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RESEARCH ARTICLE

Species delimitation in sharpnose sharks (genus *Rhizoprionodon*) in the western Atlantic Ocean using mitochondrial DNA

F. F. Mendonça · C. Oliveira · G. Burgess · R. Coelho · A. Piercy · O. B. F. Gadig · F. Foresti

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Abstract Despite Springer's (1964) revision of the sharpnose sharks (genus *Rhizoprionodon*), the taxonomic definition and ranges of *Rhizoprionodon* in the western Atlantic Ocean remains problematic. In particular, the distinction between *Rhizoprionodon terraenovae* and *R. porosus*, and the occurrence of *R. terraenovae* in South American waters are unresolved issues involving common and ecologically important species in need of fishery management in Caribbean and southwest Atlantic waters. In recent years, molecular markers have been used as efficient tools for the detection of cryptic species and to address controversial taxonomic issues. In this study 415 samples of the genus *Rhizoprionodon* captured in the western Atlantic Ocean from Florida to southern Brazil were examined for sequences of the COI gene and the D-loop and evaluated for nucleotide differences. The results on nucleotide composition, AMOVA tests, and relationship distances using Bayesian-likelihood method and haplotypes network, corroborates Springer's (1964) morphometric and meristic finding and provide strong evidence that supports consideration of *R. terraenovae* and *R. porosus* as distinct species.

Keywords Conservation · *Rhizoprionodon terraenovae* · *Rhizoprionodon porosus* · Species delimitation · Elasmobranchs

F. F. Mendonça (✉) · C. Oliveira · F. Foresti
Departamento de Morfologia, Laboratório de Biologia e Genética de Peixes, Instituto de Biociências de Botucatu, Universidade Estadual Paulista, UNESP, Distrito de Rubião Júnior, S/N, Botucatu, SP CEP 18618-000, Brazil
e-mail: fernandofm@ibb.unesp.br

G. Burgess · R. Coelho · A. Piercy
Florida Program for Shark Research, Florida Museum of Natural History, University of Florida, Dickinson Hall, Museum Road, Gainesville, FL 32611, USA

R. Coelho
Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

O. B. F. Gadig
Laboratório de Pesquisa em Elasmobrânquios, Campus Experimental do Litoral Paulista, Universidade Estadual Paulista—UNESP, Praça Infante D. Henrique, S/N, São Vicente, SP CEP 11330-900, Brazil

Introduction

The carcharhinid genus *Rhizoprionodon* is represented worldwide by seven species of small coastal sharks (Springer 1964; Compagno 1984). The three western Atlantic species are *R. terraenovae* (Atlantic sharpnose shark), which occurs from Yucatan in the Gulf of Mexico northward to the Bay of Fundy, Canada; *R. porosus* (Caribbean sharpnose shark), distributed from the Bahamas and Antilles through the Caribbean Sea to Uruguay; and *R. lalandii* (Brazilian sharpnose shark) that occurs in coastal waters from Panama to Uruguay. However, the taxonomic status of *R. terraenovae* and *R. porosus* have remained uncertain for decades as the main distinction between the two species is the number of vertebrae (58–66 in *R. terraenovae* and 66–75 in *R. porosus*; Springer 1964) This has led some to consider *R. porosus* a subspecies or a clinal variant of *R. terraenovae* (Compagno 1984).

Owing to logistic difficulty of radiographing specimens and the allopatric distributions documented by Springer (1964), most identifications made over the past 45 years were likely based solely on geographical location of capture. By contrast, some authors have suggested that these species may have some degree of sympatry with

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R. terraenovae also occurring in South America (Bigelow and Schroeder 1948; Compagno 1984; Gadig 2001). In addition to basic interest in the definition of taxonomic units, the characterization of these sharks as two distinct species and the possible occurrence of *R. terraenovae* in South America has considerable bearing on the management and conservation of these sharks, since sharpnose sharks are heavily exploited in commercial fisheries throughout most of the coastal western Atlantic.

