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Short Communication

Discrimination of Shark species by simple PCR of 5S rDNA repeats

Danillo Pinhal[1], Otto BF Gadig[2], Adriane P Wasko[3], Claudio Oliveira[1], Ernesto Ron[4], Fausto Foresti[1] and Cesar Martins[1]

[1]*Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brazil.*
[2]*Campus Litoral Paulista, Universidade Estadual Paulista, São Vicente, SP, Brazil.*
[3]*Departamento de Genética, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, SP, Brazil.*
[4]*Escuela de Ciencias Aplicadas del Mar, Universidad de Oriente, Boca del Rio, Isla Margarita, Nueva Esparta, Venezuela.*

Abstract

Sharks are suffering from intensive exploitation by worldwide fisheries leading to a severe decline in several populations in the last decades. The lack of biological data on a species-specific basis, associated with a k-strategist life history make it difficult to correctly manage and conserve these animals. The aim of the present study was to develop a DNA-based procedure to discriminate shark species by means of a rapid, low cost and easily applicable PCR analysis based on 5S rDNA repeat units amplification, in order to contribute conservation management of these animals. The generated agarose electrophoresis band patterns allowed to unequivocally distinguish eight shark species. The data showed for the first time that a simple PCR is able to discriminate elasmobranch species. The described 5S rDNA PCR approach generated species-specific genetic markers that should find broad application in fishery management and trade of sharks and their subproducts.

Key words: Chondrichthyes, PCR, species identification, 5S rDNA, sharks.

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The large worldwide increase in elasmobranch fisheries over the past decade is due not only to the intentional catch of these animals but also a result of a massive elasmobranch "bycatch" during Teleostei fisheries, leading to an unprecedented exploitation pressure on many sharks and rays populations (Bonfil, 1994). In contrast to teleost fishes, several studies have shown that elasmobranchs are remarkably susceptible to population decline and/or population collaps due to exhaustive exploitation (Kotas *et al.*, 1995; Vooren, 1997; Musick *et al.*, 2000; Baum *et al.*, 2003).

In a general context, elasmobranch stocks management is complex due to a lack of basic biological data for most species. Several recently published studies have provided a more accurate picture of the status of some populations (Simpfendorfer *et al.*, 2000; Cortés, 2002; Baum *et al.*, 2003; Baum and Myers, 2004), showing that the different life history parameters of each shark species result in a differential sensitivity to intensive exploitation (Heist and Gold, 1999; Castro *et al.*, 1999). Thus, an efficient worldwide management and conservation efforts will require fishery information on a species-specific basis. Nonetheless, this issue may not be easily achieved, due to the considerable difficulty of accurate species identification for several usually targeted species (Bonfil, 1994; Castro *et al.*, 1999). The identification problem is exacerbated by the common fishery practice of removing the head, tail, and most fins from landed sharks while still at sea to reduce required storage space for the captured animals. This practice removes the major morphological identifying characters of the animals, making it difficult to precisely recognize species and, consequently, resulting in problems of proper management (Shivji *et al.*, 2002).

The implementation of molecular biological techniques in marine forensic science has improved the development of accurate taxonomic identification of shark species by sampling biological tissues (Lavery, 1992; Heist and Gold, 1999). Several methods mainly based on protein separation by electrophoresis or high-performance liquid chromatography have been developed for species identification (Sotelo *et al.*, 1993). In recent years, new DNA analyses have been finding their way into the identification of

Send correspondence to Cesar Martins. Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista, 18618-000 Botucatu, SP, Brazil. E-mail: cmartins@ibb.unesp.br.

species, subspecies, populations, strains, hybrids and individuals. DNA amplification using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) has been used as a powerful alternative tool to protein electrophoresis, chromatography and immunological methods, due to its simplicity, specificity and sensitivity. DNA-based genetic markers, especially those related to important traits such as growth enhancement and viral and bacterial disease resistance, have been developed for aquaculture purposes primarily with the goal of improving fish stocks and strains. On the other hand, DNA markers also find application in conservation programs, specifically for stocks identification, breeding selection, analysis of loci segregation and quantitative traits, and for accessing species genetic variability (Martins *et al.*, 2004).

