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TECHNICAL NOTE

Genetic identification of the sharks *Rhizoprionodon porosus* and *R. lalandii* by PCR-RFLP and nucleotide sequence analyses of 5S rDNA

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Abstract A molecular approach based on nuclear 5S rDNA sequence variability was applied successfully to correctly identify samples from the two *Rhizoprionodon* species collected in the wild or sold in markets. The sequence of the non-transcribed spacer (NTS) of the 5S rDNA showed high interspecific variability and no intra-specific polymorphism, making it a useful marker for sharpnose shark identification. Polymorphisms in the NTS sequences of *Rhizoprionodon* sharks also created unique restriction patterns for each species after PCR-RFLP analysis. The 5S rDNA polymorphism represents a fast and non expensive tool to access species identification when rapid and unequivocal identification of shark products is needed, particularly for future management and other investigations.

Keywords Genetic markers · Non-transcribed spacer · Trade monitoring · Sharks · Species identification

Introduction

Rhizoprionodon is a genus of the family Carcharhinidae represented worldwide by seven species of small coastal sharks (Compagno 1984) that together form the basis of important commercial and recreational fisheries, as well as substantial artisanal fisheries (Motta et al. 2005). In Brazilian coastal waters, two species have been identified: *Rhizoprionodon lalandii* (Brazilian sharpnose shark) and *Rhizoprionodon porosus* (Caribbean sharpnose shark). Few biological studies are currently available for these species and they are decreasing in number through overfishing in Brazil over the last decade (Lessa et al. 2005).

Sharks are not usually sold as whole animals, but as carcasses or processed in the form of fillets, making it difficult to identify habitual commercialized species such as those of genus *Rhizoprionodon*. Although *R. lalandii* and *R. porosus* have well-defined species boundaries, their natural morphological similarity makes it difficult to distinguish between them, even when the fish are still intact. However, the common fishery practice of removing head, tail, and most fins from sharks while still at sea in order to reduce required storage space for the captured animals, also called finning, removes such major morphological identifying characteristics. This limits the precise identification of each species and, consequently, results in problems regarding their proper management (Shivji et al. 2002). In this way, we have investigated the genomic organization 5S rDNA in order to identify genetic variation patterns that could be used to distinguish the two sharpnose sharks *R. lalandii* and *R. porosus.*

Materials and methods

Tissue samples were collected from fresh, frozen or ethanol-preserved specimens caught by commercial fisheries and from frozen fillets of *Rhizoprionodon* sharks sold in markets from six distinct sampling sites along the Brazilian coast (Fig. 1). Frozen tissues were also obtained from two

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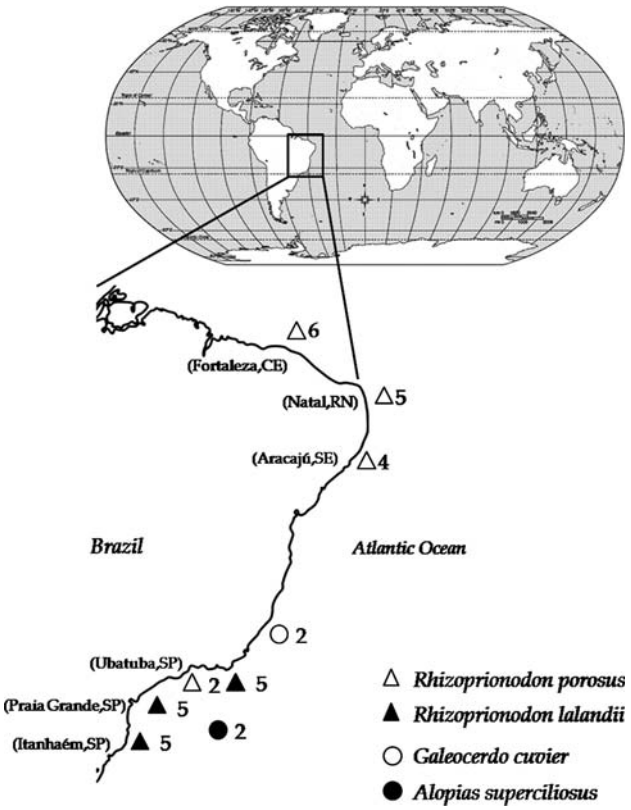

Fig. 1 Map with the sample sites of the four shark species evaluated in this study. Numbers indicate sample sizes from each location

Galeocerdo cuvier and *Alopias superciliosus* individuals for comparative analysis.

