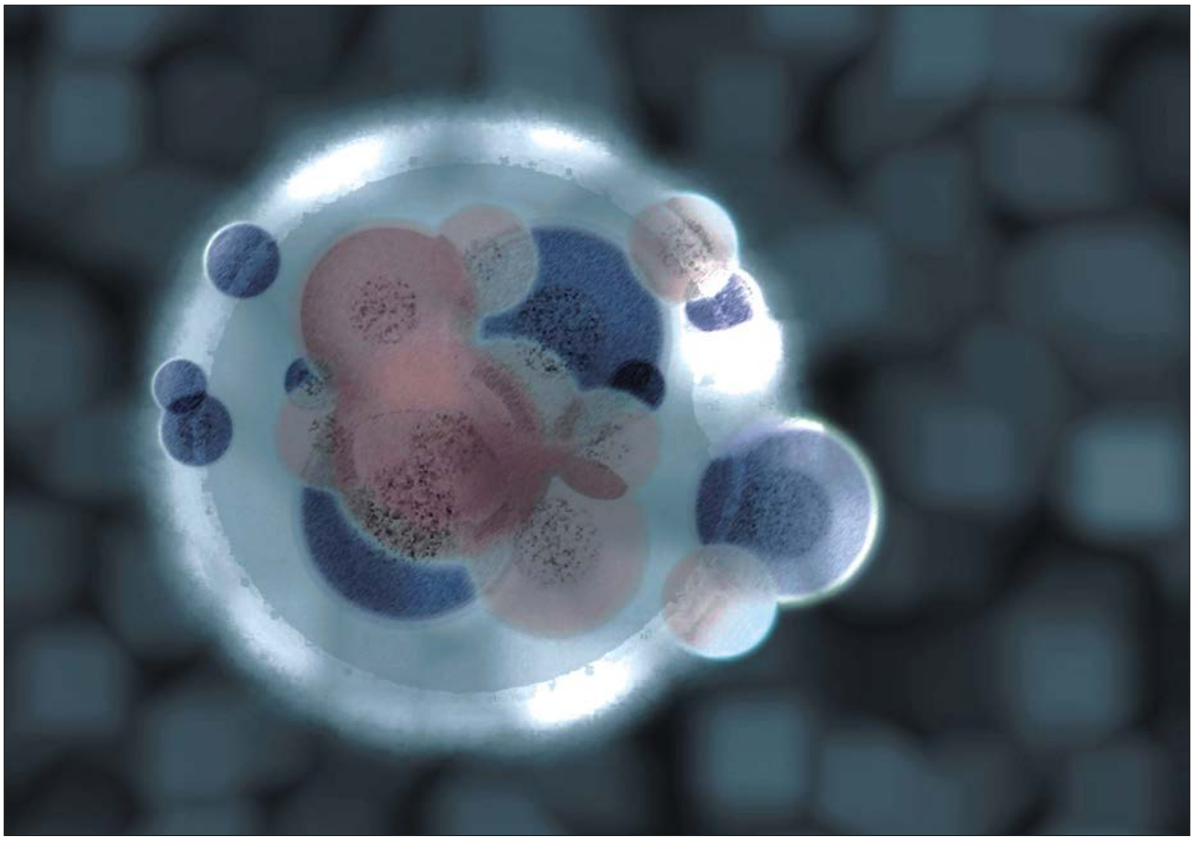

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Group I Introns and Splicing Mechanism and Their Present Possibilities in Elasmobranchs

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Abstract: Intron or other known as intervening sequence, are important in the producing of mature mRNA, rRNA or tRNA via RNA splicing mechanism. Excision of introns and ligation of the exons with the RNA processing reaction called RNA splicing. Group I introns could be accepted as in the most ancient and wide spread group of introns and are more important because of their RNA secondary structure and their self splice ability *in vitro*. Different ITS2 sizes in PCR from each order of sharks, rays, skates and ratfish indicating some interesting possibilities about the evolution of this region in elasmobranchs and a possible Group I intron insertion into ITS2 region of elasmobranchs.

Key words: Intron, splicing, elasmobranch

INTRODUCTION

The nuclear ribosomal RNA gene cluster of animals has a tandem organization, composed of a repeat unit consisting of the nontranscribed spacer (NTS), the 18S gene, the internal transcribed spacer 1 (ITS1), the 5.8S gene, internal transcribed spacer 2 (ITS2) and the 28S gene (in that order) (Lewin, 1997). There has not been any record about the presence of group I intron in a nuclear genomes of metazoans. But there isn't any report on the ITS sizes of different vertabrate groups and the possible reason of different ITS sizes in especially Elasmobranchs. Group I intron possibility in spacer region (ITS2) instead of coding region as in all other eukaryotes were suggested to explain this different size.

