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Sperm Nuclear Basic Proteins of Two Closely Related Species of Scorpaeniform Fish (*Sebastes maliger*, *Sebastolobus* sp.) With Different Sexual Reproduction and the Evolution of Fish Protamines

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ABSTRACT In this paper, we present a review of sperm nuclear basic proteins (SNBPs) in teleost fish. The distribution of the three basic groups of SNBPs [histone (H)-type, protamine-like (PL)-type and protamine (P)-type], their evolution and possible relation to the mode of fertilization are described. In this regard, we have characterized the SNBPs from two closely related species of Scorpaeniform fish: internally fertilizing *Sebastes maliger* and externally fertilizing *Sebastolobus* sp., both in the family Scorpaenidae. Despite the different reproductive behavior of these two closely related rockfish species, in both instances the SNBP consists of protamines. However, there is a significant increase in the arginine content of the protamine in the internally fertilizing rockfish. The relevance of this observation is discussed within the context of the P-type SNBP in teleosts. The rapid evolution of teleost protamines, including those in rockfish, has also allowed us to obtain a molecular phylogeny for this group of bony fish that is almost indistinguishable from that currently available from the use of conventional anatomical/paleontological markers. *J. Exp. Zool. 305A:277–287, 2006.*

SPERM NUCLEAR BASIC PROTEINS FROM FISH

From the very early attempts of chemical characterization of the chromosomal components, it became clear that the chromatin associated proteins could be mainly grouped into two different types: histones and protamines (Miescher, 1874; Kossel, 1884; Kossel, '28). Indeed it was from salmon (*Salmo salar*) sperm that the first evidence for the existence of protamines was obtained (Miescher, 1874).

The meaning of these two terms, histones and protamines, has evolved over the years and they now both describe two well-defined groups of chromosomal proteins. Histones are the major protein components of somatic chromatin. They consist generically of two main families, core histones and linker histones. Core histones (histones H2A, H2B, H3 and H4) are responsible for the arrangement of genomic DNA into discrete chromatin globular structures. Approximately

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Abbreviations: Explanation AU-PAGE, acetic acid-urea polyacrylamide gel electrophoresis; H, histone; P, protamine; PL, protamine-like protein; SNBPs, sperm nuclear basic proteins.

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146 bp are wrapped about a histone octamer ''core'' resulting in structures known as nucleosome core particles. Linker histones of the H1 family bind to the ''linker'' DNA connecting adjacent nucleosomes and contribute to stabilizing the folding of the resulting chromatin fiber (van Holde, '88). In contrast, protamines (Felix, '60; Ando et al., '73; Oliva and Dixon, '91) are only found in the sperm chromatin of certain organisms. These are highly arginine-rich proteins of relatively small size (usually smaller than 100 amino acids) (Ausió, '99). These proteins bind to DNA with high affinity and a strong electrostatic component that leads to the almost complete neutralization of the genomic DNA charge and results in a heterogeneous variety of molecular structures (Lewis et al., 2003).

From the early studies on fish (Miescher, 1874; Kossel, '28; Felix, '60), it has been clear that the sperm nuclear basic protein (SNBP) composition of this group is very heterogeneous. Whereas some fish species contain protamines in their sperm nucleus, others contain somatic-like histones.

Further analysis of the SNBPs over a larger variety of metazoans showed that this SNBP heterogeneity could be extended to other groups of organisms, including plants, and led to the realization that this protein composition could indeed be more complex (Bloch, '69; Kasinsky et al., '85). The comprehensive study by David Bloch led him to the classification of SNBPs into four major groups (Bloch, '62, '69; Kasinsky, '89): protamines, stable protamines (containing cysteine), intermediate sperm basic proteins (di- and tri-protamines), and somatic-like histones. The detailed molecular characterization of these proteins carried out more recently has allowed us to simplify the classification to three main SNBP types: histone (H)-type, protamine (P)-type, and protamine-like (PL)-type (Ausió, '95, '99).

Protamine-like proteins are an intermediate group of sperm chromosomal proteins that, ''like protamines'', can displace somatic histones from the nucleus at the end of spermiogenesis. They exhibit an amino acid composition rich in both lysine and arginine and have an enormous structural variability. All the proteins of this group appear to be evolutionarily related to the histone H1 family of chromosomal proteins (Ausió, '99; Lewis et al., 2004). This SNBP type also occurs in fish (Saperas et al., '93b; Ausió, '95; Watson and Davies, '98; Watson et al., '99).

SNBP EVOLUTION IN FISH: VERTICAL VERSUS HORIZONTAL

The SNBPs from fish have now been extensively characterized (Ando et al., '73; Kasinsky, '89; Oliva and Dixon, '91; Chiva et al., '95; Saperas et al., '97). While all the chondrichthyan (cartilaginous fish) SNBPs appear to belong to the P-type (Sautiere et al., '81; Saperas et al., '96; Wouters-Tyrou et al., '98), amongst the bony fish, representative species belong to the three types (Daisley and Davies, '82; Kasinsky, '89; Saperas et al., '94a).

The question thus arising is whether such sporadic distribution of SNBP types across fish species (Saperas et al., '93a) is random or is the result of careful adaptation under physiological constraints. Based on the findings that the genes from the rainbow trout are flanked by DNA sequences which are reminiscent of the long terminal repeats of avian retroviruses (Jankowski et al., '86), it was proposed that the appearance of protamines in fish could be the result of horizontal transmission, by which species, originally containing the H-type SNBP, would have randomly acquired the protamine genes. Indeed, viruses contain many arginine-rich proteins. However, as we shall describe next, there are other more likely alternatives to this hypothesis (Saperas et al., '94a).

The horizontal transmission hypothesis assumes that, in their evolutionary origin, the ancestors of teleost fish had the H-type SNBP (Jankowski et al., '86; Krawetz and Dixon, '88; Moir and Dixon, '88a, b; Oliva and Dixon, '91). While this is the case for agnathans (Chiva et al., '95; Saperas et al., '97), this is in contrast to the presence of protamines in the more primitive cartilaginous fish (Chiva et al., '95). Moreover, an exhaustive analysis of the occurrence of the different SNBP types in teleosts (Saperas et al., '94a) conclusively showed that the divergence amongst the SNBPs into H- and P-type has occurred repeatedly in different evolutionary lines. The relative frequency of this phenomenon is almost negligible during the differentiation of genera and species. It is very small during the differentiation of families, while being very noticeable among different orders (Saperas et al., '94a).

Based on these data, a more likely alternative is that protamines, which are prevalent in teleost fish, were already present in the bony fish ancestor, and histones occurred as a result of a reversion to the H-type found in more primitive organisms, such as echinoderms (Poccia et al., '87).