Genomic DNA was extracted from the fin clip, gills or muscles of fresh, frozen or ethanol-preserved tissues (Aljanabi and Martinez 1997) to assess the best tissue for DNA extraction. DNA was also isolated from frozen fillets to test the use of this sample kind. An elasmobranch specific set of primers Cart5S1F (5′-CAC GCC CGA TCC CGT CCG ATC-3′) and Cart5S1R (5′-CAG GCT AGT ATG GCC ATA GGC-3′), based on the nucleotide sequences of the 5S rRNA gene from the skate *Taeniura lymma* (AY278251) and the shark *Scyliorhinus caniculus* (M24954), recovered in GenBank, were designed and used to amplify the repeat units of 5S rDNA that include the 5S rRNA gene and the non-transcribed spacer (NTS) of all sharks samples (Pinhal et al. 2009). PCR-amplified products were cloned into the plasmid pGEM-T (Promega) and used to transform competent cells of *E. coli*, DH5α strain (Invitrogen). The positive clones were sequenced on an ABI Prism 3100 automatic DNA Sequencer (Applied Biosystems), following the manufacturer's instructions.

Nucleic acid sequences were subjected to BLASTN (Altschul et al. 1990) searches at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast), and the sequence alignment was performed using the computer program BioEdit (Hall 1999) and checked manually. Neighbor- Joining (NJ) and Maximum Parsimony (MP) phylogenetic analyses employing the Kimura-2-parameter genetic distance model (Kimura 1980) were conducted using the software MEGA 3.1 (Kumar et al. 2004). Nucleotide sequences of the NTSs from *R. pororus and R. lalandii* were analyzed using the software NEBcutter version 2.0 (Vincze et al. 2003) to recognize specific restriction sites for each species. Digestion of PCR products with selected endonucleases were performed in 20 µl volumes with 10 µl amplified DNA, 5 U of enzyme and 2 µl of the recommended 10× digestion buffer. Reactions were incubated for 4 h at 37°C.

Results

PCR products of 5S rDNA repeats were successfully obtained and generated agarose gel bands of ~463 bp for *R. lalandii* and *R. porosus*, ~500 bp for *G. cuvier*, and ~990 bp for *A. superciliosus*. Comparative analysis of 5S rDNA sequences acquired from the four shark species confirmed a highly conserved region (98% similarity) corresponding to the 5S rRNA gene (120 bp) and a variable NTS. Unexpectedly, the NTSs sequences of *Rhizoprionodon* species retain a very high conservation level (about 97% of nucleotide identity), indicating that *R. lalandii* and *R. porosus* have a very short evolutionary time of divergence. When the NTSs of *Rhizoprionodon* sharks were compared to *G. cuvier*, this identity decreased to about 71%, and it further decreased to 39% compared to *A. superciliosus*. Spacer sequences of *G. cuvier* were 28% similar to those of *A. superciliosus*. A very low level of divergence was found in the NTS of the two *Rhizoprionodon* species independent of sample origin and site collection. However, intraspecific sequence polymorphisms occurred in all populations assessed from both species. It was possible to detect 29 polymorphic sites for the 32 NTS sequences analyzed. Of all the polymorphic sites, those at positions 98, 211, 254, 337 and 338 were species-specific. These five polymorphic sites, representing a low but consistent interspecific variability in NTS sequences, were sufficient to separate all *R. lalandii* from *R. porosus* in 99% of the recovered NJ and MP trees (Fig. 2). Furthermore, intraspecific base divergences in NTSs could not be correlated with sample origin, since it was found that in some cases there was greater similarity between sequences of individuals from distinct localities than those belonging to the same locality. This result shows that polymorphisms detected in the NTS of sharpnose sharks are not biased by sample site and thus are an informative species diagnostic.

