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Revisiting the evolution of the somatostatin family: Already five genes in the gnathostome ancestor

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ABSTRACT

The somatostatin (SST) family members are a group of neuropeptides that are best known for their role in the regulation of growth, development and metabolism. The occurrence of six paralogous SST genes named *SST1, SST2*, *SST3*, *SST4*, *SST5* and *SST6* has been reported in vertebrates. It has been proposed that *SST1, SST2* and *SST5* arose in 2R from a common ancestral gene. *SST3* and *SST6* would have been subsequently generated by tandem duplications of the *SST1* and *SST2* genes respectively, at the base of the actinopterygian lineage. *SST4* is thought to have appeared more recently from *SST1,* in teleost-specific 3R.

In order to gain more insights into the *SST* gene family in vertebrates, we sought to identify which paralogs of this family are present in cartilaginous fish. For this purpose, we first searched the recently available genome and transcriptome databases from the catshark *Scyliorhinus canicula*. In a previous study, three *S. canicula SST* genes, called at that time *SSTa*, *SSTb* and *SSTc*, were identified and proposed to correspond to *SST1*, *SST5* and *SST2* respectively. In the present work, two additional *SST* genes, called *SSTd* and *SSTe,* were found in *S. canicula* plus two other chondrichtyan species, elephant shark (*Callorhinchus milii)* and whale shark (*Rhincodon typus)*. Phylogeny and synteny analyses were then carried out in order to reveal the evolutionary relationships of *SSTd* and *SSTe* with other vertbrates *SSTs*. We showed that *SSTd* and *SSTe* correspond to *SST2* and *SST3* respectively, while *SSTc* corresponds to *SST6* and not to *SST2* as initially proposed. Our investigations in other vertebrate species also led us to find that the so-called *SST2* gene in chicken, lungfish, sturgeons and teleosts actually corresponds to *SST6*. Conversely, the so-called *SST6* gene in actinopterygians corresponds to *SST2*.

Taken together, our results suggest that: *i) SST3* and *SST6* were already present in the gnathostome ancestor, much earlier than previously thought; *ii) SST6* was also present in the tetrapod ancestor and still occurs in living birds; with this respect, it is likely that *SST6* was independently lost several times during evolution: in amphibians, squamates and mammals; *iii) SST2, SST3* and *SST5* were probably lost in euteleosts, sarcopterygians and tetrapods, respectively.

1. Introduction

Somatostatin (currently known as somatostatin 1 or SST1) was originally isolated from ovine hypothalamus and found to inhibit the release of growth hormone from the rat pituitary (Brazeau et al., 1973). It has been subsequently shown that SST1 is widely distributed in the central nervous system and in various peripheral organs where it plays important roles in the regulation of growth, development and metabolism (Günther et al., 2018). The occurrence of SST1 was reported in all vertebrate species investigated so far. Moreover, most species were shown to possess additional SST forms. In gnathostomes (jawed vertebrates), it was demonstrated that all these SST variants are the products of at least six paralogous genes named *SST1*, *SST2* (also known as *Cortistatin* in mammals) *SST3*, *SST4*, *SST5* and *SST6* (de Lecea et al., 1996; Liu et al., 2010; Tostivint et al., 1996, 2008, 2013, 2014). SST-related peptides are thought to act through at least six SST receptor subtypes (Ocampo Daza et al., 2012). In lampreys, three *SST* genes have characterized but their phylogenetic status is still unclear (Tostivint et al., 2016), while in the hagfish *Myxine glutinosa*, the occurrence of only one *SST* gene, encoding a SST1-like peptide, is attested to date (Conlon et al., 1988).

It is currently well accepted that the evolution of the *SST* gene family, as that of many other neuropeptides (Elphick et al., 2018), was strongly shaped by the different rounds of whole-genome duplications that took place in the vertebrate history, especially the two basal rounds of vertebrate genome doubling (2R) and the teleost fish-specific genome

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doubling (3R) (Dehal and Boore, 2005; Putnam et al., 2008; Sacerdot et al., 2018). Thus, *SST1*, *SST2* and *SST5* seem to have been generated during 2R from a common ancestral gene, while *SST4* is likely to have appeared during 3R from *SST1*. The impact of 2R on the early evolution of *SST* gene family is notably supported by synteny analysis showing that *SST* genes are located on a same tetra-paralogon that contains paralogous genes of at least ten other families (Liu et al., 2010; Tostivint et al., 2013, 2014). For their part, *SST3* and *SST6* would have been independently generated by tandem duplications of *SST1* and *SST2* respectively, at the base of the actinopterygian lineage (Liu et al., 2010; Tostivint et al., 2004, 2008). Some of these genes could have been subsequently lost explaining their absence in some lineages. For example, *SST5* is believed to have been lost in tetrapods.

Cartilaginous fish (chondrichthyans) are of particular interest in evolutionary studies because of their key phylogenetic position at the base of the gnathostome lineage. They consist of two major groups: elasmobranchii (sharks and skates/rays) and holocephali (chimaeras). Until 2013, SST-related peptides were characterized in only a very few species of cartilaginous fish: SST1 from the ray *Torpedo marmorata* (Conlon et al., 1985), a SST1-like peptide exhibiting a serine residue at position 5 from the ratfish *Hydrolagus colliei* (Conlon, 1990), a SST2-like sequence from the elephant shark *Callorhinchus milii* (Tostivint et al., 2008) and finally a totally atypical SST-related sequence from both the little skate *Leucoraja erinacea* and *C. milii* (Liu et al., 2010). In 2013, three *SST* genes, called *SSTa*, *SSTb* and *SSTc*, were identified in the common dogfish (today referred to as small-spotted catshark, *Scyliorhinus canicula*) and proposed to correspond to *SST1*, *SST5* and *SST2*, respectively (Quan et al., 2013). Surprisingly, none of these genes appeared as the apparent ortholog of the atypical *SST*-like gene found in *C. milii* and *L. erinacea*. Nevertheless, these findings were regarded as in good agreement with the model described above since they support the existence of these three *SST* genes in the gnathostome ancestor (Liu et al., 2010; Tostivint et al., 2013, 2014). However, due to the lack of a fully sequenced and assembled *S. canicula* genome, this view could not be confirmed (Quan et al., 2013).