In higher eukaryotes, the 5S ribosomal DNA (5S rDNA) array consists of multiple copies of a highly conserved 120 base pairs (bp) coding sequence, separated from each other by a variable non-transcribed spacer (NTS) (Long and David, 1980) (Figure 1). The 5S rDNA represents a suitable candidate for PCR-based genetic studies due to several features: (i) head to tail organization of the 5S rDNA multigene family members; (ii) the NTS is flanked by the 5S rRNA gene copies in the 5S rDNA tandem array, thus the PCR technology can be used in the isolation of the NTSs; (iii) the 5S rRNA gene is highly conserved even among distantly related species and, consequently, it is possible to isolate the 5S rDNA repeats of one species based on the available sequence of another distantly related species with the use of PCR; (iv) repetitive units of the 5S rDNA do not exceed the length of PCR amplification range; (v) the isolation of the repeat units of 5S rDNA can be obtained from DNA of poor quality and quantity due to their tandem nature and small size (Martins and Wasko, 2004).

Moreover, the distinct genome organization patterns of the 5S rDNA tandem repeats have been also useful as genetic markers not only in evolutionary studies but also in practical approaches for the discrimination of fish species (Pendas *et al.*, 1995; Sajdak *et al.*, 1998; Céspedes *et al.*, 1999; Asensio *et al.*, 2001), even for species of the same genus (Perez and Garcia-Vázquez, 2004; Aranishi, 2005). Nonetheless, there are no data about the usefulness of the 5S rDNA as genetic marker in the elasmobranch fish group.

Hence, the present work aimed to develop a simple and reliable DNA routine method for an accurate discrimination of diverse shark species based on simple PCR amplification and agarose gel electrophoresis analysis. For this purpose, 5S rDNA tandem repeats were evaluated to generate species-specific amplified fragment patterns on eight different sharks species, including closely related species of the genus *Carcharhinus*.

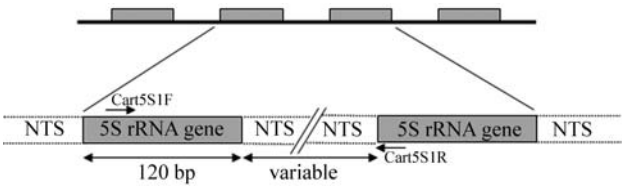

Figure 1 - Arrangement of higher eukaryotic 5S rRNA genes intercalated with non transcribed DNA segments (NTS). The primers Cart5S1F and Cart5S1R annealing regions are indicated.

Sharks belonging to the Orders Carcharhiniformes and Lamniformes were collected from several Brazilian and Venezuelan coast sites: *Sphyrna lewini* and *Isurus oxyrinchus* from the municipality Ubatuba (São Paulo State, Brazil); *Carcharhinus limbatus* and *Carcharhinus acronotus* from the municipality Fortaleza (Ceará State, Brazil); *Galeocerdo cuvier* from the Espírito Santo State (Brazil); *Alopias superciliosus* from the oceanic area in the Southeast of Brazil; *Carcharhinus leucas* from the Bombinhas beach (Santa Catarina State, Brazil), and *Carcharhinus obscurus* from Isla Margarita (Venezuela). Five to ten samples of each species were evaluated for the analyses. Previous morphological species identification was realized on all shark samples. Species identification of some samples (*C. acronotus*, *C. leucas*, *C. obscurus*, *C. limbatus*, *A. superciliosus,* and *I. oxyrinchus*) was carried out using complete specimens that were not preserved due their large size. Small specimens of *S. lewini* and *G. cuvier* were preserved in the fish collection of the Laboratório de Biologia e Genética de Peixes-UNESP, Botucatu, SP, and Instituto de Pesca, Santos, SP. The tissues were collected from specimens that were caught by commercial fisheries, or from frozen or ethanol preserved specimens. Genomic DNA was extracted from fin clip, gills and muscles according to Sambrook and Russell (2001). PCR amplifications of 5S rDNA repeats were performed as described by Martins and Galetti (2001) with some modifications. The primers, Cart5S1F (5'-CAC GCC CGA TCC CGT CCG ATC-3') and Cart5S1R (5'-CAG GCT AGT ATG GCC ATA GGC-3') (Figure 1) were designed based on the 5S rRNA gene sequence of the elasmobranchs *Taeniura lymma* (AY278251) (Rocco *et al.*, 2005) and *Scyliorhinus caniculus* (M24954) (Wegnez *et al.*, 1978). Each PCR reaction mixture contained 150 pmoles of each primer, approximately 20 ng of genomic DNA, 1x Taq buffer, 200 μM of dNTPs, and 2 U of Taq polymerase (Invitrogen) in a final reaction volume of 25 μL. Cycling times were as follows: 5 min at 94 °C; 35 cycles of 1 min at 95 °C (denaturation), 30 s at 55 °C (annealing) and 45 s at 72 °C (elongation); and a final extension step for 5 min at 72 °C. A negative control was also included to check for contamination. The PCR products were analyzed by running 3 μL of each reaction on 1.25% (w/v) agarose gels containing 1x TAE buffer (0.04 M TRIS-acetate and 1 mM EDTA, pH 8.3) and compared with a standard DNA marker (1 kb Plus Ladder - Invitrogen). Electrophoresis was carried out in 1x TAE buffer (90 min, 120 V and approximately 150 mA) at room temperature. Fragments were stained with ethidium bromide, visualized under UV illumination (Hoefer UV-25)

