

Characterization of the cDNAs Encoding Three GnRH Forms in the Pejerrey Fish *Odontesthes bonariensis* (Atheriniformes) and the Evolution of GnRH Precursors

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Abstract. Most vertebrates express two gonadotropin releasing hormone (GnRH) variants in brain tissue but there is an increasing number of fish species for which a third GnRH form has been detected. We characterized the precursors (cDNAs) of all three forms expressed in the brain of the pejerrey (silver-side) fish, *Odontesthes bonariensis* (Atheriniformes): type I (GnRH-I; 440 bp), type II (GnRH-II; 529 bp), and type III (GnRH-III; 515 bp). The expression of these GnRHs precursors was also observed in peripheral tissues related to reproduction (gonads), visual and chemical senses (eye and olfactory epithelium), and osmoregulation (gill), suggesting that in teleost fish and possibly other vertebrates GnRH mediates directly or indirectly many other functions besides reproduction. We also present a comprehensive phylogenetic analysis including representatives of all chordate GnRH precursors characterized to date that supports the idea of two main paralogous GnRH lineages with different function. A “forebrain lineage” separates evolutionarily from the “midbrain lineage” as a result of an ancient duplication (ca. 600 million years ago). A third, fish-only clade of GnRH genes seems to have originated before the divergence of fish and tetrapods but retained only in fish. Phylogenetic analyses of GnRH precursors (DNA and protein sequences) under different optimality criteria

converge on this result. Although alternative scenarios could not be statistically rejected in this study due to the relatively short size of the analyzed molecules, this hypothesis also receives support from chromosomal studies of synteny around the GnRH genes in vertebrates.

Key words: Gonadotropin releasing hormone (GnRH) — Phylogeny — mRNA expression — Brain — Reproduction — Teleosts — Pejerrey

Introduction

Gonadotropin releasing hormone (GnRH) is a pivotal neuropeptide for development and reproduction of all vertebrates. GnRH is synthesized in the hypothalamus and released into the pituitary gland via the portal vascular system in tetrapods or via direct neuronal innervations in the case of teleost fish. At the pituitary gland, the primary neurohormonal function of GnRH is to stimulate the synthesis and release of gonadotropins, which in turn, stimulate steroidogenesis and gonadal development.

Comparative endocrinological studies have revealed 14 different GnRH variants in vertebrates, defined by the amino acid sequence of the active peptide (Lethimonier et al. 2004), but all variants are decapeptides with residues 1, 4, 9, and 10 per-

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fectly conserved. Fish harbor the highest diversity in GnRHs among vertebrates: 8 of the 14 described GnRH variants have been characterized and/or detected in teleosts and six are exclusive of this lineage (see the Appendix for an account of the intricate taxonomy and nomenclature of GnRH forms used in the endocrinological literature). The early assumption that a single molecular form is expressed in all vertebrate brains has given way to the view that most vertebrates carry different GnRH genes in their genomes and express more than one GnRH variant in their brains (Fernald and White 1999). All fish species analyzed to date are known to express two or three different forms of GnRH. The most conserved form of GnRH (hereafter referred to as type II or GnRH-II, first described in birds) is found in Chondrichthyes and all other vertebrates, but all fish also express another GnRH variant related to the control of pituitary gonadotropes (type I or GnRH-I, first characterized in mammals). If a third form is present, this is a fish-specific GnRH variant, so far only described for teleosts (type III or GnRH-III).

Studies in fish species expressing three GnRH variants have shown that each variant exhibits differential distribution in the brain: the hypophysiotropic GnRH-I variant is mainly found in the preoptic-hypothalamic area, GnRH-II is expressed by midbrain *tegmentum* (MT) neurons, and GnRH-III by neurons located at the terminal nerve ganglion (TNG) (e.g., Gothilf et al. 1996; Okuzawa et al. 1997; Fernald and White 1999; Vickers et al. 2004). The distribution of these forms in different clusters of cell bodies prompted some authors to suggest the existence of three different GnRH systems with distinct physiological functions in fish (Parhar et al. 1998; Dubois et al. 2002). However, except for the hypophysiotropic function of the preoptic-hypothalamic GnRH-I form the physiological roles of other GnRHs are poorly known. Furthermore, the expression of these neuropeptides in vertebrates seems not to be restricted to the central nervous system. Several reports have demonstrated GnRH expression outside the brain, in tissues and organs such as the pituitary, skeletal muscle, heart, liver, kidney, spleen, placenta, mammary gland, ovary, and testis (Kakar and Jennes 1995; White and Fernald 1998; Nabissi et al. 2000; Uzbekova et al. 2001, 2002; Ikemoto and Park 2003). Thus, GnRHs are likely to have diverse, and so far unknown, physiological functions in addition to gonadotropin secretion.

To understand the evolution of structural and functional diversification of these regulatory molecules, comparative studies of anatomical location and taxonomic distribution of different GnRH variants should be based on a phylogenetic framework to trace

the evolution of the GnRH gene family. Unfortunately, direct phylogenetic analysis of GnRHs has been ineffective because these are short, conserved peptides with limited phylogenetic signal. Thus, analyses have been based on genomic or cDNA sequences of the precursor (prepro-GnRH) that includes a signal peptide, the GnRH peptide itself, a conserved processing tripeptide, and the GnRH-associated peptide (GAP). Phylogenetic results to date suggest that vertebrate GnRH variants fall into three main clades: clade 1, with GnRH-I variants from neurons located in the preoptic region with pituitary function; clade 2, grouping all GnRH-II variants expressed by midbrain neurons; and clade 3, with all fish-specific GnRH-III forms located either in neurons from the olfactory region and/or the telencephalon of fish (White et al. 1998; Okubo and Aida 2001; Okubo et al. 1999; 2002). The first phylogenetic analysis of GnRHs (White et al. 1998), including few representatives of fish and tetrapods, concluded that a gene duplication originating GnRH-I and GnRH-II forms must have occurred before the separation of tetrapods from the ray-finned fish lineage. However, the phylogenetic position of the GnRH-III lineage remained uncertain in this and subsequent studies due to limited taxonomic sampling and no outgroup sequences from either chondrichthyans or agnathans (but see Silver et al. 2004). In this context, two alternative hypotheses have been considered: (i) the GnRH-III lineage may have resulted from another ancient duplication predating the divergence of tetrapods and ray-finned fish, and either has been lost in tetrapods later or has not yet been discovered; and (ii) the gene duplication leading to the GnRH-III lineage occurred recently, within the ray-finned fish lineage, explaining why these variants are restricted to fish (Fig. 1; hypotheses A and B, respectively). Although different authors working in this field favor the former hypothesis (White et al. 1998; Okubo and Aida 2001; Okubo et al. 1999, 2002), accumulating evidence supporting more recent fish-specific whole-genomic duplication events (Amores et al. 1998; Christoffels et al. 2004; Hoegg et al. 2004; Van de Peer et al. 2003) would favor the latter. A comprehensive phylogenetic analysis is necessary to fully understand the evolutionary diversification of the GnRH gene family.

