Long-term evolution of 5S ribosomal DNA seems to be driven by birth-and-death processes and selection in *Ensis* razor shells (Mollusca: Bivalvia)[i]

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Abstract

A study of nucleotide sequence variation of 5S ribosomal DNA from six *Ensis* species revealed that several 5S ribosomal DNA variants, based on differences in their nontranscribed spacers (NTS), occur in *Ensis* genomes. The 5S rRNA gene was not very polymorphic, compared with the NTS region. The phylogenetic analyses performed showed a between-species clustering of 5S ribosomal DNA variants. Sequence divergence levels between variants were very large, revealing a lack of sequence homogenization. These results strongly suggest that the long-term evolution of *Ensis* 5S ribosomal DNA is driven by birth-and-death processes and selection.

Keywords: 5S ribosomal DNA; Birth-and-death evolution; *Ensis*; Mollusca; Bivalvia

Introduction

Nuclear ribosomal DNA (nrDNA) is composed of the major ribosomal genes, their spacers, the 5S ribosomal gene, and its spacer. These genomic regions has been widely used in molecular evolutionary studies, taken as an object of study itself, in an attempt to understand the evolutionary forces that shaped extant variation, or used as molecular markers in phylogenetic surveys at different levels.

The major ribosomal genes and spacers are tandemly repeated in the genome, forming long arrays, and each eukaryote species may have one or more of these arrays.

The 5S ribosomal DNA (5S rDNA) is formed by the 5S rRNA gene and a nontranscribed spacer (NTS). It is organized in the same tandemly repeated units as the major ribosomal genes in many species (Rooney 2004; Rooney and Ward 2005). However, it can also be found dispersed throughout the genome (in *Schizosaccharomyces pombe*, Wood et al. 2002, and in other fungi belonging to the subphylum Pezizomycotina, Rooney and Ward 2005), in tandem arrays, separate from the major ribosomal genes, or in both types of arrangements (in *Homo sapiens*, Little and Braaten 1989). In relation to copy number, each species genome contains many repeats of 5S rDNA (e.g., 9,000–24,000 in *Xenopus*, Brown and Sugimoto 1974; 600–4,600 in soybean, Danna et al. 1996; ~686 in rice, Li et al. 2002).

The evolution of nrDNA is usually explained by the so-called model of concerted evolution: mutations occurring in the ribosomal genes or in their spacers can spread to the other units of the array through the action of homologous and nonhomologous unequal crossovers and gene conversions. The molecular mechanism of gene conversion in multigene families is not well understood (Nei and Rooney 2005), but there is a commonly held opinion that a combination of both unequal crossovers and gene conversions gives rise to the concerted evolution of ribosomal DNA in all organisms (Eickbush and Eickbush 2007). Unequal crossovers and gene conversions have a homogenizing effect, and they significantly reduce the sequence divergence between members of the same array or between members of different arrays. Other evolutionary forces that reduce the variation produced by mutations in nrDNA are genetic drift and selection (selection is expected to have a much greater effect over the genes than over the spacers).

In several animal phyla, however, examples have been reported in which sequence divergence levels between ribosomal genes or spacers seem to be much higher than expected under a strict concerted evolution scenario (e.g., Insua et al. 2001; Leo and Barker 2002; Freire et al. 2005; Keller et al. 2006; Sword et al. 2007; Caradonna et al. 2007; López-Piñón et al. 2008).

Previous studies have demonstrated that nrDNA may evolve under the birth-and-death model (Nei and Hughes 1992). Under this model, new ribosomal gene copies are created by gene duplication, during the evolution of a particular group of organisms. These new copies can persist in the genomes as functional genes for a long time, or become pseudogenes and accumulate deleterious mutations. Organisms whose nrDNA (e.g., the 5S rDNA) is evolving under birth-and-death processes will accumulate several variants of (in this case) 5S ribosomal genes and spacers.