In other words, the acquisition of the H-type would be viewed under this proposal as a reversion of the P-type (Ausió, '99) that obviously has repeated itself several times in the course of fish evolution and is also present in other vertebrate groups, such as amphibians (Kasinsky et al., 2005).

Although the determinants for the selection of one SNBP type over the other are still not clear, it appears that the mode of fertilization is a physiological adaptation that can constrain this selection almost exclusively to the P-type preventing the reversions to the H-type, as will be discussed next.

DOES INTERNAL FERTILIZATION CONSTRAIN THE RANGE OF SNBP DIVERSITY IN TELEOST FISH?

An analysis of SNBP distribution in animals (Kasinsky et al., '85; Kasinsky, '89, '95) suggests that the mode of fertilization might serve as a constraint on SNBP diversity. For example, amongst the chordates, externally fertilizing fish show a diversity of SNBP types from somatic-like histones (H) in lamprey (Fig. 1A, lane 1), to protamine-like proteins (PL) in yellow goatfish (Fig. 1A, lane 2), to arginine-rich protamines (P) in chum salmon (Fig. 1A, lane 3). On the other hand, internally fertilizing cartilaginous fish, urodeles and amniotes (reptiles, birds and mammals) have either protamines (P) or keratinous protamines with disulfide bonds in their sperm nuclei.

Internally fertilizing sperm tend to be elongated with a more condensed nucleus (Franzen, '77; Baccetti, '82; Wirth, '84; Jamieson, '91). Kasinsky and others (Kasinsky et al., '85; Kasinsky, '89, '95) have hypothesized that SNBPs in internally fertilizing animals tend to be more protamine-like than histone-like. This may be due to the fact that during internal fertilization, the sperm have to move through the more viscous reproductive tract of the female rather than seawater. This requires the chromatin in the sperm head to be more compacted in order to resist drag forces and thereby increase the efficiency of movement. Protamines are smaller structural proteins than histones and richer in arginine side chains. Since arginine residues can bind strongly to the phosphodiester backbone of DNA with more hydrogen bonds than lysine residues, as well as by electrostatic interaction (Helene and Lancelot, '82; Ausio et al., '84), the SNBPs of internally fertilizing animals should be enriched in arginine over lysine residues with respect to SNBPs in externally fertilizing animals. This will enhance the compaction of chromatin.

To test this hypothesis, we have examined the electrophoretic patterns (Fig. 1B, C) from three of the six orders of teleost fish with internal fertilization (Fig. 1D); (Wourms, '81): order Perciformes (Nelson, '94), family Embiotocidae, *Cymatogaster aggregata* (shiner perch; Fig. 1C, lane 12); order Scorpaeniformes, family Scorpaenidae, subfamily Sebastinae, *Sebastes maliger* (quillback rockfish; Fig. 1B, lanes 6, 7); order Cyprinodontiformes, family Poeciliidae, subfamily Poeciliinae, tribe Poeciliini, *Poecilia reticulata* (black-banded guppy; Fig. 1C, lane 8), *Poecilia picta* (guppy; Fig. 1C, lane 9), *Xiphophorus helleri guentheri* (swordtail; Fig. 1C, lane 10), *Xiphophorus maculatus* (platyfish; Fig. 1C, lane 11).

Figure 1C indicates that each of the internally fertilizing teleosts in the orders Perciformes and Cyprinodontiformes have protamine as their SNBP type. This is supported (Table 1) by an arginine content of 66.7 mol% for the shiner perch *C. aggregata*. The presence of electrophoretic bands in the histone region of the gel in Fig. 1C may be due to somatic cells of the testes in these preparations. In *C. aggregata* (order Perciformes), sperm are produced in spermatophores with about 600 sperm bundled together with an extracellular capsule (Jamieson, '91). This differs from the loosely bundled sperm of poeciliids called spermatozeugmata (Jamieson, '91); for example, in *X. helleri*, where the sperm are not encapsulated (Wourms, '81) and the sperm heads are located at the periphery of a ''sperm ball'' with a core of spirally coiled flagellae. According to Jamieson ('91), this indicates clearly that internal fertilization has evolved independently in these two orders. This, in turn, suggests that the presence of protamines in internally fertilizing fish in the orders Perciformes and Cyprinodontiformes is due to convergent evolution, and that protamines must play a similar functional role in these divergent species.

In the order Scorpaeniformes, family Scorpaenidae, after the quillback rockfish, *Sebastes maliger* (subfamily Sebastinae) displays matrotrophic viviparity (Love et al., 2002); that is, internal fertilization, the embryo receives some nutrition from the mother, and is born as a larval form. On the other hand, the externally fertilizing rockfish *Sebastolobus* sp. (subfamily Sebastolobinae) is oviparous. Figure 1B indicates that both species

