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RESEARCH PAPER

Evolutionary diversification of Western Atlantic *Bathygobius* species based on cytogenetic, morphologic and DNA barcode data

Paulo Augusto Lima-Filho · Ricardo de Souza Rosa · Allyson de Santos de Souza · Gideão Wagner Werneck Félix da Costa · Claudio de Oliveira · Wagner Franco Molina

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Abstract A number of fish groups, such as Gobiidae, are highly diversified and taxonomically complex. Extensive efforts are necessary to elucidate their cryptic diversity, since questions often arise about the phylogenetic aspects of new species. Clarifications about the diversity and phylogeny of the *Bathygobius* species from the southwestern Atlantic are particularly needed. Evidence has been accumulating on the Brazilian coast regarding the possible presence of new species while doubts remain about the taxonomic status of others. The taxonomic identification of some species of *Bathygobius* has been problematic, given their generally conservative external morphology, and several species are recognized as cryptic. This situation hinders understanding the real diversity in this taxon. Taken together, genetic, cytogenetic and morphometric analyses have been effective in identifying new species of this genus. Here we describe the karyotypic features and morphological patterns of three Western South Atlantic species of *Bathygobius*. Furthermore, its cytochrome c oxidase I (COI) gene sequences were compared with those of species from Central America, North America and the Caribbean. The broad analyses performed demonstrated an unsuspected diversity, leading to the identification of an un-described new species (*Bathygobius* sp.2) and the geographic redefinition of another, *Bathygobius* sp.1, undoubtedly a branch of *B. geminatus*, hitherto inaccurately identified as *B. mystacium* on the coast of Brazil.

Keywords Biodiversity · Karyotype evolution · Geometric morphometrics · COI

P. A. Lima-Filho · A. S. Souza · G. W. W. F. da Costa · W. F. Molina (✉)
Departamento de Biologia Celular e Genética, Centro de Biociências, Universidade Federal do Rio Grande do Norte, Natal, RN 59.078-970, Brazil
e-mail: molinawf@yahoo.com.br; molinawf@pq.cnpq.br

R. S. Rosa
Departamento de Sistemática e Ecologia, CCEN, Universidade Federal da Paraíba, Campus I, João Pessoa, PB 58.059-900, Brazil

C. de Oliveira
Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Campus de Botucatu, Botucatu, SP 18.618-970, Brazil

P. A. Lima-Filho
Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Norte, Macau, RN, Brazil

Introduction

The Gobiidae, with nearly 2000 species, occupying marine, brackish and freshwater habitats, is the fish family with the greatest number of marine species, and possibly the second largest family of vertebrates (Nelson 2006). Its species generally are small-sized

and characterized by the fusion of the pelvic fins (Nelson 2006). The gobiid genus *Bathygobius* presents 29 species, six of which are known from the Western Atlantic: *B. antilliensis* Tornabene et al. 2010, *B. curacao* (Metzelaar 1919), *B. geminatus* Tornabene et al. 2010, *B. lacertus* (Poey 1860), *B. mystacium* Ginsburg 1947 and *B. soporator* (Valenciennes 1837).

The Atlantic species of *Bathygobius* are viewed as conservative with respect to external morphology, and traditional morphometric and meristic characters have been pointed as inefficient to distinguish *B. soporator, B. antilliensis* and *B. lacertus* (Tornabene et al. 2010). Subtle morphometric and meristic differences have been used to establish four subspecies in *B. soporator* and one subspecies in *B. curacao* (Ginsburg 1947). The prevalence of such conservative morphology has posed difficulties in species identification, and emphasizes the importance of using different approaches to ensure the taxonomic clarification of the genus in the western Atlantic.

Regional taxonomic revisions of the species of *Bathygobius* are available for Japan (Akihito and Meguro 1980), western Africa (Miller and Smith 1989) and the east Pacific (Ginsburg 1947; Miller and Stefanni 2001). Nonetheless, data on the systematics and phylogeny of Atlantic species of *Bathygobius* are still scarce (Ginsburg 1947; Tornabene et al. 2010; Tornabene and Pezold 2011), particularly on those occurring at the oceanic islands.

Bathygobius species present reproductive and behavioral characteristics such as adhesive eggs, short larval period and restricted movements (Tavolga 1953; Gibson and Yoshiyama 1999), which possibly favor the genetic structuring of their populations. This set of biological characteristics, associated to their conservative morphology, hinders the understanding of the real diversity of the genus in the Atlantic Ocean, and turns *Bathygobius* as a favorable model for evolutionary studies within and among the western Atlantic oceanic regions.

Recent cytogenetic and molecular analyses showed distinct patterns between continental and insular individuals from Rocas Atoll, previously identified as *B. soporator* (Lima et al. 2005; Lima-Filho et al. 2012). When compared to other marine groups within the Percomorphaceae and Eupercaria (sensu Betancur-R et al. 2013), which present high uniformity in karyotypic macrostructure, gobiids show highly variable karyotypes (Galetti et al. 2000; Galvão et al. 2011). The high chromosomal evolutionary dynamics in this family (Molina et al. 2014) supports a close relationship between chromosomal rearrangements and speciation processes. Therefore, cytotaxonomic markers are particularly useful in assessing population variability (Lima-Filho et al. 2012) and in the identification of cryptic species (Mandrioli et al. 2001; Lima-Filho et al. 2014).

We compare herein the karyotypic patterns, morphologic and morphometric data, and sequences of cytochrome c oxidase I (COI) of two nominal species of *Bathygobius*, reported from the Brazilian coast and oceanic islands to other species of *Bathygobius* from other localities of the Atlantic Ocean.