Our laboratory is working with the pejerrey fish, *Odontesthes bonariensis*, as an experimental model for the study of the physiology of reproduction and sex determination and differentiation mechanisms. In the present study we determine the cDNA sequences encoding all three GnRH forms in this species, document their expression in different brain areas and extraneural tissues and organs, and also present phylogenetic tests of the above hypotheses including representatives of all chordate GnRH precursors characterized to date.

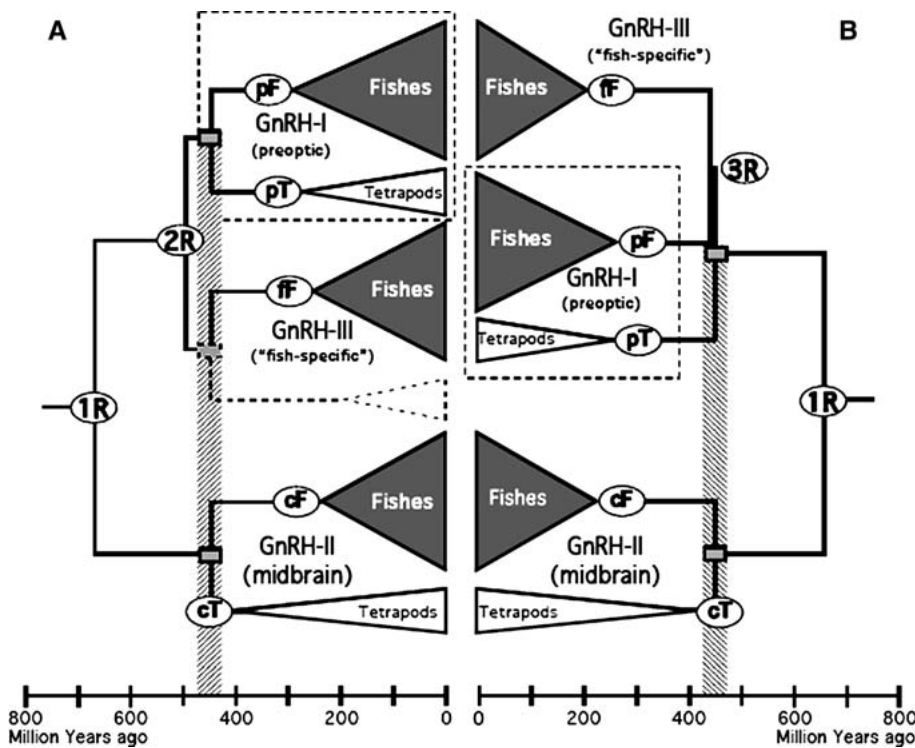


Fig. 1. Alternative hypotheses for the evolution of the main groups of GnRH genes. **A** Two gene (genome) duplications (nodes 1R and 2R) occurred before the separation of fish and tetrapod lineages (shaded bars; estimated date of divergence, 450 MYA). Dashed line indicates loss of GnRH III genes in the lineage leading to tetrapods. The fish-specific genome duplication is not implied by this hypothesis. **B** Only one duplication (1R) occurred before the separation of fish and tetrapod lineages, and the origin of GnRH III genes in the lineage follows the fish-specific genome duplication (3R). The diagram is a chronogram and estimated dates for nodes were obtained with a penalized likelihood method (see text and Table 2).

Materials and Methods

Animals and Sample Preparation

Pejerrey fish were obtained from the stock maintained under natural environmental conditions in the outdoor ponds of the IIB-INTECH aquatic facilities. All fish used for this work were 2-year-old adult specimens. At the time of tissue collection, the fish were carefully handled and sacrificed in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals (<http://www.ufaw.org.uk/pubs.htm#Lab>) and local regulations. The brains were quickly dissected out and stored in RNAlater (Sigma) at -80°C until total RNA was extracted. The gonads were excised and their weights determined to calculate the gonadosomatic index (GSI; gonadal weight/body weight $\times 100$).

In order to analyze GnRH expression in different tissues and organs, pejerrey fish of both sexes were sampled in August. Females were determined to be in midvitellogenesis and males were already spermeating according to Strüssmann (1989). The eyes, olfactory mucosa, gills, kidney, spleen, liver, heart, muscle, intestine, gonads, and brain were quickly removed and stored in RNAlater at -80°C .

Molecular Cloning of 3'-Ends

Total RNA was extracted for each sample using a commercial product, TRIzol Reagent (Life Technologies). Rapid amplification of cDNA ends (RACE) was carried out in order to isolate the different GnRH precursors in pejerrey brain. First-strand cDNA was synthesized from $2\ \mu\text{g}$ of total RNA from brain tissue using an oligo(dT)-Adaptor primer and Superscript II Rnase H- Reverse Transcriptase (Invitrogen; Life Technologies).

Six consensus sense primers were designed based on other teleost sequences and named sGnRHF1, sGnRHF2, cIIGnRHF1, cIIGnRHF2, pjGnRHF1, and pjGnRHF2 (Table 1). The first round of PCR used F1 sense primer and UAP-primer (Universal

Amplification Primer; Table 1) as antisense primer was run under these conditions: 3 min at 94°C , followed by 35 cycles of 30 s at 94°C , 30 s at 55°C , and 1 min at 72°C , ending with a 10-min extension step at 72°C . The PCR products obtained served as template for nested PCRs using the second corresponding sense primer (F2) in combination with the UAP-primer under similar conditions.

The resulting PCR products of desired sizes were cloned in a bacterial vector using the pGEM-T Easy kit (Promega), sequenced at the IIB-INTECH central sequencing facilities, and submitted to BLAST for comparison to known sequences accessible in GenBank/EMBL.

Molecular Cloning of 5'-Ends

Once the previous specific sequences were obtained, six gene-specific antisense primers were designed for isolation of 5'-ends of the corresponding cDNAs: sGSPR1, sGSPR2, cIIGSPR1, cIIGSPR2, pjGSPR1, and pjGSPR2 (Table 1). Total RNA ($2\ \mu\text{g}$) was used for reverse transcription of GnRH-III, GnRH-II, and GnRH-I using gene-specific antisense primers. First-strand cDNAs were purified using a Microcon YM-30 Centrifugal Filter Devices (Amicon/Millipore), following the instructions of the manufacturer.

For poly(A) tailing, the purified first-strand cDNAs were incubated for 2–3 min at 94°C , chilled on ice for 1 min, and then incubated at 37°C for 10 min after adding 10 units of terminal deoxynucleotidyl transferase (Invitrogen; Life Technologies). The tailing reaction was terminated by heating the reaction at 65°C for 10 min.

Tailed cDNAs obtained were amplified directly by PCR. The first round of PCR (using R1 primer and UAP-primer) was programmed as follows: 3 min at 94°C , then 35 cycles of 30 s at 94°C , 30 s at 58°C , and 1 min at 72°C . The first PCR products served as templates in nested PCRs using the second corresponding antisense nested primer (R2) in combination with the UAP-primer under similar conditions used in the first PCR. The bands of desired size were treated as already described.