The first goal of the present study was to determine the complete repertoire of the *SST* gene family in cartilaginous fish. For this purpose, we searched the newly available *S. canicula* genome and transcriptome databases (unpublished data). We also took the opportunity to explore or reexplore the genome and transcriptome databases from two additional species, the whale shark, *Rhincodon typus* (Read et al., 2017) and the elephant shark, *C. milii* (Venkatesh et al., 2014). We found two novel *S. canicula SST* genes that we propose to provisionally call *SSTd* and *SSTe* (according to the nomenclature initially proposed in Quan et al., 2013). *S. canicula SSTd* was shown to be the ortholog of the *L. erinacea* and *C. milii* atypical *SST*-like gene above-mentioned, while *S. canicula SSTe* appeared as a totally new *SST* gene. In both *R. typus* and *C. milii*, four *SST* genes were found that correspond *SSTa, SSTc, SSTd* and *SSTe*.

Taking advantage of the genome assembly of various representative species from other vertebrate groups, we then carried out phylogeny and synteny analyses in order to reveal the evolutionary relationships of chondrichthyan *SSTd* and *SSTe* with other vertebrates *SSTs*. From our investigations, it appeared that *SSTd* and *SSTe* correspond to *SST2* and *SST3*, respectively, while *SSTc* corresponds to *SST6* but not to *SST2* as initially proposed (Quan et al., 2013).

The major implication of these results is that the repertoire of the *SST* gene family in the gnathostome ancestor was already composed of five paralogs namely *SST1*, *SST2*, *SST3*, *SST5* and *SST6*. Our observations also led us to revisit the phylogenetic status of *SST2* and *SST6*. As a result, the nomenclature of these genes had to be corrected in some species.

2. Material and methods

2.1. Nomenclature

In this article, *S. canicula SSTs*, as well as their counterparts from other chondrychthyans, will be first described as *SSTa-SSTe,* according to the provisionnal nomenclature proposed by Quan et al. (2013), without prejudice to their evolutionary relationships with other vertebrates *SSTs*. Thereafter, in the light of the phylogeny and synteny data, they will be renamed according to the phylogenetic nomenclature, *SST1-SST6*, initially introducted by Tostivint et al. (2008) then extended by Liu et al. (2010). Nevertheless, the correspondence bewteen the two nomenclatures will be regularly reminded throughout the article as follows: *SSTa = SST1*; *SSTb = SST5; SSTc = SST6*; *SSTd = SST2* and *SSTe = SST3*. Note that in Quan et al. (2013) *SSTc* was initially but mistakenly referred to as *SST2*.

2.2. Identification of SSTd and SSTe sequences from cartilaginous fish

S. canicula SSTd and *SSTe* genes were sought by TBLASTN (Altschul et al., 1990) using the *C. milii* and *L. erinacea* SST2-like sequences (AGCKLFFWKTFSHC and APCKLFFWKTFSHC) as queries against the *S. canicula* genome draft assembly (version 1, unpublished). *S. canicula* full-length *SSTd* cDNA (but not *SSTe* cDNA) was also retrieved from *S. canicula* Illumina transcriptome (version 1, unpublished).

The SST repertoire of *SST* genes in *R. typus* and *C. milii* was sought by TBLASTN using the *S. canicula SST* cDNA sequences as queries against the *R. typus* and *C. milii* genome assembly databases (Read et al., 2017; Venkatesh et al., 2014).

Protein sequences were predicted by using consensus splice donor and acceptor site and by sequence identity comparison with related *SST* genes. The signal peptide cleavage site was determined using SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

2.3. Molecular cloning of the coding sequence of S. canicula SSTe cDNA

5′RACE-ready cDNAs used for this procedure were constructed as previously described (Gaillard et al., 2018). The coding sequence of the *S. canicula SSTe* cDNA was amplified by nested PCR using the Advantage 2 PCR kit (Clontech). Nested PCR was carried out in a MyCycler thermal cycler (Bio-Rad, Marne la Coquette, France) with the following primers, csSSTe Rev × Universal Primer A Mix (UPM) then csSSTe Rev Nest × Nested Universal Primer A Mix (NUP) (see Table S1) using the manufacturer's procedure. PCR amplifications were carried out for 35 cycles (denaturation 94 °C, 30 sec; annealing between 57 and 60 °C depending on the Tm of the primers, 30 sec; and extension 72 °C, 30 sec) and a final extension of 72 °C for 7 min. The product of the nested PCR was subcloned into the pGEM-T vector (Promega, Charbonnières-les-Bains, France) and sequenced (Value Read Sequencing at MWG Biotech, Ebersberg, Germany).

2.4. Synteny analysis

To generate synteny maps, genes flanking *C. milii* and *R. typus SST* genes were obtained from the NCBI database. Non-protein coding genes and genes coding unknown proteins were excluded from further analysis. Note that *SSTb* was not included in the study since it is absent in both *C. milii* and *R. typus*, while in *S. canicula*, it is located on a very short scaffold that did not contain any other genes. Genes flanking the *SST* genes in eight additional osteichthyan species, namely human, chicken (*G. gallus*), green anole lizard (*A. carolinensis*), Western clawed frog (*X. tropicalis*), coelacanth (*L. chalumnae*), spotted gar (*L. oculatus*), zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), were obtained from Genomicus (version 92.01) (Muffato et al., 2010).

2.5. Phylogeny analysis

A set of 147 vertebrate SST precursor sequences was primarily collected from Yun et al. (2015) and Tostivint et al. (2016) and supplemented by the chondrichthyan sequences characterized in the present study. All these sequences were aligned using the Muscle algorithm (Edgar, 2004) then manually adjusted. The phylogeny of SST precursors was inferred by Maximum Likelihood using PhyML (Guindon and Gascuel, 2003) with the LG model of substitution combined to the neighbor-joining interchange (NNI) method on Seaview browser (Gouy et al., 2010). The robustness of the tree was assessed by the approximate likelihood test (aLRT) SH-like branch support and by bootstrapping procedure from 500 data set replicates. The Genbank accession numbers for all sequences used in the analysis are listed in Fig. S1. The alignment is presented in Fig. S2.

2.6. Tissue expression of S. canicula SSTd and SSTe genes by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification

The expression profiles of *S. canicula SSTd* and *SSTe* genes were examined by RT-PCR, as previously described (Quan et al., 2013; Gaillard et al., 2018). Total RNA was extracted from various tissues, including telencephalon, diencephalon, mesencephalon, cerebellum, brain stem, spinal cord, skeletal muscles, heart, spleen, gills, stomach, duodenum, valvular intestine, pancreas, liver, kidney, ovary and testis and purified by using RNAeasy Plus Mini kit (Qiagen, Courtabœuf, France). For each tissue, ~330 ng of total RNA were reverse transcribed using ImProm-II Reverse Transcription System (Promega, Charbonnières, France). Gene-specific primers of *S. canicula SSTd* and *SSTe* were designed according to the predicted sequences (Table S1). PCR amplifications were carried out for 35 cycles (denaturation 94 °C, 30 sec; annealing between 57 and 60 °C depending on the Tm of the primers, 30 sec; and extension 72 °C, 30 sec) and a final extension of 72 °C for 7 min. The *S. canicula Egf1* gene was amplified in parallel with specific primers (Table S1) to verify the quality and quantity of all cDNAs samples. Negative controls were performed without cDNA template. All PCR products were electrophoresed through 2.0% agarose gel and stained with ethidium bromide and then detected under UV light with the ChemiDoc Touch Imaging System (BioRad). Three independent PCR amplifications were performed to check the consistency of amplification.