There are examples in which purifying or positive selection and birth-and-death processes are responsible for the evolution of 5S rDNA (Rooney and Ward 2005; Fujiwara et al. 2009). The 5S rDNA of these organisms is characterized by the occurrence of several 5S rDNA variants in their genomes, with low levels of nucleotide variation in the 5S rRNA gene (maintained by selection) and a highly polymorphic NTS region.

Under a birth-and-death scenario, 5S rDNA variants sampled from different species of a given group are expected to form clusters based on their sequence similarities in a phylogenetics tree. These sequences will not cluster by species (as would be expected under concerted evolution). Rather, they form several clades composed of different species (see Fig. 1 in Rooney and Ward 2005).

According to Rooney and Ward (2005), birth-and-death evolution can be detected by two means: a phylogenetic analysis of multigene family members, which will reveal a between-species gene clustering pattern; and an examination of gene sequence divergence levels, in which case a relatively high proportion of changes between gene family members will indicate a lack of homogenization. In the present study we performed such analyses in order to understand the long-term evolution of 5S rDNA in *Ensis* razor shells (Mollusca: Bivalvia). The high levels of 5S rDNA sequence divergence that we found in the species *E. directus* led us to analyze 5S rDNA variation in a broader phylogenetic context. We used sequences from five other *Ensis* species and found evidence of birth-and-death evolution of 5S rDNA, for the first time in molluscs.

Materials and methods

We analyzed six species of *Ensis* (Schumacher 1817) from the Atlantic coast and Chile: *E. directus* (Conrad 1843), *E. macha* (Molina 1792), *E. arcuatus* (Jeffreys 1865), *E. ensis* (Linné 1758), *E. siliqua* (Linné 1758), and *E. goreensis* (Clessin 1888). Specimens were preserved in ethanol, except in the case of *E. goreensis*, for which we used dried museum material (specimen code MNHN17948).

Extraction of genomic DNA was performed from foot tissue using the NucleoSpin Tissue kit (Macherey–Nagel and Co.). For PCR amplifications a pair of primers was designed from *Mytilus edulis* and *M. galloprovincialis* 5S rRNA gene sequences available in the international nucleotide sequence databases (DDBJ/EMBL/GenBank, accession nos. AJ312081–AJ312087; AJ312075–AJ312080) using GeneFisher (Giegerich et al. 1996): 5S-Univ-F (5′-ACCGGTGTTTTCAACGTGAT) and 5S-Univ-R (5′-CGTCCGATCACCGAAGTTAA). Primers had opposite orientation and were separated by 3 bp. Each reaction was performed in a total volume of 25 μl containing ~25 ng genomic DNA, 0.625 U Taq DNA polymerase (Roche Diagnostics), 5 nmol each dNTP (Roche Diagnostics), 20 pmol each primer, and the buffer recommended by the polymerase supplier. PCR conditions were as follows: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min. PCR products were run on agarose gels, stained with ethidium bromide, and imaged under UV fluorescence. Bands ranging from ~450 bp to ~1050 bp were excised from the gel and purified using a DNA Gel Extraction Kit (Millipore). Bands were cloned using Topo TA Cloning (Invitrogen). A QiaPrep Spin Miniprep Kit (Qiagen) was used to purify the plasmids.

Sequencing reactions were performed using both M13 Forward and M13 Reverse primers (included in the cloning kit) in a capillary DNA sequencer CEQ 8000 Genetic Analysis System (Beckman Coulter). The quality of the electropherograms was checked by eye in BioEdit 7.0.9.0. (Hall 1999).

A sequence-similarity search was performed in Blast to determine the similarities of the sequences obtained with other 5S rDNA sequences from DDBJ/EMBL/GenBank. For sequence alignment we used ClustalX 2.08 (Larkin et al. 2007). Tandem repeats were characterized using Tandem Repeats Finder (Benson 1999).