Table 1. Primers used for the study

Primer name	Nucleotide sequence	Usage	Location
Oligo(dt)-adapter primer	5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TVN-3'	RACE-PCR	
UAP-primer	5'-GGC CAC GCG TCG ACT AGT AC-3'	RACE-PCR	
sGnRHF1 (sense)	5'-CAG CAY TGG TCI TAY GGI TGG CT-3'	3'RACE-PCR	GnRH decapeptide
sGnRHF2 (sense)	5'-TGG TCI TAY GGI TGG CTW CCI GG-3'	3'RACE-PCR (nested)	GnRH decapeptide
cIIGnRHF1(sense)	5'-CAG CAC TGG TCI CAY GGI TGG-3'	3'RACE-PCR	GnRH decapeptide
cIIGnRHF2 (sense)	5'-TGG TCI CAY GGI TGG TAY CCI GG-3'	3'RACE-PCR (nested)	GnRH decapeptide
pjGnRHF1 (sense)	5'-CAG CAC TGG TCI TWY GGI CTG-3'	3'RACE-PCR	GnRH decapeptide
pjGnRHF2 (sense)	5'-TGG TCI TWY GGI CTG AGY CCW GG-3'	3'RACE-PCR (nested)	GnRH decapeptide
sGSPR1 (antisense)	5'-CTC CAA GTG TCC AAG TGA CT-3'	5'RACE-PCR and RT-PCR	GAP
sGSPR2 (antisense)	5'-TCT CTC TTG GAT TTG GGC ACT T-3'	5'RACE-PCR (nested)	GAP
cIIGSPR1 (antisense)	5'-CAG CGA AAG ATG GAA AGC AGT C-3'	5'RACE-PCR and RT-PCR	GAP
cIIGSPR2 (antisense)	5'-CCT CTG GGG TCT CAA GTA GC-3	5'RACE-PCR (nested)	GAP
pjGSPR1 (antisense)	5'-GGC GTC CAT TTT CCC TGT CG-3'	5'RACE-PCR and RT-PCR	GAP
pjGSPR2 (antisense)	5'-GAG ACT CGT CCG CAC AAC CCA-3'	5'RACE-PCR (nested)	GAP
sGSPF3 (sense)	5'-TGG CGT TGG TGG TTC AGG T-3'	RT-PCR	Signal peptide
cIIGSPF3 (sense)	5'-CTC TCG TAC GCC CAG CAC T-3'	RT-PCR	Signal peptide
pjGSPF3 (sense)	5'-CAA AAG AAT GGC TGT AAG AAC G-3'	RT-PCR	Signal peptide
β actF (sense)	5'-GGA YGA YAT GGA GAA GAT CTG G-3'	sqRT-PCR and expression	
β actR (antisense)	5'-GAC GGA CAG GTC ATC ACC AT-3'	sqRT-PCR and expression	

RT-PCR Analysis and Southern Hybridization for Tissue-Specific Expression of GnRH Forms

Total RNA (2 μ g) from adult male and female pejerrey tissues (eyes, olfactory mucosa, gills, kidney, spleen, liver, heart, muscle, intestine, gonads, and brain) were reverse transcribed using M-MLV (Promega Corp.) following the manufacturer's recommendations.

Specific sense primers were then designed: sGSPF3, cIIGSPF3, and pjGSPF3 (Table 1), based on the signal peptide sequence of the each cDNA precursor. The primers were tested in a PCR reaction using 1 μ l of pejerrey brain cDNA as a template in combination with the antisense specific primers (sGSPR1, cIIGSPR1, and pjGSPR1, respectively) using the following cycle conditions: a 5-min denaturing step at 95°C, 35 cycles using a cycle profile of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, ending with a 5-min extension at 72°C.

The PCR products were 214, 221, and 275 bp for GnRH-III, GnRH-II, and GnRH-I, respectively. Each primer set spanned at least one intron, such that amplification of genomic DNA using the primer sets yielded single products clearly distinguishable from cDNA-derived products (GnRH-III, ~380 bp; GnRH-I, ~500 bp; and GnRH-II, ~620 bp; data not shown).

Brain products were sequenced directly to confirm their authenticity as GnRH-specific amplicons. Products derived from other tissues were of the same size as brain-derived amplicons and were thus considered to be authentic, although they were not sequenced.

Furthermore, to confirm its identity, each RT-PCR product was transferred onto a nylon membrane (Hybond N; Amersham Biosciences) and hybridized overnight at 58°C with Church-Gilbert hybridization solution with the corresponding probe. The different probes were constructed using the brain PCR products purified and then labeled with [α -³²P]dCTP by random priming (RediPrime Random Labeling kit, Amersham Biosciences). Stringency washes were carried out at 58°C with 2 \times SSC, 1 \times SSC, and 0.1 \times SSC.

In addition, a 512-bp fragment of pejerrey β -actin was amplified from each tissue (primers β actF- β actR; Table 1) to confirm that negative results were not due to extremely low amount of cDNA or degraded RNA. These experiments were designed to be qualitative, not quantitative, in nature (Fig. 4).

Semiquantitative RT-PCR in Different Brain Areas

Semiquantitative RT-PCR was performed for each GnRH form and β -actin (as an internal control) using three samples of total RNA (2 μ g) from three different male and female fish. Each sample was divided in three areas: A (olfactory bulbs and telencephalon), B (preoptic area, hypothalamus, and rostral optic tectum), and C (cerebellum, caudal optic tectum, and medulla oblongata). All the RNA samples were treated with DNase I (Origin) and reverse-transcribed using SuperScript II RNase H⁻ (Invitrogen) and oligo(dT)₁₂₋₁₈ following the manufacturer's instructions.

Each set of primer was tested in a range from 10 to 40 cycles, using a 1:1 to 1:50 dilution of a cDNA mix derived from the samples, in order to know the number of cycles where the product accumulation was in the linear phase of the curve (27 cycles for GnRH-I, 28 cycles for GnRH-II, 27 cycles for GnRH-III, and 22 cycles for β -actin).

PCR products were resolved using the same 1.2% agarose gel and then stained by soaking the gel in a 1% ethidium bromide solution. Gels were then quantitatively analyzed using an instant imager (Gel-Pro Analyzer 3.1) and data expressed as GnRH/ β -actin ratios. The data were statistically analyzed by ANOVA and Bonferroni's multiple-comparison tests. A significance level ($p < 0.05$) was used for all tests.

Sequence Comparison and Phylogenetic Analysis

Representative GnRH sequences from jawless fish (lampreys), ray-finned fish, and tetrapods were downloaded from GenBank (see the Appendix) and trimmed to their protein-coding region encompassing the prepro-GnRH. The deduced amino acid sequences were aligned with Clustal W (Thompson et al. 1994) using default parameters (and the Gonnet matrix) and then back-translated with Mega3 (Kumar et al. 2004) to their original DNA sequences for phylogenetic analysis. Amino acid sequences of the entire GnRHs ORFs were compared against the newly obtained pejerrey sequences.

