Genetics and Molecular Biology, 37, 4, 646-651 (2014)

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Short Communication

Genetic identification of bucktooth parrotfish *Sparisoma radians* (Valenciennes, 1840) (Labridae, Scarinae) by chromosomal and molecular markers

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Abstract

Parrotfishes (Labridae, Scarinae) comprise a large marine fish group of difficult identification, particularly during juvenile phase when the typical morphology and coloration of adults are absent. Therefore, the goal of this study was to test cytogenetic markers and DNA barcoding in the identification of bucktooth parrtotfish *Sparisoma radians* from the northeastern coast of Brazil. Sequencing of cytochrome c oxidase subunit I (COI) confirmed all studied samples as *S. radians,* and all showed high similarity (99-100%) with Caribbean populations. The karyotype of this species was divergent from most marine Perciformes, being composed of 2n = 46 chromosomes. These consisted of a large number of metacentric and submetacentric pairs with small amounts of heterochromatin and GC-rich single nucleolar organizer regions (NORs) not syntenic to 5S rDNA clusters. These are the first data about DNA barcoding in parrotfish from the Brazilian province and the first refined chromosomal analysis in Scarinae, providing useful data to a reliable genetic identification of *S. radians*.

Keywords: COI, karyotype, South Atlantic fish, ribossomal genes, Robertsonian rearrangements.

Received: May 13, 2014; Accepted: July 28, 2014.

Scarinae (Labridae) encompasses about 10 genera and 99 species of marine fish, popularly known as parrotfish (Helfman *et al.*, 2009; Parenti and Randall, 2011). The members of this subfamily are widespread throughout tropical zones of the Pacific, Indian and Atlantic oceans and play a key role in ecology and fisheries of reefs (Bellwood, 1994; Streelman *et al.*, 2002; Robertson *et al.*, 2006).

Traditionally, this group has been classified as a family (Scaridae) derived from Labridae (Schultz, 1958; Bellwood, 1994). Nonetheless, molecular studies revealed that Labridae+Scaridae compose a monophyletic group, and parrotfishes were reallocated as a subfamily (Scarinae) of Labridae (Westneat and Alfaro, 2005).

The parrotfishes include some of the most colorful and typical representatives of coastal reefs (Streelman *et al.*, 2002). Indeed, some Scarinae species are morphologically identical, and coloration has been useful to distinguish them, as reported within the genus *Sparisoma* (Gasparini *et al.*, 2003; Robertson *et al.*, 2006; Pinheiro *et al.*, 2010; Rocha *et al.*, 2012). Seven out of the 15 *Sparisoma* species are reported to exist in sympatry along the Brazilian coast (*S. atomarium*, *S. amplum, S. axillare, S. frondosum, S. radians, S. rocha* and *S. tuiupiranga*), including recent descriptions based on morphological traits (Moura *et al.*, 2001; Gasparini *et al.*, 2003; Feitoza *et al.*, 2005; Pinheiro *et al.*, 2010; Rocha *et al.*, 2012).

Nonetheless, the color patterns commonly used to a precise identification of *Sparisoma* species are best observed in fresh specimens only, and they might vary according to sex and ontogenetic state (Bernardi *et al.*, 2000). Thus, the utilization of genetic markers should represent an efficient and reliable tool to identify these species, independent of the morphological aspects. Accordingly, sequencing of a portion of the COI gene has proven to be efficient in DNA barcoding at the species level in marine fish (Hubert *et al.*, 2010; Weigt *et al.*, 2012; Veneza *et al.*, 2013). Furthermore, chromosomal analyses in fish species can potentially provide informative markers for cytotaxonomy (*e.g.*, Medrado *et al.*, 2012).

In the case of Labridae, cytogenetic studies have shown that Robertsonian rearrangements and pericentric inversions accounted for the divergent karyotypes in several species when compared to the plesiomorphic condition for marine Perciformes (2n = 48 acrocentric chromosomes) (Sena and Molina, 2007a, 2007b; Molina *et al.*, 2012a). On the other hand, chromosomal data in Scarinae are available

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for only two Atlantic species: *Sparisoma axillare* and *Scarus trispinosus* (Sena and Molina, 2007b).