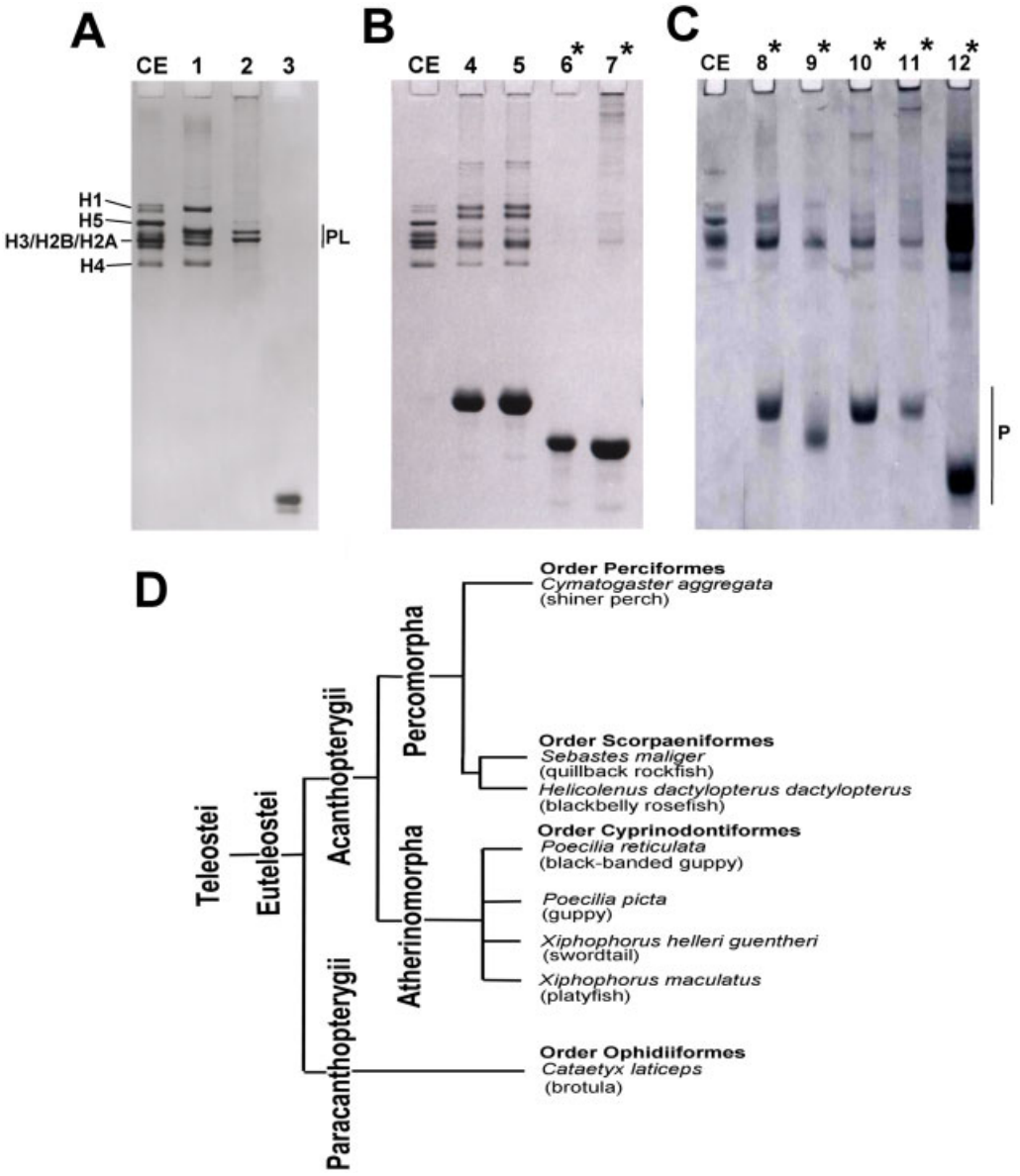

Fig. 1. Acetic acid (5%)–urea (2.5 M) (AU)-polyacrylamide gel electrophoresis (PAGE) analysis of sperm nuclear basic proteins (SNBPs) from internally and externally fertilizing fish: (**A**) (1), *Petromyzon marinus* (lamprey; E.F.); (2), *Mullus surmuletus* (yellow goatfish; E.F.); (3), *Oncorhynchus keta* (chum salmon; E.F.). CE is a chicken erythrocyte histone marker. H1/H5: linker histones; H2A, H2B, H3, H4: core histones; PL: protamine-like SNBPs; (**B**) (4), *Sebastolobus* sp. (thornhead rockfish: E.F., sperm fraction); (5) *Sebastolobus* sp. (testes fraction); (6∗) *Sebastes maliger* (quillback rockfish; I.F., sperm fraction); (7∗), *S. maliger* (testes fraction). (**C**) (8∗), *Poecilia reticulata* (black-banded guppy; I.F.); (9∗), *Poecilia picta* (guppy; I.F.); (10∗), *Xiphophorus helleri* (swordtail; I.F.); (11∗), *Xiphophorus maculatus* (platyfish; I.F.); (12∗) *Cymatogaster aggregata* (shiner perch; I.F.). SNBPs from all these organisms were prepared as described in Gimenez-Bonafe et al. (2000) and the AU-PAGE were carried out as described elsewhere (Kasinsky et al., 2005). (**D**) Phylogeny of internally fertilizing teleost fish examined for SNBPs in this paper (six species) and in the literature (two species; Saperas et al., '93b, '94a; Chiva et al., '95). Phylogeny adapted from Nelson ('94), frontispiece. Electrophoresis is from top to bottom. I.F. and E.F. denote an internally and externally fertilizing fish species, respectively. ∗I.F. species. P: protamine.

contain an electrophoretically fast protein component that runs in the region corresponding to a SNBP of the P-type.

The most striking difference between the electrophoretic patterns of these two rockfish, besides the difference in mobility of their major protamine

TABLE 1. Amino acid composition (mol%) of SNBPs from testes of internally and externally fertilizing fish

	Cymatogaster aggregata[1] (shiner perch)	*Sebastes maliger*[2] (quillback rockfish)[3]	*Sebastolobus* sp.[2] (thorny head rockfish)	*Oncorhynchus keta*[4] (chum salmon)	*Mullus surmuletus*[5] (yellow goatfish)	*Petromyzon marinus*[6] (lamprey)
	P	P	P	P	PL	H1
Lys	0.1	—	3.7	—	24.2	27.6
His	0.6	—	—	—	—	—
Arg	66.7	65.1	48.1	67.2	22.1	2.2
Asx	0.2	—	1.9	—	5.1	3.9
Thr	2.9	4.7	11.1	—	4.5	3.6
Ser	4.5	4.7	5.6	9.9	9.2	7.3
Glx	0.4	—	1.9	—	t	4.7
Pro	3.3	4.7	5.6	9.1	6.8	9.7
Gly	9.5	4.7	3.7	6.5	5.4	4.0
Ala	3.2	7.0	9.3	1.3	11.9	21.8
1/2 Cys	—	—	—	—	—	—
Val	0.3	7.0	7.4	4.7	4.9	6.8
Met	0.3	—	—	—	t	—
Ile	0.1	—	—	1.3	t	1.1
Leu	2.0	—	1.9	—	5.8	5.3
Tyr	0.3	2.3	—	—	t	0.8
Phe	7.5	—	—	—	t	1.1
Trp	—	—	—	—	—	—

[1]After high performance liquid chromatography, amino acid analysis was carried out as described by Kasinsky et al. (2005).
[2]Calculated from primary structure.
[3]Denotes an internally fertilizing fish species.
[4]Data from Ando et al. ('73).
[5]Data from Chiva et al. ('95).
[6]Data from Saperas et al. ('94b).
P, protamine; PL, protamine-like SNBP; H1, very lysine-rich H1; t, trace; SNBP, sperm nuclear basic protein.

components, is the coexistence with these proteins of a substantially larger amount of SNBP of the H-type in externally fertilizing *Sebastolobus* sp. (Fig. 1B, lanes 4, 5) when compared to internally fertilizing *S. maliger* (Fig. 1B, lanes 6, 7). While this difference could, in principle, be ascribed to differences in the extent of sexual maturity of the gonadal tissue, with the presence of histones indicating the occurrence of immature spermatogenic cells, it could also be the result of an incomplete replacement of histones by protamines during the spermatogenic process (Oliva and Dixon, '91). Hence, it could be taken as an indication of the more primitive nature of the sperm of externally fertilizing *Sebastolobus*.