All nucleotide sequence divergence and phylogenetic analyses were conducted in Mega 3.1 (Kumar et al. 2004). The extent of nucleotide sequence divergence was estimated by means of the Tamura–Nei distance (Tamura and Nei 1993), selecting the complete deletion option (i.e., not considering positions with gaps). Standard errors were calculated by the bootstrap option with 1000 replicates. Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) based on the distance matrix. The reliability of the topologies was tested by the bootstrap test (Felsenstein 1985) and by the interior-branch test (Nei et al. 1985; Li 1989; Rzhetsky and Nei 1992) with 1000 replicates. For nucleotide diversity (π, Nei 1987) calculations we used DnaSP 4.0 (Rozas et al. 2003), excluding aligned gaps.

Results

We obtained 72 sequences experimentally. Sequence-similarity searches performed in Blast showed that all our sequences matched with other mollusc 5S rDNA available in the international nucleotide sequence databases, and identification of the 5S rRNA gene was performed by comparison with these sequences.

Many sequences corresponded to monomers formed by the last portion of the gene (88 bp), the NTS, and the first portion of the contiguous gene (32 bp). In two of the species analyzed (*E. directus* and *E. ensis*), we also obtained dimer sequences formed by two contiguous monomers. To maintain the similarity with other 5S rDNA sequences from the international nucleotide sequence databases, the last 32 bp of each sequence (monomers and dimers) was moved to the beginning. We added to our analysis 28 5S rDNA sequences belonging to the species *E. macha*, which were available in DDBJ/EMBL/GenBank, so we studied a dataset of 100 sequences (Table 1).

Table 1. Sequences of 5S ribosomal DNA studied in this work

Clade	Species	Accession number	*n*	π	SD
Alpha	*Ensis directus*	AM904878–AM904918*	41	0.0086	0.0012
Beta	*Ensis macha*	FM201452*; AM906171–AM906180; AM940998–AM941004	18	0.0152	0.0023
Gamma	*Ensis directus*	AM904919–AM904929*	11	0.0217	0.0034
Delta	*Ensis directus*	AM904930–AM904933*	4	0.0019	0.0007
	Ensis arcuatus	FM201453*; FM211693*	2	0	0
Epsilon	*Ensis macha*	AM906203–AM906208; AM941005–AM941009	11	0.0161	0.0017
Zeta	*Ensis siliqua*	FM201457–FM201462*; FM211689*	7	0.0059	0.0006
	Ensis arcuatus	FM201454–FM201456*	3	0.0238	0.0068
	Ensis ensis	FM211690–FM211691*	2	0.0460	0.230
	Ensis goreensis	FM211692*	1	-	-

Accession numbers are from DDBJ/EMBL/GenBank. n, sample size; π, nucleotide diversity; SD, corresponding standard deviation

* Sequences obtained experimentally by the authors

The 5S rRNA gene displayed 22 polymorphic sites in the *Ensis* species, and its size was 120 bp in all cases. The NTS showed a high degree of variation produced by several insertion–deletion polymorphisms (indels), nucleotide substitutions, and microsatellites (in 11 *E. directus* sequences and in one *E. macha* sequence). The size of the NTS region displayed high variation, between 286 and 619 bp.

Phylogenetic analysis of *Ensis* 5S rDNA revealed the existence of six well-supported clades (confidence values: for bootstrap test P B ≥ 96% and for interior-branch test P C ≥ 87%) and three superclades (P B ≥ 97%, P C ≥ 98%) based on differences in the NTS region. Clades were named using Greek letters; alpha, beta, and zeta were given to superclade I, gamma and delta to superclade II, and epsilon to superclade III, based on mean distances between clades and phylogenetic clustering (Fig. 1, Tables 2 and 3). In the gene region, only one fixed mutation separated one clade from the others: a point mutation in position 12 (T → C) that characterized clade zeta.

