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Molecular organization of 5S rDNA in sharks of the genus *Rhizoprionodon*: insights into the evolutionary dynamics of 5S rDNA in vertebrate genomes

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Summary

In this study, we attempted a molecular characterization of the 5S rDNA in two closely related species of carcharhiniform sharks, *Rhizoprionodon lalandii* and *Rhizoprionodon porosus*, as well as a further comparative analysis of available data on lampreys, several fish groups and other vertebrates. Our data show that *Rhizoprionodon* sharks carry two 5S rDNA classes in their genomes: a short repeat class (termed class I) composed of ~185 bp repeats, and a large repeat class (termed class II) arrayed in ~465 bp units. These classes were differentiated by several base substitutions in the 5S coding region and by completely distinct non-transcribed spacers (NTS). In class II, both species showed a similar composition for both the gene coding region and the NTS region. In contrast, class I varied extensively both within and between the two shark species. A comparative analysis of 5S rRNA gene sequences of elasmobranchs and other vertebrates showed that class I is closely related to the bony fishes, whereas the class II gene formed a separate cartilaginous clade. The presence of two variant classes of 5S rDNA in sharks likely maintains the tendency for dual ribosomal classes observed in other fish species. The present data regarding the 5S rDNA organization provide insights into the dynamics and evolution of this multigene family in the fish genome, and they may also be useful in clarifying aspects of vertebrate genome evolution.

1. Introduction

Tandemly arrayed copies of 5S rDNA consist of highly conserved 120 base pair (bp) transcribing sequence, separated from each other by a variable non-transcribed spacer (NTS) (see Long & David, 1980). The high level of conservation of the 5S rRNA genes arises from their essential molecular function of enhancing protein synthesis by stabilizing the ribosome structure (Barciszewska *et al.*, 2001). While the 5S rRNA genes are conserved even among unrelated taxa, their NTSs show extensive variation, and no relationships are usually detectable between the NTSs of closely related species. Thus, the NTSs seem to be subject to rapid evolution, which makes this region both an important resource for studies concerning the organization and evolution of multigene families and genomes and also a marker to trace recent events of evolution. The intense dynamism of 5S rDNA repeats generates variant classes of 5S rDNA, which have been reported in the genomes of several vertebrates, from lampreys to mammals (Komiya *et al.*, 1986; Hallenberg *et al.*, 1994; Frederiksen *et al.*, 1997). Particularly in marine and freshwater ray-finned fishes, vast numbers of structural and functional data have demonstrated the occurrence of a dual size-class pattern of organization of the 5S rDNA (Moran *et al.*, 1996; Céspedes *et al.*, 1999; Rocco *et al.*, 1999; Deiana *et al.*, 2000; Martins & Galletti, 2001*a*; Wasko *et al.*, 2001; Martins *et al.*, 2002; Messias *et al.*, 2003; Tigano *et al.*, 2004; Alves-Costa *et al.*, 2006).

The Chondrichthyes are possibly the least-studied fish group with respect to genome structure and evolution. Although genomic studies in fish have increased in the past decade, cartilaginous fishes remain among the least examined in this aspect. For example, studies focused on 5S rDNA have been carried out only on seven species, covering less than 1% of the nearly

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1100 living species. These experiments include the characterization of the 5S rRNA gene in one carcharhiniform shark, *Scyliorhinus caniculus* (Wegnez *et al*., 1978), and more comprehensive analyses of the entire 5S rDNA repeats in six members of Rajiformes: *Raja polystigma* and *Taeniura lymma* (Rocco *et al*., 2005); and *Raja asterias*, *Raja clavata*, *R. polystigma, Raja miraletus* and *Dipturus oxyrinchus* (Pasolini *et al*., 2006). In the last study, the authors reported the presence of two size classes of 5S rDNA in Rajidae, suggesting that a dual system, such as that detected in bony fishes, could be a common pattern for the 5S rDNA organization in elasmobranchs.

Rhizoprionodon is a genus of the family Carcharhinidae represented worldwide by seven species of small coastal sharks (Compagno, 1984). Since they are abundant and globally distributed from tropical to subtropical oceans, *Rhizoprionodon* sharks form the basis of important commercial and recreational fisheries as well as substantial artisanal fisheries (Motta *et al*., 2005). *Rhizoprionodon* sharks have accounted for about 60% of all sharks landed in Brazil (Motta *et al*., 2005), and intensive coastal fishing has led these species to be given 'vulnerable' status (Rosa *et al*., 2004). Several features allow for the discrimination of *Rhizoprionodon lalandii* from *Rhizoprionodon porosus*, such as head and nose curvature, dorsal fin length and position relative to pectoral fins, and body colour; however, it is difficult to distinguish these two species from one another once the head and fins are removed at the fisheries (Gadig, 2001). Currently, there are no molecular data on mitochondrial or nuclear markers concerning population or forensic analysis in these two sharpnose sharks. This prevents inspection programs from properly identifying unprocessed products and inhibits these programs' ability to enforce endangered species laws. Moreover, it makes it impossible to estimate catch and trade on a species-specific basis and thus to assess the sustainability and environmental impact of fisheries. To ameliorate these difficulties, increasing efforts have been directed to the discovery of molecular markers that help in the identification of shark species.

Here, in order to advance understanding of the dynamics and evolution of 5S rDNA arrays in Chondrichthyes, and also to contribute to the development of genetic markers that help in the management of sharks, an evaluation of the genetic structure of the 5S rDNA in the genome of *R. lalandii* and *R. porosus* was conducted. In addition, we carried out a comparative analysis of our data with the available data on lampreys, elasmobranchs and several model organisms such as humans, chickens, *Xenopus* and fugu, among other vertebrates, in an attempt to elucidate the evolutionary patterns that guide the 5S rDNA arrays.