Therefore, the goal of this study was to evaluate the applicability of chromosomal markers and DNA barcoding for the identification of *Sparisoma radians* from the Brazilian coast. In addition, more refined methods of chromosomal analyses were carried out to be added to the karyotypic data available for Scarinae and further inferences about chromosomal evolution in parrotfish.

Five individuals of *Sparisoma radians* (three females and two juveniles of undetermined sex) were collected in Camamu Bay at the southern coast of Bahia, Brazil. Mitotic chromosomes were obtained based on the procedure described by Netto *et al.* (2007). Heterochromatin and active NORs were detected by C-banding (Sumner, 1972) and silver nitrate staining (Howell and Black, 1980), respectively. Base-specific fluorochrome staining (Schweizer, 1980) was used to reveal GC-rich and AT-rich regions.

The 18S and 5S rDNA cistrons were simultaneously mapped on chromosomes by fluorescence *in situ* hybridization (double-FISH) as proposed by Pinkel *et al.* (1986), with a stringency level of 77%. Ribossomal probes were obtained via PCR according to Hatanaka and Galetti Jr (2004) for 18S rDNA and Martins *et al.* (2006) for 5S rDNA. The 18S rDNA probe was labeled with biotin-16-dUTP and signals were detected by fluorescein-conjugated avidin (FITC) while 5S rDNA probes were labeled with digoxygenin-11-dUTP and detected by a anti-digoxygenin-rhodamine system (Roche Applied Science). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI) included in Vectashield mounting medium (Vector).

Micrographs were captured using epifluorescence Olympus BX-51 and Olympus BX-61 microscopes equipped with Image Pro-Plus v. 6.2. software (Media Cybernetics), followed by edition in Adobe Photoshop v. 7.0. The karyotypes were organized based on chromosomal measurements and classified as proposed by Levan *et al.* (1964).

For DNA barcoding, total DNA was extracted from liver or muscle tissue using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. A fragment of nearly 650 bp at the 5' end of COI was amplified via PCR using the primers Fish F2 5'TCGACTAATCATAAAGATATCGGCAC-3' and Fish R2 5'ACTTCAGGGTGACCGAAGAATCAGAA-3' (Ward *et al.*, 2005). Each reaction comprised 0.2 mM of dNTPs, 1x PCR buffer, 2 mM of $MgCl_2$, 0.4 μM of each primer, 0.7 U of *Taq* DNA polymerase, 50 to 100 ng of template DNA and ultrapure water to a final volume of 15 μL. The PCR protocol consisted of an initial step at 95 °C for 5 min, 35 cycles of 40 s at 94 °C, 40 s at 55 °C and 40 s at 72 °C, and a final extension step for 7 min at 72 °C. Subsequently, the reaction products were purified and sequenced in an ABI 3500XL Genetic Analyzer automatic sequencer (Applied Biosystems). All sequences were deposited in the Barcode of Life Database (BOLD) platform (http://www.boldsystems.org) along with details of collected specimens and identified as HG3012/BAHIA-102-14, HG3015/BAHIA-105-14, HG3017/BAHIA-106-14, HG3029/BAHIA-117-14, and HG3014/BAHIA-118-14.

The sequences were then submitted to BLAST (basic alignment search tool) searches in the NCBI (National Center for Biotechnology Information) database to check putative homologies with other sequences and confirmation of taxonomic identification of the samples. Using ClustalW Multiple Alignment in the software BioEdit, the obtained sequences were aligned with six COI sequences available in GenBank (NCBI) for Caribbean samples of *S. radians* (access numbers JQ839590, JQ839899), as well as *S. cretense* (KC501552, GQ341598), *S. chrysopterum* (JQ841012) and *S. rubripinne* (GU225442). The aligned sequences (about 650 bp) were exported to Mega v.5 (Tamura *et al.*, 2011) to generate a genetic distance matrix and a tree topology using Neighbor-Joining (NJ) algorithm and the Kimura-2-parameter substitution model, with 1000 bootstrap replications.