Molecular diagnostic techniques are now commonly employed in species identification and often are applied to shed light on situations where traditional morphological- and meristic-based analyses have proven difficult or unsatisfactory. Identification via DNA is based on the assumption that intra-specific genetic divergence is usually lower than inter-specific divergence (Meyer and Paulay 2005). Hebert et al. (2003) proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of animal species, and proposed the use of the mitochondrial DNA gene cytochrome oxidase subunit I (COI) as a global bioidentification system for animals. The COI gene was utilized as the determiner in several shark species characterization studies (Shivji et al. 2002; Abercrombie et al. 2005; Mendonça et al. 2009a; Mariguela et al. 2009; De-Franco et al. 2009). Also known as D-loop, the control region is the most rapidly evolving region of mtDNA (Hoelzel et al. 1991; Lopez et al. 1997) and it is one of the most commonly used markers for addressing evolutionary relationships of closely related species and/or subspecies (Meyer et al. 1990; Rocha-Olivares et al. 1999; Murgra et al. 2002). The D-loop also proved effective in the characterization of shark species (Corrigan et al. 2008; Quattro et al. 2006; Sandoval-Castillo et al. 2004).

In the present study, we evaluated mitochondrial nucleotide sequences in samples of *Rhizoprionodon* sharks with the objective of determining the taxonomic status of species *R. terraenovae* and *R. porosus* in the western Atlantic Ocean and assessing the possible occurrence of *R. terraenovae* in South America. The results of this study will help elucidate the separation and distribution of these two closely related taxa, providing fundamental baseline information for fisheries biologists and managers to assess the status and manage those species more efficiently.

Materials and methods

Sample characterization

A total of 415 samples of the genus *Rhizoprionodon* were analyzed: 64 samples from *R. terraenovae* (Richardson, 1836) specimens caught in Florida, USA (37 from the Gulf of Mexico and 27 from the Atlantic coast); 321 samples from

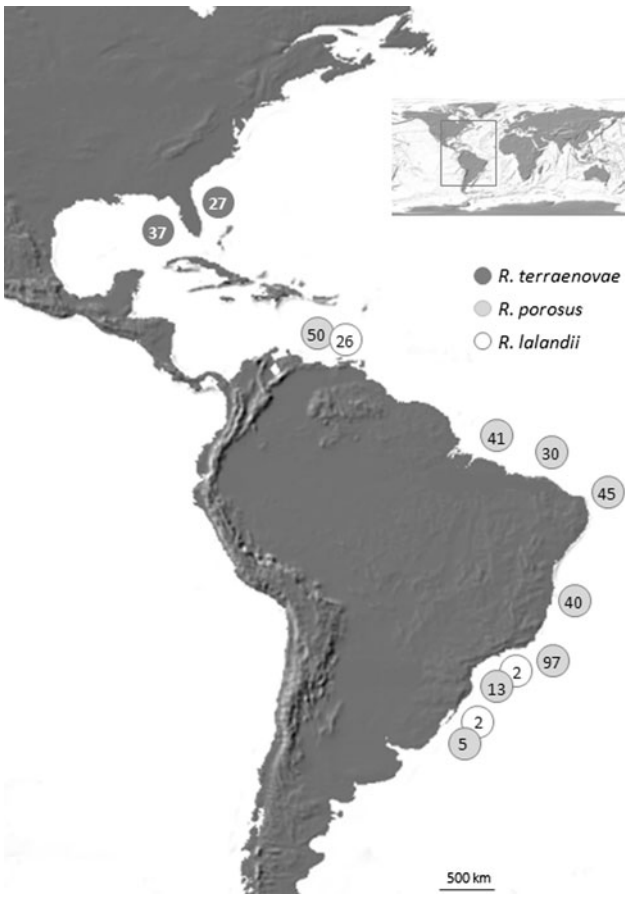

Fig. 1 Sampling localities of the *Rhizoprionodon* sharks used during this study. The *numbers in the circles* represent the sample size at each location

R. porosus (Poey, 1861) specimens collected over a wide latitudinal range, from the Caribbean (Isla Margaritta, Venezuela) to the extreme south of Brazil; and 30 samples from *R. lalandii* (Műller and Henle, 1839) collected from the Caribbean and southern Brazil (Fig. 1). The samples of *R. terraenovae* were collected between 2003–2005 and in 2009 by staff of the Florida Program for Shark Research at the Florida Museum of Natural History, University of Florida, Gainesville, Florida, USA. The samples of *R. porosus* and *R. lalandii* were collected from 2005 to 2008, mostly during field work carried out by researchers of the Laboratório de Biologia e Genética de Peixes of the Instituto de Biociências, Universidade Estadual Paulista, São Paulo, Brazil, and Laboratório de Pesquisa em Elasmobrânquios, Universidade Estadual Paulista, São Paulo, Brazil.