For this past decade, there has been an information about that many genes or coding regions so-called exons are interrupted by stretches of DNA known as intervening sequences or introns. Introns have divided into 4 major groups, which are group I, group II, spliceosomal introns in nuclear pre-mRNA and nuclear tRNA introns. Group I introns are distributed widely in protists, bacteria and bacteriophages while Group II introns are found in fungal and land plant mitochondria, algal plastids, bacteria and Archaea. Group II and spliceosomal introns have a common splicing mechanism and might be related to each other. The tRNA and/or archaeal introns are found in the nuclear tRNA of eukaryotes and in archaeal tRNA, rRNA and mRNA. The broad and sporadic distribution of these introns suggests that they have successfully spread into different genomes and genic sites and that they are prone to loss over evolutionary time (Haugen *et al*., 2004, 2005; Henrik *et al*., 2003). The size of group I intron changes from 258 bases to over 2 kb. The larger size reflects that intron has extra nucleotides like open reading frame or non-coding sequences (Cech, 1988; Collins, 1988).

There has been a lot of study carried out by many researchers about group I intron. First rRNA group I intron were discovered in large subunit (LSU) rRNA of the protist *tetrahymena thermophila* by Zaug *et al.* (1983) and *Physarum polycephalum* by Muscarella and Vogt (1989). Numerous recently gained introns were identifyed in nematodes (Roy, 2004). More recent studies reported presence of the group I intron in nuclear small subunit (SSU) rDNA, in the benthic seaweed *Urospora penicilliformis* by Van Oppen *et al.* (1993), in the yeast *Ustilago maydis* by De Wachter *et al.* (1992), in the marine red alga *Porphyra spiralis* by Oliveira and Ragan (1994), in the Lichen Fungus numerous group I intron were found with a variable distributions by DePriest and Been (1992), gave almost same result, which group I intron were determined in the 18S rRNA. A possible inhibition of group I intron self-splicing by using short oligonucleotides in mammalian pathogens Pneumocystis carinii and Candida albicans determined by Disney *et al.* (2004).

All these research and more others determine the presence and widespread phylogenetic distribution of group I intron in mitochondrial and chloroplast genomes and nuclear ribosomal RNA genes, but there has not been any record about the presence of group I intron in a nuclear genomes of metazoans. There has not been any reported cases of a survey of ITS sizes across a wider taxonomic group such as a class, across different

Table 1: Examples of ITS sizes across various vertebrate taxa taken from Genbank (Dosay, 2000)

Taxon	ITS Size
Tetraodon (pufferfish)	450 bp (ITS1)
Hyla (treefrog)	150 bp (ITS1)
Acantholingula (salmonid teleost)	570 bp (ITS2)
Petromyzon (sea lamprey)	300 bp (ITS2)
Cyprinus (carp)	367 bp (ITS1); 385 bp (ITS2)
Cichlid fishes of Lake Victoria	520 bp (ITS1)
Human	1094 (ITS1); 1154 (ITS2)
Hominoid primates	Approximately 1000 bp (ITS1 and 2)

vertebrate groups Table 1. If this study indicate group I intron insertion possibilities to the genome of elasmobranch this will be possible first observation of group I intron like elements, interrupts spacer region (ITS2) instead of coding region as in all other eukaryotes.

MATERIALS AND METHODS

This study was carried out in the Quenn's University of Belfast, School of Biology and Biochemistry, UK between 1997-1998 and revised in 2006. In DNA extraction, some of the samples were arrived as a genomic DNA, already extracted. Others were send in a 70% ethanol, about 1 to 5 g of liver or muscle tissue samples. All samples were kept in a refrigerator. Tissue samples were later extracted to obtain genomic DNA.

Either, phenol/water/chloroform method, based on ABI manual DNA extraction kit or QIAamp tissue kit method from QIAGEN company was used in a DNA extraction. All buffers and materials that extraction requires comes with the kit. 0.2 to 0.5 g. tissue for first method and about 25 mg of tissue in a second method, which approximately 0.2-1.2 mg of DNA was obtained from 1 mg of tissue with second method, were used each time. After extraction, Genomic DNA was stored in a 4°C refrigerator.