3. Results

3.1. Structure of S. canicula SSTd (SST2) and SSTe (SST3) precursor cDNAs and genes

The nucleotide and deduced amino acid sequences of *S. canicula SSTs* including *SSTd* and *SSTe* are shown in Fig. S3. The coding region of the *SSTd* cDNA consists of 315 nucleotides (nt) that encode a 105-amino acid (aa) protein (PSSTd) including a 20-residue potential signal sequence (Petersen et al., 2011). The primary structure of PSSTd contains a monobasic processing site at position 91 (Lys) potentially yielding the mature 14-aa peptide SSTd (SST2), TPCKLFFWKTFSHC, plus one dibasic and one monobasic processing sites at positions 87–88 (Arg-Arg) and 79 (Arg) respectively, potentially yielding 17- and 26-aa NH2-extended forms of SSTd.

The coding region of the *SSTe* cDNA consists of 321 nt that encode a 106-aa protein with a 18-residue potential signal sequence. The SSTe sequence is flanked by a pair of basic residues (Arg-Lys) at position 92–93 potentially yielding the mature 14-aa peptide SSTe (SST3), NCKNFFWKTYTLC, plus two monobasic processing sites at positions 85 (Arg) and 90 (Arg) respectively, potentially yielding 17- and 22-aa NH2-extended forms of SSTe.

Comparison of the cDNAs with genomic sequences revealed that *SSTd* and *SSTe* genes are each composed of two exons and one intron (Fig. S4). For each gene, exon 1 encodes the 5′ UTR, the signal peptide and the N-terminus of the prepro-peptide while exon 2 encodes the C-terminus of the precursor, including the putative mature peptide.

3.2. Repertoire of SST cDNAs and/or genes in cartilaginous fish

The entire repertoire of *SST* cDNAs and/or genes found from *S. canicula*, *R. typus* and *C. milii* databases is shown in Figs. S3–S8. *S. canicula* SST family appears as the richest with no less than five *SST* genes: the three genes previously reported in Quan et al. (2013), namely *SSTa, SSTb* and *SSTc,* plus two additional genes characterized in the present study, *SSTd* and *SSTe*. All these genes, with the exception of *SSTb,* were retrieved from *R. typus* and *C. milii*. Only a partial sequence of the *SSTd* gene could be detected from *L. erinacea* (already reported in Liu et al., 2010).

3.3. General organization of the chondrichthyan SST genes and SST precursors

The molecular organization of the SST precursors is the same in all species examined, with a signal peptide followed by a long central segment, a conserved pair of basic residues and, finally, the SST sequence at their C-terminus (Fig. 1). All putative SST possess a cyclic conformation ensured by the presence of two Cys residues, and their cyclic region encompasses the Phe-Trp-Lys motif. The structure of the corresponding genes is also very well conserved with two exons and one intron (Figs. S4, S5, S7). Note that the first exon of *S. canicula SSTd* and that of *S. canicula SSTa (SST1)* exhibit almost the same nucleotide sequence (Fig. S4). This sequence identity was also found in the corresponding cDNA identified by RT-PCR ruling out an artefact of genome assembly (Fig. S3).

3.4. Structural features of the chondrichthyan putative SST tetradecapeptides

3.4.1. SSTa (SST1)

The primary structure of SSTa is AGCKNFFWKTFTSC both in *S. canicula* and *R. typus*. In *C. milii*, it exhibits one substitution, Ser *vs* Asn, at position 5, as previously reported in the ratfish *H. colliei* (Conlon, 1990) (Table S2). It is to note that the genomic *SSTa* sequence encodes for a Leu in place of Phe at position 6 that was not retrieved in the cDNAs (Figs. S3 and S4). Chondrichthyan prepro-SSTa exhibit high sequence identity (ranging from 66.3 to 74.1% between one another) (Table S3).

3.4.2. SSTb (SST5)

As mentioned above, the SSTb sequence could only be found in *S. canicula* (see Quan et al., 2013 for a complete description of the precursor). Just as a reminder, *S. canicula* SSTb exhibits the same primary structure as SSTa (SST1) (Table S2).

3.4.3. SSTc (SST6)

SSTc exhibits two substitutions at positions 1 and 2 when compared to SSTa (Table S2). The residue at position 2 is a Pro, except in *R. typus*. The residue at position 1 is more variable. Chondrichthyan prepro-SSTc exhibit high sequence identity (ranging from 53.8 to 86.7% between one another) (Table S3).

3.4.4. SSTd (SST2)

When compared to that of SSTa, the primary structure of SSTd can be distinguished by the Ser-His-Cys (SHC) motif at its C-terminus (Table S2). Variable residues are present at position 1 and 2. However, the residue at position 2 is generally a Pro, except in *C. milii*. Chondrichthyan prepro-SSTd exhibit high sequence identity (ranging from 51.9 to 76% between one another) (Table S3).

Fig. 1. Alignment of the amino acid sequences of prepro-SST1 (PSSTa), -SST2 (PSSTd), -SST3 (PSSTd) and -SST6 (PSSTc) characterized from cartilaginous fish. (*) denotes conserved residues. SST peptide sequences are in red. Potential cleavage sites are in bold. Signal peptides are in grey. (p), partial sequences. Sequences of *S. canicula SST2 (SSTd)* and *SST3 (SSTe)* cDNAs have been deposited in the GenBank database under the accession numbers MK491629 and MK491630, respectively.

3.4.5. SSTe (SST3)

In all species examined, the primary structure of SSTe exhibits the unique Tyr-Thr-Leu-Cys (YTLC) motif at its C-terminus (Table S2). Only the residue at position 1 is different depending on the species. *S. canicula* and *C. milii* prepro-SSTe exhibit only 38.1% identity (Table S3).