DNA extraction, amplification through PCR and sequencing

The genomic DNA was extracted from epithelial cells, using the saline extraction method described by Aljanabi

and Martinez (1997). Amplification reactions of the cytochrome oxidase gene subunit I (COI) were carried out using the primers F1 5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′ and R1 5′-TAG ACT TCT GGG TGG CCA AAG AAT CA-3′, described by Ward et al. (2005). Amplifications of the D-loop were carried using the primers D-loop F 5′-CTC CCA AAG CCA AGA TTC TG-3′ and D-loop R 5′-GGC TTA GCA AGG TGT CTT CTT GG-3′ described by Mendonça et al. (2009b). Those amplifications were carried out in a PCR thermal cycler using 25 µl of solution 0.8 mM of dNTP, 1.5 mM of $MgCl_2$, enzyme buffer Taq DNA polymerase (Tris–HCl 20 mM pH 8.4 and KCl 50 mM), 1 unit of enzyme Taq Polymerase (Invitrogen) and 0.5 mM ng of primers. Each amplification cycle through PCR was formed by denaturation at 95°C for 30 s, hybridization at 50°C to the COI gene and 55°C to the D-loop for 30 s and extension at 72°C for 1 min, with 35 repetitions. The amplified DNA segments were visualized on agarose gel at 2%, stained with ethidium bromide, under ultraviolet light. PCR products were labeled using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). Each cycle sequencing reaction mixture consisted of 5.0 µl of 10% trehalose, 0.917 µl of ultrapure ddH_2O, 1.917 µl of 5× buffer (400 mm Tris–HCl pH 9.0 and 10 mm $MgCl_2$), 1.0 µl of primer 0.167 µl of BigDye (Applied Biosystems, Inc.), and 1.5 µl of PCR product. The sequences were obtained using ABI 3730 capillary sequencer (Perkin-Elmer) with the kit DYEnamicTM ET Terminator Cycle Sequencing (Amersham Biosciences), and were then manually analyzed and lined using the program CLUSTAW-Macvector 65 (1998) for identification of polymorphic sites among the species. The haplotype sequences were deposited in GenBank. COI gene sequences with accession numbers HM991184–HM991199 to *R. terraenovae*, numbers HM991200–HM991208 to *R. porosus* and HM991209 and HM991210 to *R. lalandii*. Control region mitochondrial DNA with accession numbers HM802332–HM802351 to *R. terraenovae*, GU318244–GU318296 to *R. porosus* and HM446216–HM446227 to *R. lalandii*.

Analysis

Arlequin 3.0 (Excoffier et al. 2005) was used to generate relative nucleotide composition, number of polymorphic sites, haplotype diversity (h), nucleotide diversity (π), and number of pairwise nucleotide differences among species. In order to estimate the levels of genetic divergence among species of *R. terraenovae*, *R. porosus* and *R. lalandii*, the diversity measure F_{ST} was calculated using an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) under the parameters of the Tamura and Nei (1993) nucleotide substitution model. F_{ST} estimates were tested nonparametrically (1.000 bootstrapped replicates) by ARLEQUIN 3.0 and adjusted for simultaneous pairwise comparisons by the sequential Bonferroni procedure (Rice 1989).