and the gel image was retrieved by using EDAS program (Electrophoresis Documentation and Analysis System 120 - Kodak Digital Science 1D).

PCR amplification of 5S rDNA repeats from shark specimens generated a distinct agarose gel fragment pattern for each analyzed species. We did not find variation among different samples of the same species (Figure 2). Fragment sizes ranged from approximately 130 bp for the minor band in the blacktip shark *C. limbatus* to approximately 1,000 bp for the largest band in the mako *I. oxyrinchus*, and the unique band in the bigeye thresher *A. superciliosus*. The tiger shark *G. cuvier* showed a single band of approximately 520 bp band. The bands for the scalloped hammerhead *S. lewini* were approximately 220 bp and 480 bp, respectively. *I. oxyrinchus* showed two r amplified fragments of approximately 300 bp and 400 bp. The requien sharks, genus *Carcharhinus*, exhibited very distinct PCR band patterns, with one, two, three or four fragments in agarose gels. The blacknose shark *C. acronotus* presented only a single band of approximately 450 bp, while fragments of approximately 130 bp and 520 bp were obtained for *C. limbatus*. The dusky shark *C. obscurus* and the bull shark *C. leucas*, had similar three bands of approximately 450, 500 and 540 bp, with an additional band of 230 bp in *C. leucas*. The distinct amplified fragment patterns of the analyzed shark species, detected by agarose gel electrophoresis, reflect a high variability in the 5S rDNA genomic architecture. This is probably due to major differences in the sharks' NTS organization, since the coding region was found to be conserved in other cartilaginous fishes (Pasolini *et al.*, 2006). Nucleotide sequence analyses based on the NTS have shown that the great variability found in this region can be due to insertions/deletions, minirepeats, and pseudogenes

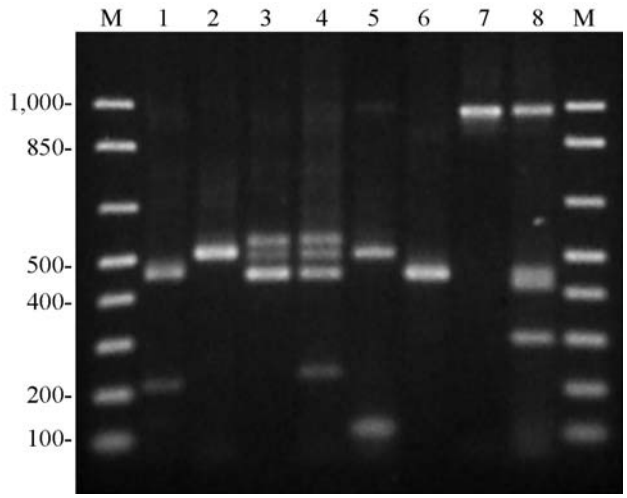

Figure 2 - Agarose electrophoresis profiles of 5S rDNA-PCR products of *Sphyrna lewini* (1), *Galeocerdo cuvier* (2), *Carcharhinus obscurus* (3), *Carcharhinus leucas* (4), *Carcharhinus limbatus* (5), *Carcharhinus achronotus* (6), *Alopias superciliosus* (7), *Isurus oxynchus* (8). M, molecular mass marker in bp.