Materials and methods

Collection areas and cytogenetic preparations

Cytogenetic analyses were performed in *Bathygobius soporator* (n = 15; 7♂/8♀), in specimens from the coast of Rio Grande do Norte State (RN) previously identified as *B. mystacium* in faunal surveys and labeled herein as *Bathygobius* sp.1 (n = 10; 5♂/5♀), and in specimens herein labeled as *Bathygobius* sp.2 (n = 20; 7♂/13♀) from Rocas Atoll (RA) (Figs. 1, 2). The specimens were subjected to overnight mitotic stimulation in vivo by intramuscular inoculation of a complex of bacterial and fungal antigens (Molina et al. 2010). Mitotic chromosomes were obtained from the cell suspensions of the anterior kidney (Gold et al. 1990).

The diploid numbers were established by the analysis of thirty metaphases stained with Giemsa 5 %, diluted in phosphate buffer (pH 6.8). The heterochromatic regions were analyzed by C-banding (Sumner 1972). The chromosomes were stained with base-specific fluorochromes CMA_3 and DAPI (Sola et al. 1992). The nucleolar organizer regions (NORs) were identified by silver staining, according to Howell and Black (1980). Dual-color FISH (*fluorescence in situ hybridization*) was performed according to Pinkel et al. (1986). In all samples 18S rDNA and 5S rDNA sites were mapped. The 18S rDNA probe consisted of a sequence tandem labeled by nick translation with digoxigenin-11-dUTP (Roche, Germany, Mannheim), while the 5S rDNA probe was

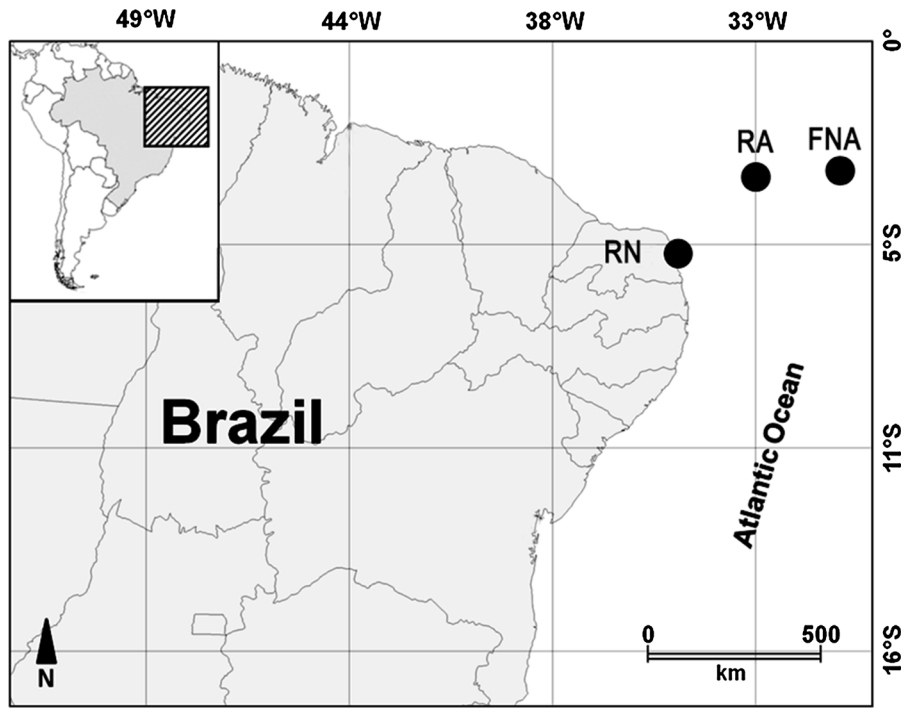

Fig. 1 Collection sites for *Bathygobius*, Rio Grande do Norte (RN), Rocas Atoll (RA) and Fernando de Noronha Archipelago (FNA)

labeled with biotin-14-dATP (Invitrogen, USA, CA, San Diego), both obtained from *Lutjanus analis* (Teleostei, Eupercaria sensu Betancur et al. 2013). The best metaphases were photographed under a microscope BX50 OlympusTM coupled to an Olympus DP73 digital capture system, using the CellSens software (Olympus, Japan) and used for illustrating the karyotype. The chromosome morphology was classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) according to the position of the centromere (Levan et al. 1964).

Mitochondrial DNA analysis: cytochrome oxidase I

Mitochondrial DNA analysis of the samples was conducted for *B. soporator* from Rio Grande do Norte State (n = 4), *Bathygobius* sp.1 from Rio Grande do Norte State (n = 4) and *Bathygobius* sp.2 from Rocas Atoll (n = 6) and the Fernando de Noronha Archipelago (n = 5). Fragments of muscle and/or liver tissue from the samples were stored into microtube (1.5 mL) containing 95 % ethanol and stored at -20 °C. Total DNA was extracted according to the protocol of Sambrook et al. (1989).

PCR reactions for amplification of sequences of the COI gene were performed in a final volume of 25 µl. Each reaction consisted of 1 µl of total DNA, 0.5U Taq polymerase (Bio-Line USA, Boston, Massachusetts), 0.4 µl of 50 mM $MgCl_2$, 1 µl of 10 × buffer, 0.5 µl 10 mM dNTP, 0.3 µl of 10 µM of the primers FISH-BCL (5′-TCA ACY AAT CAY AAA GAT ATY GGC AC-3′) and FISH-BCH (5′TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Baldwin et al. 2009). Ultrapure was added to complete the final volume of the reaction. Thermal cycling was initiated with denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 45 s with a final extension of 5 min at 72 °C. The PCR amplification products were purified with enzyme ExoSAP-IT (USB, Cleveland, OH) following the manufacturer's protocol. Subsequently, the samples were sent for sequencing by the company ACTGene Análises Moleculares using the equipment ABI-PRISM 3100 *Genetic Analyzer* (Applied Biosystems, USA, CA, Foster City).