2. Material and methods

(i) *Animal sampling and DNA isolation*

Two shark species, *R. lalandii* and *R. porosus*, were collected from different areas of the Brazilian coast, in the western South Atlantic. All the specimens of *R. lalandii* were from the São Paulo coast in southern Brazil ($n=30$), whereas those of *R. porosus* were from distant sampling points, presumably from different populations (São Paulo in southern Brazil, and Sergipe and Ceará in northern Brazil; $n=28$). Fin clips and gill tissues were collected from fresh or recently frozen animals and immediately immersed in tubes containing pure ethanol. Individuals were identified and voucher specimens were preserved in the fish collection of the Laboratório de Biologia e Genética de Peixes–UNESP, Botucatu, SP, Brazil, under collection numbers LBP-3001 (*R. lalandii*) and LBP-3155 (*R. porosus*). Genomic DNA was successfully isolated from shark samples based on the protocol of Aljanabi & Martinez (1997), which is an alternative procedure without environmentally hazardous reagents such as phenol and chloroform.

(ii) *PCR amplification, cloning and sequencing*

The entire 5S rRNA gene and NTS were amplified from the total genomic DNA by PCR. The set of primers 5SA (5′-TAC GCC CGA TCT CGT CCG ATC-3′) and 5SB (5′-CAG GCT GGT ATG GCC GTA AGC-3′), based on the 5S gene sequence of *Salmo gardnerii*, described by Komiya & Takemura (1979) and applied successfully to other fish species (Martins & Galetti, 2001*a*; Wasko *et al*., 2001), was used for PCR. The primers 5SA and 5SB were designed to amplify the entire NTS and 118 bp of the 5S rRNA gene. After cloning and sequencing of the PCR fragments obtained, a second set of primers Cart5S2F (5′-TGG GAG ACC GCC TGG GAA-3′) and Cart5S2R (5′-CCA AGT ACT AAC CAG GCC-3′) was designed to amplify the annealing region of the primers 5SA and 5SB, allowing the complete sequencing of the 5S rRNA gene. The PCR reactions had 25 μl of total volume, which contained 2 units of *Taq* polymerase, 1 × *Taq* buffer, 1·5 mM of $MgCl_2$, 200 μM of dNTPs, 100 pmol of each primer and ~30 ng of genomic DNA. The background reactions were 35 cycles of 1 min at 95 °C, 30 s at 55 °C and 45 s at 72 °C, with a 5-min final extension at 72 °C. A negative control was also included to test for any contamination. PCR products were assessed by electrophoresis in 1·25% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination. The amplified DNA fragments were purified using the kit GFX PCR Purification (GE Healthcare) and inserted into pGEM-T plasmids (Promega) that were used to transform *Escherichia coli* DH5α

competent cells (Invitrogen), according to Sambrook & Russel (2001). Positive clones were sequenced on the ABI Prism 3100 automatic DNA sequencer (Perkin-Elmer) with the kit BigDye Terminator Cycle Sequencing (Perkin-Elmer), following the manufacturer's instructions.

(iii) *Sequence analysis*

Individual sequences were subjected to BLASTn (Altschul *et al*., 1990) searches on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast). All sequences were aligned using the software DAMBE (Xia & Xie, 2001), using the options: gap open penalty = 10 and gap extension penalty = 0·1. The final alignment was checked visually and corrected when necessary. The 5S rDNA sequences retrieved from *Rhizoprionodon* sharks have been deposited in GenBank under the accession numbers FJ517166–FJ517257. Additional molecular data were obtained from different vertebrate taxa available at NCBI database and from the sharks *Galeocerdo cuvier* and *Alopias superciliosus* (present paper, GenBank accession numbers FJ539130–FJ539131) and were used in the comparative and evolutionary analysis.

Sequence composition and diversity among clones were examined by means of the program BioEdit 7.0 (Hall, 1999). Nucleotide saturation, substitution patterns and genetic distance were examined in MEGA 4 (Tamura *et al*., 2007). Nucleotide saturation was observed by plotting the absolute number of transitions (Ti) and transversions (Tv) against genetic distance values. Maximum-likelihood (ML) phylogenetic relationships among 5S sequences were analysed using the website version of the program PhyML (Guindon & Gascuel, 2003; Guindon *et al*., 2005). ML phylogenetic analyses were carried out using the Tamura–Nei nucleotide substitution model (Tamura & Nei, 1993), incorporating rate variation (G) and PINVAR with four G-distributed rate classes (Swofford *et al*., 1996). This model was selected based on a hierarchical hypothesis test of alternative models implemented with Modeltest 3.6 (Posada & Crandall, 1998). The Ti/Tv ratio, gamma shape parameter and proportion of non-variant sites were estimated by ML from a neighbour-joining tree (BIONJ). Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes, using 500 replicates with random additions and TBR branch swapping. The Bayesian-likelihood method of phylogenetic analysis (Huelsenbeck *et al*., 2001) was used to evaluate tree topologies of vertebrates through the estimation of probabilities using MrBayes v.3.0 (Ronquist & Huelsenbeck, 2003). Four chains were run simultaneously for 1 000 000 generations using MrBayes analysis. Every 100th generation was sampled, and the asymptote of likelihood score was detected with the SUMP command. The above procedure was repeated twice. All the sampled topologies beneath the asymptote (12 000 generations in the present study) were discarded from the population of trees considered in the subsequent majority-rule consensus. Consensus trees were produced with the program TreeExplorer implemented in MEGA 4 (Tamura *et al*., 2007).

