TTAGGG)_n$ (Ijdo et al. 1991). Probes were labeled through polymerase chain reaction (PCR), using biotin-16-dUTP (Roche Applied Science) for rDNA 18S, or digoxygenin-11-dUTP (Roche Applied Science) for rDNA 5S and $(TTAGGG)_n$ probes. Polymerase chain reaction labeling using specific primers was performed for rDNA clones using 20 ηg of template DNA, 1X Taq Reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 40 μM dATP, dGTP and dCTP, 28 μM of dTTP, 12 μM biotin-16-dUTP or digoxygenin-11-dUTP, 1 μM primers, 2 mM $MgCl_2$ and 2 U of Taq DNA polymerase (Invitrogen) under the following conditions: 5 min at 94 °C; 35 cycles: 1 min at 90 °C, 1 min 30 s at 52 °C and 1 min 30 s at 72 °C; and a final extension step at 72 °C for 5 min. The reaction for labeling the telomeric probe involved 1× Taq Reaction buffer, 40 μM dATP, dGTP, and dCTP, 28 μM of dTTP, 12 μM of digoxygenin-11-dUTP, 0.2 μM primer (TTAGGG) 5, 0.2 μM primer (CCCTAA)5, 2 mM $MgCl_2$, and 2 U of Taq DNA polymerase, under the following conditions: the first amplification was performed with low stringency: 4 min at 94 °C, 12 cycles of 1 min at 94 °C, 45 s at 52 °C and 1 min 30 s at 72 °C, followed by 35 high stringency cycles: 1 min a 94 °C, 1 min 30 s at 60 °C, and 1 min 30 s at 72 °C.

The overall hybridization procedure followed the protocol described by Pinkel et al. (1986), under high stringency conditions (2.5 mg/μl from each probe, 50 % deionized formamide, 10 % dextran sulfate, 2XSSC, pH 7.0–7.2, at 37 °C overnight). After hybridization, slides were washed in 15 % formamide/0.2XSSC at 42 °C for 20 min, 0.1XSSC at 60 °C for 15 min, and 4XSSC/0.05 % Tween at room temperature for 10 min, the latter consisting of two 5-min washes. Signal detection was performed using streptavidin-alexa fluor 488 (Molecular Probes) for 18S rDNA, and anti-digoxigenin-rhodamine (Roche Applied Science) for 5S rDNA and $(TTAGGG)_n$ probes.

One-color FISH was performed to detect (TTAGGG), while 5S and 18S rDNA were detected by dual-color FISH.

Chromosome analyses

Chromosomes were analyzed under an epifluorescence microscope (Olympus BX41) coupled to an image capturing system (Olympus DP71). Approximately 30 metaphases of each species were analyzed to establish the diploid number and karyotype structure, as well as to map rDNA sites and eventual ITS (Interstitial telomeric sites) on the chromosomes. Hybridization signals exhibiting 100 % repeatability were considered genuine. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a), based on the system proposed by Levan et al. (1964).

Morphometrics analyses

Geometric morphometrics methods were used to compare body patterns of *S. brownii* ($n = 20$), *S. setapinnis* ($n = 15$), *S. vomer* ($n = 17$), and *Oligoplites palometa* (Cuvier 1832) ($n = 15$), from the coast of Natal, Rio Grande do Norte state and *Caranx lugubris* (Poey 1860) ($n = 15$), from the São Pedro and São Paulo Archipelago. In view to minimize possible distortions due allometric effects, only adult individuals were used to the measurements.

Specimens of each species were individually photographed (right side view), with an 8.1 megapixel Sony DSC-H10 digital camera. Body shapes were analyzed using 12 landmarks (Fig. 2), defined by tps Dig 2 1.40 software (Rohlf 2004). Configurations of body landmarks were overlapped using generalized procrustes alignment (Rohlf and Slice 1990; Dryden and Mardia 1998). Analyses of canonical variates were performed to discriminate and quantify the magnitude of morphological divergence. Intergroup significance levels were determined by Hotelling T2 statistic and the permutation test (1,000 times). Interspecies morphological differences were illustrated by the thin-plate spline method, using wireframe graphs. All analyses were carried out with MorphoJ 1.02 software (Klingenberg 2008).

