



Brief Communication

Centromeric Satellite DNA in Flatfish (Order Pleuronectiformes) and Its Relation to Speciation Processes

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Abstract

Two new centromeric satellite DNAs in flatfish (Order Pleuronectiformes) have been characterized. The *Sacl*-family from *Hippoglossus hippoglossus*, restricted to this species, had a monomeric size of 334 base pair (bp) and was located in most of the centromeres of its karyotype. The *Pvull*-family, with a monomeric size of 177 bp, was initially isolated from the genome of *Solea senegalensis*, and fluorescent in situ hybridization (FISH) localized the repeat to centromeres of most of the chromosomes. This family could only be amplified in 2 other species of the genus *Solea* (*Solea solea* and *Solea lascaris*). Molecular features and chromosomal location indicated a possible structural and/or functional role of these sequence repeats. The presence of species-specific satellite-DNA families in the centromeres and their possible role in the speciation processes in this group of fishes is discussed.

Subject area: Molecular systematics and phylogenetics

Keywords: centromeric satellite DNA, flatfishes, molecular evolution, speciation

Flatfish (Pleuronectiformes) comprise a vast group of fishes that are economically and ecologically important. The origin of flatfishes possibly dates to the Eocene period (Verneau et al. 1994), and their phylogenetic relationships are not well established, as the taxonomic classification has been based on morphological characteristics and on only a few molecular studies, mainly using mitochondrial DNA markers (Infante et al. 2004; Pardo et al. 2005). The most representative morphological trait of members of this group is their body asymmetry, which results from metamorphosis in the early stages of larval development.

At the molecular level, all flatfishes have small genomes in relation to most other teleosts, with C-values ranging from 0.39 to 1.10

pg (381–1076 Mb). While this is comparable to a value of about 0.38 pg (370 Mb) for the pufferfish *Tetraodon nigroviridis* (Jaillon et al. 2004) it is 6-fold smaller than the salmon *Salmo salar*, the teleost with the highest C value (Gregory 2016).

Eukaryotic genomes are characterized by the presence of satellite DNA sequences (Plohl et al. 2008), at least in the centromeric and/or subtelomeric region of their chromosomes (Charlesworth et al. 1994). These repeats have been found even in smaller fish genomes, such as the pufferfishes *T. nigroviridis* (Fischer et al. 2000) and *Fugu rubripes*, which has a genome size of 400 Mb (Brenner et al. 1993). In flatfish, only 2 satellite DNA families have been isolated thus far, one in *Achirus lineatus* (Azevedo et al. 2005) and another in *Dicologlossa*

cuneata (de la Herrán et al. 2008). Despite both being centromeric DNA, no sequence similarity has been detected (de la Herrán et al. 2008). The recurrent presence of this kind of repetitive DNA in the centromeres of eukaryotic chromosomes has been historically considered to be a hint of their possible role in cell division processes. In fact, numerous research studies have suggested that these genome components could play different roles in heterochromatin assembly, epigenetic regulatory processes or as a source of siRNA (reviewed in Pezer et al. 2012). Its conserved presence and distribution in eukaryotic cells contrasts with the high variability of satellite DNA, this being one of the most dynamic components of genomes, evolving through concerted evolution (Dover 1982). This rapid mechanism promoting evolution, in the case of the (peri)centromeric satellite DNA families, has been related to the speciation process because it may cause reproductive isolation and ultimately species radiation (Pohl et al. 2012).

In the present study, 2 new centromeric satellite DNA families in flatfish were isolated: *SacI* from *Hippoglossus hippoglossus* and *PvuII* from the genomes of several *Solea* species (*Solea senegalensis*, *Solea solea*, and *Solea lascaris*). Molecular features, chromosomal location, and the evolutionary conservation of these satellite DNAs could shed light on their possible role in the speciation processes of this group of fish.

Materials and Methods

Sampling and Genomic DNA Extraction

Larvae and juvenile samples of *S. senegalensis* (Soleidae) were taken from the experimental hatchery center “Agua del Pino” at the Andalusian Institute of Agricultural Research and Training (IFAPA, Junta de Andalucía, Spain).