Modeltest software (Posada and Crandall 1998) was used to objectively determine the best model of sequence evolution and the accompanying parameter values for these data. The HKY nucleotide substitution model (Hasegawa et al. 1985) was utilized for all likelihood analyses based on a hierarchical hypothesis test of alternative models implemented with Modeltest 3.7 (Posada and Crandall 1998). The Bayesian-likelihood method of phylogenetic analysis (Huelsenbeck and Ronquist 2001) was used to evaluate the tree topologies through the estimation of posterior probabilities using MrBayes v.3.0 (Ronquist and Huelsenbeck 2003). Four chains were run simultaneously for 5.000.000 generations using MrBayes analysis. Every 100th generation was sampled and the asymptote of likelihood score was detected with the SUMP command. All sampled topologies beneath the asymptote were discarded from the population of trees considered in the subsequent majority-rule consensus. For Maximum Likelihood (Tamura et al. 2004) all analyses used tree-bisection-reconnection (TBR) as the branch swapping algorithm in the program PAUP (Swofford 2004). Bootstrapping was performed on 1.000 replicates of the data sets. Starting trees were obtained by stepwise addition, and AsIs sequence addition was used. Using also the UPGMA method (Sneath and Sokal 1973) the evolutionary distances were computed and are expressed as of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was realized with 1,000 replicates. Consensus trees were produced with the software TreeExplorer in MEGA 4 (Tamura et al. 2007).

A minimum-spanning haplotype network was estimated using the program TCS 1.21 (Clement et al. 2000), which implements the statistical parsimony method of Templeton et al. (1992). The haplotype network was manually nested into increasingly inclusive clades (or nestings) following the rules described by Templeton et al. (1992).

Results

The sequencing of shark samples resulted in 580 analyzable nucleotides of COI gene (30 samples—16 *R. terraenovae*, 9 *R. porosus* and 5 *R. lalandii*) and 802 analyzable nucleotides for the D-loop (415 samples—64 *R. terraenovae*, 321 *R. porosus* and 30 *R. lalandii*). These sequences showed higher levels of genetic variability in the species *R. terraenovae* when considering the composition of the COI gene and greater variability of the species *R. lalandii*

Table 1 Basic parameters of the COI gene and D-loop of mitochondrial DNA in *R. terraenovae*, *R. porosus* and *R. lalandii*

DNA sequences	Species	n	A	T	C	G	N	h	π
COI	*R. terraenovae*	16	0.257	0.346	0.247	0.150	4	0.775	0.00324
	R. porosus	9	0.260	0.349	0.244	0.147	2	0.556	0.00096
	R. lalandii	5	0.259	0.345	0.252	0.145	2	0.667	0.00236
	All	30					8	0.884	0.00920
D-loop	*R. terraenovae*	64	0.312	0.351	0.205	0.131	20	0.749	0.00306
	R. porosus	321	0.306	0.352	0.209	0.133	53	0.881	0.00277
	R. lalandii	30	0.313	0.335	0.202	0.129	11	0.899	0.00252
	All	415					84	0.923	0.00849

n Number of individuals; nucleotide composition: *A* adenine, *T* thymine, *C* cytosine, *G* guanine; *N* number of haplotypes; *h* haplotype diversity; π nucleotide diversity

Table 2 Divergences of the nucleotide composition of the COI gene and D-loop sequences between *R. terraenovae*, *R. porosus* and *R. lalandii*

		R. terraenovae (%)	*R. porosus* (%)
COI	*R. porosus*	1.2	–
	R. lalandii	1.3	1.5
D-loop	*R. porosus*	1.3	–
	R. lalandii	2.2	3.5

when considered the nucleotide sequence of the D-loop region. On indexes of diversity considering the three species, the results were similar for both regions of mitochondrial DNA (Table 1). In comparisons between pairs of species were also observed similar levels of divergence based on COI gene sequences and the D-loop. A lower genetic differentiation was observed between species *R. terraenova* and *R. porosus* based on COI gene (1.2%) and D-loop sequences (1.3%) and the largest genetic distance was obtained between the species *R. porosus* and *R. lalandii* with 1.5% in the COI gene and 3.5% in the D-loop sequences (Table 2).