The obtained sequences were edited with BioEdit v. 5.0.6 software (Hall 1999). In total, 565 base pairs (bp) of the gene cytochrome c oxidase subunit I (COI) were obtained, and subsequently multiple-aligned by the software ClustalW (Thompson et al. 1997). The

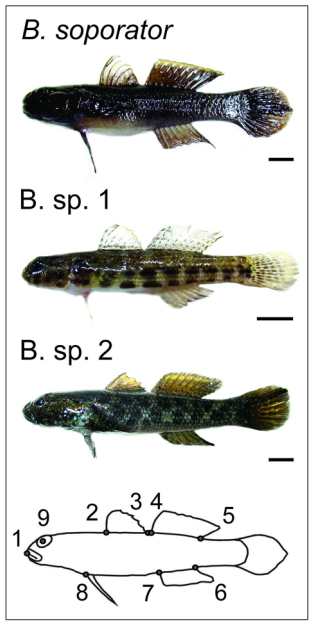

Fig. 2 Representative specimens of the *Bathygobius* species collected on the coast of Brazil and oceanic islands. Bar = 1 cm. Homologous landmarks used in geometric morphometric analysis are shown in the generic body plan of *Bathygobius*: *1* Distal extremity of the premaxillary bone, *2* origin of the first dorsal-fin base, *3* distal margin of the first dorsal-fin base, *4* origin of the second dorsal fin base, *5* distal margin of the second dorsal-fin base, *6* distal margin of the anal-fin base, *7* origin of the anal-fin base, *8* origin of the pelvic fin, *9* center of eye pupil

obtained sequences were then compared with the sequences of these and other Atlantic species deposited in GenBank (Table 1).

Sequences were analyzed with the MEGA 6 software (Tamura et al. 2013), seeking comparisons with other species of the genus *Bathygobius* (Tornabene et al. 2010). The model of nucleotide substitution used for inter- and intraspecific analysis was Kimura 2-parameter (K2P) (Kimura 1980). The representation of the genetic distance between species was based on the neighbor-joining method (Saitou and Nei 1987), which has proved effectivity in studies of the relationships between species using the COI gene (e.g. Nei and Kumar 2000; Barrett and Hebert 2005; Rivera and Currie 2009).

Geometric morphometrics analysis

Intact adult individuals were used in the morphometric analysis. The specimens were photographed in left lateral view with a Sony H10 (Japan, Tokyo, Konan Minato-ku) digital camera, 8.1 megapixels, under standardized distance and position. A total of 10 landmarks (defined in Lima-Filho et al. 2012) were digitalized using the software v2.16 TPSdig (Rohlf 2010a) and the images were arranged in a single file with the TPS format using the tpsUtil software (Rohlf 2010b). The Procrustes superposition was done, followed by an analysis of Canonical Variables (CV) and the discriminant function (DFA) performed with the software MorphoJ 1.02b (Klingenberg 2011). Where the resulting Mahalanobis distances (D2) enabled the measurement of the variation in body shape between *Bathygobius* species. The robustness of the assignment was assessed through a leave-one-out resampling cross-validation procedure. Warped outlines were obtained from the canonical variable of greatest influence on the morphology, to identify the vector variations of deformation grids between species.

Results

Chromosome structure

Despite sharing the same diploid number (2n = 48 chromosomes), *B. soporator* (2 m + 6st + 40a, NF = 56), *Bathygobius* sp.1 (2 m + 4st + 42a, NF = 54) and *Bathygobius* sp.2 (28st + 20a, NF = 76) exhibit conspicuous differences in the karyotype macrostructure (Fig. 3), chromosome banding patterns and localization of 18S rDNA and 5S rDNA (Fig. 4). The heterochromatic blocks are mainly distributed in the centromeric regions of chromosomes of the species, and are more conspicuous in *Bathygobius* sp.2 (Fig. 3).

The Ag-NORs sites, the only CMA_3^+ regions, are located in terminal position on the short arms of submetacentric pairs 1 and 4 in *Bathygobius soporator* and *Bathygobius* sp.2 and in an interstitial position in the acrocentric pair 1 in *Bathygobius* sp.1.

Dual-color FISH revealed that the 5S and 18S rDNA genes are located on different chromosomes. The 18S rDNA sites for all species, are single and coincide with the markings Ag-NORs. On the other hand, the 5S rDNA sites may be simple or multiple. They are located in the terminal portion of the long arms of pairs 2 and 10 in *B. soporator,* in centromeric

Table 1 COI sequences of *B. soporator*, *Bathygobius* sp.1, *Bathygobius* sp.2, and of other Atlantic species deposited in GenBank

Species	GenBank	Geographical origin
B. soporator (n = 5)	KM248292–KM248296	Rio Grande do Norte State (Brazil)
Bathygobius sp.1 (n = 6)	KM248286–KM248291	Rio Grande do Norte State (Brazil)
Bathygobius sp.2 (n = 6)	KM248280–KM248285	Rocas Atoll (Brazil)
Bathygobius sp.2 (n = 4)	KM248276–KM248279	Fernando de Noronha Archipelago (Brazil)
B. soporator (n = 11)	HM748382; HM748386; HM748387; HM748388; HM748390; HM748394; HM748395; HM748405; HM748424–HM748426	USA; Venezuela; Puerto Rico; Panama
B. mystacium (n = 10)	HM775936–HM775945	Belize
B. geminatus (n = 10)	HM748367–HM748369; HM748373–HM748375; HM748377–HM748379; HM748389	USA; Puerto Rico
B. antilliensis (n = 10)	HM748333; HM748392; HM748393; HM748404; HM748406-HM748411	Bahamas; Puerto Rico; Trinidad and Tobago
B. lacertus (n = 1)	HM775923	Belize
B. curacao (n = 1)	HM7759201	Belize