3.5. Synteny analysis of SST genomic regions

In both *R. typus* and *C. milii*, *SSTe* (SST3) and *SSTa* (SST1) are tandemly arranged on the same scaffold (Fig. 2, Table S4). However, this scaffold is very short and does not contain any other genes. On the basis of our previous results suggesting that *SSTa* probably corresponds to *SST1* (Quan et al., 2013), we aligned the chondrichthyan *SSTa-SSTe* scaffold with the *SST1* locus from the other species analyzed. In teleosts, *SST3* is localized in tandem with *SST1*, as *SSTe* and *SSTa* in cartilaginous fish.

In spotted gar, the *SST1-SST3* locus bears a third *SST* gene, here called *SST4,* surrounded by *Nyap2* and *Irs1* (Fig. 2, Table S4). In teleosts, the *SST* known as *SST4* is located on a different chromosome than that bearing *SST1* and *SST3*. Its closest neighbors are *Nyap2b* and *Fgf12a* (Fig. 2, Table S4). The co-orthologs of these two latter genes, *Nyap2a* and *Fgf12b,* are present in the vicinity of both *SST1 and SST3*. Thus, the teleost *SST4* can be view as either an ortholog to gar SST4 (as this is what its name implies) or a 3R duplicate of either *SST1* or *SST3*. These three possibilities are illustrated in Fig. 2.

R. typus and *C. milii SSTd* (named *SST2* in Fig. 3) were found in a genomic region containing several genes, *Kif1b*, *Pgb*, *Cenps* and *Dffa*, that are also present in the vicinity of the human *SST2* gene (Fig. 3, Table S4). It is to note that the gar and zebrafish *SST* genes present in the syntenic region correspond to genes previously known as *SST6*.

The neighborhood of the *R. typus* and *C. milii SSTc* (named *SST6* in Fig. 3) is composed of several genes, *Tardbp*, *Masp2* and *Srm*, that, in the other species examined, were present in the vicinity of *SST* genes previously known as *SST2,* notably in chicken, spotted gar, zebrafish and medaka (Fig. 3, Table S4).

3.6. Phylogeny analysis of SST precursors

Fig. 4 displays an unrooted tree constructed from 147 selected vertebrates SST precursor sequences using maximum likehood phylogeny analysis. In the resulting tree, osteichthyan SST sequences cluster into six main clades, SST1-SST6. Bootstrap support values for these six groups were 53, 10, 97, 29, 68 and 75%, respectively. The only discrepancy of this patterning is the presence of the gar SST4 sequence into the SST3 clade. It is to note that the actinopterygian SST sequences belonging to the SST2 clade were previously known as SST6. Likewise, chicken, lungfish, sturgeon and teleost SST sequences included in the SST6 clade were previously known as SST2.

Chondrichthyan SSTb, SSTc and SSTd branch at the base of the SST5, SST6 and SST2 clades, respectively. Gnathotostome SST2-SSTd

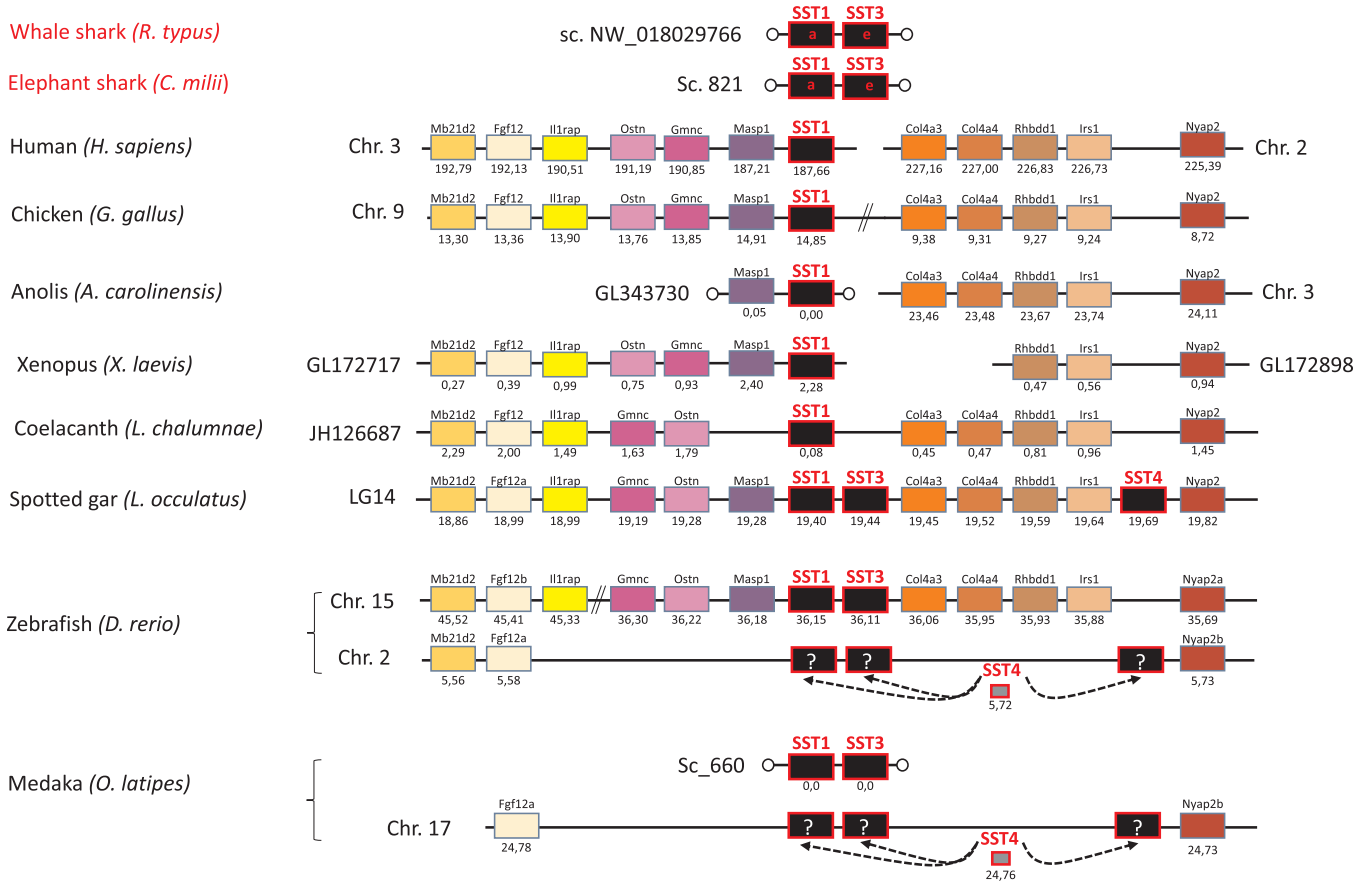

Fig. 2. Synteny of genes in the *SST1-SST3* locus in *R. typus* and *C. milii* plus eight selected bony vertebrate species (human, chicken, anole lizard, western clawed frog, coelacanth, spotted gar, zebrafish and medaka). Genes are represented by block arrows. Genes with conserved synteny are coloured. For the teleost *SST4* gene, three possible positions are proposed with respect to the gar SSTs (see the text for more details). Position of the genes (in megabases -Mb) is displayed below each box, according to the Ensembl database. Empty circles indicate the end of scaffolds. Letters inside the boxes refer to the specific and provisional nomenclature of *S. canicula SSTs* initially proposed in Quan et al. (2013). The detailed chromosomal locations of genes displayed in this map are included in Table S4.