The resulting phenogram based on 8 sequences of the COI gene (Fig. 2) and 84 sequences of the D-loop (Fig. 3), using the Bayesian-likelihood, Maximum likelihood and UPGMA methods shows yielded similar topologies that there are three major clades: *R. terraenovae*, *R. porosus* and *R. lalandii*. Using the COI gene sequences of the species *R. terraenovae* and *R. porosus* were separate with bootstrap values of 99–100%. With the D-loop sequences of these same species are separated with bootstrap values of 95–100%.

The AMOVA analysis denoted a strong level of genetic structure between the three species. For the COI gene, and considering the overall sample, $F_{ST} = 0.863$ ($P < 0.00001$), with a percentage of variation between species of 82.56% and a percentage of population variation of 15.80%. For the D-loop, $F_{ST} = 0.842$ ($P < 0.00001$), with a percentage of variation between species of 79.46% and a percentage of population change of 13.66%. The levels of F_{ST} between pairs are shown in detail in Table 3.

In the haplotypes generated from the sequences of the COI gene and D-loop, distinct clusters were observed among the three clades, without the existence of shared haplotypes, with clusters characterized by several mutational steps and missing haplotypes between clades (Figs. 4, 5). Haplotypes characteristic of *R. terraenovae* were not found in the samples collected between the coasts of the Caribbean and southern Brazil. Likewise, haplotypes corresponding to *R. porosus* were not found in the areas sampled in the Gulf of Mexico and eastern Florida.

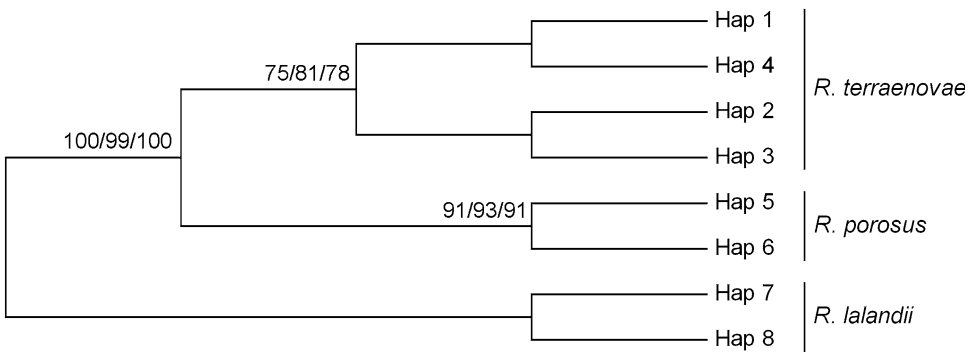

Fig. 2 Phylogenetic relationships between *R. terraenovae*, *R. porosus* and *R. lalandii* based on COI gene. *Numbers along branches* are bootstrap values for Bayesian-likelihood, maximum likelihood and UPGMA distance methods respectively