The genomic DNA and chromosomes of *H. hippoglossus* (Pleuronectidae) were obtained from the National Research Council-Institute for Marine Biology in Halifax (Canada), thanks to Dr M. Reith. Tissue samples of *S. lascaris*, *S. solea*, *D. cuneata*, *Microchirus azevia*, and *Sinaptura lusitanica* (Soleidae); *Scophthalmus maximus*, and *Scophthalmus rhombus* (Scophthalmidae); *Arnoglossus laterna* and *Arnoglossus thori* (Bothidae); and *Citharus linaguatula* (Citharidae) were donated by the Dr P. Martínez group from the Department of Genetics, Faculty of Veterinary Science, University of Santiago de Compostela (Spain). Genomic DNA was isolated from muscle and larval tissue as described by Sambrook and Russell (2001).

Isolation of Satellite DNA Families

Restriction Enzymes

Ten micrograms of *H. hippoglossus* genomic DNA were digested overnight at 37 °C with 15 U each of the following restriction enzymes: *HindIII*, *AluI*, *PvuII*, *RsaI*, *HaeIII*, *BamHI*, *PstI*, *DraI*, *SacI*, and *EcoRI* (Roche). The fragments generated were separated by electrophoresis on a 3% agarose gel.

Genomic Self-priming PCR

This method was used to isolate the *PvuII* satellite DNA family from *S. senegalensis*, according to the protocol described in Buntjer and Lenstra (1998).

PCR with Specific Primers

The *PvuII* satellite DNA family was isolated from the genome of the rest of species found in the genus *Solea* by specific primers, designed from previously characterized monomeric sequences from *S. senegalensis*: *PvuII*-F 5'-ACA TCC CAG CAG TGA ATT CAT-3' and *PvuII*-R 5'-TTT TTC TAT TAG TGT CTC-3'.

Cloning, Sequencing, and Hybridization Analysis of Satellite DNA Families

The purified fragments obtained by enzyme restriction (*SacI*-family in *H. hippoglossus* and *PvuII*-family in *S. senegalensis*) were ligated to vector pUC18 (Roche). The amplified monomers of satellite DNA (*PvuII*-family in *S. lascaris* and *S. solea*) were cloned using the TOPO TA Cloning® Kit PCR® 2.1 (Invitrogen, Carlsbad, CA, USA).

Positive clones were sequenced using an ABI 3100 Avant sequencer system in both strands using a Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems). Repetitive fragments isolated from *H. hippoglossus* and *S. senegalensis* were used as Southern blot probes to check for the tandem organization of these repetitive sequences in the genome.

For FISH analysis, the mitotic chromosomes were obtained from larvae by following the protocol described by Garrido-Ramos et al. (1994), with FISH performed according to the Schwarzacher and Heslop-Harrison (2000) protocol.

Conservation, Variability, and Phylogenetic Analyses

Multiple alignments of our sequences against reference sequences from the GenBank database were performed using ClustalX software (Thompson et al. 1997) and Geneious (v.4.8.4) (available from <http://www.geneious.com/>). Phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 (v.6.0.5) (Tamura et al. 2013). Sequence divergence was calculated according to the Jukes-Cantor method and the resulting distance trees by the neighbor-joining method (Saitou and Nei 1987).

Results

Isolation and Characterization of *SacI*-Satellite DNA from *H. hippoglossus*

Restriction digestion of *H. hippoglossus* gDNA with *SacI* revealed a single prominent 350 bp band. These fragments were excised and cloned as candidate repeat units of a satellite DNA family. Subsequently, an aliquot of *SacI* fragments was hybridized against genomic DNA of *H. hippoglossus*, digested with different restriction enzymes and blotted onto a nylon filter. A typical ladder pattern with a 350 bp repeat unit of was detected, supporting the idea of the tandem organization of this repeat DNA family.

Sequence analyses of 5 recombinant clones of *SacI* (accession numbers in data availability) revealed a consensus length of 334 bp with monomer variation in size ranging from 331 to 336 bp and an AT percentage of 47%. Intraspecific variability (Π) was 6.3%. The sequence showed short internal repeats and several centromeric-like motifs, with emphasis on the regions between 231 and 248 bp with a 67% similarity to the CENP-B motif of other vertebrate and from 193 to 218 bp with a 64% similarity to CDEIII from *Saccharomyces cerevisiae* (Figure 1A).