The amino acid sequences of the two main protamine components in *S. maliger* and *Sebastolobus* are remarkably similar (Fig. 2A), as would be expected for two closely related species. However, careful comparison of the two sequences (Fig. 2B) indicates that selection for internal fertilization has increased the arginine content of protamine in internally fertilizing *S. maliger* to 65.1 mol% from 48.1 mol% in the protamine of externally fertilizing *Sebastolobus* sp., (Table 1) and has also removed two lysine residues. Arginine residues not only bind DNA more tightly than lysine residues by ionic interactions, but also display more flexibility in hydrogen bonding (Cheng et al., 2003). The observation that *S. maliger* sperm has a lower histone content than *Sebastolobus* sperm, as well as a lower molecular weight protamine that lacks asparagine and glutamine residues, suggests that the more ''primitive'' externally fertilizing *Sebastolopus* sp. also possesses a ''primitive'' protamine. By the same token, the more ''advanced'' internally fertilizing *S. maliger* has acquired an ''advanced'' protamine to cope with tighter binding to DNA.

Thus far, the data provide support for the hypothesis that the evolution of internal fertilization in vertebrate organisms imposes a strong constraint on the range of SNBP diversity, selecting for arginine-rich protamines (Kasinsky et al., '85, Kasinsky, '89, '95) as a result of different structural and functional advantages of

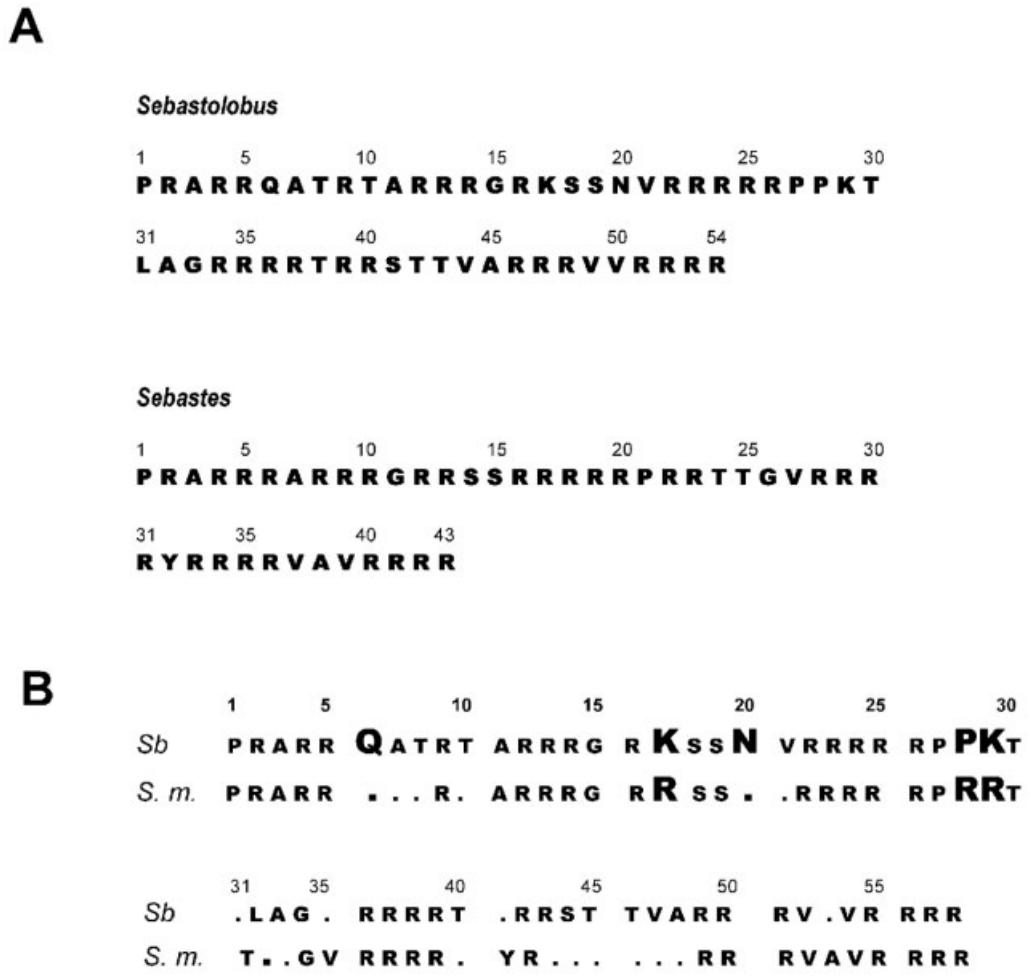

Fig. 2. (**A**) Primary structures of the main protamine components of the sperm of the two rockfish analyzed in this paper. Protein sequencing was performed as described in Jutglar et al. ('91). (**B**) Visual alignment of the sequences shown in (A) highlighting the amino acid similarity and differences between the two sequences. Externally fertilizing *Sb*: *Sebastolobus* sp. and internally fertilizing *S.m.*: *Sebastes maliger*. The squares point to amino acid deletions and the bold letters point to the disappearance of Q, K, N and P from the *Sebastolobus* sp. protamine and their partial replacement by R in the protamine of *S. maliger*. During this transition, the arginine content of the protamine increases from 48.1% (mol/mol) in *Sebastolobus* sp. protamine to 65.1% in *S. maliger* protamine (Table 1).

arginine. This is also indicated by the presence of a protamine (Saperas et al., '94a) in internally fertilizing (Wourms, '81; Wourms and Lombardi, '92) *Helicolenus dactylopterus dactylopterus* (order Scorpaeniformes, family Scorpaenidae, subfamily Sebastinae; (Nelson, '94). This is the same subfamily that includes internally fertilizing *S. maliger*.

There is one exception, however. Internally fertilizing *Cataetyx laticeps* (order Ophidiiformes, family Bythitidae) is a benthic, deep-sea viviparous fish. This is the only internally fertilizing fish, amongst the eight species from four orders examined so far, whose sperm contain an arginine-enriched PL basic protein (Saperas et al., '93a; Chiva et al., '95), along with histones, but no protamine. This may be related to the particular reproductive biology of such deep-sea brotulas. These teleosts have a progenetic tendency (Marshall, '84) towards relatively short larval stages (Moyle and Cech, '88) in order to facilitate the settling of a small number of young in their benthic environment.