Table 2 Average genetic distances between COI sequences of *Bathygobius* from the western Atlantic Ocean based on the substitution model of Kimura-2 parameters

	B. soporator	*B. soporator*[a]	*B.* sp1	*B. mystacium*[a]	*B. geminatus*[a]	*B.* sp2 RA	*B.* sp2 FNA	*B. antilliensis*[a]	*B. curacao*[a]	*B. lacertus*[a]
B. soporator		0.0021	0.0184	0.0172	0.0184	0.0175	0.0175	0.0175	0.0164	0.0113
B. soporator[a]	0.0076		0.0181	0.0171	0.0181	0.0174	0.0173	0.0172	0.0164	0.0110
B. sp1	0.1609	0.1609		0.0185	0.0034	0.0174	0.0172	0.0161	0.0181	0.0185
B. mystacium[a]	0.1552	0.1539	0.1625		0.0183	0.0175	0.0170	0.0184	0.0186	0.0177
B. geminatus[a]	0.1646	0.1643	0.0133	0.1648		0.0178	0.0176	0.0162	0.0178	0.0182
B. sp2 RA	0.1559	0.1557	0.1448	0.1557	0.1547		0.0005	0.0100	0.0173	0.0176
B. sp2 FNA	0.1563	0.1558	0.1447	0.1525	0.1547	0.0005		0.0100	0.0172	0.0175
B. antilliensis[a]	0.1540	0.1528	0.1317	0.1619	0.1406	0.0539	0.0544		0.0169	0.0189
B. curacao[a]	0.1331	0.1348	0.1516	0.1699	0.1547	0.1390	0.1398	0.1336		0.0163
B. lacertus[a]	0.0747	0.0726	0.1671	0.1499	0.1675	0.1564	0.1568	0.1700	0.1362	

Below diagonal = Genetic distance; Above diagonal = Standard error

[a] Sequences obtained from GenBank

RA Rocas Atoll, *FNA* Fernando de Noronha Archipelago

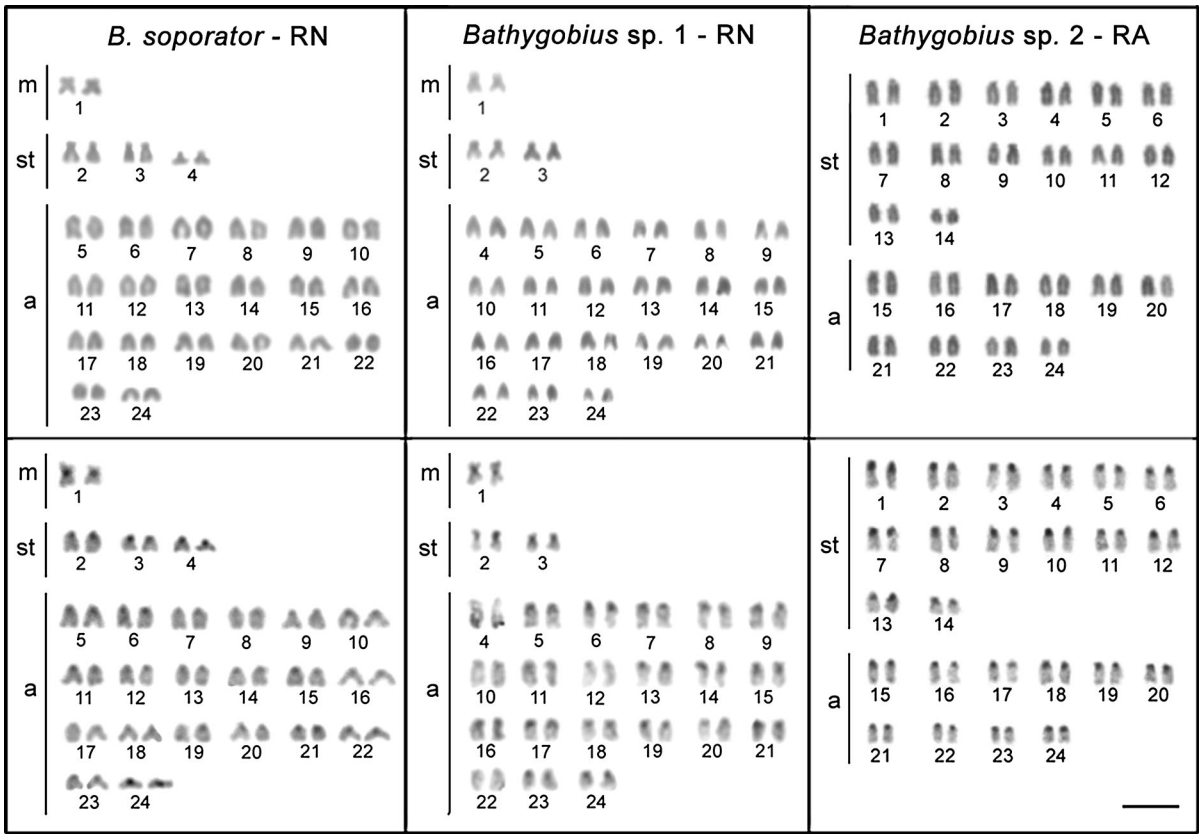

Fig. 3 Karyotypes of *B. soporator*, *Bathygobius* sp.1 and *Bathygobius* sp.2 (Rocas Atoll), with conventional Giemsa staining (*top row*) and C-banding (*bottom row*). Bar = 5μm