Phylogenetic analyses of aligned DNA and protein sequences were conducted under the maximum parsimony (MP) and maximum likelihood (ML) criteria using PAUP 4.0b10 (Swofford 2002)

and Treefinder (Jobb 2005) or PHYML (Guindon and Gascuel 2003; Guindon et al. 2005), respectively. Preliminary trees were constructed by neighbor joining (NJ; Saitou and Nei 1987) using ML distances in PAUP. Bayesian phylogenetic inference (BI) was conducted with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). For MP analyses only DNA sequence data were used in heuristic searches repeated 100 times with starting trees obtained by random stepwise addition and TBR branch swapping. Confidence in the results was assessed by performing 100 bootstrap replicates (with one repetition each of TBR swapping). BI and ML analyses were conducted on the amino acid sequences using the JTT model (Jones et al. 1992) with gamma correction (G) for among-site rate heterogeneity (Yang 1994), allowing a fraction (I) of sites to be invariant. This model was selected by PROTTEST under the AIC and BIC criteria (Abascal et al. 2005). For DNA analysis, Modeltest 3.7 (Posada and Crandall 1998) was used to select the appropriate substitution model and implemented in Treefinder and MrBayes. The resulting GTR+I+G model was applied to a single partition (ML and BI) and also to three data partitions corresponding to each codon position independently (BI). Two runs with Treefinder were done for each analysis to check for convergence; approximate bootstrap support for reconstructed edges was computed by applying Local Rearrangements of tree topology around an edge with the Shimodaira-Hasegawa test with RELL approximation (LRSH; Shimodaira and Hasegawa 1999). For BI, Markov Chain Monte Carlo (MCMC) simulations were run for at least 2 million generations with four chains each time, and each run was repeated multiple times to check for convergence as suggested by the authors. Trees were sampled every 1000 steps and burnin values (typically around 1000) were set conservatively based on the likelihood plot. Posterior probability (pp) values were recorded for each node to assess robustness of the results and to compare with the phylogenies obtained by the other methods.

Alternative hypotheses arising from these analyses also were compared under ML using the SH test (Shimodaira and Hasegawa 1999) implemented in PAUP. ML ratio tests were performed with PAUP to test for rate constancy across lineages (the molecular clock hypothesis). Rejection of the molecular clock hypotheses led to the application of penalized likelihood methods to estimate ages of divergence among the main lineages (Sanderson 2002). Following several authors (e.g., Carroll 1988; Benton 1990; Zhu et al. 1999; Hedges and Kumar 2003), separation of land vertebrates (tetrapods) and fish lineages was fixed at 450 million years ago (MYA) to calibrate the chronogram and estimate divergence ages for the other nodes. The program r8s version 1.7 (Sanderson 2003, 2004) was used to determine the appropriate level of smoothing (to account for non-clock behavior) with the cross-validation approach under penalized likelihood (PL) and the truncated Newton algorithm (TN). Several runs of this program with different starting points were performed to guarantee convergence on the resulting time estimates. A second cross-validation routine using two fossil-fixed ages also was applied to obtain a second estimate for the value of the smoothing parameter.

Results

Molecular Cloning of the GnRH Precursors

The pejerrey type III GnRH cDNA is 515 bp in length, not including the poly(A) tail and containing two possible ATG translational start codons at positions 73 and 94. Nevertheless, the ATG in position 73 is expected to be the functional translational starting site as indicated by NetStart 1.0 (genome.cbs.dtu.dk/services/NetStart; Pedersen and Niel-

sen 1997), in agreement with the initiation sites of all other analyzed species. Therefore, the pejerrey type III prepro-GnRH comprises (i) 72 bp on the 5'UTR; (ii) a 276-bp open reading frame encoding 91 amino acids (aa), including the signal peptide (23 aa) and the GnRH decapeptide, the processing tripeptide, and the GAP (55 aa); and (iii) 167 bp on the 3'UTR (Fig. 2A).

The cDNA encoding for pejerrey type I GnRH is 440 bp in length, not including the poly(A) tail. This sequence also has two putative ATG translational starting codons, in positions 36 and 48, but only the ATG in position 48 is expected to be a functional translational starting codon. Therefore the pejerrey type I prepro-GnRH contains (i) 47 bp on the 5'UTR; (ii) a 285-bp open reading frame encoding 94 aa including the signal peptide (22 aa), the GnRH decapeptide, the processing tripeptide, and the GAP (59 aa); and 108 bp on the 3'UTR (Fig. 2B).

The pejerrey GnRH-II cDNA is 529 bp in length without the poly(A) tail, which includes 99 bp on the 5'UTR, a 252-bp open reading frame (83 aa, which includes the 21-aa signal peptide, the GnRH decapeptide, the processing tripeptide, and the 49-aa GAP), and 178 bp on the 3'UTR (Fig. 2C).

The 3'-untranslated region of each cDNA contains a consensus polyadenylation signal (Fig. 2). The nucleotide and the deduced amino acid sequences of pejerrey types I, II, and III GnRH cDNA have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database with accession numbers AY744689, AY744687, and AY744688, respectively.

Tissue-Specific Expression of Prepro-GnRH Variants

The expression of pejerrey type III GnRH was observed in the eyes, the olfactory mucosa, and the brain of males. Faint signal were also observed in all other tissues sampled, however, it is difficult to know if those signals represent GnRH-III expression. In females, GnRH-III expression was only evident in the eyes, the ovary, and the brain (Fig. 3).

The expression of pejerrey type I GnRH was observed in the eyes, spleen, and brain of both sexes. Male fish also expressed GnRH-I in the gills and heart. In females GnRH-I expression was also observed in the ovaries (Fig. 3).

The expression of pejerrey type II GnRH was restricted to the brain of both sexes at this particular gonadal stage (Fig. 3).