The restriction site assessment by PCR-RFLP allowed the characterization of enzymes that had cleavage in only

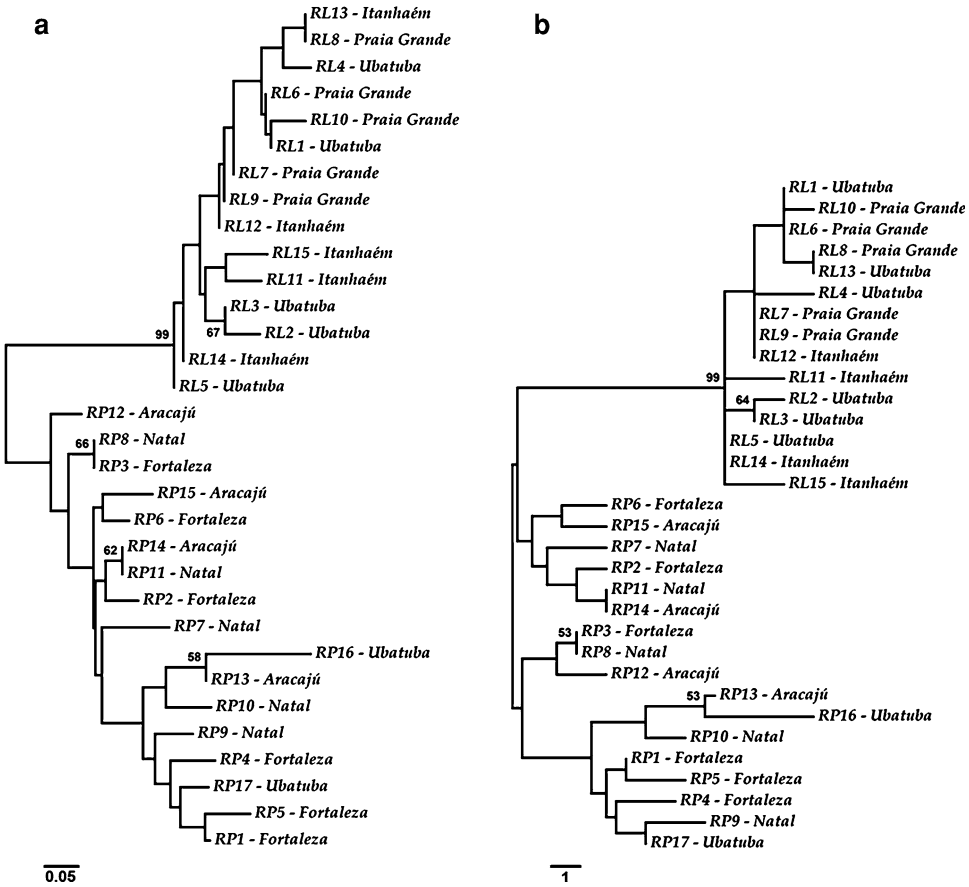

Fig. 2 Trees of maximum parsimony (**a**) and neighbor joining (**b**) from NTS sequences of *R. lalandii* (RL) and *R. porosus* (RP). Branch lengths are proportional to evolutionary distance (scale bar), and the numbers at each node indicate the percentage recovery (>60%) of the particular node (1,000 bootstrap replicates) in which the same internal branch was recovered