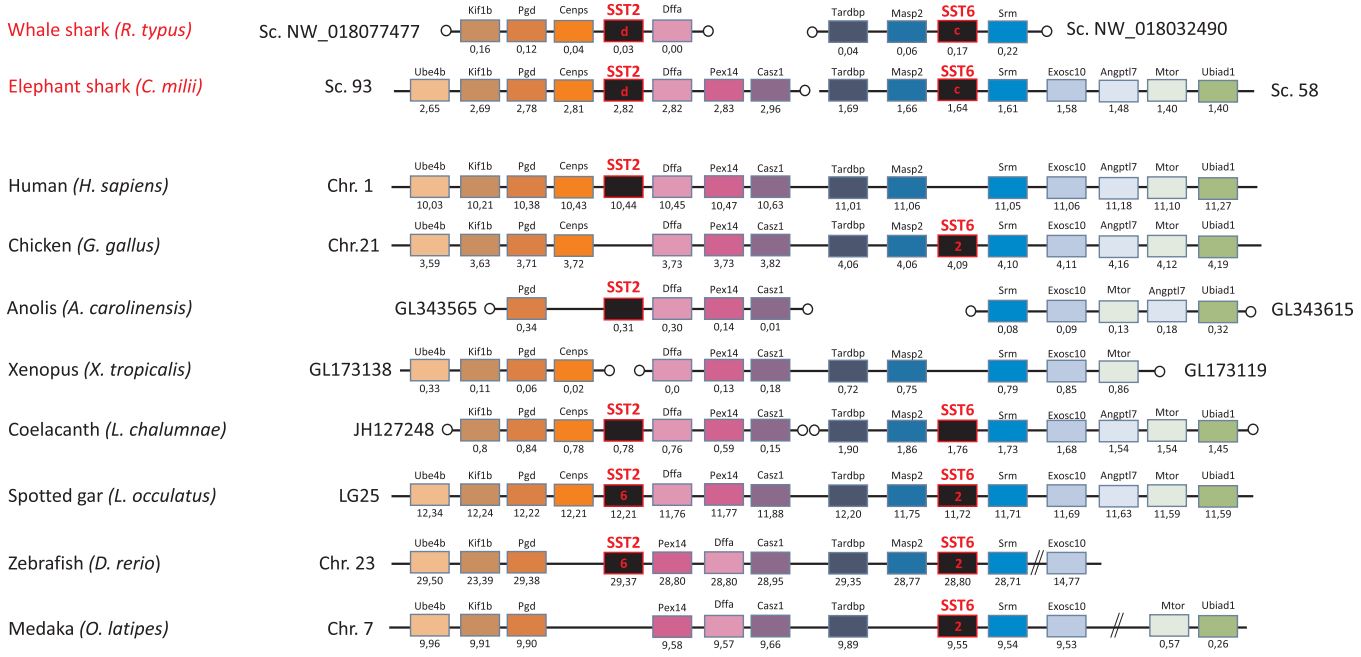

Fig. 3. Synteny of genes in the *SST2-SST6* locus in *R. typus* and *C. milii* plus eight selected bony vertebrate species (human, chicken, anole lizard, western clawed frog, coelacanth, spotted gar, zebrafish and medaka). Letters inside the boxes refer to the nomenclature of *S. canicula SSTs* proposed in Quan et al. (2013) while numerals refer to the previous nomenclature of vertebrate *SST* genes (Tostivint et al., 2013, 2014). Legends are the same as in the Fig. 2.