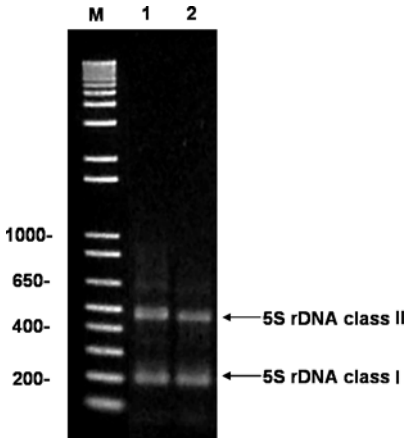

Fig. 1. Agarose gel showing the two 5S rDNA types of *R. lalandii* (1) and *R. porosus* (2) amplified by PCR using the primers 5SA and 5SB. M refers to the 1 kb plus molecular weight marker in base pairs (bp).

3. Results

(i) *Sequence analysis of 5S rDNA arrays from* Rhizoprionodon *sharks*

The pairs of primers 5SA and 5SB, and Cart5S2F and Cart5S2R allowed the amplification by PCR of the entire 5S rDNA repeats in *Rhizoprionodon*. Two fragments of roughly 185 and 465 bp (hereafter referred to as class I and class II, respectively) were obtained for both species (Fig. 1). Nucleotide sequencing and BLASTn searches confirmed that the obtained sequences correspond to 5S rDNA repeat units. These units consisted of a 120 bp coding region (5S gene) as well as a NTS of variable length and composition (Fig. 2).

Sequencing of 46 recombinant clones showed that each class of 5S rDNA was characterized by distinct NTS and 5S rRNA gene sequences (Fig. 2), which showed variable levels of genetic divergence (Table 1). The GC contents in the 5S genes of class I and class II of *R. lalandii* were 55·2 and 53·1%, respectively, whereas *R. porosus* showed rates of 54·7% for class I and 52·5% for class II. The 5S genes of sharks contained some class-specific base substitutions. Regarding *R. porosus*, the class I and class II coding sequences contained six and two base substitutions, respectively, whereas in *R. lalandii*, the sequences contained four substitutions for each class. Common

Table 1. *Genetic distance of the 5S genes and NTS classes among* Rhizoprionodon *sharks. NC, number of clones; L, length in base pairs; GD, genetic distance; SE, standard error*

	5S rDNA class I			5S rDNA class II		
	NC	L	GD ± SE	NC	L	GD ± SE
5S gene						
R. lalandii	7	120	0·011 ± 0·007	19	120	0·025 ± 0·010
R. porosus	4	120	0·066 ± 0·023	16	120	0·023 ± 0·012
All	11		0·042 ± 0·013	35		0·024 ± 0·009
NTS						
R. lalandii	7	64–68	0·009 ± 0·008	19	338–345	0·008 ± 0·004
R. porosus	4	67–72	0·187 ± 0·084	16	340–348	0·017 ± 0·005
All	11		0·389 ± 0·096	35		0·025 ± 0·007

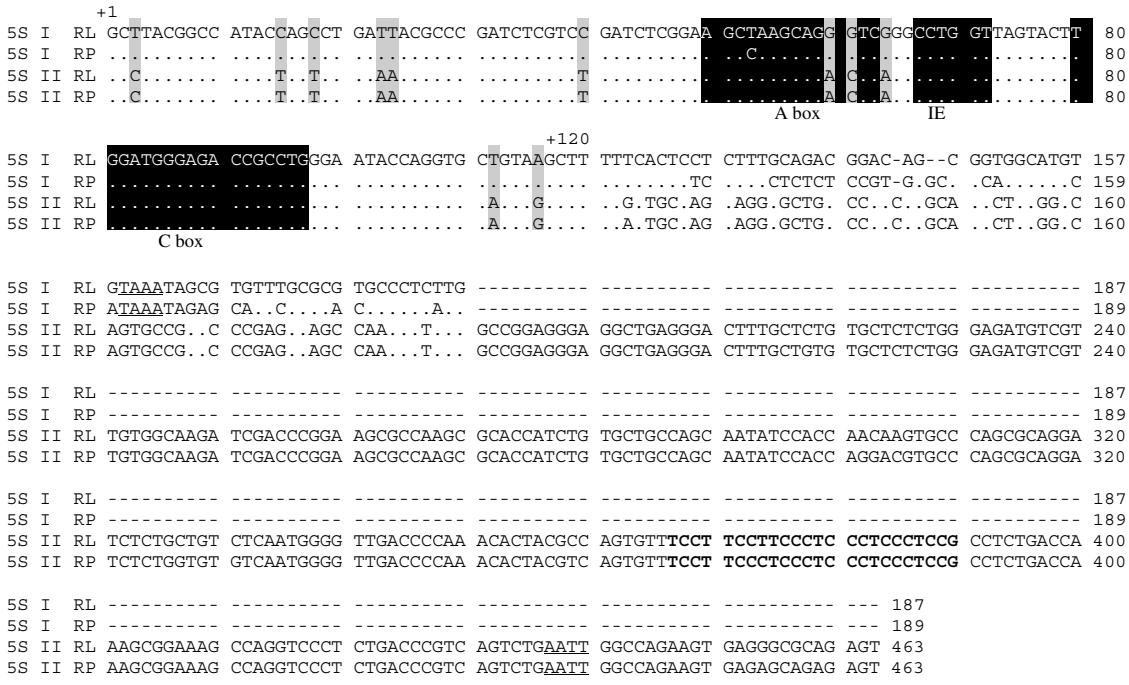

Fig. 2. Representative sequences of 5S rDNA class I (5S I) and class II (5S II) from *R. lalandii* (RL) and *R. porosus* (RP). The start and end points of transcription are indicated by +1 and +120, respectively; the nucleotide substitutions of specific classes are indicated in grey shading, and the ICRs (A box, IE and C box) are indicated in black shading. TATA-like sequences are underlined. SSRs are in boldface. Hyphens represent gaps, and dots indicate identical nucleotides.