one species. Hence, 30 enzymes were characterized with particular cleavage sites in the *R. porosus* NTS sequences (*Ple*I, *Aci*I, *Bfa*I, *EcoP*15I, *Psp*GI, *Bst*NI, *Fai*I, *Mnl*I, *Bss*SI, *Sty*D4I, *Bss*KI, *Psp*GI, *Bst*NI, *Scr*FI, *Afl*III, *Bsp*1286I, *Bsi*HKAI, *Hpy*CH4IV, *Pml*I, *Bmg*BI, *Bsa*AI, *Tst*I, *Mbo*II, *Bbs*I, *Alw*NI, *Bsa*HI, *Zra*I, *Hpy*99I, *Mnl*I and *Hpy*CH4V), while 8 enzymes presented only cleavage in *R. lalandii* (*Eco*RV, *Hpy*AV, *Bsm*FI, *Ava*II, *Ppu*MI, *Hin*P1I, *Hpy*CH4III and *Hha*I). Several of these enzymes were checked and always produced fragments of different sizes between the species (Fig. 3), undoubtedly distinguishing the two sharpnose species.

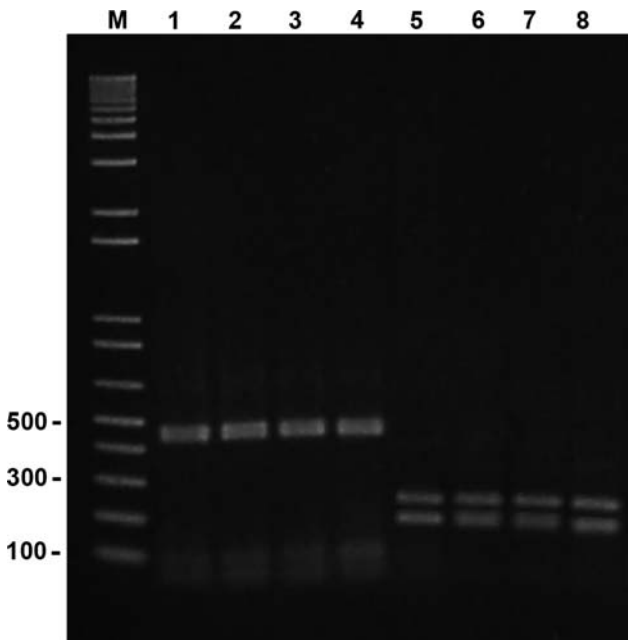

Fig. 3 PCR-RFLP profiles of the 5S rDNA amplicons digested with *Eco*RV. *Rhizoprionodon porosus* samples: lanes 1, 2, 3, 4; and *R. lalandii* samples: 5, 6, 7, 8; *M* molecular weight marker in bp

Discussion

True identification of species is a prerequisite for forensic investigations regarding species management and conservation as well as in taxonomic/systematic studies (Heist and Gold 1999). Among the many nuclear and mitochondrial markers so far studied, the 5S rDNA is of special interest in species identification because of its inherent characteristics, which make it an ideal species-specific

marker in higher eukaryotes. The fact that the organization of 5S rDNA presents rapid rates of base substitution, no intraspecific polymorphism, and high interspecific variability makes it a very good candidate for comparison of close related species, such as *Rhizoprionodon* sharks (Pinhal et al. 2009).

In the present study, we used DNA sequencing and PCR- restriction length fragment techniques to discriminate between congeners *Rhizoprionodon* species. Previous 5S rDNA data have reported that sequence polymorphisms in the ribosomal NTS were sufficient to distinguish subspecies of mice *Mus musculus domesticus* from *M. m. musculus* (Suzuki et al. 1994), suggesting that this region was evolving at an appropriate rate for distinguishing close related species. In the same way, the NTS region has been extensively used for fish species discrimination, either through a simple PCR amplification analysis (Cespedes et al. 1999; Pendás et al. 1995; Pinhal et al. 2008) or in combination with PCR-RFLP analysis (Aranishi et al. 2005; Carrera et al. 2000). NTSs seem to bear consistently low intraspecific variability and high interspecific variability independent of the vertebrate group analyzed. Our data show that the NTS spacers of sharks seem to evolve as quickly as those of mice and other fish, since their polymorphisms allowed differentiation between congeners, representing the first report of genetic data analysis of 5S rDNA applied to Caribbean and Brazilian sharpnose sharks identification. In addition, the PCR-RFLP provides a practical and rapid species-specific diagnostic for these species and enlarges the forensic potential of NTSs as molecular markers.