RAPID EVOLUTION OF FISH PROTAMINES

In contrast to other SNBP types, the proteins of the P-type appear to be present in organisms at the tips of the evolutionary branches of both

protostome and deuterostome organisms (Ausió, '95, '99; Lewis et al., 2003) and are noted for being among the proteins with the highest rate of evolution (Oliva and Dixon, '91; Oliva, '95). This trait, which is shared with other reproductive proteins (Wyckoff et al., 2000; Swanson and Vacquier, 2002), makes protamines a very valuable tool as precise markers for evolution (Hunt et al., '96; Ausió et al., '99; Lemke et al., '99; Gimenez-Bonafe et al., 2000; Lewis et al., 2003).

The protamine sequences of the two species of rockfish (Fig. 2), one internal fertilizer (*S. maliger*) and the other an external fertilizer (*Sebastolobus* sp.), represent the first protein sequence information on protamines from the order Scorpaeniformes. We have included this information within a broader taxonomic context encompassing other previously published protamine sequences from externally fertilizing species of fish in six orders (Saperas et al., '94a) to reconstruct the first comprehensive phylogenetic tree of teleost fishes based on protamines (Figs. 3 and 4). The internally fertilizing chondrichthyan *Scyliorhinus canicula*, was chosen as the outgroup. The presence of

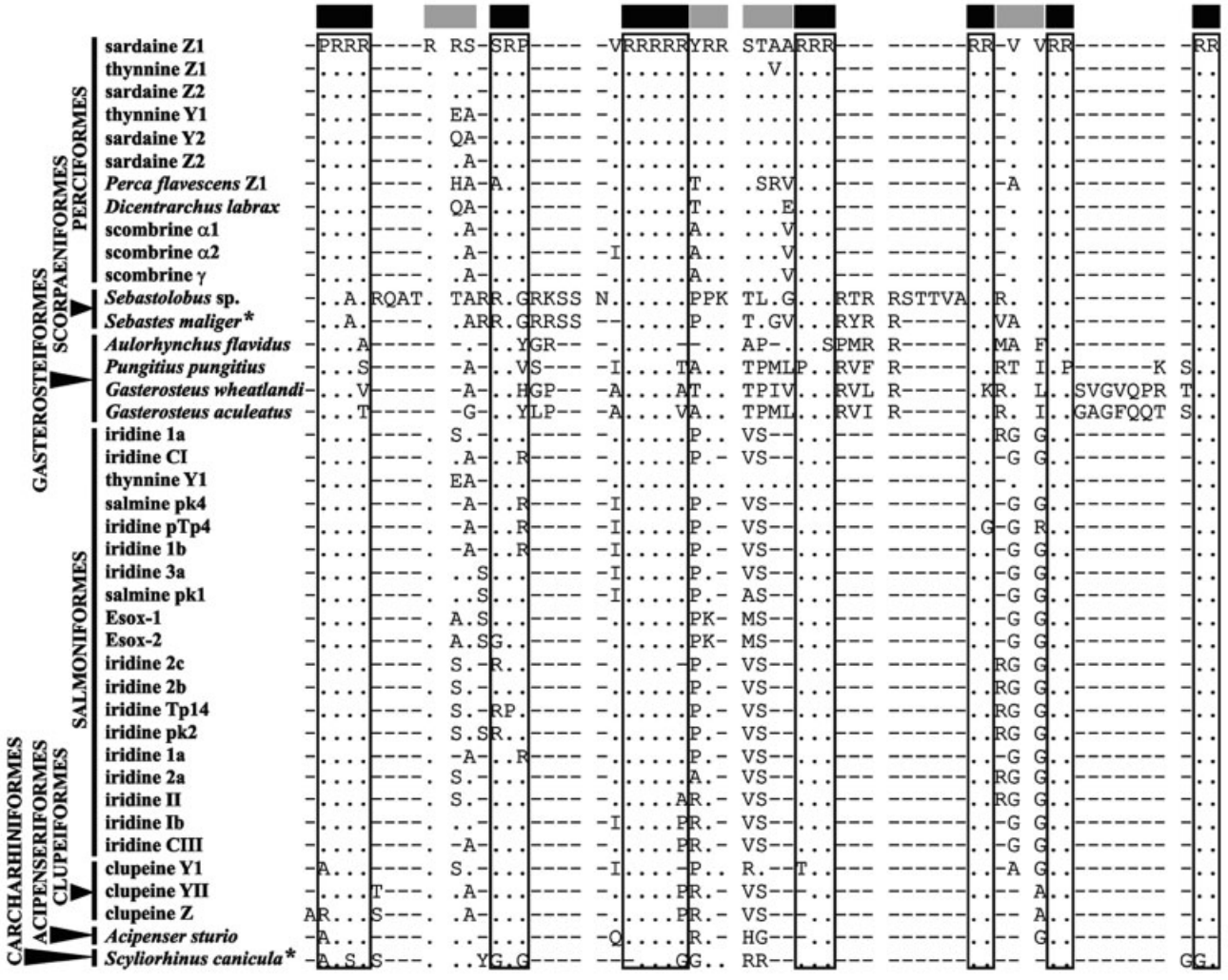

Fig. 3. Sequence alignment of protamines from teleost fish from the orders Perciformes, Scorpaeniformes, Gasterosteiformes, Salmoniformes, Clupeiformes, and Acipenseriformes (Nelson, '94). The protamine of the internally fertilizing chondrichthyan *Scyliorhinus canicula* (order Carcharhiniformes) is also included in the analysis as an outgroup. Sequences were aligned using the default parameters given by the program CLUSTAL_X (Thompson et al., '97) and were visually inspected for errors. Similar residues are indicated by squares and new amino acids are indicated by the corresponding amino acid replacements, taking sardaine Z1 protamine as a reference. Indel events are designated by dashes and regions of high and low homology are identified by black and gray bars, respectively. *An internally fertilizing fish species.

highly conserved homologous regions in teleosts can easily be ascertained from inspection of the alignments shown in Fig. 3, which have been used to reconstruct the maximum parsimony tree shown in Fig. 4. The phylogeny thus obtained closely matches the taxonomic relationships obtained from anatomical characters (Nelson, '94) and underscores once again the validity and relevance of protamines as evolutionary markers, most likely due to their rapid evolution. These results also provide further support to the theory of the vertical evolution of protamines (Ausió, '99). Although still controversial (Clark and Civetta, 2000), the rapid evolution of protamines has been ascribed recently to a positive Darwinian (adaptive) selection process (Rooney and Zhang, '99; Wyckoff et al., 2000; Swanson and Vacquier, 2002), rather than to a relaxation in the evolutionary constraints as had been proposed earlier (Retief and Dixon, '93). It is very likely that this adaptive process has been determined by the functional constraints imposed by chromatin condensation in the sperm nucleus. The term positive selection refers here to selection ''in relation to sex'', common to many genes expressed in male reproductive tissues (Eberhard, '85; Wyckoff et al., 2000).