alterations detected in the 5S genes of class I in relation to those of class II are listed in Table 2. A transition at position 116 (A→G) caused the 5S class II gene of sharks to lose a *Hind*III restriction site, and it is extensively conserved among bony fishes and previously detected in the 5S rDNA of Rajidae (Pasolini *et al.*, 2006). In general, these base shifts were detected outside internal control regions (ICRs). When in ICRs, they are present in Box A and/or Box C, but never in the intermediate element (IE) (Fig. 2). Another clear disparity between the 5S rDNA classes was the number of thymidine residues located in the

Table 2. *Diagnostic nucleotide sites present in 5S gene class I and class II from* R. lalandii *and* R. porosus

	Nucleotide sites of 5S gene										
	3	15	18	23	24	40	60	61	64	112	116
Rhizoprionodon class I	T	C	C	T	T	C	G	G	G	T	A
Rhizoprionodon class II	C	T	T	A	A	T	A	C	A	A	G

T-rich tail region of the 5S genes: five thymidines in class I and four in class II (Fig. 2). These repeated sequences correspond to transcriptional terminators (Korn & Brown, 1978). TATA-like elements, believed to be regulatory regions for 5S gene transcription, also appear in the NTSs of sharks. These elements were found to be modified to TAAA in NTS I and AATT in NTS II (Fig. 2).

Phylogenetic analyses of 5S rDNA sequences were carried out between and within each *Rhizoprionodon* species, analysing the entire 5S rDNA and the 5S gene and NTS sequences separately (Fig. 3*a–d*). Both approaches supported the existence of a 5S rDNA dual pattern in all specimens checked. Additionally, no differences were found among paralogous copies of 5S genes or NTSs from the same 5S rDNA class of *Rhizoprionodon* sharks, even from different populations. This result was confirmed by the clustering of 5S rDNA classes into two distinct branches on all assembled ML trees (Fig. 3*a*). Our data also showed that it was not only variability in the NTS that contributed to the separation of 5S rDNA into two classes. In addition, 5S rRNA genes bearing consistent variability also increased the difference between the two 5S rDNA classes, as illustrated by the 96% divergence of relationship trees (Fig. 3*d*).

Comparing *R. lalandii* and *R. porosus*, it is feasible to assert that these sharks carry similar paralogous copies of 5S genes in their respective 5S rDNA classes, given that the sequences are randomly distributed in tree branches of the phylogenetic reconstructions (Fig. 3*a*, *d*). In addition, the base-to-base comparison of the 5S genes from both 5S rDNA classes did not permit us to recognize species-specific nucleotide sites.

Regarding NTSs, relationships among the different classes between or within species were only slight because these classes were separated into individual branches on ML trees by a 100% bootstrap value (Fig. 3*c*). NTS class I had 64–68 bp, and class II had 335–343 bp; mean values of GC content were as follows: class I = 49·6% and class II = 61·4% in *R. porosus*; class I = 53·4% and class II = 62·1% in *R. lalandii*. Considering all clones, the mean genetic distance of NTS sequences was very different between classes (NTS I: $0{\cdot}389 \pm 0{\cdot}096$; NTS II: $0{\cdot}025 \pm 0{\cdot}007$). While NTS class I was highly variable even within individuals from the same population in a species, large identical sequences were shared by NTS class II in the *Rhizoprionodon* species (e.g. positions 29–97). Besides this unexpected interspecific similarity, five species-specific sites could be detected (at positions 98, 211, 254, 337 and 338), enough to separate all *R. lalandii* sequences from those of *R. porosus* (Table 3). The length of NTS II paralogous sequences varied only slightly in *R. porosus*, ranging from 335 to 343 bp, whereas all sequences in *R. lalandii* were 343 bp. The variation of the former species was related to the presence of microsatellites [TCCC]*n* at positions 251–270 in this species.

(ii) *Comparative analysis of 5S rDNA arrays in vertebrata*

Coding sequences of 5S rDNA arrays from *Rhizoprionodon* sharks were aligned and compared with those of a large number of extant vertebrates, providing information about their relationships. Bayesian analysis was used to construct the phylogenetic relationships among paralogous and orthologous 5S rRNA gene sequences of eight elasmobranch species (two orders), several teleost orders, amphibians, reptiles, birds and mammals (see Supplementary Material at http://journals.cambridge.org/grh). In the Bayesian method, *Rhizoprionodon* sharks clustered according to the 5S rDNA classes on a consensus tree obtained from the analysis of 1000 bootstrap replicates (Fig. 4). *Rhizoprionodon* 5S rRNA genes of class I clustered close to bony fishes, to *Raja* type I and to Vertebrata 5S genes, showing it to be the most widespread variety in existence. On the other hand, *Rhizoprionodon* 5S genes of class II always grouped with other elasmobranch species in an isolated clade, bootstrapping by 91% similarity. Despite the high level of base substitutions in 5S genes of class II (Table 2), we found a higher level of transitions versus transversions ($R = 2.1$) in all sequences, suggesting that homogenizing forces were acting across the transcribed region, thus preserving its function in the ribosome structure.