Techniques here applied, 5S rDNA sequencing and PCR-RFLP, permit the tracking of two economically important species: the Brazilian sharpnose shark and the Caribbean sharpnose shark, currently one of the most exploited and common traded species of sharks. In addition, the analysis of relationships between such close related species by means of a species-specific element may be useful to infer evolutionary relationships between other members of the elasmobranch group. Given the relatively ancient phylogenetic radiation of elasmobranch species, and the consequent high levels of interspecific differentiation, the use of 5S rDNA sequences as molecular markers, particularly the highly polymorphic NTS regions, can provide secure and easily reproducible data. Thus, both techniques represent an efficient way to differentiate the two closely related shark species and can be used to reinforce morphological identification, allowing effective traceability of *Rhizoprionodon* sharks in future management studies.

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References

Aljanabi SM, Martinez I (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res 25:4692–4693

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aranishi F, Okimoto T, Izumi S (2005) Identification of gadoid species (Pisces, Gadidae) by PCR-RFLP analysis. J Appl Genet 46:69–73

Carrera E, Garcia T, Céspedes A, Fernández A, Hernández PE, Martín R (2000) Differentiation of smoked *Salmo solar*, *Onconhynchus mykiss* and *Brama raii* using the nuclear marker 5S rDNA. Int J Food Sci Tech 35:401–406

Cespedes A, Garcia T, Carrera E, Gonzalez I, Fernandez A, Hernández PE, Martin R (1999) Identification of sole (*Solea solea*) and greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rDNA gene. J Agric Food Chem 47:1046–1050

Compagno LJV (1984) FAO species catalogue, vol 4. Sharks of the world. Part 2. Carcharhiniformes. FAO fish. Synopsis 125:251–655

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Heist EJ, Gold JR (1999) Genetic identification of sharks in the US Atlantic large coastal shark fishery. Fish Bull 97:53–61

Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinfo 5:150–163

Lessa RPT, Vooren CM, Araújo MLG, Kotas JE, Almeida PC, Rincón G, Santana FM, Gadig OBF, Sampaio C (2005) Brazilian national plan for conservation and management of elasmobranch fishes stocks. SBEEL, Recife, pp 100

Motta FS, Gadig OBF, Namora RC, Braga FMS (2005) Size and sex compositions, length–weight relationship, and occurrence of the Brazilian sharpnose shark, *Rhizoprionodon lalandii*, caught by artisanal fishery from southeastern Brazil. Fish Res 74:116–126

Pendás AM, Móran P, Martínez JL, Garcia-Vasquez E (1995) Applications of 5S rDNA in Atlantic salmon, brown trout, and in Atlantic salmon x brown trout hybrid identification. Mol Ecol 4:275–276

Pinhal D, Gadig OBF, Wasko AP, Oliveira C, Foresti F, Martins C (2008) Discrimination of shark species by simple PCR of 5S rDNA repeats. Genet Mol Biol 31:361–365

Pinhal D, Araki CS, Gadig OBF, Martins C (2009) Molecular organization of 5S rDNA in sharks of the genus *Rhizoprionodon*: insights into the evolutionary dynamics of 5S rDNA in vertebrate genomes. Genet Res 91:1–12

Shivji MS, Clarke S, Pank M, Natanson L, Kohler N, Stanhope M (2002) Genetic identification of pelagic shark body parts for conservation and trade monitoring. Conserv Biol 16:1036–1047

Suzuki H, Moriwaki K, Sakurai S (1994) Sequences and evolutionary analysis of mouse 5S rDNAs. Mol Biol Evol 11:704–710

Vincze T, Posfai J, Roberts RJ (2003) NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res 31:3688–3691