(Nelson and Honda, 1985; Leah *et al.*, 1990; Sajdak *et al.*, 1998).

The NTS regions seem to be subject to intense evolution, which makes this region an important source for studies concerning the organization and evolution of multigene families and genomes and also as markers to trace recent evolutionary events. Previous studies have shown the presence of different 5S rDNA arrays in the fish genome (Martins and Wasko, 2004). Similarly, the multiple bands detected for some of the analyzed shark species suggest the presence of different 5S rDNA classes also among cartilaginous fishes. One interesting characteristic of the 5S rDNA repeats is the tendency of homogenization of the different copies that are arrayed in the same cluster, which can differ extensively from the copies of a second 5S cluster (Martins and Wasko, 2004). Multigene families are thought to evolve according to homogenization processes governed by molecular drive and concerted evolution, resulting in sequence similarity of the repeat units that is greater within than between species (Dover, 1986; Elder and Turner, 1995). This process was clearly observed in the organization of the 5S rDNA in the Nile tilapia *Oreochromis niloticus* (Martins *et al.*, 2002) and in the South American species of the genus *Leporinus* (Martins and Galetti, 2001).

Particularly among fishes, different NTS lengths have been used as efficient genetic markers for sex identification and for inspection programs that intend to access species, hybrids, or identity of smoked products. In the rainbow trout (*Oncorhynchus mykiss*), chromosome hybridization analyses on male and female metaphase spreads revealed a 5S rDNA chromosome sex-specific pattern (Morán *et al.*, 1996). PCR amplified products of 5S rDNA clearly discriminate the Atlantic salmon (*Salmo salar*), the brown trout (*Salmo truta*), and their hybrids (Pendás *et al.*, 1995), and also several Neotropical fish species of the genus *Brycon* (Wasko *et al.*, 2001). PCR was also applied in the identification of the flatfishes *Solea solea* and *Reinhardtius hippoglossoides* (Céspedes *et al.*, 1999) and for the identification of smoked fillets of salmon, rainbow trout, and bream (*Brama raii*) (Carrera *et al.*, 2000). The present results also demonstrated that 5S rDNA repeats represent good markers for shark species identification at several taxonomic levels.

Previous molecular analyses on shark species discrimination were based on a multiplex PCR assay using both nuclear (ribosomal internal transcribed spacer 2) and mitochondrial (cytochrome *b*) loci simultaneously (Pank *et al.*, 2001; Shivji *et al.*, 2002; Chapman *et al,.* 2003; Shivji *et al.*, 2005; Abercrombie *et al.*, 2005; Clarke *et al.*, 2006), or on mitochondrial gene sequences (Heist and Gould, 1999; Greig *et al.*, 2005). However, these approaches are not only time consuming but also expensive. The PCR amplification of 5S rDNA repeats and agarose gel electrophoresis analysis showed to be a simple routine and low cost methodology to achieve shark species identification. Moreover, this prac-

tice can be used to corroborate the usual morphometric and morphological identification of these animals (Last and Stevens, 1994; Naylor and Marcus, 1994) and can be also used to recognize "cryptic" species, like those of the genus *Carcharhinus*, in which morphological identification can not be easily done.

Particularly in fishery management and conservation, the 5S rDNA PCR approach does not require intensive or expensive labor to be implemented. Additionally, such technology could be applied to body parts that are commonly sold in markets with the generic name of "shark meat", allowing assessment of shark catch and trade on a species-specific basis to detect potential overexploitation of individual species.

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