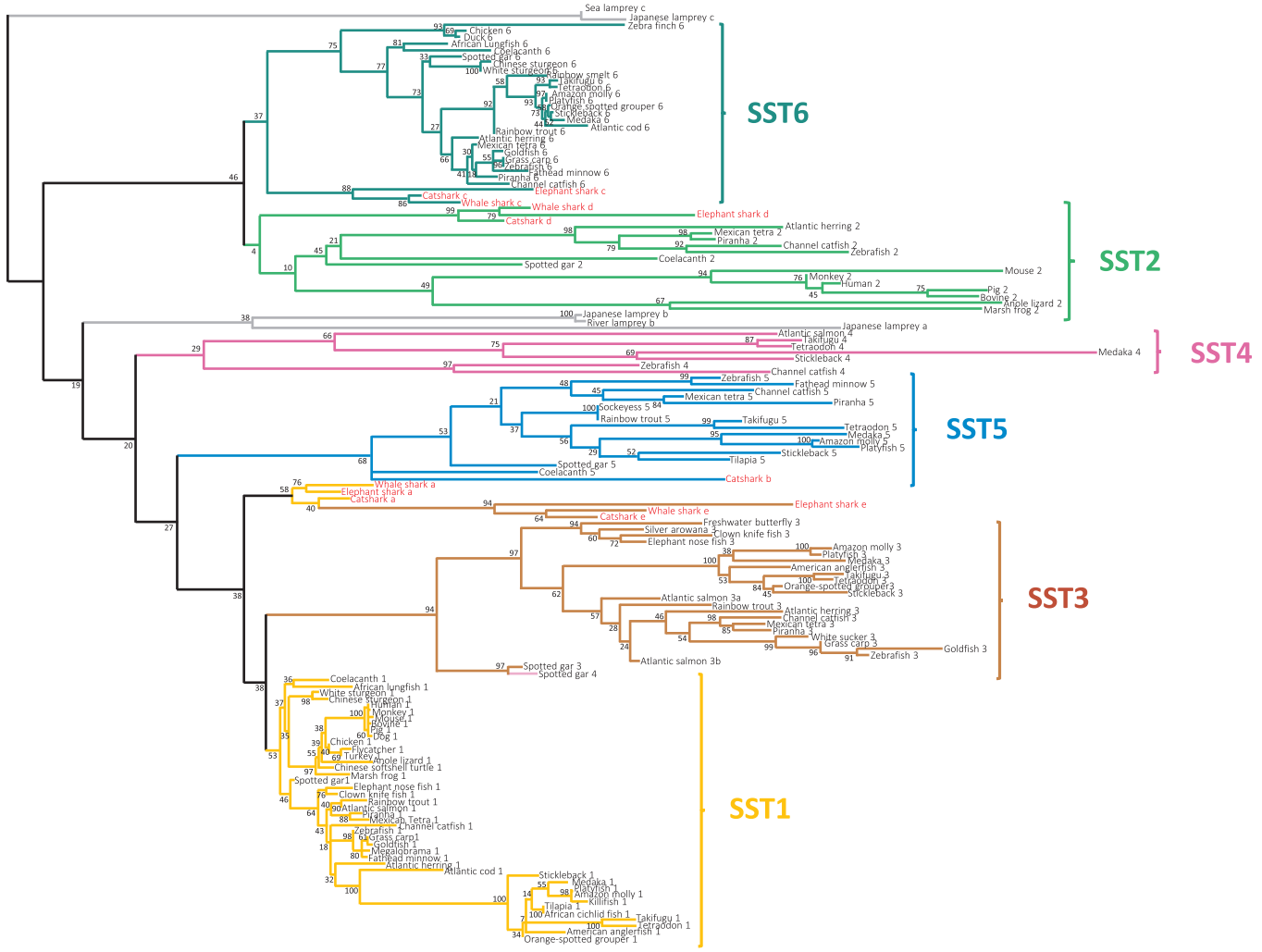

Fig. 4. Phylogenetic tree of vertebrate SST precursor sequences. Phylogenetic analysis of 147 vertebrate SST amino acid sequences was performed using Maximal Likelihood, with 500 bootstrap replicates. The number shown at each branch node indicates in percentage the bootstrap value. Sequence references and alignment are given in Figs. S1 and S2, respectively.

and SST6-SSTc appeared as two sister groups as well as osteichthyan SST1 and SST3. For their part, chondrichthyan SSTa and SSTe formed a monophyletic group that branch at the base of the osteichthyan SST1-SST3 clade.

As previously reported (Tostivint et al., 2016), lamprey SST sequences did not group to any of the gnathostome SST clades.

3.7. Tissue-specific expression of S. canicula SSTd (SST2) and SSTe (SST3) genes

The distribution of *S. canicula SSTd* and *SSTe* mRNAs in various tissues was examined by RT-PCR. Fig. 5 shows the results of one representative experiment out of three performed with identical results. *SSTd* mRNA was detected primarily in the brain (notably in the diencephalon, mesencephalon and brainstem) and spinal cord. In contrast, *SSTe* mRNA appeared particularly abundant in the central nervous system (in all subdivisions examined except in the cerebellum) as well

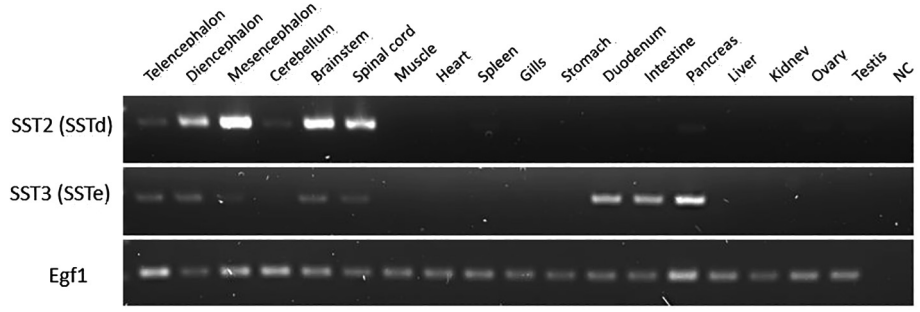

Fig. 5. Tissue distribution of *S. canicula SST2 (SSTd) and SST3 (SSTe)* mRNAs using RT-PCR. Parallel amplification of dogfish *Egf1* mRNA served as internal control. NC, non- template control.

as in the intestinal tract and pancreas. Note that traces of *SSTd* mRNA were also seen in the spleen, gills, pancreas and gonads.

4. Discussion

The main aim of the present study was to determine the repertoire of the *SST* gene family in cartilaginous fish. For this purpose, the genome and/or transcriptome databases from three representative chondrichthyan species were examined, namely the small-spotted catshark *S. canicula* (unpublished, see Gaillard et al., 2018 for more details), the whale shark, *R. typus* (Read et al., 2017) and the elephant shark *C. milii* (Venkatesh et al., 2014). In *S. canicula*, two new *SST* genes, named *SSTd* and *STTe* were found, in addition to the three *SST* genes, *SSTa*, *SSTb* and *SSTc*, previoulsly identified (Quan et al., 2013). All these genes, with the exception of *SSTb,* were retrieved from *R. typus* and *C. milii*. It is noteworthy that the occurrence of partial sequences of the *SSTc* (Tostivint et al., 2008) and *SSTd* genes (Liu et al., 2010) were already reported in both *L. erinacea* and/or *C. milii*. In contrast, *SSTe* appeared as a totally novel gene.

The nomenclature used above (*SSTa-SSTe*) was initially (and provisionally) proposed to designate the *S. canicula SST*s without prejudice to their evolutionary relationships with other vertebrate *SST*s (Quan et al., 2013). Synteny comparisons and phylogeny analysis were subsequently carried out in order to reveal the phylogenetic status of all these genes.

Synteny comparisons suggest that *SSTd* is the chondrichthyan counterpart of *SST2* since both *S. canicula* and *C. milii SSTd* reside in the same chromosomal environment as that of human *SST2*. For the same reasons, *SSTc* can be legitimately viewed as the chondrichthyan counterpart of *SST6.* These conclusions are quite consistent with our phylogeny analysis because SSTd and SSTc precursor sequences branch at the base of the SST2 and SST6 clades, respectively, although the bootstrap support values of the new clades are extremely weak (4% and 37% respectively).