The ribosomal internal transcribed spacer ITS 2 were amplified by using the Polymerase Chain Reaction (PCR) with these primers listed in Table 2.

For all PCR amplifications, either Perkin Elmer DNA Thermal Cycler 480 or a PTC-100TM Programmable Thermal Controller (MJ Research, Inc.) were used. The same two primers were used both PCR amplification and sequence of the ITS2 region. ITS1 region PCR amplification was carried with external primers, 18S3 and 5.8S5. In the sequence of the ITS1 region, same primers and internal primer, 5.8S4 were used. After extraction of tissue sample, the double stranded genomic DNA was used in a 30 cycles of PCR amplification. Five minutes initial denaturing at 94°C, followed by 1 min at 94°C and

Table 2: Primers used in for PCR amplification and sequencing of ITS2 region. L-strand primer forward and H- strand for reverse primer

ITS2F L	CTACGCCTGTCTGAGTGTC	ITS2
ITS2R H	ATATGCTTAAATTCAGCGGG	ITS2

Primers were designed from fish sequence that were obtained from genbank

either 1 min annealing at 50-55°C, followed by 2 min extension at 65°C or 3 min annealing plus extension at 60-65°C, after 30 cycle in final 10 min extension at 65°C. Fifty microliter reaction contains, 200 ng μL^{-1} of two external primers, 10 mM of each nucleotide (dATP, dCTP, dGTP and dTTP) ultrapure dNTP set from Pharmacia Biotech, 17.5 mM $MgCl_2$ and 1.75 U of Taq polymerase (Boehringer Mannheim) or Tag polymerase (Expand™ High Fidelity PCR system, Boehringer Mannheim) and at least 300 ng genomic DNA. After that, this PCR product was run out on a 0.7% agarose gel, stained with ethidium bromide solution, visualised under low intensity ultraviolet light and photographed. After determine approximate size of this fragment, PCR sample was run on a 1% low melt agarose gel. Then the band was visualised under low intensity ultraviolet light, excised and melted in a 65°C heating block. This excised amplified band amplified again with same PCR technique by using same or internal primers. The final fragments were purified for sequence by using high pure PCR product purification kit (Boehringer Mannheim), according to kit instruction.

Sequencing: Either a Perkin Elmer DNA Thermal Cycler 480 or a PTC-100TM Programmable Thermal Controller (MJ Research, Inc.) were used in a cycle sequence reaction. The PCR amplified products were sequenced directly by using dideoxy chain termination method. Twenty microliter dye-termination reaction contains 300-500 ng of purified PCR amplified product, 5 pmol each primer and 20 mM $MnSO_4$. The cycle includes first, denaturation at 96°C for 1 min, follow by 25 cycle of 50 sec at 96°C, 4 min at 62°C and 20 sec at 50°C. After that, the reactions were loaded onto ABI 373A automated sequencers. When, 300-400 bp long good quality sequence were obtained with using each primer every time, new internal primers were designed differently for each species to complete sequence of all fragment.

The vast majority of the resulting PCR products were sequenced, at least partially, to verify that they were actually the ITS2 region. The 5 prime end of the resulting PCR fragments had 20-30 bp of 18S sequence and the 3 prime end had at least 50 bp of 5.8S sequence, both regions show high similarities across vertebrate groups (Table 3).