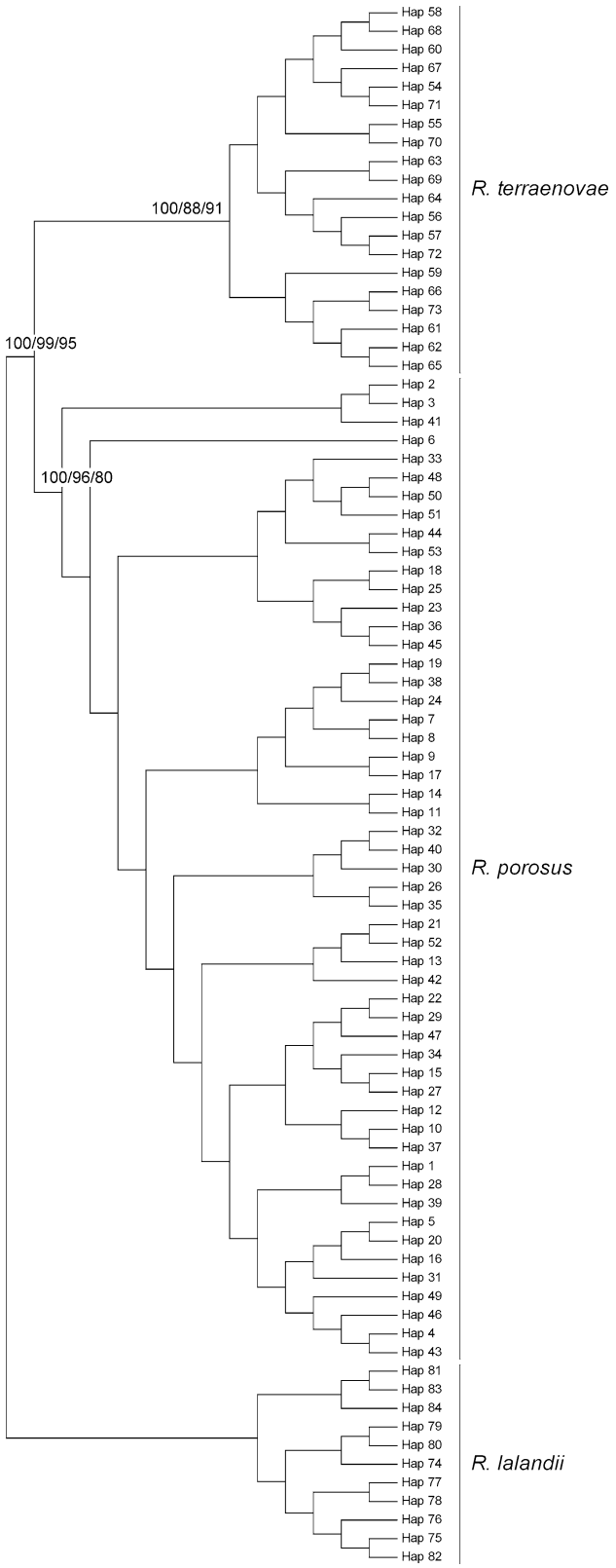

Fig. 3 Phylogenetic relationships between *R. terraenovae*, *R. porosus* and *R. lalandii* based on D-loop sequences. *Numbers along branches* are bootstrap values for Bayesian-likelihood, maximum likelihood and UPGMA distance methods respectively

Table 3 Estimate of pairwise F_{ST} values (and the respective *P* values) using Tamura and Nei (1993) genetic distances based on COI gene and the D-loop

		R. terraenovae	*R. porosus*
COI	*R. porosus*	0.85357, $P < 0.00001$	–
	R. lalandii	0.87488, $P < 0.00001$	0.91852, $P < 0.018$
D-loop	*R. porosus*	0.82525, $P < 0.00001$	–
	R. lalandii	0.84934, $P < 0.00001$	0.87262, $P < 0.00001$

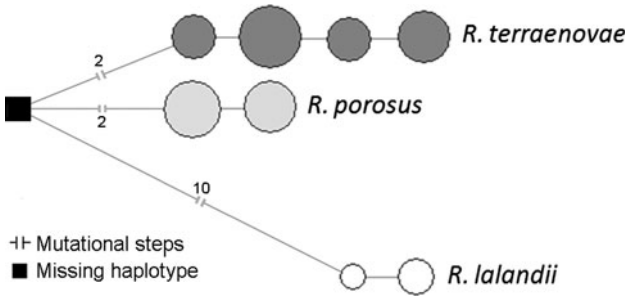

Fig. 4 Statistical parsimony network of haplotypes between *R. terraenovae*, *R. porosus* and *R. lalandii* species based on COI gene. The size of the *circle* in the network is proportional to haplotype frequency