Semiquantitative RT-PCR in Different Brain Areas

The expression of GnRHs in different brain areas from male and female pejerrey was also analyzed by semiquantitative RT-PCR (Fig. 4). The brain samples were

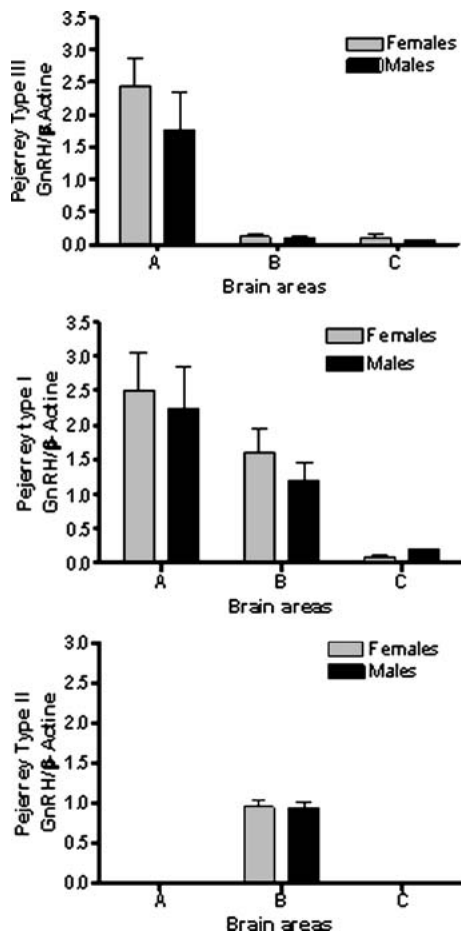


Fig. 4. Expression of GnRHs in different brain areas from males and females pejerrey analyzed by semiquantitative RT-PCR. The samples were divided into three areas as follows: A (olfactory bulbs and telencephalon), B (preoptic area, hypothalamus, and rostral optic tectum), and C (cerebellum, caudal optic tectum, and medulla oblongata).

sequences formed a well-supported monophyletic group ($pp=1.0$ and $bootstrap=100\%$). This group includes two GnRH variants from *Petromyzon marinus* (Suzuki et al. 2000; Silver et al. 2004), indicating that a gene duplication along the lamprey lineage is the most likely explanation for their origin. Among jawed vertebrates, the three clades originally described by White et al. (1998) were supported in most analyses (as shown in Fig. 5); furthermore, the results suggest a close relationship between clade 1 and clade 3 (GnRH-I and GnRH-III), consistent with the hypothesis shown in Fig. 1A. This clade (I + III) received strong support from Bayesian inference using DNA sequences under all models tested ($pp > 0.95$) but marginal support from MP analyses ($bootstrap = 59\%$) and ML analysis of amino acid sequences ($bootstrap = 68\%$).

All analyses unambiguously supported the monophyly of clade 3 (GnRH-III) containing all fish-specific GnRH variants with high bootstrap and pp support (100% and 1.0, respectively). In contrast, not

all analyses resulted in high support for the monophyly of clade 1 (containing the preoptic GnRH-I peptides with pituitary function). Support for clade 1 was variable: BI analysis resulted in pp values between 0.70 and 0.85, ML analysis on amino acid sequences recovered the monophyly with $bootstrap = 81\%$ (Fig. 5), but MP did not support this grouping. The alternative hypothesis (Fig. 1B), grouping fish-only GnRH-I sequences (clade 1 fish sequences) and fish-specific GnRH-III sequences (clade 3) to the exclusion of tetrapod GnRH-I sequences, was weakly supported by MP and ME analyses only (but with $bootstrap < 50\%$). An explicit SH test of these two alternatives was nonsignificant ($p=0.47$). Therefore, the sequence data do not contain strong phylogenetic signal to discriminate statistically between these two hypotheses (Figs. 1 A and B).

The type II GnRH variants (clade 2) failed to form a monophyletic group in most DNA analysis (Fig. 5), probably because of the high divergence of mammalian sequences from the relatively conserved fish and basal tetrapod sequences (causing potentially long branch attraction between mammalian sequences and the outgroup). Interestingly, only one replicate run under BI resulted in marginal support ($pp = 53$) for GnRH-II clade monophyly and the NJ tree also showed this grouping (results not shown). PHYML analyses on protein sequences with the JTT+I+G model also did not recover the monophyly of the GnRH-II clade (in agreement with the topology shown in Fig. 5). In spite of this, for all subsequent tests to obtain estimates of divergence times, the monophyly of GnRH variants was enforced, given the high degree of conservation of their mature peptides and homogeneous anatomical expression in the known cases. Furthermore, phylogenetic hypotheses constrained with monophyly of GnRH-II clade were not statistically different from the topology shown in Fig. 5 (SH test, $p=0.75$).

The molecular clock hypothesis was rejected under ML (likelihood ratio test, $p < 0.01$), thus penalized likelihood models were applied using the program r8s to estimate age of divergence for critical nodes in the phylogeny. Hypotheses A and B (Fig. 1) were used to compare estimated dates of the putative gene (or genome) duplication events. Several taxa (17) were pruned from the data set (marked with asterisks in Fig. 5) to eliminate terminal branches with length close to zero. The phylogeny used to study hypothesis A is the best BI hypothesis (Fig. 5) without the pruned taxa. The phylogeny to study hypothesis B was obtained under BI by constraining the monophyly of clade 3 + fish-only GnRH I sequences (i.e., excluding tetrapod-only GnRH I sequences). Branch lengths for both hypotheses were estimated under ML (with the GTR + I + G model) using PAUP. Tree