4. Discussion

(i) *Double-class structure of 5S rDNA in* Rhizoprionodon *sharks: is this a rule in sharks?*

Although extensive taxon sampling from shark lineages is indispensable to defining the number of 5S rDNA classes, our data provided new insights into the genomic organization of 5S rDNA in elasmobranchs. PCR and sequence analysis unequivocally demonstrated for the first time that sharks can bear two size classes of 5S rDNA arrays carrying distinct NTS sequences and, surprisingly, highly variable 5S genes. Different units of 5S rDNA, especially those characterizing a double-class pattern, have also been previously documented in several teleost species (Pendás *et al*., 1995; Sadjak *et al*., 1998; Martins & Galetti, 2001*a*; Alves-Costa *et al*., 2006) and some elasmobranchs of the family Rajidae (Pasolini *et al*., 2006).

Sharks exhibited a higher similarity in their 5S rDNA units within a specific class between two species than between the two classes in the same species. This probably occurred because 5S rDNA sequence tends to homogenize the different copies that are arrayed

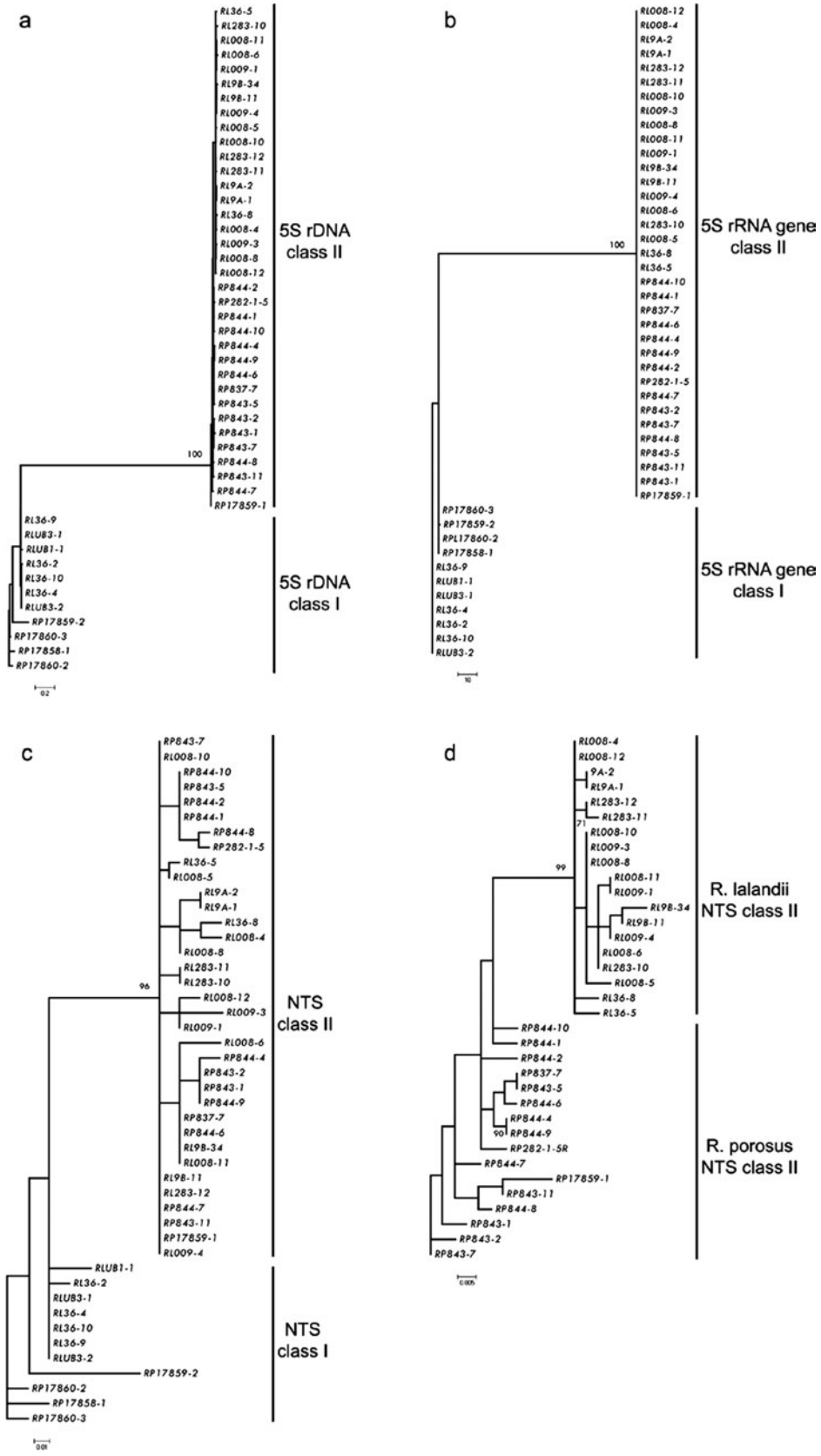

Fig. 3. Recovered ML trees for (*a*) the entire 5S rDNA sequences, (*b*) 5S gene only, (*c*) all NTS sequences and (*d*) NTS II only. Note the existence of two main branches in the trees discriminating the two 5S rDNA classes. In these trees, sequence names correspond to the first two letters of their species names (RL, *R. lalandii* and RP, *R. porosus*) and the number of the clones analysed. The scale bar indicates genetic distance based on the Tamura–Nei model analysis. Bootstrap values under 70 were omitted.