Our investigations on the chondrichtyan *SSTc* and *SSTe* also led us to redefine the relationships between *SST2* and *SST6*. The *SST2* gene was been first characterized in frog (Tostivint et al., 1996) and mammals (de Lecea et al., 1996, 1997a), then in most other vertebrates, including chicken (Trabucchi et al., 2003), lungfish (Trabucchi et al., 1999) and sturgeons (Trabucchi et al., 2002; Li et al., 2009) (see Tostivint et al., 2008 for review). All peptides encoded by *SST2* possessed a Pro residue at position 2 and most of them exhibited the APCKNNFWKTFTSC sequence (Quan et al., 2013), except in mammals and some other species such as frog (Vaudry et al., 1992; Tostivint et al., 2008 for review) (Table S2). The *SST6* gene was initially characterized in only one teleost species, the zebrafish (Liu et al., 2010), then in spotted gar (Tostivint et al., 2013) and coelacanth (Yun et al., 2015) (Table S2). In the present study, we found an ortholog of this gene in several other teleost species belonging to various groups (particularly from otocephala) but not from euteleosts. Peptides encoded by *SST6* also shared a Pro at position 2, suggesting a common origin with *SST2*. In support of this latter view, in both zebrafish and gar, *SST2* and *SST6* are closely linked on the same chromosome, indicating that they most likely arose by tandem duplication. Our phylogeny and synteny analyses showed that most of the non-mammalian *SST* sequences previously referred as to *SST2* are not orthologous to *cortistatin* (the other name of the mammalian *SST2*) but actually correspond to *SST6*. Such is notably the case for birds (chicken) and fish (lungfish, sturgeons, bony and cartilaginous fish), but not for squamates (lizard) and amphibians (marsh frog). Conversely, all *SST* sequences previously referred to as *SST6* appeared to correspond to *SST2*. As a result, all the *SST* genes concerned with these observations were renamed accordingly. It is to note that the nomenclature adopted in the present article was already proposed in Yun et al. (2015) but without explicit justification.

In *R. typus* and *C. milii*, *SSTe* and *SSTa* are tandemly arranged suggesting that both genes arose by local duplication of a common ancestral gene. Synteny analysis of the *SSTa-SSTe* locus was inhibited by the fact that *SSTa* and *SSTe* were the only genes present on their scaffold. However, based on previous results showing that *SSTa* probably corresponds to *SST1* (Quan et al., 2013), the most credible assumption is that *SSTe* corresponds to *SST3* because in all species possessing both *SST1* and *SST3*, these genes are systematically localized in tandem (Tostivint et al., 2004, 2008, 2013, 2014). Phylogeny analysis is in agreement with close evolutionary relationships between *SSTa* and *SSTe,* on the one hand, and *SST1* and *SST3,* on the other hand. However, it does not fully support the orthology between *SST1* and *SSTa,* on one hand, and *SST3* and *SSTe,* on the other hand, since chondrichthyan *SSTa* and *SSTe* sequences did not strictly group with osteichthyan SST1 and SST3, respectively. One explanation to reconcile these contradictory results would be that *SST1/SSTa* and *SST3/SSTe* genes were subjected to concerted evolution, a genetic process that leads to homogenize repetitive DNA sequences over time (Liao, 1999). Indeed, the major hallmark of concerted evolution is that paralogous genes within species are more similar to one another than they are to their orthologous counterparts in closely related species (Liao, 1999). DNA recombination mechanisms, especially unequal crossover and gene conversion, are responsible for concerted evolution (Liao, 1999). Interestingly, we have shown that nucleotide sequences of the first exon of *S. canicula SSTa* and *S. canicula SSTe* are almost identical (93.5% of identity), while those of the second exon are much more divergent (only 61% of identity). Such level of similarity may be a good indicator of a recent recombination event occurring between the two genes.

The phylogenetic status of the *S. canicula SSTd* and *SSTe* genes suggested by synteny and phylogeny analyses is consistent with their tissue expression pattern since *SSTd,* as *SST2*, is primarily expressed in the central nervous system (SNC) (de Lecea et al., 1997b; Lin et al., 1999; Trabucchi et al., 1999, 2003), while *SSTe*, as *SST3,* is expressed both in the SNC and the gastrointestinal tract (Moore et al., 1995; Xing et al., 2005). Further studies at the cellular level will be needed to speculate about the functions of the corresponding peptides.

In contrast to the other *SST* genes found in cartilaginous fish, *SSTb* was detected only in *S. canicula* and not in *R. typus* and *C. milii*. Phylogeny analysis showed that SSTb grouped into the SST5 clade, as previously reported (Quan et al., 2013). However, the orthology relationships between *SSTb* and osteichthyan *SST5* could not be confirmed by synteny analysis since the *S. canicula SSTb*-bearing scaffold was too short.

Among the six *SST* paralogs characterized so far in gnathostomes, *SST4* is the only one not to exist in cartilaginous fish. Up to now, the *SST4* gene was considered as a *SST1* paralog generated by 3R (Liu et al., 2010), in agreement with the fact that it was found only in teleosts (Fig. S9A). Recently, however, Yun et al. (2015) reported the occurrence of a so-called *SST4* ortholog in spotted gar. This finding is clearly in conflict with the Liu's view since the gar lineage is known to have diverged from teleosts before the 3R (Fig. S9B) (Braasch et al., 2016). In the present study, synteny analysis showed that teleost *SST4* can be equally viewed as orthologous to the gar *SST4,* or co-orthologous to either teleost *SST1* or teleost *SST3*, without the possibility to decide between these three conceptions. Indeed, in both zebrafish and medaka, the *SST4*-bearing chromosome block contains only very few paralogs of the neighboring genes of *SST1* and *SST3* (such as *Fgf12* and *Nyap2*), making it impossible to precisely place the teleost *SST4* in relation to gar *SSTs*. Examination of other teleost species whose genome is available in Ensembl failed to resolve the problem (data not shown). What is clear, however, is that phylogeny analysis absolutely does not support the orthology of gar *SST4* and teleost *SST4* since gar SST4 groups within the gnathostome SST3 clade, thus without any obvious relationships with the teleost SST4 clade. Rather, the very high level of sequence similarity between gar SST3 and gar SST4 (91% nucleotide identity) strongly suggests that gar *SST4* arose very recently by tandem duplication of *SST3*. It therefore appears unlikely that gar *SST4* and teleost *SST4* are orthologous unless gar *SST4* and *SST3* were subjected to concerted