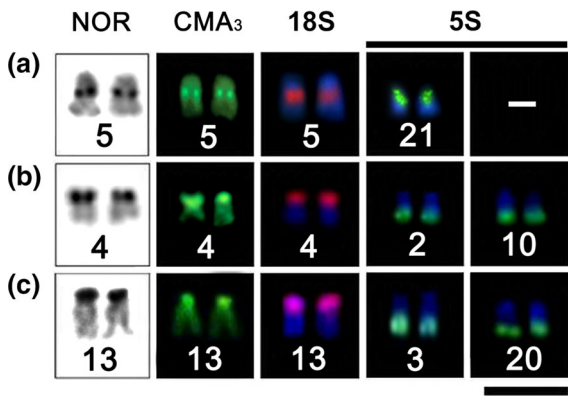

Fig. 4 Chromosome pairs bearing ribosomal sites in **a** *Bathygobius* sp.1 **b** *B. soporator* and **c** *Bathygobius* sp.2 (Rocas Atoll) using the Ag-NOR technique, CMA_3 staining and mapping of 18S rDNA and 5S rDNA sequences. Bar = 5μm

position in par 21 in *Bathygobius* sp.1, and in terminal position in pairs 3 and 20 in the karyotype of *Bathygobius* sp.2 (RA).

Mitochondrial DNA

Phylogenetic reconstruction from COI sequences using the neighbor-joining method revealed seven different genetic lineages in Atlantic *Bathygobius* (Fig. 5). Samples identified as *B. soporator* from the Brazilian coast are genetically similar to samples of *B. soporator* from North America, Central American and Caribbean (K2P d = 0.007 %) (Table 2; Fig. 5). On the other hand, samples of *Bathygobius* sp.1 from the coast of Rio Grande do Norte showed a high similarity with *B. geminatus* from the Caribbean and North America (K2P d = 0.013 %).

Bathygobius sp.2 from RA and FNA showed high similarity, indicating they belong to the same species (K2P d = 0.0005 %), but are differentiated from *B. antilliensis* (K2P d = 0.054 %), the species that it has the closest phylogenetic relationship with, and from *B. soporator* (K2P d = 0.155 %), the species with which