The similarity levels of the five COI sequences obtained in the present study and those available in GenBank for specimens of *Sparisoma radians* from the Caribbean (Weigt *et al.*, 2012 and unpublished data) ranged from 99 to 100%. As a result, the NJ tree clustered both Brazilian and Central America specimens of *S. radians* apart from *S. rubripinne*, *S. cretense* and *S. chrysopterum* (Figure 1). The mean distance within *S. radians* was 0.2%, while the distance values of this group in relation to *S. cretense*, *S. chrysopterum* and *S. rubripinne* were equal to 11.9, 12.9 and 14%, respectively. Therefore, the COI sequences efficiently distinguished *Sparisoma* species, including samples from the closely related *S. radians* (present study, Weigt *et al.*, 2012) and *S. rubripinne* (Valdez-Moreno *et al.*, 2010)

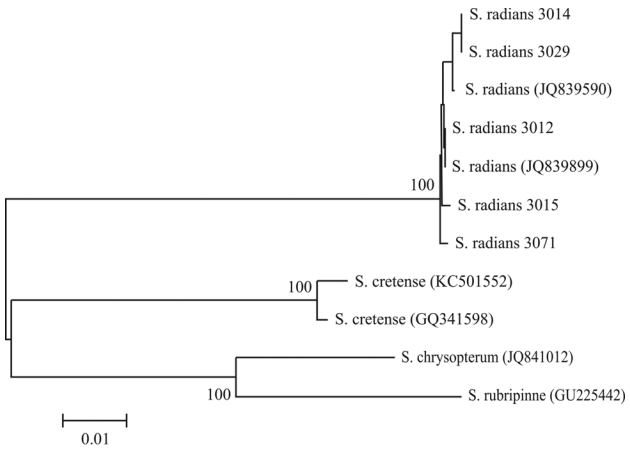

Figure 1 - Neighbor-Joining tree of COI sequences comparing *S. radians* from Brazilian coast and other populations and species of *Sparisoma*. The numbers in parentheses refer to GenBank database (see text for details).

by revealing a barcode gap largely superior to 10 times the mean intraspecific variation, as recommended by Hebert *et al.* (2003).

All specimens of *S. radians* shared a diploid number of 2n = 46 and a karyotype composed of 24 meta-submetacentric (m/sm) and 22 subtelo-acrocentric (st/a) chromosomes (Figure 2A). Heterochromatin was distributed over pericentromeric regions of most chromosomes and telomeric regions on the short arms of pair 14 (Figure 2B). Ag-NORs were located at terminal position on the short arms of pair 14 (Figure 2C), interspersed with GC-rich heterochromatin segments that were positively stained by chromomycin A_3 (CMA_3) (Figure 2D).

The physical mapping of 18S and 5S rDNA cistrons revealed non-synteny between both the ribossomal genes (Figure 3). As expected, the location of 18S rDNA sites was overlapping with Ag-NORs, confirming the existence of a single NOR-bearing pair in *S. radians*. The 5S rRNA genes were located at interstitial region on the smallest chromosomal pair (Figure 3).

The karyotypic pattern of *S. radians* (2n = 46) is very similar to that reported for *S. axillare* (Table 1), suggesting a differentiated karyoevolution pathway of parrotfishes (Galetti Jr *et al.*, 2006). The chromosomal number and the presence of large metacentric pairs in the few species of *Sparisoma* analyzed so far also contrasts with the numerical conservativeness (2n = 48) observed in *Scarus* and other Labridae subfamilies, such as Bodianinae and Corinae (Table 1).