Table 3. *Diagnostic nucleotide sites present in NTS class I sequences of* Rhizoprionodon *sharks*

	Nucleotide position of NTS class I				
	98	211	254	337	338
R. lalandii	C	C	T	G	C
R. porosus	G	G	C	A	G

in the same cluster, which can differ extensively from the copies of a second 5S cluster (Martins & Galetti, 2001*a*; Martins & Wasko, 2004). This observation was clearly documented 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 & Galetti, 2001*a*). It has been suggested that multigene families evolve according to homogenization processes governed by molecular drive and concerted evolution, resulting in a sequence similarity of the repeat units that is greater within than between species (Dover, 1986; Elder & Turner, 1995). Molecular pathways that lead to such concerted evolution are unequal crossing-over, gene conversion and replication slippage, but the rate at which the variant repeat types become homogenized depends on other aspects such as the number of repeats in an array, the strength of natural selection and the effective population size (Schlötterer & Tautz, 1994). Thus, the discrepancy observed between the 5S rDNA classes obtained (i.e. variable length and base composition) must be the product of normal levels of divergence between orthologous sequences in the *Rhizoprionodon* genome.

Although the existence of variant copies of 5S rDNA has been reported in several mammals and amphibians (Komiya *et al*., 1986; Little & Braaten, 1989; Leah *et al*., 1990; Frederiksen *et al*., 1997), these copies did not form distinct repetitive classes in the species investigated. In contrast, the presence of two 5S rDNA classes seems to be a general trend for teleost fishes (Martins & Galetti, 2001*b*; Martins & Wasko, 2004). Moreover, it is likely that the two 5S rDNA classes correspond to two distinct subfamilies of 5S genes. These findings uphold the complex organization of this repeated element in fishes. Furthermore, the distinct 5S rDNA classes detected in teleost fishes were well characterized by highly variable classes of NTSs and a single, highly conserved class of 5S gene.

Conversely, the present data, added to those of Rajidae from Pasolini *et al*. (2006), not only account for a double-class 5S rDNA pattern, but also for a double form of the 5S gene shared by elasmobranchs. This assumption is based on the consistent mutations observed in sequences responsible for the clustering of

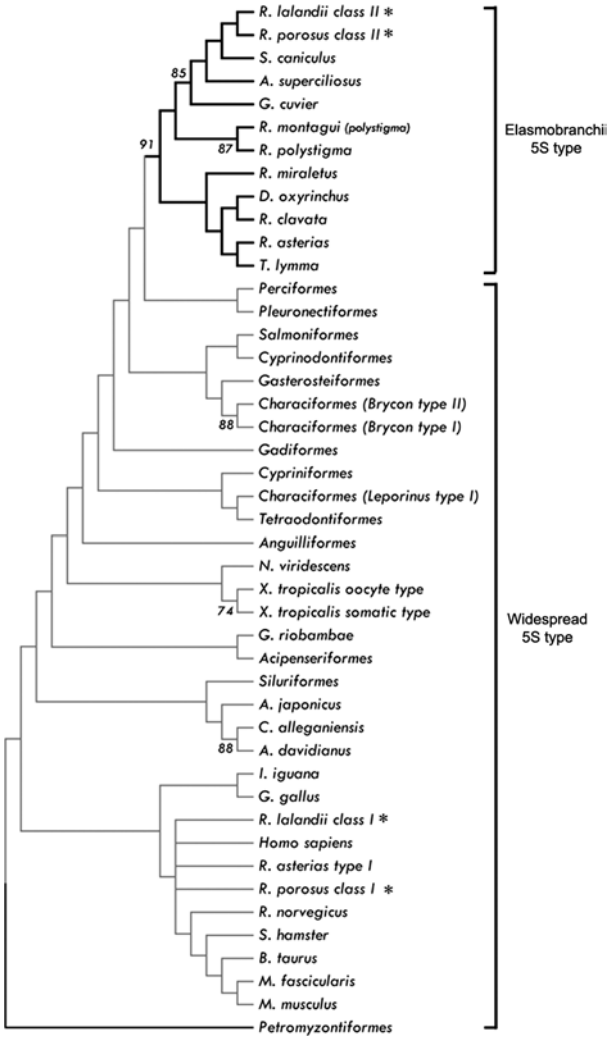

Fig. 4. A phylogenetic tree of 5S rRNA genes from Elasmobranchii, Teleostei and several other vertebrates using the Bayesian-likelihood method. Bootstrap values under 70 were omitted.

5S genes of class II from sharks and rays into an 'elasmobranch clade' on relationship trees (Fig. 4) and is supported by a large dataset that we generated for sharks (46 complete 5S gene sequences of two species) plus other data from Rajidae acquired in GenBank (30 complete 5S gene sequences of six species). The presence of control elements in the NTS-I and NTS-II, the T-rich tail in the 3′ end of the gene, and conserved ICRs in 5S genes of both 5S rDNA classes of *Rhizoprionodon* species suggest that both classes of 5S rDNA might be functional in sharks. The occurrence of 5S genes that are differently regulated in somatic and oocyte cells has been previously described for several vertebrates, including lampreys and teleost fishes (Komiya *et al*., 1986), and it could explain the dual 5S rDNA pattern that is widely reported among teleosts. However, both somatic and oocyte types are similar to the genes found in the 5S rDNA class I of sharks. Considering that a dual 5S

rDNA system, similar to that detected among vertebrates, was also described for plants of distant taxa such as Liliopsida and Coniferopsida (Shibata & Hizume, 2002; Besendorfer *et al*., 2005), it seems probable that the presence of a dual system of repeat units of 5S rDNA has a major biological role in the cells. Although the information on *Rhizoprionodon* needs to be corroborated by further studies on a larger number of Elasmobranchii, including many sharks and other freshwater and marine rays, the present evidence suggests a group-specific evolutionary history of 5S rDNA in these animals. Additional studies will be helpful to clarify their expression and functionality.