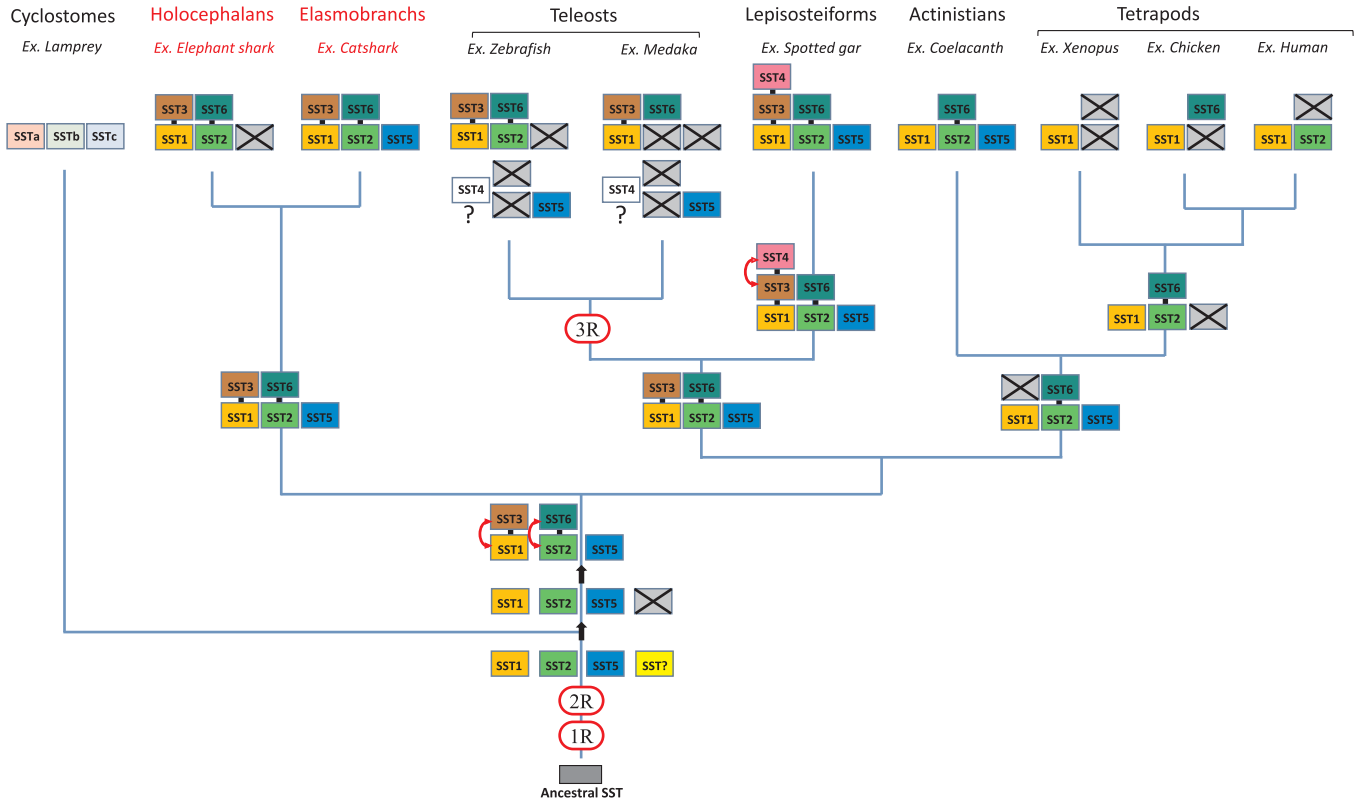

Fig. 6. A proposed evolutionary scheme for the evolution of the *SST* gene family in gnathostomes with special emphasis on chondrichthyan species (in red). The names of the different paralogous genes are given in the boxes. Note the unresolved status of the teleost *SST4*. Gar *SST4* is shown here to have arisen independently of teleost *SST4*. Boxes connected by a vertical line represent linked genes. Crossed-out boxes represent lost genes. Double-headed arrows represent local duplications. R, rounds of whole genome duplication.

evolution, as shown above for *S. canicula SST1/SSTa* and *SST3/SSTe*.

5. Concluding remarks

Over the past few years, significant advances have been made in the understanding of the evolution of the *SST* family. One of the most important results has been the demonstration that the current diversity of the family was initially caused by 2R (Liu et al., 2010; Tostivint et al., 2013, 2014). According to this view, the last common ancestor of vertebrates possessed four *SST* genes, three of which, *SST1, SST2* and *SST5,* are still present in all extant vertebrate species. A fourth *SST* gene (referred to as *SST?* in Fig. 6) is likely to have been generated by 2R but since this gene has never been found in extant vertebrate species, it is assumed to have been lost very early after 2R. Conversely, the rest of the diversity of the family, represented by *SST3*, *SST4* and *SST6*, was considered restricted to only some groups of bony fish (Tostivint et al., 2013, 2014): actinopterygians for *SST3* and *SST6* and teleosts for *SST4*.

The principal result of the present study is the demonstration of an unexpected diversity of *SST* genes in cartilaginous fish. Thus, in *S. canicula*, not less than five *SST* genes, *SST1, SST2, SST3*, *SST5* and *SST6,* have been identified, while only three of them were characterized so far (Quan et al., 2013). Therefore, contrary to what was previously though, it is likely that all these five genes were already present in the gnathostome ancestor (Fig. 6).

It is to note that our study did not provide any new data supporting the impact of 2R in the evolution of the *SST* gene family due to the small size of the scaffolds from the three chondrichthyan species examined. Thus, within the regions containing *SST* genes, *Masp 1* and *Masp 2* were the only genes indicating that *SST1* and *SST3,* on the one hand and *SST2* and *SST6,* on the other hand, belong to the same paralogon. In contrast, our study confirms the idea that *SST3* and *SST6* likely arose by tandem duplications from *SST1 and SST2*, respectively (Fig. 6). In strict logic, however, we must admit that it is currently impossible to know which of *SST1* and *SST3,* on the one hand and *SST2* and *SST6,* on the other hand are the ancestral genes. The uncertainty on this matter will remain as long as the phylogenetic status of lamprey *SSTs* is unsolved (Tostivint et al., 2016) because the lamprey *SST* sequences are the only ones that could be currently used as outgroups. Thus, in Fig. 6, *SST1* and *SST2* are displayed as ancestral genes in a totally arbitrary manner.

Another important result of the present study was the finding that *SST6* was not totally lost in sarcopterygians, as previously thought, because it is still present in coelacanth and birds (Fig. 6). In this context, it is noteworthy that chicken *SST6* was initially but mistakenly reported as *SST2* (Trabucchi et al., 2002), now refuted by synteny and phylogeny analysis. In a general way and for the same reasons, all teleost genes previously known as *SST6* had to be renamed *SST2*, and *vice versa*. Notably, the true *SST2* is also present in amphibians (but not in *Xenopus*), sauropsids (but not in birds) and mammals (Fig. 6). Taken together, our result show that *SST2* and *SST6* have been lost independently and repeatedly during evolution: in euteleosts, birds and some amphibians for *SST2* and amphibians, squamates and mammals for *SST6* (Fig. 6). After their appearance, *SST3* and *SST5* were also secondarily lost in sarcoperygians and tetrapods, respectively.

Finally, the present work was unable to precisely determine the phylogenetic status of teleost *SST4*. In particular, whether teleost *SST4* and gar *SST4* are truly orthologous or not remains to be investigated. Further studies in other species than those examined here will be needed to address this issue.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.02.022.

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