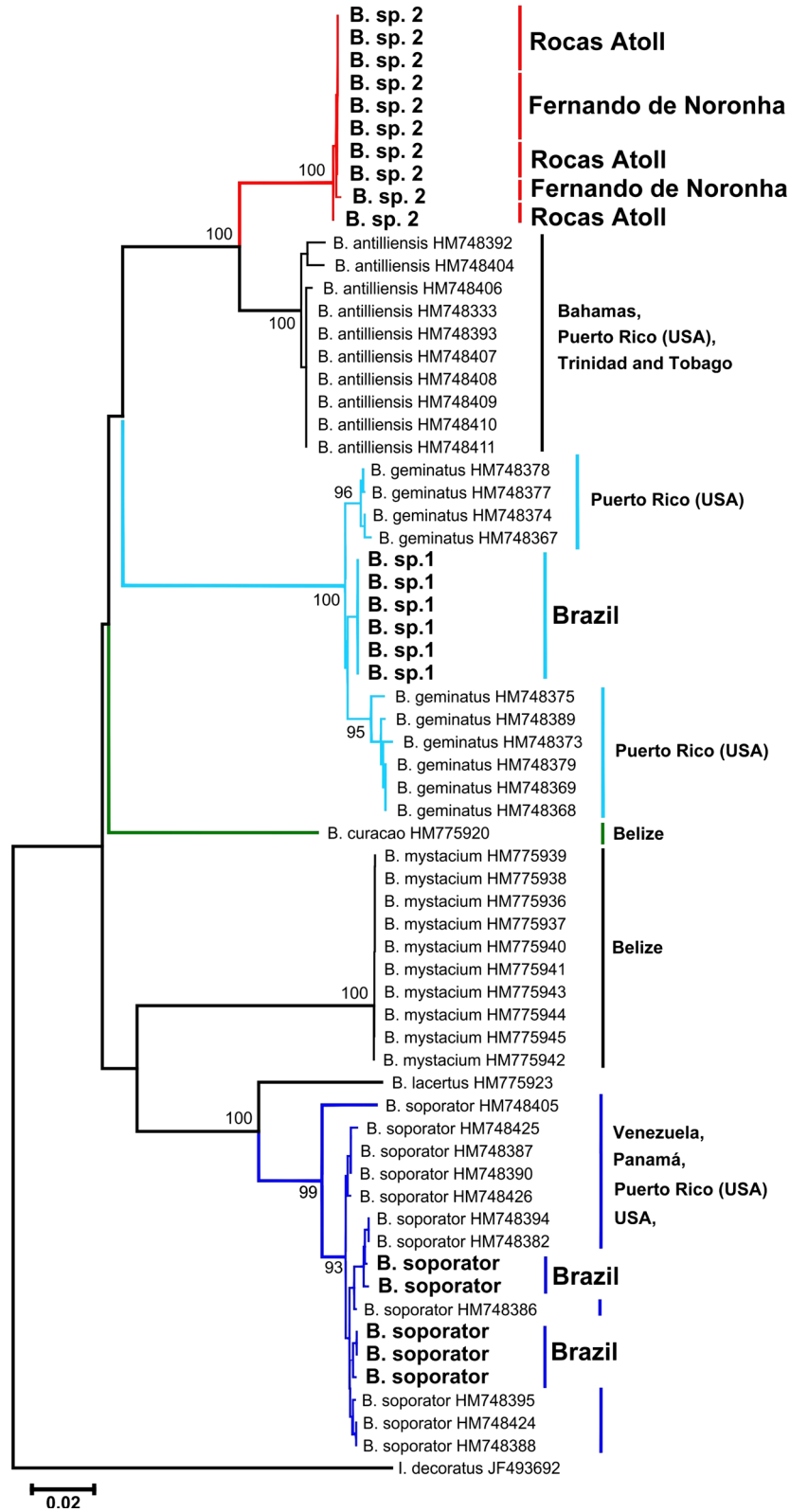

Fig. 5 Neighbor-joining tree from COI sequences of *Bathygobius* in the western Atlantic. *Bold names* correspond to the species of the northeastern coast of Brazil (RN) and of the Rocas Atoll (RA) and Fernando de Noronha Archipelago (FNA)

it has been taxonomically confounded in previous publications (Lima et al. 2005; Mendes 2006). The remarkable genetic differentiation of *Bathygobius* sp.2 from the Brazilian coastal species, as well as from those in the Caribbean, suggests that it constitutes an undescribed species, genetically differentiated from all other Atlantic species.

Morphometric analysis

The canonical variables 1 and 2, based on body form data, showed the largest contribution to the differentiation between species *Bathygobius* (CV1 = 70.54 %; CV2 = 24.49 %; $p < 0.001$), representing together 95 % of the variation in body form. A discriminant function analysis between *B. soporator*, *Bathygobius* sp.1, *Bathygobius* sp.2 RA and *Bathygobius* sp.2 FNA, using Mahalanobis distances to quantify morphological variation and Hotelling test (Table 3), showed complete discrimination between them. Correct assignments were cross-validated indicating a high accuracy between *B. soporator* (92 %) versus *Bathygobius* sp.2 FNA (88 %), and a complete sucess rate (100 %) for all the others comparisons.

The warped outlines generated to identify the variation in body shape between species of *Bathygobius* from CV1 (CV1 = 70.54 %) showed a high morphological diversification (Fig. 6), highlighting variations related to the position of the eyes and mouth and body length (see landmarks 1, 5, 6 and 9) and positions of the dorsal and anal fins (landmarks 4, 5, 7 and 8).

Discussion

The species identified as *B. soporator*, *Bathygobius* sp.1, and *Bathygobius* sp.2 clearly represent distinct lineages, based on their cytogenetic, genetic and morphological

Table 3 Statistical comparison of variation in body shape among *Bathygobius* species

	B. soporator	*Bathygobius* sp.1	*Bathygobius* sp.2 RA	*Bathygobius* sp.2 FNA
B. soporator	–	423.04	399.74	92.63
Bathygobius sp.1	5.81		379.23	775.79
Bathygobius sp.2 RA	5.65	5.50		417.92
Bathygobius sp.2 FNA	2.72	7.87	5.78	–

Mahalanobis distances (bellow diagonal) and Hotelling test (above diagonal) (all p values <0.001; values were generated after 10.000 random permutations)

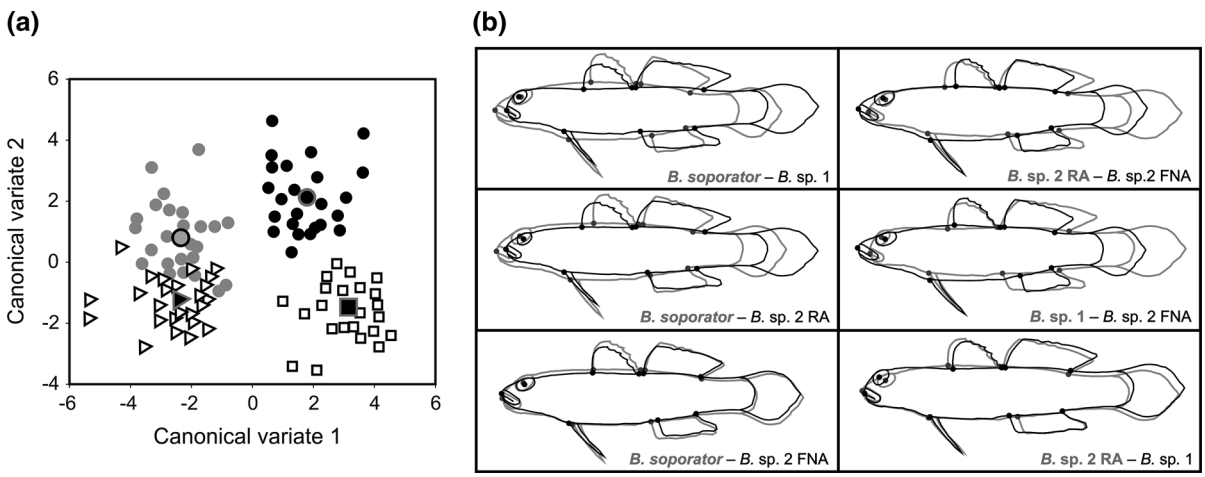

Fig. 6 Body shape variation among *Bathygobius* species examined: **a** canonical variables of body *B. soporator* (*right pointing triangle*), *Bathygobius* sp.1 (*open square*), *Bathygobius* sp.2 RA (*black filled circle*) *Bathygobius* sp.2 FNA (*grey filled circle*), between CVI and CVII axes (CVI = 70.5 % and CVII = 24.5 %). Larger symbols indicate the morphometric mean for each species. **b** warped outlines of CVI showing body shape differences in *Bathygobius* spp

traits. Of these, *Bathygobius* sp.1 and *Bathygobius* sp.2 show different taxonomic status from previous identifications, confirming the necessity for taxonomic reassessment of species on the Brazilian coast. The different approaches used highlight the particular evolutionary patterns of each lineage and clarify the phylogenetic relationships with other Western Atlantic species.