(ii) *NTS features*

Numerous studies have shown that the 5S rDNA is a valuable molecular marker for fish species (Pendás *et al*., 1995; Sadjak *et al*., 1998; Carrera *et al*., 2000), essentially because of the low intra-specific and high inter-specific variability of NTS domains. Among mammals, sequence differences in the ribosomal NTS were sufficient to distinguish the mouse subspecies *Mus musculus domesticus* from *M. m. musculus* (Suzuki *et al*., 1994), suggesting that this region evolved at an appropriate rate for differentiating closely related species. The substitution rate of 0·58 %/Myr within the NTS of mice (Suzuki *et al*., 1994) is as rapid as that of mammalian pseudogenes and non-functional sequences (Li *et al*., 1985).

Among the two *Rhizoprionodon* sharks, a variable degree of similarity was found within each NTS class. Whereas the NTS II showed a highly conserved pattern, the NTS I was diverse and appeared to have evolved faster than the other class. However, NTS II also separated *R. lalandii* from *R. porosus* in phylogenetic trees, due to five consistent base substitutions, showing that the NTS II can be useful for phylogenetic inference in shark species. This result is in agreement with those obtained for the neotropical characiform *Leporinus* (Ferreira *et al*., 2006) and *Brycon* (Wasko *et al*., 2001), in which the NTS effectively separated closely related species, but it contrasts with the results obtained by Pasolini *et al*. (2006) for the equivalent NTS II existing in rays, as well as those by Robles *et al*. (2005) for sturgeons and by Sajdak *et al*. (1998) for *Coregonus*. In view of these contradictory results, NTS sequences must be used with caution in evolutionary surveys.

Apart from these sequence variations, it is interesting that the remaining 340 nucleotides of NTS II of *R. lalandii* and *R. porosus* matched almost perfectly. Given the high mutation rates of NTS observed in every fish genome so far studied (for review see Martins & Wasko, 2004), as well as in mammals (Suzuki *et al*., 1994), the large sequence conservation of NTS II in the *Rhizoprionodon* genome indicates that these non-coding domains are not as free from selective pressure as other non-coding sequences. In this context, it has been recently demonstrated that the presence of conserved elements located within this NTS also plays an important role in the regulation of the 5S gene expression in mammals (Nederby-Nielsen *et al*., 1993; Hallenberg & Frederiksen, 2001). In fishes, a conserved TATA-like sequence has been observed upstream of the 5S gene in several teleosts, including *Salmo salar* (Pendás *et al*., 1994), *Carassius auratus* (Murakami & Fugitani, 1998), *Coregonus* (Sajdak *et al*., 1998), *Gasterosteus aculeatus* (Rocco *et al*., 1999), *Acheilognathus tabira* and *Cyprinus carpio* (Inafuku *et al*., 2000), *O. niloticus* (Martins *et al*., 2000), *Brycon* (Wasko *et al*., 2001) and *Leporinus* (Martins & Galetti, 2001*a*), suggesting a possible influence on the transcription level of this gene. In elasmobranchs, such sequences were also detected in Rajidae (Pasolini *et al*., 2006), but no data regarding sharks have been reported previously. In the present study, the analysis of upstream sequences of 5S genes from *Rhizoprionodon* identified an upstream TATA-like control element at positions −25 in NTS II and −30 in NTS I; interestingly, these are the same positions recognized in Rajidae (Pasolini *et al*., 2006). Although the role of these sequences for 5S gene transcription is still unknown, the high degree of conservation of TATA-like sequence positions in the two elasmobranch species currently studied is intriguing. This subject suggests some structural pattern for these elements shared by elasmobranchs, in opposition to ray-finned fishes, where the TATA-like sequences had an irregular position in different genera.

Microsatellite elements in the NTSs have also been found in some fish species (Deiana *et al*., 2000; Gornung *et al*., 2000; Alves-Costa *et al*., 2006), including elasmobranchs (Pasolini *et al*., 2006). These elements could be linked to regulatory functions of the 5S rRNA gene, which is not yet fully elucidated (Ota *et al*., 2003). A similar example was found in *Hox* cluster sequences of sharks and mammals, which share simple repetitive elements in the intervening sequence between coding genes, implying conservation of large-scale structural properties of non-coding regions (Kim *et al*., 2000). In *Rhizoprionodon* sharks, the single typical microsatellite (TCCC)*n* recovered in NTS class II was highly conserved, especially in the *R. porosus* genome, where the same number of repeats was recovered in all the clones studied (Fig. 2). However, in general, the scarcity of microsatellites in NTS of *Rhizoprionodon* sharks compared with their abundance in rajid rays (Pasolini *et al*., 2006) eliminates the possibility of some global biological role of the 5S rDNA in elasmobranchs, but it does not negate its usefulness to exemplify the dynamic evolution experienced by NTS domains, even in related genomes.

(iii) *Comparative analysis of 5S genes among several vertebrate lineages*

According to many studies, modern elasmobranchs represent the vertebrate basal condition because of their long (about 400 mya) evolutionary history, although their relationships with subsequent vertebrate groups are unclear. Cartilaginous fishes have been believed to correspond to the sister group of Actinopterygii and Sarcopterygii (including Tetrapoda), and to have diverged from these vertebrate lineages about 500 mya (Kumar & Hedges, 1998; Nelson, 2006). The ancient condition of elasmobranchs, together with their remarkable endurance, explains the interest in this group as pivotal in understanding early vertebrate evolution (Grogan & Lund, 2004).