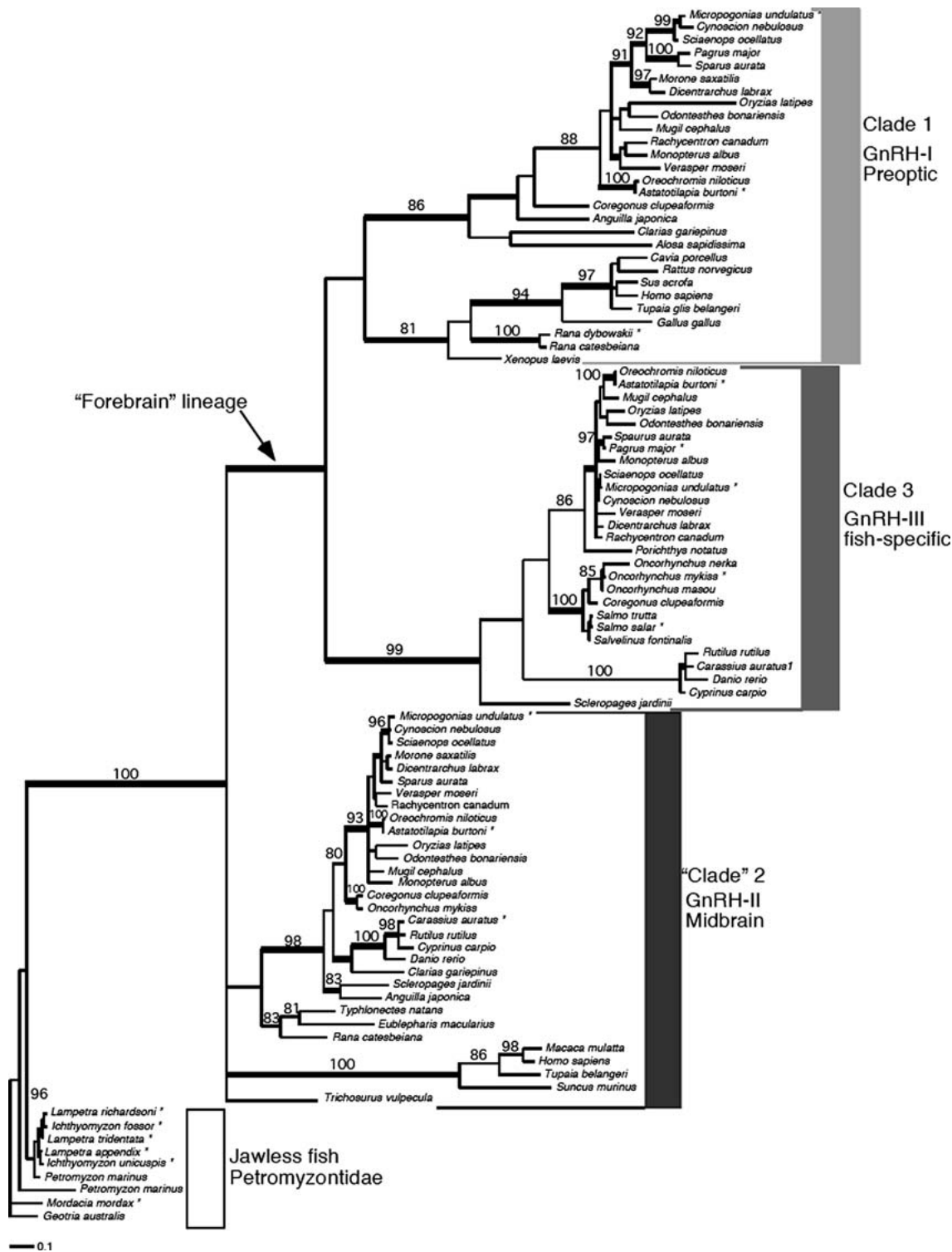


Fig. 5. Phylogenetic tree of prepro-GnRH sequences obtained by Bayesian inference of DNA sequences under the GTR + I + G model (for details see Results). Thick branches indicate posterior probability support $p > 0.95$. Numbers above branches are bootstrap values ($> 80\%$) obtained by maximum parsimony analysis of DNA sequences.

descriptions with branch lengths generated by PAUP were used as input for r8s. The cross validation routines were run several times for both hypotheses, resulting in smoothing parameter values ranging approximately from 4000 to 8000. When the fossil

calibration cross validation approach was used (fixing fish-tetrapod divergences at 450 MYA), smoothing parameters about one order of magnitude lower were obtained (ranging from 400 to 500). Thus, as a conservative approach, ranges of estimated ages reported

Table 2. Estimation of divergence times (millions of years ago; MYA) under penalized likelihood

Node	Hypothesis A	Hypothesis B
1R	653–676	653–684
2R or 3R	492–501	448
fF	234–289	195–209
pF	280–316	263–272
pT	256–291	252–262
cF	183–295	173–265
cT	405–411	410–415

Note. Range of values shown was obtained with different smoothing parameter values (see text). Hypotheses A and B and node labels are indicated in Fig. 1. Divergence time between fish and tetrapod lineages was fixed at 450 MYA.

here reflect the application of the most divergent smoothing parameter values obtained (Table 2). In most cases the effect was minimal (e.g., for node 1R, 2R, 3R, or cT in Fig. 1), but in other instances the estimated ages were quite discrepant (nodes cF and fF in Fig. 1). Under either hypothesis (A or B), the ancestral genome duplication for jawed vertebrates (1R) was estimated to have occurred between 653 and 684 MYA. Under hypothesis A, the second round of genome duplication (2R) was estimated at around 500 MYA. Constraining the origin of fish specific GnRH-III variants to after the split between fish and tetrapods (hypothesis B) generated an internal branch length (between 3R and the fish-tetrapod divergence; see Fig. 1) close to zero (actual value was 5×10^{-7}); such value was interpreted as effectively zero by r8s and the program failed to run. In order to get results for hypothesis B the value of this branch was incremented to 10^{-3} ; under these conditions, the estimated age of node 3R (the putative fish-specific duplication event) was 448 MYA (Table 2), effectively indistinguishable from the fixed age of divergence between fish and tetrapod lineages (450 MYA). Taken together, the estimated ages of divergence for GnRH genes are more consistent with two rounds of duplication before the divergence of fish and tetrapod lineages than with the proposed fish-specific duplication event putatively dated at 320 MYA (Hoegg et al. 2004; Van de Peer et al. 2003).

Discussion

Three different prepro-GnRH cDNAs were characterized from brain tissue of pejerrey fish. The deduced amino acid sequences of the decapeptides encoded by these precursors correspond to the previously characterized GnRH orthologues first isolated from salmon, pejerrey, and chicken (Montaner et al. 2001; Somoza et al. 2002b). These three cDNAs share the structural organization of other vertebrate GnRH cDNA sequences, consisting of

the 5'UTR, a signal peptide, the GnRH peptide itself, the processing site, the GnRH-associated peptide, and the 3'UTR.

Many studies have documented GnRH gene expression in the central nervous system of vertebrates; however, only recently has the presence of GnRH been documented in many tissues outside the brain. In pejerrey, only two forms (GnRH type III and GnRH type I) were found to be expressed in different extrabrain tissues and organs. The type III form was clearly expressed in the eyes of males and females, the male olfactory mucosa, and the ovary.

It has been suggested that the primary role of GnRH-III from the terminal nerve ganglion in teleosts is to coordinate olfactory and visual inputs related to the control of reproduction (Kudo et al. 1996; Parhar et al. 1994). The expression of the GnRH-III variant in gonadal tissue of pejerrey is consistent with many reports for other ray-finned fish species: midshipman (Grober et al. 1995), goldfish (Lin and Peter 1996; Pati and Habibi 1998), rainbow trout (von Schalburg et al. 1999; Uzbekova et al. 2001), African cichlid (White and Fernald 1998), and seabream (Nabissi et al. 2000). The expression of type III GnRH in gonadal tissue also has been shown to vary following the sexual cycle in rainbow trout and has been implied in gonadal autocrine and/or paracrine regulation (von Schalburg et al. 1999; Uzbekova et al. 2001).

On the other hand, pejerrey type I GnRH was found to be expressed in sites such as the eyes, gills, spleen, ovary, and heart.

RT-PCR experiments in the cichlid *Astatotilapia burtoni* provided the first evidence of extraneural expression of type I GnRH in heart, liver, spleen, kidney, and testis of adult males (White and Fernald 1998). Nabissi et al. (2000) also documented GnRH-I expression in the ovary of sea bream and recently, Moncaut et al. (2005) have shown expression in a wide variety of tissues and organs: gills, heart, head, kidney, and gonads from the same species. However, the role of GnRH-I in peripheral tissues is unknown, but it is particularly interesting that this form is expressed in tissues related to osmoregulation such as gills and kidney.

Finally, expression of GnRH-II forms outside the brain also has been reported in different vertebrate groups: mammals (White et al. 1998), reptiles (Ikemoto and Park 2003), and fish (Yu et al. 1998; White and Fernald 1998; von Schalburg et al. 1999; Okubo et al. 1999; Bogerd et al. 2002). In contrast, pejerrey type II GnRH mRNA was not detected in any tissue examined outside the brain; at least, at the single reproductive stage tested. Thus, it is possible that GnRH-II expression outside the brain could exhibit seasonal and stage-dependent variation.