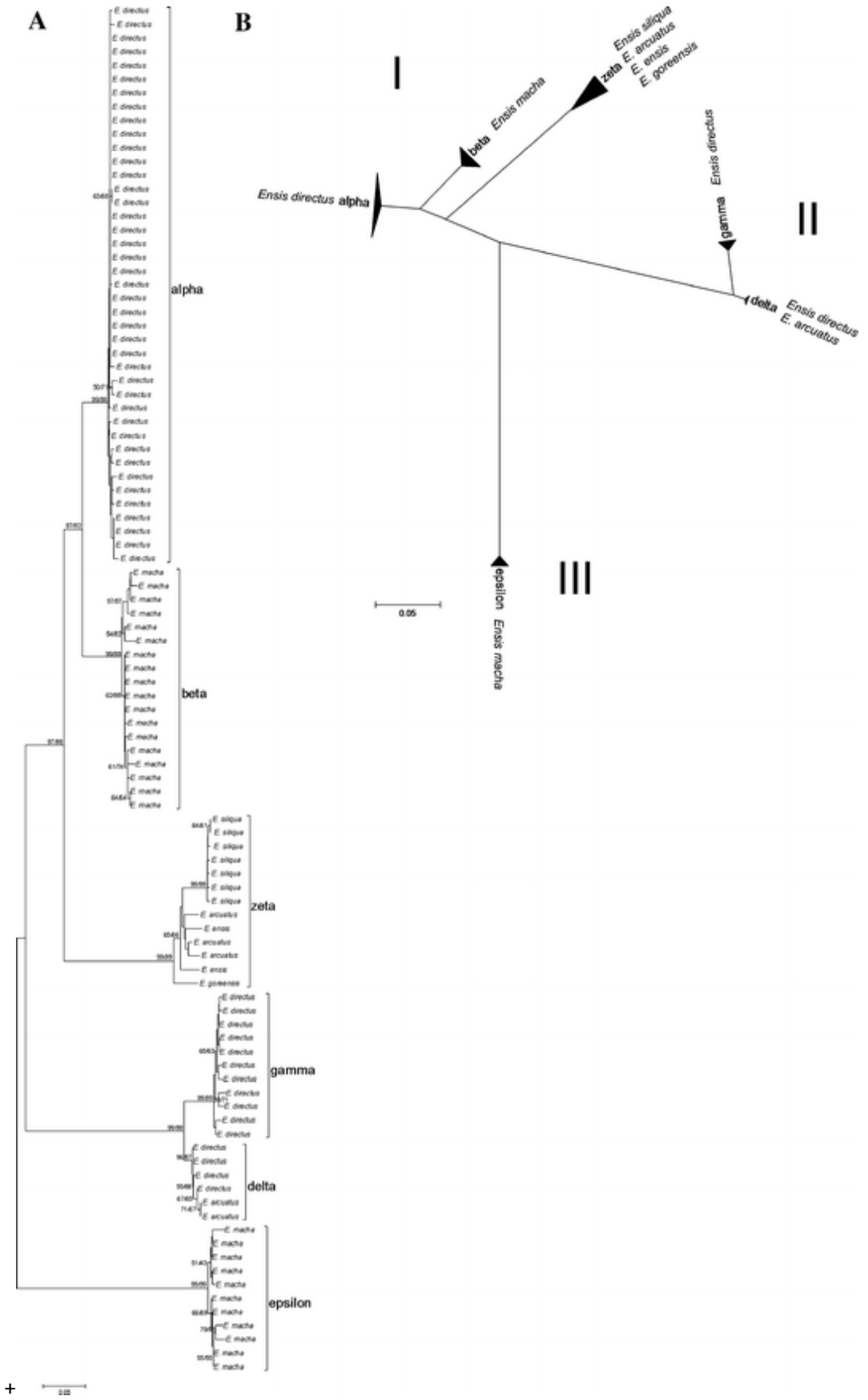

Figure 1. Phylogenetic relationships of 5S rDNA variants in *Ensis* species reconstructed by means of a neighbor-joining phylogenetic tree. Six major clades, based mainly on differences in the NTS region, were identified and named using Greek letters. The tree is unrooted. **A** Decondensed tree with confidence values (P_B and P_C) shown at the internal nodes when $P_B \geq 50$. **B** Condensed tree displaying phylogenetic relationships between the six clades. Clades form three superclades designated I–III