In a Southern blot hybridization against genomic DNA from several Pleuronectiformes species (*S. senegalensis*, *S. lascaris*, and *D. cuneata*; *S. maximus* and *S. rhombus*) digested with different restriction enzymes, a positive signal was detected only for *H. hippoglossus* itself (data not shown). No DNA sequence similarity was detected when the complete unit was searched for in the EMBL/GenBank databases.

FISH analysis, using the *SacI* repeat as a probe, demonstrated that this repetitive family localizes to the centromeric region of most of the chromosomes from the *H. hippoglossus* karyotype ($2n = 48$), except for 2 pairs of chromosomes that had undetectable hybridization signals (Figure 2A).



Figure 1. (A) Consensus sequence (Cs) of satellite DNA *SacI* of *Hippoglossus hippoglossus*. The sequences highlighted in grey (16 bp) and in italics (20 bp) correspond to internal repetitions with main homologies of 52.7% (grey) and 55.1% (italics). The *SacI* enzyme restriction site is underlined. In both figures, boxed sequences present similarities with centromeric motives as CENP-B (solid line), CDEIII (broken line), and PJ α (dotted line). (B) Consensus sequence (Cs) of satellite-DNA *PvuII* of *Solea senegalensis*. The sequences highlighted in grey (18 bp) correspond to an internal repetition with main homologies of 66.7%.

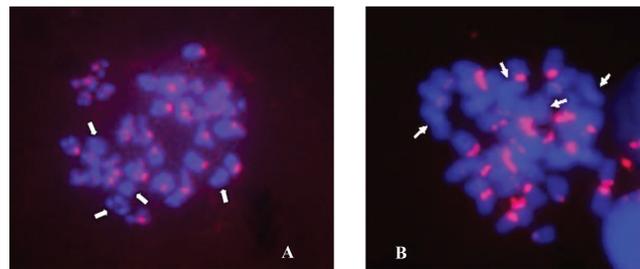


Figure 2. (A) Chromosomal locations of *SacI*-family satellite DNA by FISH in mitotic metaphase of *Hippoglossus hippoglossus*. Arrows indicate chromosomes lacking hybridization signals. (B) Chromosomal locations of *PvuII*-family satellite DNA by FISH in mitotic metaphase of *Solea senegalensis*. Arrows indicate chromosomes lacking hybridization signals.

Isolation and Characterization of *PvuII*-Satellite DNA from *S. senegalensis*

After self-DNA amplification and restriction with several enzymes, a single band of approximately 200 bp was visible in an agarose gel, corresponding to the fragment excised by the *PvuII* enzyme. This band was purified and used as a probe in Southern blot hybridization against digested genomic DNA of *S. senegalensis* with several restriction enzymes. In the *PvuII*-lane, a clearly visible typical ladder pattern characteristic of satellite DNAs was found. This repetitive DNA was cloned and sequenced. Alignment of 6 monomers revealed a consensus sequence of 177 bp. The sequence was composed of short stretches of adenine and thymine (AT content of 66%) and short direct or inverted repeated motifs and a region (positions 152–175) with DNA sequence similarity to the centromeric motif CDEIII of *S. cerevisiae* of 66.6% (Figure 1B). The variability of the cloned sequences was estimated by nucleotide diversity ($\Pi = 6.8\%$).

Southern blot hybridization showed that the *PvuII*-family is present in species from the genus *Solea* (*S. solea* and *S. lascaris*) but not in the other Pleuronectiformes species sampled. After a search in the EMBL/GenBank databases, no similarity was detected for this monomeric sequence.

FISH analysis, using the *PvuII* sequence as a probe, revealed that this satellite DNA was located in the centromeres from most of the chromosomes of *S. senegalensis* (Figure 2B). Similar to halibut, no hybridization signals were detected in 2 pairs of chromosomes of this species' karyotype.

Isolation and Characterization of *PvuII*-Satellite DNA from Other *Solea* Species and Phylogenetic Analyses

Repetitive units from species with Southern blot positive hybridizations (*S. solea* and *S. lascaris*), were amplified by PCR and subsequently cloned. Six clones from each species were sequenced, showing lengths ranging from 173 to 176 bp and $\Pi = 13.3\%$ for *S. solea* and lengths ranging from 155 to 161 bp and $\Pi = 8.9\%$ for *S. lascaris*.

Interspecific divergence values were calculated between species. In all the cases, intraspecific variability was lower than interspecific divergence (Table 1). These data were used for a phylogenetic study of these 3 *Solea* species, grouping the monomeric sequences in species-specific clusters (Figure 3).