With regard to the mammalian protamines P1 and P2 from primates and old world monkeys, positive selection was inferred from the significantly greater non-synonymous substitution rates detected in comparison with rates at neutral (intron and synonymous positions) sites (Rooney and Zhang, '99). From a more general perspective, the selection of arginine-rich protamines in the vertebrate lineage may have had its origin through a frameshift mutation in PL-I, a sperm-specific lysine-rich histone H1 (PL-I) in a primitive chordate (Lewis et al., 2004). This mechanism involves a few rapid changes at the protein level, which later were positively selected.

It thus appears that the driving force behind protamine evolution in vertebrates is a selection process that acts at the amino acid level and favors a high arginine content. Three structural/functional determinants are possibly responsible for this selection. First, an increase in arginine content (probably at the expense of lysine residues) increases the affinity of a protein for DNA (Puigdomenech et al., '76; Ausio et al., '84) and

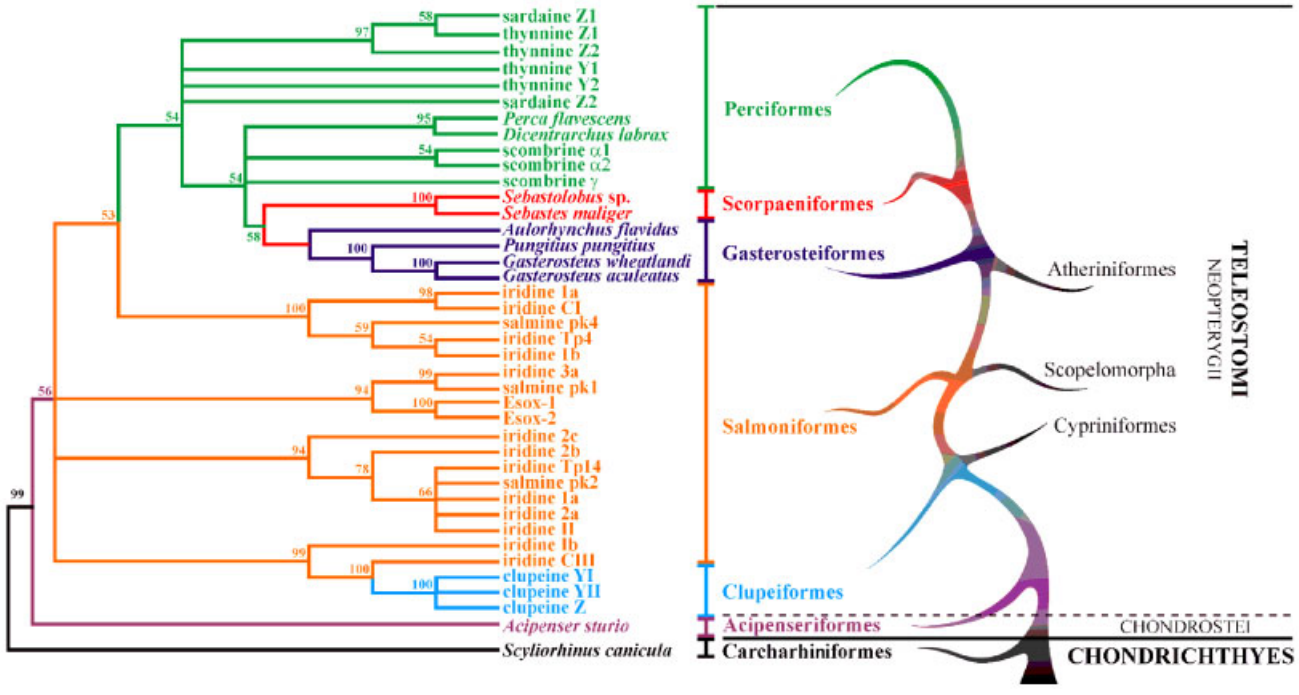

Fig. 4. (**A**) Phylogenetic relationships among teleost protamines revealed by maximum parsimony. The numbers at the branching points represent the consensus value for the corresponding nodes, obtained from the 1,148 MP trees found in the search process, and are only shown when the value is higher than 50%. The consensus tree was rooted with the sequence of the internally fertilizing chondrichthyan *Scyliorhynus canicula*. (**B**) Schematic ''tree of life'' of teleost fish evolution adapted from Nelson ('94) to highlight the good correlation between the two phylogenetic approaches.

confers a greater flexibility in the formation of hydrogen bonds with the DNA backbone (Cheng et al., 2003), thus allowing a tighter chromatin condensation in the sperm nucleus. Second, it has been shown that arginine plays a special role at the time of sperm–egg fertilization. For instance, salmon protamine is able to activate the regulatory protein casein kinase II (CK-II) in fertilized eggs by way of polyarginine clusters (Ohtsuki et al., '96). In contrast, polylysine clusters cannot activate CK-II, suggesting that constraints other than the basic charge are responsible for the arginine selection. Finally, the sperm competition hypothesis has been invoked in hominoid and old world monkeys in order to account for an arginine-rich selection in determining different shapes for the sperm nucleus (Harcourt, '91; Rooney and Zhang, '99).

In the case of the rapidly evolving protamines of teleost fish (Lemke et al., '99; Gimenez-Bonafe et al., 2000), clues for selection favoring arginine can also be found. In the two species highlighted in this review, the protamine of the internally fertilizing rockfish *S. maliger* exhibits a higher arginine content (65.1 mol%) (Table 1) than that of the externally fertilizing *Sebastolobus* (48.1 mol%). In addition, the *S. maliger* sperm nucleus has a lower histone content (Fig. 1B) and the protamines are of lower molecular weight and lack asparagine and glutamine residues (Fig. 2). These observations can be interpreted in the light of the primitive nature of protamines in *Sebastolobus*. The more advanced protamines of *S. maliger* would represent an adaptation to cope with its more advanced mode of internal fertilization, as has already been discussed in the preceding section.