Results

Cytogenetic analyses

Selene brownii exhibited karyotype composed of $2n = 48$ acrocentric chromosomes (FN = 48) (Fig. 1; left column). Reduced heterochromatic blocks are present in the pericentric region of most chromosomes and, in some pairs, in the terminal region. Dual-color FISH with 18S and 5S rDNA showed non-syntenic location between these ribosomal subunits. The 18S rDNA sites are unique and coincident with the Ag-NOR$^+$/C-band$^+$/CMA$^+$/DAPI$^-$ region of chromosome 1. The 5S rRNA genes are positioned in the pericentromeric region of chromosome pair 9, co-located with a conspicuous heterochromatic region.

Selene setapinnis showed karyotype with $2n = 46$ (FN = 48), 44 acrocentric chromosomes and two large metacentric chromosomes (Fig. 1; central column). Small heterochromatic blocks are present in the pericentromeric regions of most chromosomes. *In situ* hybridization with 18S and 5S rDNA probes indicates the location of these ribosomal subunits in different chromosome pairs. The 18S sites are located proximally to the centromere, on the long arm of the large metacentric pair, coincidentally with the Ag-NOR$^+$/C-band$^+$/CMA$^+$/DAPI$^-$ region. The 5S rDNA sites were mapped in the terminal position of chromosome pair 9, co-located with heterochromatic bands. Probes $(TTAGGG)_n$ showed no ectopic sequences in any of the chromosome pairs, in addition to the telomeric sites expected.

Selene vomer showed karyotype with $2n = 48$ chromosomes (FN = 50), consisting of 46 acrocentric chromosomes and two subtelocentric chromosomes (Fig. 1; right column). Reduced heterochromatic sites are present in the pericentromeric regions of the chromosomes. As in other *Selene* species, a dual-color FISH with 18S and 5S rDNA probes showed that these sequences occupy distinct chromosome pairs. The 18S rDNA sequences are located in the Ag-NOR$^+$/C-band$^+$/CMA$^+$/DAPI$^-$ region of the short arm on the only subtelocentric pair (pair 1). As with the other species, 5S rDNA sites were mapped in the pericentromeric position of pair 9. Telomeric sequences are located only in the terminal portions of all chromosomes.

Dual base-specific staining in all species revealed no additional positive CMA_3/negative DAPI staining beyond that already registered for the NOR region. In all species analyzed, FISH with telomeric probes $(TTAGGG)_n$ exhibited signs of hybridization only in the terminal portions of chromosomes. In the Fig. 1d (center column) is shown the hybridization pattern with telomeric probe for the nucleolus organizer pair of *S. setapinnis*.

Morphometrics analyses

Data obtained through analysis of canonical variates demonstrate significant morphological separation between *S. brownii*, *S. setapinnis,* and *S. vomer,* primarily those of *O. palometa* and *C. lugubris* (Table 1; Fig. 2). Canonical variates one and two jointly explain 93.44 % of the variation observed. Canonical variate one contributed to 85.82 % of total variation, clearly separating species from