Table 3: Determined ITS2 size in Elasmobranchs

Species name	Order/Family	Common name	ITS2 size
C. ventriosum	Carcharhiniformes/Scyliorhinidae	Swell shark	1.2 kb
C. altimus	Carcharhiniformes/Carcharhinidae	Bignose	1.4 kb
C. brevipinna	Carcharhiniformes/Carcharhinidae	Spinner	1.4 kb
C. acronotus	Carcharhiniformes/Carcharhinidae	Blacknose	1.4 kb
C. falciformis	Carcharhiniformes/Carcharhinidae	Silky	1.4 kb
C. isodon	Carcharhiniformes/Carcharhinidae	Finetooth	1.4 kb
C. leucas	Carcharhiniformes/Carcharhinidae	Bull	1.4 kb
C. limbatus	Carcharhiniformes/Carcharhinidae	Blacktip	1.4 kb
C. obscurus	Carcharhiniformes/Carcharhinidae	Dusky	1.4 kb
C. plumbeus	Carcharhiniformes/Carcharhinidae	Sandbar	1.4 kb
C. perezi	Carcharhiniformes/Carcharhinidae	Carib. reef	1.4 kb
C. porosus	Carcharhiniformes/Carcharhinidae	Smalltail	1.4 kb
C. P. glauca	Carcharhiniformes/Carcharhinidae	Blue	1.4 kb
C. G. cuvier	Carcharhiniformes/Carcharhinidae	Tiger	1.4 kb
C. N. brevirostris	Carcharhiniformes/Carcharhinidae	Lemon	1.4 kb
C. R. terraenovae	Carcharhiniformes/Carcharhinidae	Atshnose	1.4 kb
S. mokarran	Carcharhiniformes/Sphyrnidae	G.H.H	800 bp
S. tiburon	Carcharhiniformes/Sphyrnidae	Bonnethead	800bp
G. cirratum	Orectolobiformes/Ginglymostomatidae	Nurse	1.4 kb
S. acanthias	Squaliformes/Squalidae	Spinydogfish	1050 bp
S. californica	Squatiniformes/Squatinidae	Pacific angel sh	1000 bp
H. fraucisci	Heterodontiformes/Heterodontidae	Horn sh	1.4 kb
H. griseus	Hexanchiformes/Hexanchidae	Bltnose sixgill	800bp
P. japonicas	Pristoriformes/Pristiophoridae	Jap. saw sh	1000bp
P. nudipinnis	Pristoriformes/Pristiophoridae	Shnose saw sh	1000bp
Isurus paucus	Lamniformes/Lamnidae	Longfin mako	1.2 kb
Isurus oxyryuchus	Lamniformes/Lamnidae	Shortfin mako	1.2 kb
Lamna nasus	Lamniformes/Lamnidae	Porbeagle	1.2 b
Carcharodon carcharias	Lamniformes/Lamnidae	White shark	1.2 kb
Alopias vulpinus	Lamniformes/Alopiidae	Thresher shark	1.2 kb
Carcharias taurus	Lamniformes/Odontaspididae	Sandtiger shark	1.2 kb
U. ionaiceusis	Rajiformes/Family	Yellow st ray	350 bp
A. narinari	Rajiformes/Myliobatidae	Sp.eagle ray	350 bp
R. eglauteria	Rajiformes/Rajidae	Clearnose skate	1.0 kb
H. colliei	Chimeras/Chimaeridae	Ratfish	420 bp

RESULTS AND DISCUSSION

The ITS size of different samples indicate, all the members of the morphologically proposed superorder Galeomorphii have ITS2 sizes of roughly similar size at about 1.4 kb. This 1.4 kb ITS2 reflects a molecular synapomorphy for that superorder, with a 200 bp deletion on Lamniformes. Except one taxon, swell shark, which is currently classified within the order Carcharhiniformes, but has an ITS2 size the same as Lamniformes. In fact, the swell shark ITS2 sequence aligns much better against the lamniforms than it does against the Carcharhinidae, suggesting the possibility that it might actually be more closely related to lamniforms than the Carcharhiniformes.

There is a big differences in ITS sizes of skate and the two rays (1000 bp vs. 350). Ratfish has a very similar ITS2 size to that of rays (420 bp), suggesting the ancestral ITS2 was about 350-400 bp. After that, ITS2 size turns to support branching arrangement, separation to rays and skate node with 1000 bp of increased size of ITS2. Continuaty of this suggestion, the larger size of ITS2 was obtained by the sharks, with a minor differences within the different orders and families. In summary this 1000 bp ITS2 size, indicate skate as a possible sister group of shark.

There is another point in the PCR size of ITS2 region is large difference between hammerheads and carcharhinids (800 vs 1400 bp). However, alignments between this two groups reveal a 600 bp deletion in hammerhead sharks, right in the middle of the ITS2 region. This 600 bp region from the carcarhinids was submitted to BLAST similarity searches on GenBank, with no resulting significant homologies. But it indicates it can be a group I intron (Fig. 1). Such elements are common in lower plants, but have not been reported in any animal system. Group I introns differ from nuclear protein encoding introns in that their RNA secondary structure can fold to form an active site to accomplish their own RNA splicing, which means they can self splice *in vitro*. However, it needs a double check and secondary structure modelling to confirm this is a Group I intron. But this prefindings still suggests a possibility that mature ribosomal RNA process may be occurs differently in sharks from all other vertebrates.