The presence of GnRH forms outside the brain-pituitary-gonadal axis of vertebrates suggests that

this hormone could also be involved in various non-reproductive functions.

Traditionally, the distribution of the three GnRH forms in bony fish was considered to be restricted to specific brain areas (Gothilf et al. 1996; Okuzawa et al. 1997; White and Fernald 1998; Stefano et al. 2000). However, González-Martínez et al. (2001) described for the first time in the European sea bass (*Dicentrarchus labrax*) an overlapping distribution of GnRH-III and GnRH-I forms in cells of the olfactory bulbs to the preoptic region. Similar distribution schemes have been subsequently reported in different species such as the white fish, *Coregonus clupeaformis* (Vickers et al. 2004), the Atlantic croaker, *Micropogonias undulatus* (Mohamed et al. 2005), and the South American cichlid, *Cichlasoma dimerus* (Pandolfi et al. 2005).

The present results show the expression of both pejerrey GnRH type I and III forms in the anterior brain and in the preoptic and midbrain area, suggesting that they are expressed by neurons located in both regions. Although in situ hybridization studies are needed to further analyze the exact localization of GnRH-expressing neurons in anterior brain areas of pejerrey fish, these results are consistent with the idea of overlapping GnRH-expressing neurons in the pejerrey forebrain. On the other hand, GnRH-II expressing neurons have been localized only in the midbrain tegmentum in several bony fish species (Gothilf et al. 1996; Stefano et al. 2000; González-Martínez et al. 2001; Vickers et al. 2004; Pandolfi et al. 2005). The goldfish (*Carassius auratus*) is the only fish species in which an extra-midbrain GnRH-II expression has been demonstrated (Kim et al. 1995).

Evolution of GnRH Genes

Gene and genome duplications have long been linked to the evolution of vertebrate diversification and novel gene function (e.g., Ohno, 1970), and most recently, the hypothesis of two rounds of genome duplications within the vertebrate lineage has been proposed (Spring 1997; reviewed by Van de Peer et al. 2003). Recent comparison of human and pufferfish (*Tetraodon nigroviridis*) genomes shows that whole-genome duplication occurred in the teleost fish lineage, subsequent to its divergence from tetrapods (Jaillon et al. 2004). The phylogenetic timing of this event was more accurately placed at the base of the teleost lineage (Hoegg et al. 2004), before the origin of Osteoglossiformes (bony tongues, arowana), but after the divergence of Acipenseriformes (sturgeons) and Semionotiformes (gars) among basal actinopterygians. The absolute age of the duplication event was estimated at around 320 MYA based on analysis

of 50 duplicated chromosomal blocks of Fugu (Vandepoele et al. 2004).

The phylogeny of GnRH genes (Fig. 5) and the estimated dates of divergence for the main lineages (Fig. 1 and Table 2) are consistent with the hypothesis that the GnRH-II clade diverged from the rest of the GnRHs clades (GnRH-I and GnRH-III) through a duplication event older than 600 MYA, one of the two proposed whole-genome duplication events within the vertebrate lineage (Spring 1997). None of the jawless vertebrate sequences was affected by this event, since lamprey GnRH duplicated genes cluster together among the other agnathan sequences (as reported by Silver et al. 2004). The exact age of divergence between lamprey and gnathostome lineages is not certain but has been placed at around 550 MYA (Janvier 1999; Hedges and Kumar 2003), still allowing this scenario to remain viable, given the intrinsic error of dating estimates.

According to our results, less certainty surrounds the origin of the fish-specific GnRH-III clade. A close phylogenetic relationship between the GnRH-III clade and the preoptic lineage (GnRH-I clade) has already been suggested by O'Neill et al. (1998), Okubo and Aida (2001), and Dubois et al. (2002). The close relationship between clade 1 and clade 3 would imply that transcription factors binding sites involved should be conserved between the corresponding promoters. In this context, reporter gene studies confirmed the importance of this region for cell specific expression in zebrafish, because the promoter of human type I GnRH was able of drive cell-specific reporter gene expression in transgenic zebrafish (Torgersen et al. 2002).

The preferred hypothesis obtained (Figs. 5 and 1A) suggests a second round of duplication (node 2R in Fig. 1A) dated at around 500 MYA, again before the divergence of tetrapods from the fish lineage. This duplication gave origin to isoforms GnRH-I and GnRH-III, so that the ancestor of ray-finned fish and tetrapods would have carried three copies of GnRH in its genome. But this hypothesis also implies that GnRH-III genes would have been lost or that they have not yet been detected in tetrapods (White et al. 1998; Okubo and Aida 2001). Recent chromosomal analyses (Kuo et al. 2005) of genomic regions surrounding the GnRH genes in human, chicken, zebrafish, and pufferfish (fugu and tetraodon) are consistent with the secondary loss of type III GnRH genes in the tetrapod lineage. The synteny around the location of the GnRH-III gene is conserved in tetraodon and zebrafish, and this region also is conserved in the human and chicken genomes but the latter lack the GnRH-III gene. The predicted location for GnRH-III gene in tetrapods falls at a breakpoint of an inversion shared by both human and chicken, which may have precipitated

the loss of this variant in the tetrapod lineage (Kuo et al. 2005). Therefore, our working hypothesis predicts the evolution of all three types (I, II, and III) before the split of lobe-finned (leading to tetrapods) and ray-finned fish with subsequent loss of type III genes in tetrapods. The estimated time of divergence between genes encoding for GnRH-I and GnRH-III (492–501 MYA; Fig. 1A and Table 2) also supports this view.

Finally, also according to the phylogenetic results, the proposed fish-specific genome duplication event (Jaillon et al. 2004) would have had no effect on the origin and the current distribution of GnRH genes. If the fish-specific genome duplication really occurred and created another set of GnRH genes in fish, these could have been lost after the event; otherwise, a fourth group of GnRH genes would be predicted to exist in fish but have not been detected. Two copies for each of the GnRH-III or GnRH-II genes have been observed, but only in some tetraploid species (cyprinids and salmonids) presumed to have experienced more recent and independent tetraploidization events (Gray et al. 2002; Uzbekova et al. 2002). Estimates of the half-life of duplicated genes (ca. 4.0 MY, according to Lynch and Connery 2000) make gene loss a viable explanation.