Discussion

Molecular Characterization

The length of the repetitive units found in both families (177 bp -*PvuII*- and 334 bp -*SacI*-) were typical of these types of repetitive sequences, with these sizes associated to a structural function, such as nucleosomic organization (Sharma and Raina 2005; Plohl et al. 2008), heterochromatin condensation, or centromeric function (Henikoff et al. 2000), and even with a regulatory function as transcripts, for which size may be a limiting factor (revised in Ugarkovic 2005). The existence of double-length units has been explained by the rapid evolution that these sequences can undergo, involving

Table 1. Intraspecific variations (diagonal) and interspecific divergence among *PvuII* sequences isolated from *Solea senegalensis* (Ssen), *Solea solea* (Ssol), and *Solea lascaris* (Slas).

	Ssen	Slas	Ssol
Ssen	0.076		
Slas	0.159	0.093	
Ssol	0.180	0.209	0.149

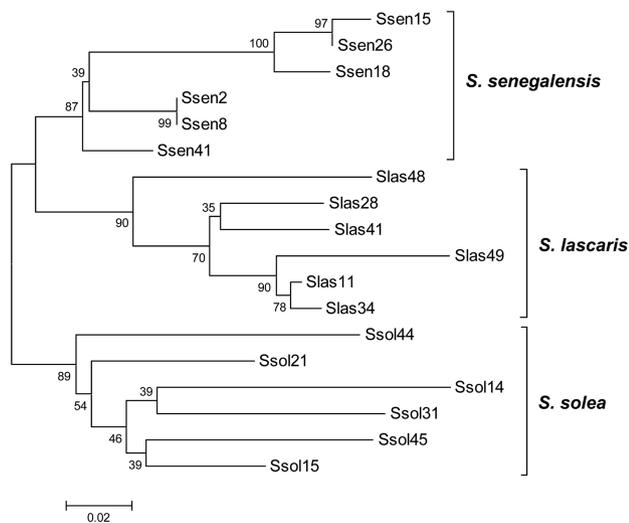


Figure 3. Unrooted neighbor-joining tree of *PvuII* sequences from *Solea* species. The numbers next to the name of the species indicates the clone analyzed. Numbers are bootstrapping indices for the level of support for individual nodes.

duplication and divergence phenomena. Thus, the *SacI*-family unit would originate from the shortest units (about 170) in a single duplication event. This mechanism is supported by the equidistance between smaller internal repetitive motifs present in the monomers (Figure 1A).

Additionally, the presence of these short internal repeats in the sequences of both satellite DNAs, supports the idea of a possible origin of the main unit from the duplication of an ancestral sequence motif (Figure 1A, B). In fact, this is true of the satellite DNA *DraI* from *D. cuneata*, generated from an initial sequence of 9 nucleotides (de la Herrán et al. 2008).

Both satellite DNAs showed a high monomeric intra-specific homology (mean 90% in *PvuII* and 99.8% in *SacI*). These data seemed normal in the centromeric-satellite families in this group of flatfish, as the 2 satellite DNAs analyzed prior to this work also showed a high homology between their sequences: 97% in *D. cuneata* (de la Herrán et al. 2008) and 95.2% in *A. lineatus* (Azevedo et al. 2005). Such high identity between repeat units in the same species has been explained by the presence of a monomeric “canonical” sequence in the genome of these species (López-Flores and Garrido-Ramos 2012) and, by the intrinsic molecular processes that operate in this type of repeat sequence, such as unequal crossover between chromatid sisters and nonsisters, gene conversion, and transposition (Charlesworth et al. 1994).

Chromosomal Localization

The centromeric location of both satellite DNAs is supported by molecular data: AT percentage, short stretches of adenine and

thymine, direct and inverse repetitive motifs, and the presence of centromeric motifs that are similar to others described in vertebrates (Singer 1982; Garrido-Ramos et al. 1995; Choo 1997). This location is shared with another 2 satellites that were previously characterized in flatfish. The sharing of a single satellite DNA family in the centromeres of related species is most frequently the case, according to previous studies (Oliveira and Wright 1998; de la Herrán et al. 2001; Canapa et al. 2002). However, these 2 new centromeric satellite DNA families did not show sequence similarity between them or with any previously described fish in this group, even between closely related species such as *D. cuneata* and *Solea* species.