Group I introns could be accepted as in the most ancient and wide spread group of introns. They can form conserved secondary structure which is essential for splicing. Splicing occurs in two transesterification steps, first breaking the exon/intron bond, then joining the two

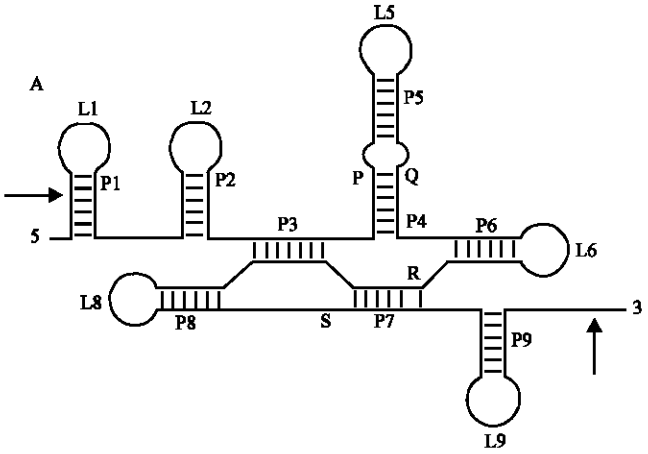

Fig.1: Structural diagram for group I introns (Burke *et al*., 1987)

exons and releasing the intron in linear form (Burke *et al*., 1987; Burke, 1988; Dujon, 1989; Weaver and Hedrick, 1992). Transcription of these interrupted genes, to produce a pre-mRNA, pre-rRNA, or pre-tRNA, needs excision of introns or removing the intervening noncoding sequences and ligation of the exons, the coding sequences with the RNA processing reaction called RNA splicing (Singh, 2002). Each of these groups has their own RNA splicing mechanism. Group II and nuclear mRNA introns splicing mechanisms are very similar to each others. But group I and group II introns are more important than the others for their RNA structure, because they can fold to form an active site to accomplish their own RNA splicing, which means they can self splice *in vitro*. Also, another important aspect for group I intron is their ability to reproduce themselves with inserting at already known positions into intronless sites of genes. (Dujon, 1989; Weaver and Hedrick, 1992; Cech, 1988; Van Oppen *et al*., 1993).

Group I intron's self splicing mechanism firstly demonstrated in *tetrahymena thermophila* and *Physarium polycephalum*. They have been observed at different locations in mitochondrial, chloroplast or nuclear genetic system in eukaryotes and bacteriophages. However, there is no information about their presence in any animal nucleus or in intergenic regions. There are some evidence suggests that they may be originated from mobile elements like retroviruses and transposons. This could be important for the evolution of eukaryotes (De Wachter *et al*., 1992). Introns were present at the earliest stages of evolution of eukaryotes and the original, catastrophic intron invasion accompanied the emergence of the eukaryotic cells (Rogozin *et al*., 2005).

Although the widespread proliferation of introns in eukaryotic protein-coding genes remains one of the most poorly understood, evidence supports the idea that spliceosomal introns were present in the stem eukaryote and also diverged into at least two distinct classes very early in eukaryotic evolution (Lynch and Richardson, 2002). Eukaryotes have evolved elaborate splicing mechanisms to remove introns or not to destroy the protein-coding capacity of genes. The presence of a splicing apparatus in the protist *Trichomonas vaginalis* and same RNA motifs found in yeast and metazoan introns are required for splicing were demonstrated and also described the first introns in this deep-branching lineage, indicating the conservation of intron splicing signals across large evolutionary distances, reveal unexpected motif conservation in deep-branching lineages, suggesting a simplified mechanism of splicing in primitive unicellular eukaryotes and supporting the presence of spliceosomal introns (lost and gained) in the earliest eukaryote before its divergence from its common ancestor with plants and animals (Vanacova *et al*., 2005).

All these studies and more others determine the presence and widespread phylogenetic distribution of other group of introns and group I intron distrubutions in mitochondrial and chloroplast genomes and nuclear ribosomal RNA genes, but there has not been any record about the presence of group I intron in a nuclear genomes of metazoans up to know, especially interrupting spacer region (ITS2) instead of coding region as in all other eukaryotes.

The different ITS2 sizes across representatives of each order of sharks, rays, skates and ratfish indicating there is a differences in evolution of this region in elasmobranchs. I could find none informations on different sizes in spacer region of the any metazoans and a possible causing explanations to different size in any coding or noncoding regions of the Elasmobranchs, relating to the group I introns in my Genbank research.

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