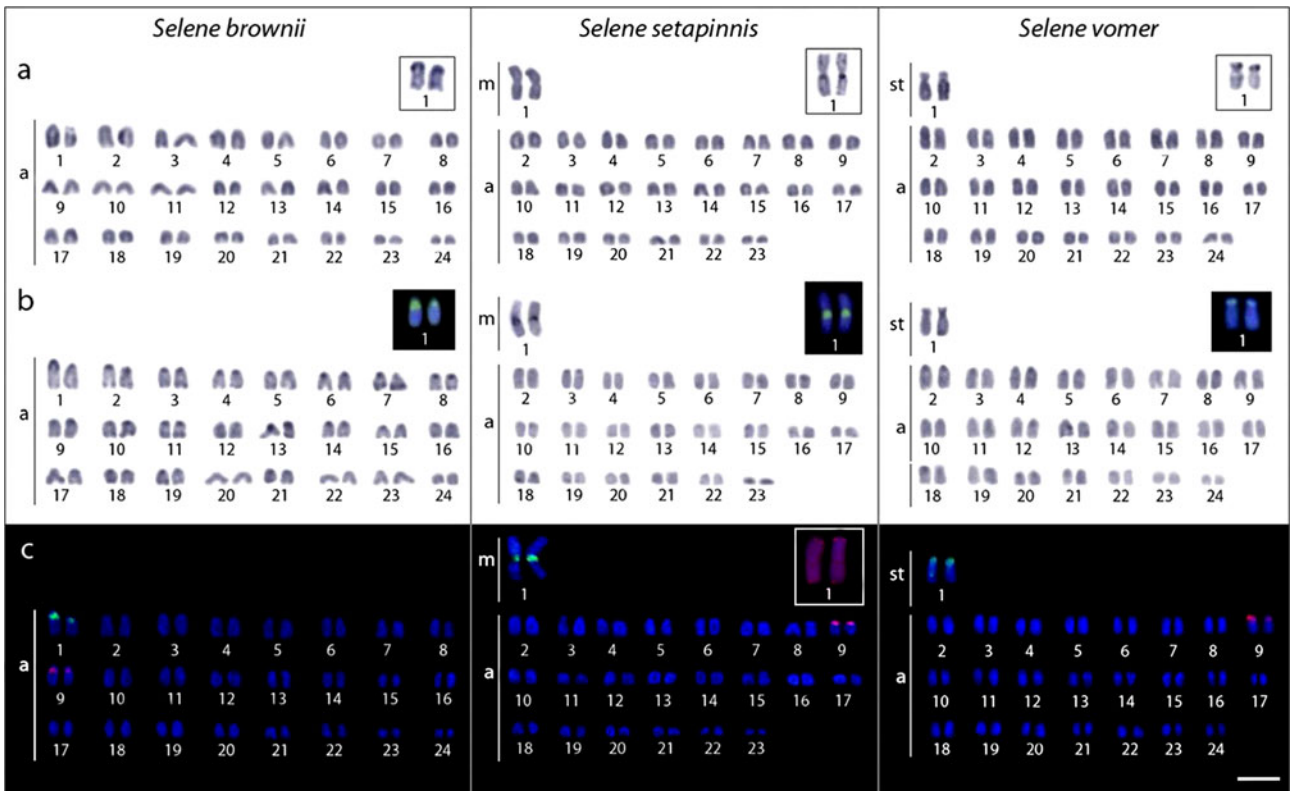

Fig. 1 Karyotypes of *S. brownii*, *S. setapinnis*, and *S. vomer*. **a** Conventional staining highlighting Ag-NOR sites in chromosome 1; **b** C-banding highlighting the NOR region with $CMA_3^+/DAPI^-$ staining; **c** dual-color FISH with 18S rDNA (*green*) and 5S probes (*red*), showing the location of 18S rDNA sites in the first pair and 5S rDNA in chromosome pair 9 in the karyotypes; **d** In the center column, FISH with $(TTAGGG)_n$ probes showing telomeric sites in the nucleolus organizer pair of *S. setapinnis*. Bar = 5 μm (color figure online)

the genus *Selene* of *O. palometa* and *C. lugubris*, while species *S. brownii* and *S. setapinnis* are entirely distinguishable from *S. vomer* by canonical axis 2, which explains 7.62 % of total variation.

Wireframe graphs for each species, compared with standard consensus, indicate that the distinguishing characteristic for species from the genus *Selene* in relation to *O. palometa* and *C. lugubris* is their deep body. In turn, *S. vomer* is clearly separated from *S. brownii* and *S. setapinnis* by the shape of its head, exhibiting a less steep profile. In addition, *S. brownii* and *S. setapinnis* differ from *S. vomer* by the deeper and more oval anteroposterior shape of their head (Fig. 2).