Table 2. Mean sequence divergence[a] between and within clades of *Ensis* 5S rDNA

Clade	Alpha	Beta	Gamma	Delta	Epsilon	Zeta	Distance within clade	Standard error
Alpha	-	0.016	0.038	0.036	0.039	0.028	0.006	0.002
Beta	0.090	-	0.042	0.040	0.041	0.028	0.012	0.003
Gamma	0.330	0.370	-	0.012	0.048	0.048	0.012	0.003
Delta	0.296	0.337	0.055	-	0.048	0.047	0.004	0.002
Epsilon	0.348	0.365	0.459	0.455	-	0.043	0.012	0.003
Zeta	0.223	0.227	0.438	0.422	0.439	-	0.038	0.006

[a] Calculated using the Tamura–Nei distance. Below the diagonal, distance; above the diagonal, corresponding standard error

Table 3. Mean sequence divergence[a] between and within superclades of *Ensis* 5S rDNA

Superclade	I	II	III	Distance within superclade	Standard error
I	-	0.036	0.038	0.097	0.011
II	0.349	-	0.049	0.032	0.006
III	0.369	0.458	-	0.012	0.003

[a] Calculated using the Tamura–Nei distance. Below the diagonal, distance; above the diagonal, corresponding standard error

Sequences of the species *E. directus* clustered in three clades (alpha, gamma, and delta) and in two superclades (I and II). The *E. macha* sequences clustered in beta (superclade I) and in epsilon (superclade III). Sequences from *E. arcuatus* clustered in zeta (superclade I) and in delta (superclade II). All *E. siliqua*, *E. ensis*, and *E. goreensis* sequences were in clade zeta (superclade I) (Fig. 1).

Tamura–Nei mean distances within clades were small (0.004–0.038) but larger between clades (0.055–0.459) (Table 2). In the same way, mean distances within superclades were small, but distance values between superclades were very large (0.349–0.458) (Table 3). Nucleotide diversity (π) was calculated by species within each of the six clades, resulting in small values of 0–0.0460 (Table 1), in comparison with π values obtained for other 5S rDNA bivalve sequences available in DDBJ/EMBL/GenBank (data not shown).

Regarding the organization of 5S rDNA dimers, we found that in the species *E. directus*, in some cases, sequences belonging to different clades were organized in tandem in the same clone. We sequenced 12 dimers alpha–alpha, but also one dimer alpha–gamma and two dimers alpha–delta. Contrarily, the dimer sequenced from *E. ensis* was composed of two zeta monomers.

Discussion

In the present study we found that several 5S rDNA variants, based on differences in the NTS region, occur in the genomes of *Ensis* razor shells. Three *Ensis* had more than one variant in their genomes, and the presence of more variants in the genomes of all the species cannot be discarded.

The 5S rDNA was used to design a genetic identification methodology (Fernández-Tajes and Méndez 2007) by means of PCR-RFLP, for some of the razor shells that we studied in the present work. These authors sequenced a small number of clones per species ($n = 2$), and sequences were not uploaded to DDBJ/EMBL/GenBank. Thus, we cannot compare their sequences with our own. The sequence sizes they provided, however, made us think they amplified only some of the 5S rDNA variants that we found, probably because their extension times in PCR (45 s) were shorter than ours, so shorter variants may have been favored in the amplification process.

The presence of several 5S rDNA variants has been reported in other bivalve molluscs, such as *Mytilus* mussels (Insua et al. 2001), the scallop *Aequipecten opercularis* (López-Piñón et al. 2008), and the cockle *Cerastoderma glaucum* (Freire et al. 2005), and in echinoderms (Caradonna et al. 2007), arthropods (Keller et al. 2006), and chordates (Martins and Galetti 2001; Daniels and Delany 2003). In other examples, 5S rDNA was found to be homogenized within the genome (Brown and Sugimoto 1974). Thus, the presence of multiple variants cannot be considered the rule, but given the examples listed above, neither is it the exception, especially because it was reported in four different animal phyla.