Chromosomal diversification

Species diversity observed in the Gobiidae has been mainly explained by vicariant processes leading to allopatry (Huyse et al. 2004). The chromosome data presented herein seem to suggest an evolutionary scenario of population fragmentation followed by chromosome differentiation, as leading events in the differentiation process in *Bathygobius* (Lima-Filho et al. 2012).

The three analyzed *Bathygobius* species show the same diploid number (2n = 48), but with remarkable karyotype diversity. In fact, the presence of 2n = 48 in these and other species of *Bathygobius* (Arai and Sawada 1974; Lima-Filho et al. 2012) and other Gobiidae (Vasil'ev and Grigoryan 1993), suggests that this is a plesiomorphic condition for the genus. Moreover, *Bathygobius* sp.1, *B. soporator* and *Bathygobius* sp.2 have conspicuously different karyotypic formulas, with different values in the number of chromosome arms (NF = 54, 56 and 76, respectively). Such changes are putatively due to processes of pericentric inversions, which have been pointed out as one of the main mechanisms of karyotypic diversification in Gobiidae (Vasil'ev and Grigoryan 1993).

Along with structural diversification, mapping analyses of repetitive sequences in some species of Gobiidae have shown remarkable quantitative and qualitative differentiation of repetitive DNA in some species (Mandrioli et al. 2001; Lima-Filho et al. 2012, 2014). In fact, unlike other families of the Percomorphaceae and Eupercaria, in which the heterochromatic patterns are mainly centromeric, the Gobiidae show more variable heterochromatic blocks. In this aspect, mapping of 5S and 18S rDNA sites in all three species is effective in their citotaxonomic differentiation.

Chromosomal characters have been successfully used in the identification of cryptic species of fishes (Moreira-Filho and Bertollo 1991; Bertollo et al. 2004). The high level of karyotypic diversity observed among the species analyzed in this study supports the complete distinction between them.

In Gobiidae, the intense dynamism of karyotypic macrostructure (Caputo et al. 1997; Galvão et al. 2011) and of specific sequences on chromosomes is possibly related to the high level of speciation found in this family (Lima-Filho et al. 2012). The karyotypic differences observed herein do not represent transient polymorphisms as in other species of the family (Thode et al. 1988; Amores et al. 1990), but on the contrary, reveal fixed patterns, leaving almost no doubt that they represent distinct species.

Diversity in the COI sequences

Comparison of the sequences of the COI between different species of *Bathygobius* allowed the confrontation of the taxonomic definitions of previously established species of the Brazilian Province with their counterparts in the Caribbean.

The sequences of *B. soporator* distributed along the Brazilian coast are similar to those described for other areas of occurrence of the species, validating its occurrence in the Brazilian Province. On the other hand, individuals of *Bathygobius* sp.1 revealed a striking molecular divergence with *B. mystacium*, the species with which it had been confounded (Carvalho-Filho 1992; Moura et al. 1999). In fact, *Bathygobius* sp.1 reveals a deep genetic similarity with *B. geminatus* (Tornabene et al. 2010) in Central America, which had previously not been cited for the Brazilian Province. The level of genetic similarity between *Bathygobius* sp.1 and *B. geminatus* suggests that they are the same species. Although we can not rule out the existence of *B. mystacium* in Brazilian waters, the occurrence of *B. mystacium* in the Brazilian coast demand new studies aimed at redefining the limits of geographic distribution of both species.

Bathygobius sp.2, which so far had been identified as *B. soporator* in several studies of Atlantic fishes (Rosa and Moura 1997; Mendes 2006) has a distinct genetic pattern from *B. soporator*. These data confirm previous indications of the remarkable genetic differentiation between *Bathygobius* sp.2 and *B. soporator* in enzymatic and cytogenetic analyses (Lima et al. 2005; Lima-Filho et al. 2012). The COI sequence data grouped *Bathygobius* sp.2 and the Caribbean species *B. antiliensis* in the same clade. However, the level of genetic divergence between them, suggests that *Bathygobius* sp.2 is a distinct and an undescribed species. Despite the high colonizing ability of *B.*

soporator as reflected in its extensive geographical distribution (US–Brazil), *B. antiliensis* possibly does not occur on the Brazilian oceanic islands.

Most species of Gobiidae are benthic inhabitants of coral reefs, whose high productivity (Fraser and Currie 1996), high spatial (Lingo and Szedlmayer 2006) and ecological complexity have been implicated in high levels of diversity (Bellwood and Wainwright 2002). The distribution in multiple habitats of islands and coastal areas of the Brazilian and Caribbean biogeographic provinces seems to contribute to the genetic diversity displayed by the species of *Bathygobius*. These factors promote a high rate of cladogenesis, which allied with vicariant paleogeographic events, favor the speciation of resident fishes (Alfaro et al. 2007).

Cytogenetic and morphological data from different populations of the broadly distributed *B. soporator* on the coast of Brazil, showed conspicuous variations between some of them (Lima-Filho et al. 2012). Such evidence corroborates population fragmentation that causes the genetic diversification and the development of local adaptive patterns for this species.