A reduced 2n value combined with a large number of biarmed chromosomes in *Sparisoma*, including *S. radians*, indicates that both Robertsonian rearrangements and pericentric inversions took place during karyoevolution in this group. Actually, inversions have been commonly reported in Labridae from the South Atlantic, leading to increased fundamental arm numbers and conserved diploid values (Table 1). On the contrary, fusions are rarely observed in marine fish and, thus, the presence of large metacentric pairs associated with lower 2n values in relation to basal

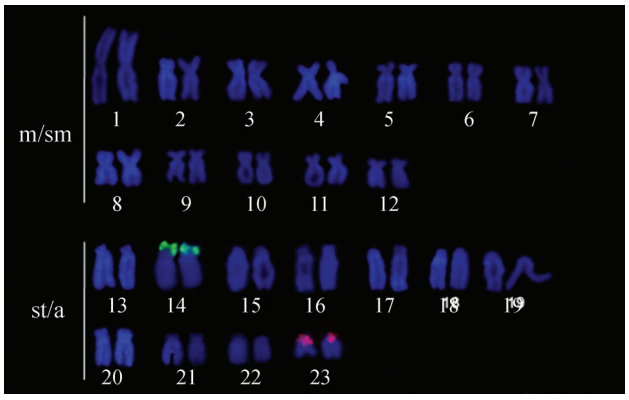

Figure 3 - Distribution of 18S rDNA (in green) and 5S rDNA (in magenta) in DAPI-stained karyotype of S. radians after double-FISH.

karyotype (2n = 48) can be interpreted as a synapomorphic feature of *Sparisoma* (Table 1). Considering that, in spite of identical 2n values, the fundamental number in *S. radians* was higher than that reported in *S. axillare* (Sena and Molina, 2007a), we infer that the former has undergone additional pericentric inversions and should be placed as a more derived species than the latter.

The presence of single NORs is regarded as a basal and widespread trait in Perciformes (Galetti Jr *et al.*, 2006), including *S. radians*. Likewise, other labrids (Molina *et al.*, 2012b), including Scarinae (Sena and Molina, 2007a), share a single NOR-bearing pair. However, multiple NORs seem to be an apomorphic feature in the subfamily Corinae, as reported in *Halichoeres poeyi*, *H. radiatus* (Sena and Molina, 2007b) and *Coris julis* (Mandrioli *et al.*, 2000). The NOR-bearing pair of *S. radians* (pair 14) seems to be homeologous to that observed in *S. axillare* (pair 11) (Sena and Molina, 2007a), given their similarity in type, size and location of NORs.

Heterochromatin in fish chromosomes is usually distributed over centromeric and pericentromeric regions in several marine fish groups such as Gobiidae (Lima-Filho *et al.*, 2012), Haemulidae (Motta-Neto *et al.*, 2011a, 2011b),

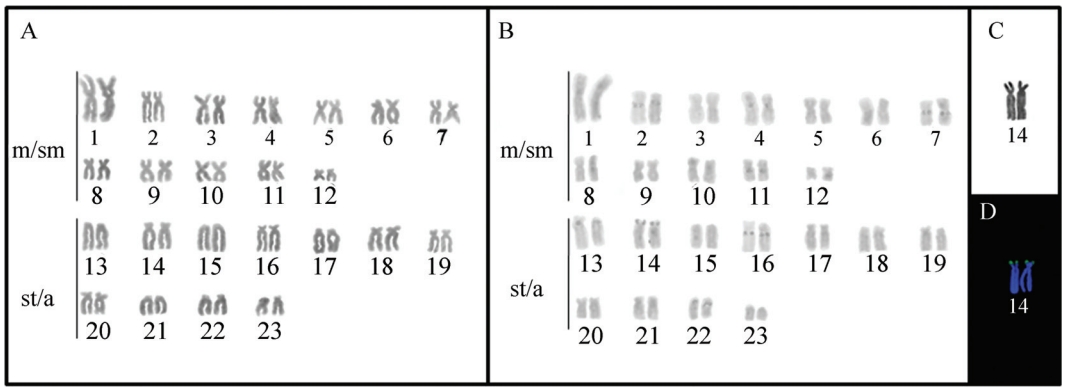

Figure 2 - Karyotype of *Sparisoma radians* (2n = 46) after Giemsa staining (A) and C-banding (B). The NOR-bearing pair is shown in detail after silver nitrate (C) and fluorochrome staining (D) revealing CMA_3^+ sites coincident with NORs.