Molecular studies have included sharks or rays when describing the structure and expression of particular genes and proteins in model organisms (e.g. human, mouse and chicken) to gain perspective on conservation of function (Martin, 2001). A more complete characterization of the cartilaginous fish genome is, therefore, essential for gaining insights into the ancestral condition of other vertebrate genomes. Towards such a characterization, our analyses on sharks showed that the clusters bearing the 5S rDNA classes I and II of *Rhizoprionodon* seem to evolve rapidly and independently. This can be confirmed by examination of the variability found in NTS and, surprisingly, in 5S gene sequences. Both the 5S rDNA classes of teleosts as well as the different copies of 5S rDNA arrays, which comprise a unique class in the amphibians, reptiles, birds and mammals thus far studied, seem to have arisen from the same ancestral 5S rDNA class I found in *Rhizoprionodon* sharks, Rajidae rays and lampreys. Conversely, the 5S rDNA class II probably embodies derived sequences of Elasmobranchii, as confirmed by the Bayesian analysis (Fig. 4). Interestingly, these 5S rDNA class II sequences from sharks have no recognizable homologues in lampreys, which also bear the ancestral 5S rDNA class I. It remains unclear if lampreys may also have two classes of 5S rDNA, as detected in Elasmobranchii and Teleostei, but it is probable that they contain variant copies corresponding to a single class, as is well documented in tetrapod genomes (Rosenthal & Doering, 1983; Suzuki *et al*., 1994; Frederiksen *et al*., 1997; Jensen & Frederiksen, 2000). Regarding the number of 5S rDNA classes in the genome of sharks, agarose electrophoresis profiles of 5S rDNA-PCR products from *Sphyrna lewini*, *G. cuvier*, *Carcharhinus obscurus*, *Carcharhinus leucas*, *Carcharhinus limbatus*, *Carcharhinus acronotus*, *A. superciliosus* and *Isurus oxyrinchus* have indicated the extensive variability present in 5S rDNA unit size (Pinhal *et al*., 2008). Each species showed an exclusive pattern with one to four bands of different molecular weights, possibly referring to distinct paralogous copies of 5S rDNA arrays. We believe that these copies correspond to the two classes of 5S rDNA, whereas extra copies might represent variants and pseudogenes, similar to those previously detected in rat, mouse and human cells (Emerson & Roeder, 1984; Little & Braaten, 1989; Leah *et al*., 1990; Nederby-Nielsen *et al*., 1993) and in fish genomes (Murakami & Fujitami, 1998; Roest-Crollius, 2000; Martins *et al*., 2002; Robles *et al*., 2005). Species of sharks that showed merely one band on gels (e.g. *G. cuvier* and *A. superciliosus*) could either carry two classes of a similar mass or one single class, although more than PCR data are necessary to test these hypotheses. The loss of a cluster might have occurred secondarily in fish taxa that bear only one class of 5S rDNA, as reported for *Synbranchus marmoratus* (Messias *et al*., 2003), *Acipenser sturio* (Tagliavini *et al*., 1999) and *G. aculeatus* (Rocco *et al*., 1999), perhaps caused by a strong reduction of the NTS (less than 60–80 bp length) below the minimum required to sustain the array and the dynamics of the 5S rDNA in the genome (Martins & Galetti, 2001*b*). To date, the short-class array length of 5S rDNA in the *R. lalandii* and *R. porosus* genomes has always been found to be greater than the apparently critical 60 bp length (Table 1), the same length as found for *G. cuvier* and *A. superciliosus* (data not shown but available upon request).

Considering the three round hypothesis (named '3R') of genome duplications in the evolutionary history of chordates (McLysaght *et al*., 2002; Donoghue & Purnell, 2005), it is likely that such events created new paralogous copies in 5S rDNA families. Indeed, recent molecular studies have suggested that the genome duplication event took place in the teleostean stem lineages, and many genes constitute more than two families in teleostean genomes (Hoegg *et al*., 2004; Crow *et al*., 2005), making it feasible for 5S rDNA arrays. Nevertheless, almost all studies on this subject have claimed that 5S rDNA arrays in the teleostean genome evolved into a double-class (or double-family) system (see Martins & Wasko, 2004). Therefore, we can conclude that the extra copies found dispersed throughout their genomes probably represent variant or even inactive forms and do not constitute a third class of 5S rDNA. The shark genome, in contrast, has been suggested to have undergone only two rounds of duplications (Froschauer *et al*., 2006), and duplicated segments encompassing genes and non-codifying sequences could evolve independently in individual species, being lost or maintained in subsequent generations, as described in other fish taxa (Crollius & Weissenbach, 2005). These conclusions provide support for the different varieties of 5S rDNA classes currently detected in these animals.

Although extensive taxa sampling is needed to deduce the origin of 5S rDNA classes of sharks in vertebrate evolution, we used corresponding sequences available from model organisms such as human, *Xenopus*, chicken and fugu, added to those of basal taxa such as lampreys, to generate a consistent phylogenetic report. In such an evolutionary scenario, the 5S rDNA class I in the shark and lamprey genome seems to represent the widespread ancestral condition, retained in the course of evolution and still extant in tetrapod genomes (Fig. 4), whereas the 5S rDNA class II apparently evolved in parallel in Elasmobranchii, explaining the existence of the paralogous copies of 5S genes in these animals.

Additional studies including representatives from the family *Potamotrygonidae* of freshwater stingrays, additional lineages of *Vertebrata*, and shark species such as those of the orders *Lamniformes* and *Squatiniformes*, and including sequences of different genes will be very valuable for testing the relationship pattern found in the current study and for improving our knowledge of the evolution of 5S rDNA.

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