Table 1 Mahalanobis distance between Carangidae species (lower diagonal) and *P* values with permutation tests of (×1,000) (upper diagonal)

	C. lugubris	*O. palometa*	*S. brownii*	*S. setapinnis*	*S. vomer*
C. lugubris	–	<0.001	<0.001	<0.001	<0.001
O. palometa	18.14	–	<0.001	<0.001	<0.001
S. brownii	30.12	41.51	–	<0.001	<0.001
S. setapinis	33.59	44.57	78.2	–	<0.001
S. vomer	27.88	37.78	14.36	16.71	–

Discussion

Morphological diversification between Perciformes groups was, in many cases, not accompanied by pronounced karyotype diversification (Molina et al. 2002; Motta-Neto et al. 2011c). This has been reported in several families, including Haemulidae, which exhibits extensive numerical and structural chromosome conservatism among its species (Motta-Neto et al. 2011a, b, c). Nevertheless, a number of families are characterized by chromosomal diversification, either in regard to diploid value or aspects of karyotype macrostructure (Caputo et al. 1996; Araújo et al. 2010).

In contrast with other fish families (Motta-Neto et al. 2011a), cytogenetic and morphological analyses indicated that high morphological differentiation among *Selene* species was also accompanied by some discernible chromosome changes, primarily diploid number variation and structural changes in one pair of chromosomes. Comparatively, *S. brownii* and *S.setapinnis* exhibited greater morphological similarity between them. However, the karyotype of *S. brownii* maintains more basal characteristics of the order

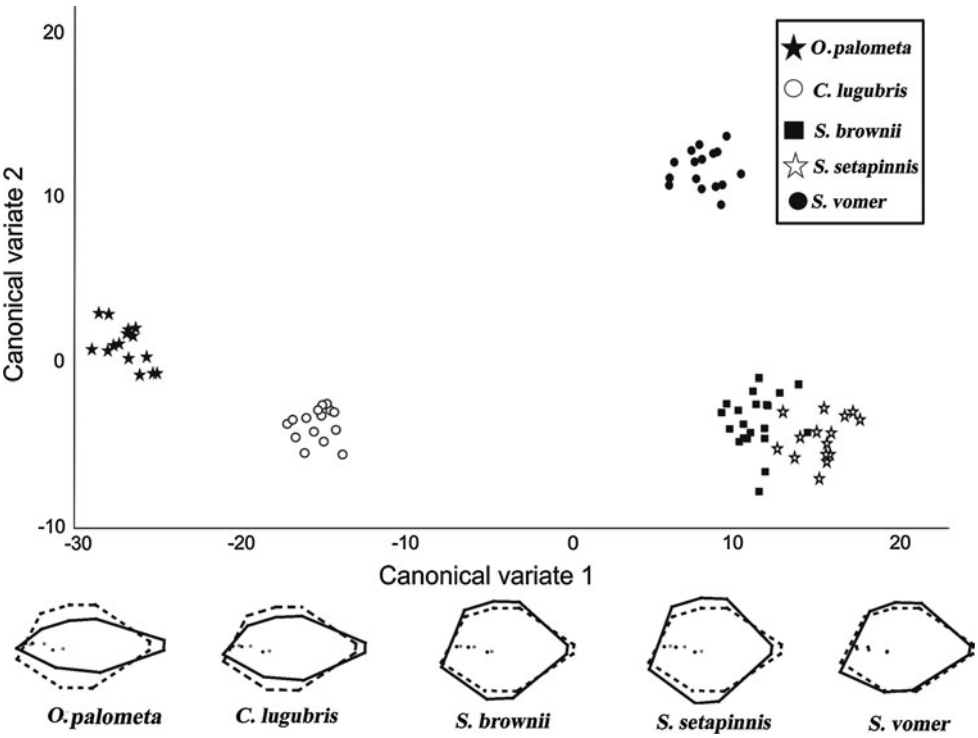

Fig. 2 Canonical variates projection of body shape measurements for *O. palometa* (*black stars*), *C. lugubris* (*white circles*), *S. brownii* (*black squares*), *S. setapinnis* (*white stars*), and *S. vomer* (*black circles*). Schematic drawings show comparisons between species and consensus shape

Perciformes, such as $2n = 48$ acrocentric chromosomes, simple ribosomal sites, and reduced heterochromatin (Brum 1996). The karyotype of *S.setapinnis* diverges from S. *brownii* in that it displays a lower diploid value ($2n = 46$) and large metacentric pair, suggesting a clear Robertsonian translocation events as the origin of the pair.