CONCLUDING REMARKS

Despite the protein divergence resulting from the high rate of evolution, it is important to note that the relative proportion of arginine residues remains fairly constant (50–70 mol%) in protamines from different taxonomic groups (Rooney et al., 2000), including both internally and externally fertilizing teleost fish (Table 1). In mammalian protamines, both the total number of amino acids and the positions of arginine residues have substantially changed during evolution. This pattern differs from that of most proteins, where site-specific amino acid conservation is maintained and the non-synonymous variation is much lower than the synonymous variation (Kimura, '83; Nei, '87). This phenomenon was analyzed by Rooney et al. (2000) in terms of nucleotide frequencies and substitutions in mammalian protamine P1. All six codons for arginine have G in the second position. Selection pressure must operate therefore so as to maintain G at a high frequency in the second position in order to elevate arginine levels in protamines. In addition, non-synonymous substitution rates are not as low as expected under ''canonical'' purifying selection. These results, which were also verified in the case of several non-mammalian vertebrate protamine genes and even in an insect species, led these authors (Rooney et al., 2000) to suggest that protamines are under an ''unusual'' form of purifying selection that maintains a high arginine content at the protein level, rather than conserving the position of arginine residues.

It seems clear now that in protamine evolution, both positive Darwinian (adaptive) evolution and an unusual form of purifying selection are tumbling the dice under strict constraints imposed by structural and functional demands. As a result, protamines emerge as a valuable marker that allows us to trace the phylogenetic history of different vertebrate groups, including teleost fish (Fig. 4), in a way that mirrors the results obtained using other more conventional molecular or anatomical markers.

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LITERATURE CITED

Ando T, Yamasaki M, Suzuki K. 1973. Protamines: isolation, characterization and function. New York, NY: Springer.

Ausió J. 1995. Histone H1 and the evolution of the nuclear sperm specific proteins. In: Jamieson BGM, Ausió J, Justine JL, editors. Advances in spermatazoal phylogeny and taxonomy, Vol. 166. Paris: Memoires de Museum National d'Histoire Naturelle. p 501–514.

Ausió J. 1999. Histone H1 and evolution of sperm nuclear basic proteins. J Biol Chem 274:31115–31118.

Ausio J, Greulich KO, Haas E, Wachtel E. 1984. Characterization of the fluorescence of the protamine thynnine and studies of binding to double-stranded DNA. Biopolymers 23:2559–2571.

Ausió J, Soley JT, Burger W, Lewis JD, Barreda D, Cheng KM. 1999. The histidine-rich protamine from ostrich and tinamou sperm. A link between reptile and bird protamines. Biochemistry 38:180–184.

Baccetti B. 1982. The evolution of the sperm tail. Symp Soc Exp Biol 35:521–532.

Bloch DP. 1962. Symposium: Synthetic processes in the cell nucleus. I Histone synthesis in non-replicating chromosomes. J Histochem Cytochem 7:137–144.

Bloch DP. 1969. A catalog of sperm histones. Genetics 61 (Suppl.):93–111.

Cheng AC, Chen WW, Fuhrmann CN, Frankel AD. 2003. Recognition of nucleic acid bases and base-pairs by hydrogen bonding to amino acid side-chains. J Mol Biol 327: 781–796.

Chiva M, Saperas N, Caceres C, Ausió J. 1995. Nuclear basic proteins from the sperm of tunicates, cephalochordates, agnathans and fish. In: Jamieson BGM, Ausió J, Justine JL, editors. Advances in spermatazoal phylogeny and taxonomy, Vol. 166. Paris: Memoires de Museum National d'Histoire Naturelle. p 501–514.

Clark AG, Civetta A. 2000. Evolutionary biology. Protamine wars. Nature 403:261–263.

Daisley SL, Davies PL. 1982. Divergence of protamine gene sequences in fish. Biochim Biophys Acta 698:271–279.

Eberhard WG. 1985. Sexual selection and animal genitalia. Cambridge, MA: Harvard University Press.

Felix K. 1960. Protamines. Adv Protein Chem 15:1–56.

Franzen A. 1977. Sperm structure with regard to fertilization biology and phylogenetics. Verhandl Deutsch Zool Ges 1977:123–138.

Gimenez-Bonafe P, Laszczak M, Kasinsky HE, Lemke MJ, Lewis JD, Iskandar M, He T, Ikonomou MG, White FM, Hunt DF, Chiva M, Ausió J. 2000. Characterization and evolutionary relevance of the sperm nuclear basic proteins from stickleback fish. Mol Reprod Dev 57: 185–193.

Harcourt AH. 1991. Sperm competition and the evolution of non-fertilizing sperm in mammals. Evolution 45:314–328.

Helene C, Lancelot G. 1982. Interactions between functional groups in protein–nucleic acid associations. Prog Biophys Mol Biol 39:1–68.

Hunt JG, Kasinsky HE, Elsey RM, Wright CL, Rice P, Bell JE, Sharp DJ, Kiss AJ, Hunt DF, Arnott DP, Russ MM, Shabanowitz J, Ausió J. 1996. Protamines of reptiles. J Biol Chem 271:23547–23557.

Jamieson BGM. 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge, UK: Cambridge University Press.

Jankowski JM, States JC, Dixon GH. 1986. Evidence of sequences resembling avian retrovirus long terminal repeats flanking the trout protamine gene. J Mol Evol 23: 1–10.

Jutglar L, Borrell JI, Ausió J. 1991. Primary, secondary, and tertiary structure of the core of a histone H1-like protein from the sperm of *Mytilus*. J Biol Chem 266:8184–8191.

Kasinsky HE. 1989. Specificity and distribution of sperm basic proteins. In: Hnilica LS, Stein GS, Stein JL, editors. Histones and Other Basic Nuclear Proteins. Boca Raton, FL: CRC Press Inc. p 73–163.

Kasinsky HE. 1995. Evolution and origins of sperm basic proteins. In: Jamieson BGM, Ausió J, Justine JL, editors. Advances in spermatazoal phylogeny and taxonomy, Vol. 166. Paris: Memoires de Museum National d'Histoire Naturelle. p 447–462.

Kasinsky HE, Mann M, Lemke M, Huang SY. 1985. Diversity of sperm basic chromosomal proteins in vertebrates: a phylogenetic point of view. In: Reeck GR, Goodwin GH, Puigdomenech P, editors. Chromosomal proteins and gene expression. New York, NY: Plenum Press. p 333–359.

Kasinsky HE, Frehlick LJ, Su HW, Ausio J. 2005. Protamines in the internally fertilizing neobatrachian frog *Eleutherodactylus coqui*. Mol Reprod Dev 70:373–381.

Kimura M. 1983. The neutral theory of molecular evolution. Cambridge, UK: Cambridge University Press.

Kossel A. 1884. Uber einen peptonartigen bestandteil des zellkerns. Z Physiol Chem 8:511–515.

Kossel A. 1928. The protamines and histones. London, New York, Toronto: Longmans, Green and Co.