Table 1 - Cytogenetic data in Labridae from South Atlantic.

Species	2n	Karyotype	FN	C-banding	Ag-NOR	Reference
Bodianinae						
Bodianus rufus	48	6m+12sm+14st+16a	80	cen, per	10p	Molina *et al*., 2012b
Bodianus pulchellus	48	4m+12sm+14st+18a	78	cen, per	9p	Molina *et al*., 2012b
Bodianus insularis	48	4m+12sm+14st+18a	78	cen, per	9p	Molina *et al*., 2012b
Corinae						
Halichoeres brasiliensis	48	48a	48	cen, per	9q	Sena and Molina, 2007a
Halichoeres poeyi	48	4m+44st	52	cen, per	1-2 a pairs	Sena and Molina, 2007a
Halichoeres radiatus	48	48a	48	cen, per	2 a pairs	Sena and Molina, 2007a
Scarinae						
Scarus trispinosus	48	6m+10sm+24st+8a	88	cen, per	9p	Sena and Molina, 2007b
Sparisoma axillare	46	6m+14sm+4st+22a	70	cen, per	11p	Sena and Molina, 2007b
Sparisoma radians	46	24m/sm+22st/a	84	cen, per, ter	14p	Present study

2n =diploid number; FN = fundamental number; m = metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric; cen = centromeric; per = pericentromeric; t = terminal; p = short arms; q = long arms.

Carangidae (Accioly *et al.*, 2012; Jacobina *et al.*, 2012) and Grammatidae (Molina *et al.*, 2012a). Following this trend, *S. radians* was characterized by a preferential distribution of C-bands close to centromeres, in addition to a terminal GC-rich heterochromatin segment equivalent to NORs on pair 14 (Figure 2). As this is the first report about GC content in Scarinae, further inferences can currently not be made, even though the pattern herein described agrees with that observed in other labrids of the subfamily Bodianinae (Molina *et al.*, 2012b).

Similarly, the present study provides the first data concerning the location of 18S and 5S ribosomal genes in parrotfishes. The lack of synteny of each rDNA class suggests an independent evolution of the two ribosomal gene families (Pendás *et al.*, 1993). As expected, 18S rDNA clusters in *S. radians* were coincident with Ag-NORs and CMA_3^+ regions, supporting the occurrence of single NORs as a basal condition of Labridae (Molina *et al.*, 2012b). The interstitial location of 5S rDNA on long arms, as observed in *S. radians*, is also considered an ancestral trait of teleosteans (Martins and Galetti Jr, 2001; Martins and Wasko, 2004). On the other hand, the presence of a single pair bearing 5S rRNA genes, as reported in the studied species, is a less frequent condition in fish (Martins *et al.*, 2011). Unfortunately, there are only few studies on the distribution of 5S rDNA clusters in Labridae, this impairing a reliable comparative analysis.

In spite of the scarcity of cytogenetic data about Scarinae, the currently available reports suggest that repeated pericentric inversions and centric fusions have taken place during the evolutionary history of this subfamily. Such a divergent karyotypic trend in relation to other marine Perciformes might have been favored by vicariance events combined with habitat differentiation and specialization of parrotfishes (Bernardi *et al.*, 2000; Robertson *et al.*, 2006). To test this hypothesis, additional cytogenetic analyses in other Scarinae representatives are highly encouraged.

Finally, the present study challenges the traditional view of conserved chromosomal patterns in marine Perciformes, which, to our view, represents an overstatement caused by limited data. In addition, the combination of chromosomal analyses and DNA barcoding have proven to be an efficient strategy to identify sibling or morphologically very similar species, as commonly observed within *Sparisoma*.

Acknowledgments

This study was supported by FAPESB (PNE0019/2011 and APP0064/2011), UESB, "Rede de DNA barcoding da ictiofauna do Brasil" and "INCT de Ambientes Marinhos Tropicais". Specimens were collected after authorization provided by ICMBio/SISBIO (license numbers 31360-1 and 27027-2).

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Associate Editor: Yatiyo Yonenaga-Yassuda