Ribosomal sites, located proximally to the centromere on the long arm of this pair and identified by Ag-NOR and mapping of 18S rDNA sequences, are compatible in size and position with those found in the conserved karyotype of *S. brownii*, which reinforced this hypothesis. Thus, *S.brownii* and *S.setapinnis* are completely distinct with regard to their cytogenetic patterns. Involvement by an ancestral chromosome pair, bearing NOR sites, in the formation of a single metacentric pair in *S. setapinnis* suggests a more derived condition for this species in relation to *S. brownii*. Unlike *S.brownii* and *S.setapinnis*, the chromosome bearing NOR sites in *S. vomer* is subtelocentric, indicating the occurrence of pericentric inversion in the formation of this pair, exclusive to this species. In conjunction, these results suggest chromosome rearrangement is favored by the presence of ribosomal sites. In fact, there are indications that heterochromatin congruent to NORs may play a role in evolutionary rearrangements involving NOR-bearing chromosomes (Fujiwara et al. 1998).

In the family Carangidae, approximately 85 % of species display $2n = 48$ chromosomes. Of these, 30 % have karyotypes formed exclusively by acrocentric chromosomes (Chai et al. 2009), representing a basal characteristic for Perciformes. As such, numerical and structural alterations in this pattern are indicative of more derived karyotypes (Molina 2007), as in *S.setapinnis* and *S. vomer*. On the other hand, presence of bibrachial chromosomes demonstrates evolutionarily more dynamic karyotypes in Carangidae when compared with some other marine Perciformes families, such as Chaetodontidae and Sciaenidae (Galetti et al. 2006; Accioly and Molina 2008).

Among primary karyotype diversification events, pericentric inversions play an important role in the family Carangidae. However, chromosome polymorphisms related to Robertsonian translocations in *Trachurus* and *Seriola* (Vitturi et al. 1986) indicate these occurrences also contribute to karyotype diversification in Carangidae and may become established in some species, as observed in *S. setapinnis*.

In light of the extensive karyotypical conservatism presented by some groups of Perciformes, NORs may be inefficient cytotaxonomic markers for some species (Molina and Galetti 2004). However, NORs located in chromosomes involved in rearrangements, as in Carangidae (Caputo et al. 1996; Sola et al. 1997; Rodrigues et al. 2007; present study), make them potentially effective taxonomic and population approaches for this group. Although species analyzed contained a nucleolar organizer pair as a specific marker, the genus *Selene* displayed several conservative traits in its karyotypes. These include a large number of acrocentric chromosomes, simple 18S rDNA sites not syntenic with 5S-rRNA genes, GC-rich regions coincident with NORs, 5S rDNA sites situated in chromosomal pairs corresponding to the presence of low heterochromatic content primarily in

pericentromeric regions. These characteristics have been identified as basal to Perciformes (Galetti et al. 2006; Molina 2007) and indicative of significant chromosome conservatism with low evolutionary dynamics. (Motta-Neto et al. 2011a, b, c). Similarly to NORs, 5S rDNA sites have proven to be phylogenetically discriminatory markers between several species and populations of marine fish (Motta-Neto et al. 2011a, b). Some cases have identified possible participation by 5S sites in chromosome rearrangements (Molina and Galetti 2002). Nevertheless, among *Selene* species, 5S sequences have demonstrated a conserved condition with low evolutionary dynamics.

Mapping of telomeric sequences has shown chromosomal fusion points in some fish species (Phillips and Reed 1996; Fontana et al. 1998). *Selene brownii, S. setapinnis* and *S. vomer* sequences $(TTAGGG)_n$ are exclusively located in the terminal portions of chromosomes. In *S. setapinnis* ($2n = 46$), the reduced diploid number observed in the ancestral condition ($2n = 48$) is attributed to centric fusion. In such cases, acrocentric/telocentric chromosomes, which are now united by their centromeres, may maintain fragments of their telomeric DNAs at the points of the fusion, with the occurrence of interstitial telomeric sites (ITS). However, several centric fusion events did not indicate the presence of ITS, as observed in the large metacentric pair of *S. setapinnis*, likely due to loss of these sequences during fusion (Slijepcevic 1998). Nevertheless, the 18S rDNA site in this chromosomal pair is adjacent to the point of fusion, that is, proximal to the centromere. A similar situation was recorded in fish from the genus *Chromis*, where 5S sites were contiguous to chromosome fusion regions and no ITS sites were detected (Molina and Galetti 2002). Similarly, decondensed 5S rDNA sites, interspaced by ITS sites, are indicative of chromosome fusions (Rosa et al. 2012).