Krawetz SA, Dixon GH. 1988. Sequence similarities of the protamine genes: implications for regulation and evolution. J Mol Evol 27:291–297.

Lemke MJ, Chiva M, Coyle R, Kulak D, MacKay M, Kasinsky HE. 1999. Variability of sperm nuclear basic proteins in the three-spine stickleback and related species of gasterostoidei. Comp Biochem Physiol 122B:339–353.

Lewis J, Song Y, de Jong M, Bagha S, Ausió J. 2003. A walk though vertebrate and invertebrate protamines. Chromosoma 111:473–482.

Lewis JD, Saperas N, Song Y, Zamora MJ, Chiva M, Ausio J. 2004. Histone H1 and the origin of protamines. Proc Natl Acad Sci USA 101:4148–4152.

Love MS, Yoklavich M, Thorsteinson L. 2002. The rockfish of the Northeast Pacific. Berkeley, CA: University of California Press.

Marshall NB. 1984. Progenetic tendencies of deep-sea fishes. In: Pott GW, Wooton RJ, editors. New York: Academic Press. p 91–101.

Miescher F. 1874. Das Protamin eine neue organische Base aus den Samenfäden des Rheinlachses. Berichte 7:376–379.

Moir RD, Dixon GH. 1988a. Characterization of a protamine gene from the chum salmon (*Oncorhynchus keta*). J Mol Evol 27:8–16.

Moir RD, Dixon GH. 1988b. A repetitive DNA sequence in the salmonid fishes similar to a retroviral long terminal repeat. J Mol Evol 27:1–7.

Moyle PB, Cech JJ. 1988. Fishes: an introduction to icthiology. Upper Saddle River: Prentice-Hall.

Nei M. 1987. Molecular evolutionary genetics. NY: Columbia University Press.

Nelson JS. 1994. Fishes of the world. New York, NY: Wiley.

Ohtsuki K, Nishikawa Y, Saito H, Munakata H, Kato T. 1996. DNA-binding sperm proteins with oligo-arginine clusters function as potent activators for egg CK-II. FEBS Lett 378: 115–120.

Oliva R. 1995. Sequence, evolution and transcriptional regulation of avian-mammalian P1 type protamines. In:

Jamieson BGM, Ausió J, Justine JL, editors. Advances in spermatazoal phylogeny and taxonomy, Vol. 166. Paris: Memoires du Museum national d'Histoire naturelle. p 537–548.

Oliva R, Dixon GH. 1991. Vertebrate protamine genes and the histone-to-protamine replacement reaction. Prog Nucleic Acid Res Mol Biol 40:25–94.

Poccia DL, Simpson MV, Green GR. 1987. Transitions in histone variants during sea urchin spermatogenesis. Dev Biol 121:445–453.

Puigdomenech P, Martinez P, Palau J, Bradbury EM, Crane-Robinson C. 1976. Studies on the role and mode of operation of the very-lysine-rich histones in eukaryote chromatin. Nuclear-magnetic-resonance studies on nucleoprotein and histone phi 1-DNA complexes from marine invertebrate sperm. Eur J Biochem 65:357–363.

Retief JD, Dixon GH. 1993. Evolution of pro-protamine P2 genes in primates. Eur J Biochem 218:1095.

Rooney AP, Zhang J. 1999. Rapid evolution of a primate sperm protein: relaxation of functional constraint or positive Darwinian selection? Mol Biol Evol 16:706–710.

Rooney AP, Zhang J, Nei M. 2000. An unusual form of purifying selection in a sperm protein. Mol Biol Evol 17: 278–283.

Saperas N, Lloris D, Chiva M. 1993a. Sporadic appearance of histones, histone-like proteins and protamines in sperm chromatin of bony fish. J Exp Zool 265:575–586.

Saperas N, Ribes E, Buesa C, Garcia-Hegart F, Chiva M. 1993b. Differences in chromatin condensation during spermiogenesis in two species of fish with distinct protamines. J Exp Zool 265:185–194.

Saperas N, Ausió J, Lloris D, Chiva M. 1994a. On the evolution of protamines in bony fish: alternatives to the ''retroviral horizontal transmission'' hypothesis. J Mol Evol 39:282–295.

Saperas N, Chiva M, Ribes E, Kasinsky HE, Rosenberg E, Youson JH, Ausio J. 1994b. Chromosomal proteins of the sperm of a cephalochordate (*Branchiostoma floridae*) and an agnathan (*Petromyzon marinus*). Compositional variability of the nuclear sperm proteins of deuterostomes. Biol Bull 186:101–114.

Saperas N, Buesa C, Abian J, Vandekerckhove J, Kasinsky HE, Chiva M. 1996. The primary structure of a chondrichthyan protamine: a new apparent contradiction in protamine evolution. J Mol Evol 43:528–535.

Saperas N, Chiva M, Pfeiffer DC, Kasinsky HE, Ausió J. 1997. Sperm nuclear basic proteins (SNBPs) of agnathans and chondrichthyans: variability and evolution of sperm proteins in fish. J Mol Evol 44:422–431.

Sautiere P, Briand G, Gusse M, Chevaillier P. 1981. Primary structure of the protamine isolated from the sperm nuclei of the dog-fish *Scylliorhinus caniculus*. Eur J Biochem 119: 251–255.

Swanson WJ, Vacquier VD. 2002. The rapid evolution of reproductive proteins. Nat Rev Genet 3:137–144.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

van Holde KE. 1988. Chromatin. New York, NY: Springer.

Watson CE, Davies PL. 1998. The high molecular weight chromatin proteins of winter flounder sperm are related to an extreme histone H1 variant. J Biol Chem 273:6157–6162.

Watson CE, Gauthier SY, Davies PL. 1999. Structure and expression of the highly repetitive histone H1-related sperm chromatin proteins from winter flounder. Eur J Biochem 262:258–267.

Wirth U. 1984. Die struktur der Metazoen-spermien und ihre bedeutung fur die phylogenetik. Ver Hamburg (NF) 27: 295–303.

Wourms JP. 1981. Viviparity: the maternal–fetal relationship in fishes. Am Zool 21:473–515.

Wourms JP, Lombardi J. 1992. Reflections on evolution of piscine viviparity. Am Zool 32:276–293.

Wouters-Tyrou D, Martinage A, Chevaillier P, Sautiere P. 1998. Nuclear basic proteins in spermiogenesis. Biochimie 80:117–128.

Wyckoff GJ, Wang W, Wu CI. 2000. Rapid evolution of male reproductive genes in the descent of man. Nature 403: 304–309.