In spite of being located respectively at 340 and 270 km from the Brazilian tropical coast, the Fernando de Noronha archipelago and the Rocas Atoll belong to the same biogeographic province (Rocha 2003). However, isolation from the mainland and the local environmental conditions give these islands a high degree of endemism, from where new species continuously have been described (Sazima et al. 1998; Sampaio et al. 2004; Rangel and Mendes 2009). The great similarity between the fish fauna of these islands has been attributed to the presence of shallow seamounts between them, which allows their connection (Sampaio et al. 2004).

Geographic isolation and the oceanic currents should contribute to the species diversification between the Brazilian coast and islands. Additionally, it has been suggested that island and coastal populations are subject to divergent selection pressures, sufficient to overcome the effect of homogenization of sporadic gene flow and lead to divergence between lineages (Rocha 2003). In fact, cases of non-allopatric speciation (ecological) were reported for the gobiid genus *Gobiodon* where the diversification of species was not consistent with the geographic pattern, but with changes on the host coral (Munday et al. 2004). In some cases, as in populations of the gobiid *Elacatinus evelynae*, separated by only 20 km, a sharp genetic discontinuity has been identified, with differences in morphological patterns that have played an important role in their diversification (Taylor and Hellberg 2003).

Analysis using mitochondrial sequences have proven useful in determining the inter-specific variability in the genus *Bathygobius* (Tornabene et al. 2010; Tornabene and Pezold 2011). However, even if molecular characters allow the identification of new species (Sperling and Hickey 1994; Wells et al. 2001; Hebert et al. 2003a, b), the delimitation of species ideally requires confirmation from other approaches (Funk and Omland 2003; Dayrat 2005; Roe and Sperling 2007; Castro et al. 2014), as those used here in the differentiation of species of *Bathygobius*.

Morphological variation

The geometric morphometrics analysis completely evidenced the distinction among *B. soporator*, *Bathygobius* sp.1 and *Bathygobius* sp.2 (RA). However *Bathygobius* sp.2 (FNA) showed greater morphological similarity with *B. soporator* than *Bathygobius* sp.2 (RA), to which it is genetically closer (K2P d = 0.0005 %).

The populations of *Bathygobius* sp.2 from RA and FNA possibly share with other species of *Bathygobius* (Tavolga 1953; Peters 1983) the presence of limited movements associated with reproductive characteristics as adhesive eggs and short pelagic larval period. Despite the close proximity between *Bathygobius* sp.2 from FNA and RA, the individuals of these regions vary regarding the body shape. Such conditions in island environments governed by different factors may favor disruptive selection of morphotypes adapted to the local conditions of these environments. In fact, in response to environmental characteristics, many fish species have shown considerable increment of phenotypic plasticity that promotes fitness to a particular environment (Mérona et al. 2009). When compared to other island ecosystems, the Rocas Atoll has low habitat diversity (Rocha 2003), and this condition may have driven morphological changes in the population of *Bathygobius* sp.2 due to specific differences in conditions of the system resources (Mittelbach et al. 1992; Walker 1997).

Variations in body shape among the analyzed species of *Bathygobius* were more significant in

relation to the positioning of the dorsal and anal fins and body height. These differences suggest ecomorphological adaptations related to the process of swimming, with direct implications for habitat use (Collar et al. 2008), biotic interactions (Werner 1977) and foraging (Webb 1986). On the other hand, the morphological changes related to the size and positioning of the mouth could reflect different degrees of feeding specializations, particularly regarding the type and size of the potential preys (Piorski et al. 2005). The phenotypic plasticity identified among populations of *B. soporator* (Lima-Filho et al. 2012) and in island populations of *Bathygobius* sp.2 RA and FNA support a strong environmental effect on local adaptive patterns of each population. This condition confirms the difficulty in defining the precise taxonomic status of some species based only on morphological characters.

The use of mitochondrial markers has proved adequate to identify morphologically cryptic forms within the genus *Bathygobius*. Although *B. soporator*, *B. antilliensis* and *B. lacertus* are almost morphologically indistinguishable, analysis of the COI sequences indicates that they are not a monophyletic group (Tornabene et al. 2010). The similarity in morphology among the three species could be due to convergent evolution. The observed phenotypic plasticity among populations of *B. soporator* probably indicates morphological patterns in response to prevailing environmental conditions (Lima-Filho et al. 2012). Nonetheless, they could also roughly indicate the conservatism of a basal morphological pattern. The homoplasic nature associated with small body size in the genus *Bathygobius* poses difficulties in phylogenetic analyzes based only on the morphological dimension (Tornabene and Pezold 2011). The combined use of genetic and morphological analyzes have been increasingly useful in understanding the processes involved in phylogenetic diversification both in Gobiidae (Taylor and Hellberg 2003, 2005; Tornabene et al. 2010; Lima-Filho et al. 2012) as well as in different groups of organisms (Cheverud 1989; Doebley and Stec 1993; Larson 1998).

In a general context, the joint use of chromosomal, molecular and body form characters proved to have a high potential resolution in evolutionary studies of the genus *Bathygobius*. In fact, the set of approaches led to the identification of an undescribed new species (*Bathygobius* sp.2) and the geographic redefinition of another, *Bathygobius* sp.1, clearly a branch of the *B. geminatus* hitherto inaccurately identified as *B. mystacium* in the coast of Brazil. The successful combination of these techniques potentially consolidates its application to other representatives of this taxon, primarily as an aid in improving the knowledge of its actual species composition.

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