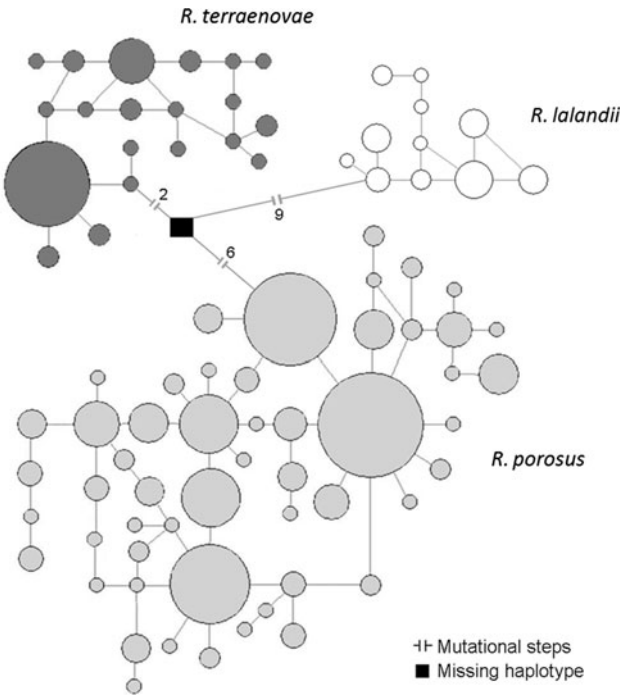

Fig. 5 Statistical parsimony network of haplotypes between *R. terraenovae*, *R. porosus* and *R. lalandii* based on D-loop sequences. The size of the *circle* in the network is proportional to haplotype frequency

Discussion

In this study, mitochondrial DNA sequences of the COI gene and D-loop of 415 samples of the genus *Rhizoprionodon* from the western Atlantic Ocean showed levels of divergence compatible with the separation of *R. terraenovae* and *R. porosus* in two different species. Here, the *R. lalandii* is presented in all analysis and discussion as a measure of genetic divergence among species of the genus *Rhizoprionodon*.

In the nucleotide composition of the COI gene the divergences between the three species were relatively similar, with the lowest difference found between *R. terranovae* and *R. porosus* (1.2%), followed by the differences between *R. terraenovae* and *R. lalandii* (1.3%), and the greatest differences observed between *R. porosus* and *R. lalandii* (1.5%). These values are generally lower than the obtained by Moura et al. (2008) between sharks species of the genus *Centrophorus* and *Centroscymnus* (3%) and are presented within the range of divergence observed by Ward et al. (2008) between several species of Australian fishes, including 41 sharks species, where differences between taxa of the same genus ranged from 0 to 20.63% with distance mean of 9.93%. Between three sharks species of the genus *Squalus* the rate of nucleotide difference was 4.1%. According to Ward et al. (2008), these differences in nucleotide compositions in different groups of fish help explain the multimodal distribution of GC content at third base in each codon. This reflects the fact that most synonymous mutations occur at the 3rd position, with a few at the 1st position and none at the 2nd.

The data regarding the genetic diversity within species is especially relevant to population studies and for species of economic importance for the determination of different stocks. In this study these aspects replicate some population characteristics contained in each species and thus may help to distinguish between them, especially between *R. terraenovae* and *R. porosus*. Significant differences between the three species were also observed in the nucleotide composition of the D-loop, with divergences of 1.3% found between *R. terraenovae* and *R. porosus*, 2.2% between *R. terraenovae* and *R. lalandii* and 3.5% between *R. porosus* and *R. lalandii*. The rates of divergence of the mitochondrial DNA D-loop of the genus *Rhizoprionodon* were similar to those found among species of the genus *Rhinobatos* (2.5%) rated by Sandoval-Castillo et al. (2004); slightly lower than the rates found among cryptic species of hammerhead sharks, *Sphyrna* spp. (5.3%) reported by Quattro et al. (2006); and also slightly lower than the indices found by Corrigan et al. (2008) between species of the genus *Chiloscyllium* (4.8%). The rates of divergence found in the compositions of the D-loop between elasmobranch species may be justified by the nature of these non-coding sequences and may be associated still, with the time elapsed since the period of speciation with the relatively low divergence values found for the *Rhizoprionodon* genus on the western Atlantic Ocean.