The absence of hybridization in some chromosome pairs of a centromeric satellite DNA, as in our case for both families, is no exception. This has also been found in other fish species (Phillips and Reed 1996; Lanfredi et al. 2001; Viñas et al. 2004; Fontana et al. 2008), including the satellite DNA *DraI* of other flatfish such as *D. cuneata* (de la Herrán et al. 2008). This suggests that processes of genomic turnover that homogenize individual chromosomal subsets of satellite DNA are more efficient than processes that spread and homogenize these sequences throughout the genome. The lack of a signal could also be due to the fact that the amount of satellite DNA in these pairs was not large enough for the fluorescence signal to be seen, due to the different intrachromosomal expansion rates. Additionally, this result could be explained by the presence of other centromeric satellite families or to the absence of satellite repeats in these centromeres. In fact, in some animals and plants, the complete lack of satellite DNA in their centromeres has been demonstrated (Piras et al. 2010; Shang et al. 2010; Locke et al. 2011; Gong et al. 2012).

Conservation and Evolution

The *PvuII* family is conserved among the *Solea* species analyzed, but not in the rest of the Pleuronectiformes used in this research work. However, *SacI* is restricted to halibut. This is not new in Pleuronectiformes, as the other 2 satellite DNAs that were previously isolated from this group seemed to be species-specific. In both families, the homogenization rate was high (99.8% for *SacI* and 90% for *PvuII*). In the cases of (peri)centromeric satellite DNA, a high intraspecific homogenization, due to the maintenance of a sequence homogeneity that is crucial for centromere stability, accompanied by rapid divergence between repeats of different species, led to these satellite DNAs being species-specific (Plohl et al. 2012) or being present in a reduced group. The new variants in the (peri)centromeric regions of the different species can be established in different ways. In our case, the different species showed species-specific centromeric satellite DNA, or shared with a phylogenetically close group of species such as species from the genus *Solea* (Infante et al. 2004; Pardo et al. 2005). Gradual evolution can be ruled out because a slow change is not consistent with the presence of different centromeric satellite families in this fish group. However, the library hypothesis could explain the fast emergence of species-specific satellite DNAs in a related group of species by differential amplifications and/or contractions within a pool of sequences shared by their genomes (Fry and Salser 1977). In addition, this faster change in the homogenization rate in the centromeric satellite DNA could be favored by the small genomes of flatfish species and could contribute to a stronger effect of these changes.

In fact, this evolutionary process would explain why, despite the recent origin attributed to this group of fish (Verneau et al. 1994), Pleuronectiformes show high numbers of species and

genera (716 and 123, respectively; Munroe 2005). The rapid evolution of the satellite DNA in Pleuronectiformes contrasts with the low divergence found in other markers such as mitochondrial DNA genes (Infante et al. 2004; Pardo et al. 2005) encountered with gradual evolution, confirming the involvement of this centromeric satellite DNA in the speciation process of this group. On the other hand, in relation to the *PvuII*-family shared among the members of *Solea* species, our data support a concerted mode of evolution for this family, given that homology between repeat units of the same species were higher than between different species, despite being closely related species. Thus, phylogenetic inference methods such as neighbour joining and UPGMA grouped the repeats by species (Figure 3). Additionally, in the evolutionary context of this group, the presence of a shared centromeric satellite DNA among the 3 *Solea* species, their low interspecific divergence and the high homology found in mitochondrial markers by other authors (Infante et al. 2004; Pardo et al. 2005), would support a close relationship between them and the recent origin of the *Solea* group.

In conclusion, we have characterized 2 new satellite DNA families in the order Pleuronectiformes with a centromeric location and presence restricted to one species (in the case of the *SacI* satellite-DNA family) and a few closely related species (*PvuII* satellite-DNA family). The comparison studies with different satellite DNA families that were previously found in other flatfish species point to species-specific evolution. In this sense, the role of these centromeric sequences in the speciation process could be related to the high diversity of this group of fishes.

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Data Availability

The accession numbers for GenBank for the new reported sequences are: HG977008–HG977012 (*SacI*-satellite DNA from *H. hippoglossus*), HG976990–HG976995, HG976996–HG977007 (*PvuII*-satellite DNA from *S. senegalensis*, *S. sole*, and *S. lascaris*, respectively).

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