Morphologically, Carangidae are characterized by a compressed, though highly variable, body shape, whereas species from the genera *Decapterus* and *Trachurus* have slender bodies, and those from *Selene* exhibit taller bodies (Gushiken 1988; Reed et al. 2002). As such, three body patterns are found in the genus *Selene*: (1) steep head profile with elongated dorsal and anal fins, seen in *S. vomer*, (2) head profile rounded on the top and very steep, with short dorsal and anal fins, present in *S. brownii* and S. *setapinnis* and, (3) and intermediary profile to the previous two, found in *S. osterdii* (Smith-Vaniz 1984). Although these body characteristics appear to indicate substantial derivation among carangids, no study has established how morphological evolution with in the group occurred. Phylogenetic inferences, as yet unconfirmed, based on Cit B sequences (Reed et al. 2001), suggest that the short dorsal and anal fins, such as those found in *S. brownii* and *S. setapinnis*, are derived. However, this hypothesis is not substantiated by cytogenetic data, since *S. brownii* displays a karyotype with characteristics considered the most basal among the three species analyzed here.

As such, in light of the apparent inconsistency between cytogenetic and molecular data, aspects of body shape were quantified in order to determine possible relationships between the species. Comparison of the body pattern of *Selene* species, polarized by members of the genus *Oligoplites* (*O.palometa*), which are phylogenetically more basal and *Caranx* (*C. lugubris*), a more modern group (Reed et al. 2002), enabled quantification of the morphological amplitude between these species (Table 1). Morphological patterns are notably different among *Selene* and representatives of the genera *Oligoplites* and *Caranx*. *Selene brownii* and *S.setapinnis* show greater morphological proximity in species analyzed. In other words, *S. setapinnis* exhibits a more divergent pattern in relation to the basal external group, followed by *S. brownii,* while *S. vomer* differs more from these two species and is less divergent from *O.palometa* and *C. lugubris*. Thus, if these patterns are an accurate representation of phylogenetic relationships with in the family, this would suggest that chromosome rearrangements occur independently between species and, as such, would not be valid phylogenetic markers.

Morphological patterns are often indicators of the lifestyle of a species (Karr and James 1975; Wainwright and Reilly 1994). In fact, strong associations have been observed between basic shape and ecological function (Winemiller 1992). Morphological analyses in *Selene* suggest its steeper head profile is a derived characteristic, effectively separating *S. brownii* and *S. setapinnis* from *S. vomer*, which exhibits a less steep profile. However, significant differences in the anteroposterior axis are also discriminatory for the species. Therefore, the oval and deeper body characteristic of *S. setapinnis*, followed by a more oblong pattern in *S. brownii*, is sequentially enhanced in *S. vomer, C. lugubris,* and *O. palometa.* In association with these features, differences in the caudal peduncle were also detected in the five species investigated. This is a discriminating characteristic in juvenile phases of *Selene*, as well as other species (Winans and Nishioka 1987; Lima-Filho et al. 2006).

Data obtained in this study appear to indicate a lack of evolutionary synchrony between morphological changes and karyotype alterations in *Selene* species, which has also been suggested for other families of Perciformes (Molina et al. 2002; Motta-Neto et al. 2011c). However, this is not a universal occurrence, since distinct situations have also been observed among Perciformes. Thus, integrating cytogenetic approaches with body patterns has shown combined variation in both parameters for other species, such as *Bathygobius soporator* (Lima-Filho et al. 2012). In addition to corroborating molecular evidence identifying *S. brownii* and *S. setapinnis* as distinct taxa (Reed et al. 2001,

2002), cytogenetic aspects and body shape patterns shown here also highlight divergences of these two species with the Atlantic lookdown, *S. vomer*. The reduced species diversity observed in the genus *Selene*, combined with morphological peculiarities and low general variation of karyotypes among its species, makes this taxon an effective and interesting evolutionary model within the family Carangidae, deserving of more detailed investigation.

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