High levels of structuring among the three species were found when using the AMOVA analysis ($F_{ST} = 0.863$ to the COI gene; $F_{ST} = 0.842$ to the D-loop). When pairwise comparisons were carried out, the greatest differentiation was found between *R. porosus* and *R. lalandii* ($F_{ST} = 0.918$ to the COI gene; $F_{ST} = 0.872$ to the D-loop) and a smaller genetic distance between *R. terranovae* and *R. porosus* ($F_{ST} = 0.853$ to the COI gene; $F_{ST} = 0.825$ to the D-loop). Between *R. terraenovae* and *R. lalandii* the genetic structuring index was $F_{ST} = 0.874$ to the COI gene and $F_{ST} = 0.849$ to the D-loop. Amongst marine teleosts, including silver scabbardfish of the genus *Lepidopus* and John Dory (*Zeus faber*), distinct species also showed high values of molecular variance ($F_{ST} = 0.84$ and $F_{ST} = 0.96$) (Ward et al. 2008). In elasmobranchs, an AMOVA analysis was applied by Smith et al. (2009) to characterize the existence of new species in the butterfly ray genus *Gymnura* with a value of $F_{ST} = 0.81$. There are no studies addressing the rates of F_{ST} in distinguishing shark species, however, the values of molecular variance found in this study between *R. lalandii* and the two other clades suggest that this is a robust method of evaluating interspecific differences also for this group of fishes. Although this study used a discrete number of individuals for the analysis of the COI gene, the parameters obtained were very similar to those observed in the use of the D-loop with populations fairly represented. Still, due to the conservative evolutionary characteristics, analyses using the COI gene for assessment among species typically involve a small number of individuals of each taxon.

The haplotype network and the topologies of phylogenetic trees originating from sequences of the COI gene and D-loop, show the levels of relationships between the three species of the genus *Rhizoprionodon* occurring in the western Atlantic Ocean. In both tests *R. terranovae* and *R. porosus* showed a closer phylogenetic relationship compared to *R. lalandii*. However, the conformation of the haplotype network and the identification of mutations in the three groups reinforce the determination of *R. terraenova* and *R. porosus* as distinct species. In the phylogenetic trees presented, these species were placed into three distinct branches, strongly supported by high bootstrap values (95–100%).

Considering all parameters analyzed for the three clades addressed in this study, we provide strong evidence that supports consideration of *R. terraenovae* and *R. porosus* as distinct species. This verifies Springer's (1964) taxonomic revision based on morphological and meristic characters.

Rocha et al. (2005) in a survey of mtDNA sequences of five congeneric west Atlantic reef fishes (genus *Halichoeres*) with similar dispersal potential, observed phylogeographical patterns that contradict expectations of geographical isolation and indicate a role for ecological speciation. In *Halichoeres bivittatus* was observed strong partitions (3.4% divergence) between adjacent and ecologically distinct habitats, but high genetic connectivity between similar habitats separated by thousands of kilometers. This habitat partitioning is maintained even at a local scale where *H. bivittatus* lineages are segregated between cold- and warm-water habitats in both Bermuda and Florida. In a study seeking to characterize divergences and connections in two tropical Atlantic reef fishes, the specie *Epinephelus adscensionis* showed a deep divergence between the southeastern United States and seven other localities from the Bahamas to the south, central and east Atlantic ($F_{ST} = 0.867$) (Carlin et al. 2003). However the geographic distribution of the two lineages is highly unusual in genetic studies of Caribbean Sea reef fishes, because those lineages are separated by less than 250 km of open water within a major biogeographic region.

In the two studies presented above there is a deep genetic partition in reef fishes, with the characterization of distinct biogeographic areas between the southeastern United States and Caribbean. These results strongly resemble those obtained with the sharks *R. terraenova* and *R. porosus* distributed also in these same areas and help corroborate the hypothesis of evolutionary partitions with habitat types, rather than conventional biogeographical barriers, indicating parapatric ecological speciation, in which adaptation to alternative environmental conditions in adjacent locations overwhelms the homogenizing effect of dispersal.

The identification of a taxon at the species level is imperative not only for proper attribution of life history and ecological traits, but also is needed for enlightened fishery management. Among the difficulties that currently hinder the development of efficient plans for the management of elasmobranch fishes, perhaps the most important difficulty involves the lack of species-specific catch and landing data. In addition, individual species may differ in their life history characteristics and therefore in their susceptibility to exploitation. In tandem, these gaps in knowledge limit the ability to assess stocks at both the species and population levels. Many of these shark species are over-exploited and potentially threatened, but our results may now be used by fisheries biologists to more efficiently manage these populations.

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