Nucleotide sequence divergence between the main *Ensis* 5S rDNA lineages (superclades I–III) is very large. This indicates that they split long ago, and probably were already present in the genome of the most common ancestor of these species (ancestral polymorphism). New sequence variants continued to emerge during evolution (in superclade I, alpha, beta, and zeta clades were differentiated, and the same happened in superclade II, with gamma and delta clades). The emergence of new variants gives support to the idea that birth-and-death processes are responsible for the extant variation of this multigene family in *Ensis*.

Sequences of 5S rDNA belonging to different *Ensis* species clustered together in the same clades and superclades in the neighbor-joining tree. The lack of homogenization between 5S rDNA variants was evident from the analysis of distances between clades and superclades. Both features suggest again the involvement of birth-and-death processes in the long-term evolution of *Ensis* 5S rDNA. On the other hand, nucleotide diversity values calculated by species within each clade were rather small (e.g., $\pi = 0.0086$ in *E. directus* sequences from clade alpha). This suggests that homogenizing mechanisms are also taking part, reducing sequence divergence within each 5S rDNA variant in each species.

The presence of pseudogenes in a multigene family strongly suggests that the family evolves under a birth-and-death process (Rooney and Ward 2005). In *Ensis*, no evidence of pseudogenes was found. This is probably because both primers anneal in the 5S rRNA gene, and the presence of mutations in the gene would have considerably reduced the chance of amplification, cloning, and sequencing of pseudogenes.

The analysis of the 5S rRNA gene revealed only one diagnostic position (a cytosine in position 12) that was shared by all zeta sequences. Even though we found 22 polymorphic sites within the gene, all of them were point mutations (no indels in the gene) and could be selectively neutral. Purifying selection may have played a big role in maintaining the integrity of the 5S rRNA gene, as was found by Rooney and Ward (2005) and Fujiwara et al. (2009) in filamentous fungi and bitterlings, respectively.

Although the organization of 5S rDNA in *Ensis* chromosomes is still to be investigated, our data provide a very interesting feature in relation to *E. directus* sequences. Dimer sequences sampled from this species showed a striking organization: different 5S rDNA variants were organized in tandem in the same clone. The same has also been reported in two different fish groups, sturgeons (Robles et al. 2005) and bitterlings (Fujiwara et al. 2009), and it was suggested in gray mullets (Gornung et al. 2007). We cannot provide any certain explanation for the organization and the evolutionary processes that are maintaining this apparent organization of 5S rDNA in *E. directus*, until in situ hybridizations using specific probes are performed.

In short, (1) we have found several 5S rDNA variants in *Ensis* species, (2) 5S rDNA main lineages probably split a long time ago and may be a consequence of ancestral polymorphism, (3) new 5S rDNA sequence variants emerged during *Ensis* evolution, (4) phylogenetic analyses revealed a between-species clustering of *Ensis* 5S rDNA variants, (5) a lack of homogenization between variants was evident, (6) homogenizing mechanisms may be taking part within each variant in each species, and (7) scarce variation in the 5S rRNA gene was detected.

We conclude that birth-and-death processes are responsible for the extant variation of *Ensis* 5S rDNA. The low levels of variation found in the 5S rRNA gene are probably a consequence of selective pressures (i.e., purifying and positive selection). In filamentous fungi (Rooney and Ward 2005), sturgeons (Robles et al. 2005), and bitterlings (Fujiwara et al. 2009), the evolution of 5S rDNA was reported to be a consequence of several evolutionary processes. In the same way, the long-term evolution of *Ensis* 5S rDNA seems to be driven by a combination of birth-and-death processes and selection.

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