The alternative hypothesis (Fig. 1B) was not favored by the phylogenetic results of this study (Fig. 5) but could not be rejected altogether based on statistical tests. According to this hypothesis, an ancient genome duplication (again, older than 600 MYA) would have given rise to clades 1 and 2, and much later, the fish-specific genome duplication (around 320 MYA) would have originated clade 3 in fish only. This scenario is consistent with the presence of GnRH-I in basal ray-finned fish but no GnRH-III co-occurring with GnRH-II in Chondrostei, Ginglymodi, and Halecomorphi (O'Neill et al. 1998). This hypothesis (Fig. 1B) also is the most parsimonious explanation because it does not involve two gene losses as above. However, based on the sequence data, the estimated date of the fish-specific duplication (3R; ca. 448 MYA) was indistinguishable from the fossil-fixed date for the fish-tetrapod split (450 MYA). Moreover, chromosomal synteny analyses mentioned above (Kuo et al. 2005) also are not consistent with this scenario. Interestingly, in their comparison of paralogues between zebrafish and Fugu, Taylor et al. (2003) found evidence for the fish-specific duplication in only 30% of their comparisons. The majority of paralogues compared (70%) were consistent with two rounds of duplication prior to the separation of fish and vertebrate lineages (older than 500 MYA), in agreement with the results obtained in this study (Figs. 5 and 1A). As in those cases, GnRH evolution may have been affected by more localized gene or local chromosomal region duplications.

Whether the origin of clade GnRH III preceded or not the fish-tetrapod divergence, the additional gene found in teleosts has unique consequences for the diversification of function in these important signaling molecules. The expression of two different GnRH genes in the anterior brain of fish is unique to this group and may be the result of the process of sub-functionalization (Lynch and Force 2000).

In summary, the present work has reported for the first time the cDNA sequence of three GnRH variants in an artherinid fish, the expression of these three transcripts in multiple extraneuronal tissues and organs, suggesting multiple physiological roles. Furthermore, the phylogenetic results presented in this work support the idea of two main paralogous GnRH lineages, constituted by the “forebrain GnRH” (clades GnRH I and GnRH III) and “midbrain GnRH” (clade GnRH II). This phylogenetic analysis contributes, together with anatomical and embryological data (see Guilgur et al. 2006), to the assumption of the existence of two main GnRH systems in gnathostoms, as originally proposed by Muske (1993; 1997): the “forebrain GnRH system,” which in some teleost species contains two different molecular forms of GnRH, and the “midbrain GnRH system,” with only one form.

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Appendix 1

Nomenclature of the GnRH Variants Used in the Endocrinological Literature and Their Primary References

Usually, the different GnRH variants are known by the name of the species in which they were first isolated, except for the first characterized GnRH from mammalian hypothalamic tissues (pig and sheep), now generally referred to as mammalian GnRH (mGnRH). Eight out of the 14 vertebrate GnRH variants known to date have been characterized and/or detected in teleosts and six are exclusive of this lineage. The latter include: salmon GnRH (sGnRH; Sherwood et al. 1983), catfish GnRH (cfGnRH; Bogerd et al. 1992; Ngamvongchon et al. 1992), seabream GnRH (sbGnRH; Powell et al. 1994), herring GnRH (hrGnRH; Carolsfeld et al. 2000), pejerrey GnRH (pjGnRH; Montaner et al. 2001; also named

as mdGnRH; Okubo et al. 2000) and whitefish GnRH (wfGnRH; Adams et al. 2002). Two other forms present in bony fish species also are found in mammals and other tetrapods: mammalian GnRH (mGnRH) and chicken GnRH-II (cGnRH-II). In fishes, mGnRH was first identified in eels (King et al. 1990; Okubo et al. 1999), and the chicken form (cGnRH-II) in the goldfish (Yu et al. 1988), and subsequently in all teleost species examined to date (see Somoza et al. 2002a).

Accession numbers of the GnRH sequences from jawless fishes, ray-finned fishes, and tetrapods downloaded from GenBank.

GnRH I clade: *Alosa sapidissima*: [AF536381](#); *Anguilla japonica*: [AB026989](#); *Astatotilapia burtoni*: [U31865](#); *Cavia porcellus*: [AF033346](#); *Clarias gariepinus*: [X78049](#); *Coregonus clupeaformis*: [AY245104](#); *Cynoscion nebulosus*: [AY796308](#); *Dicentrarchus labrax*: [AF224279](#); *Gallus gallus*: [X69491](#); *Homo sapiens*: [NM_000825](#); *Micropogonias undulatus*: [AY324668](#); *Monopterus albus*: [AY858056](#); *Morone saxatilis*: [AF056314](#); *Mugil cephalus*: [AY373450](#); *Odontesthes bonariensis*: [AY744689](#); *Oreochromis niloticus*: [AB101665](#); *Oryzias latipes*: [AB041333](#); *Pagrus major*: [D86582](#); *Rachycentron canadum*: [AY677175](#); *Rana catesbeiana*: [AF188754](#); *Rana dybowskii*: [AF139911](#); *Rattus norvegicus*: [NM_012767](#); *Sciaenops ocellatus*: [AY677172](#); *Sparus aurata*: [U30320](#); *Sus scrofa*: [L32864](#); *Tupaia belangeri*: [U63326](#); *Verasper moseri*: [AB066360](#); *Xenopus laevis*: [L28040](#).

GnRH II clade: *Anguilla japonica*: [AB026990](#); *Astatotilapia burtoni*: [AF076962](#); *Carassius auratus*: [U30386](#); *Clarias gariepinus*: [X78047](#); *Coregonus clupeaformis*: [AY245102](#); *Cynoscion nebulosus*: [AY796309](#); *Cyprinus carpio*: [AY147400](#); *Danio rerio*: [AF511531](#); *Dicentrarchus labrax*: [AF224281](#); *Eublepharis macularius*: [AB104485](#); *Homo sapiens*: [AF036329](#); *Macaca mulatta*: [AF097356](#); *Micropogonias undulatus*: [AY324669](#); *Monopterus albus*: [AY786183](#); *Morone saxatilis*: [AF056313](#); *Mugil cephalus*: [AY373451](#); *Odontesthes bonariensis*: [AY744687](#); *Oncorhynchus mykiss*: [AF125973](#); *Oreochromis niloticus*: [AB101666](#); *Oryzias latipes*: [AB041330](#); *Rachycentron canadum*: [AY677174](#); *Rana catesbeiana*: [AF186096](#); *Rutilus rutilus*: [U60668](#); *Sciaenops ocellatus*: [AY677171](#); *Scleropages jardinii*: [AB047326](#); *Sparus aurata*: [U30325](#); *Suncus murinus*: [AF107315](#); *Trichosurus vulpecula*: [AF193516](#); *Tupaia belangeri*: [U63327](#); *Typhlonectes natans*: [AF167558](#); *Verasper moseri*: [AB066359](#).

GnRH III clade: *Astatotilapia burtoni*: [AF076963](#); *Carassius auratus*: [AB012721](#); *Coregonus clupeaformis*: [AY245103](#); *Cynoscion nebulosus*: [AY796310](#); *Cyprinus carpio*: [AY189960](#); *Danio rerio*: [NM_182887](#); *Dicentrarchus labrax*: [AF224280](#); *Micropogonias undulatus*: [AY324670](#); *Monopterus albus*: [AY858055](#); *Mugil cephalus*: [AY373449](#);

Odontesthes bonariensis: [AY744688](#); *Oncorhynchus masou*: [S44614](#); *Oncorhynchus mykiss*: [AF232212](#); *Oncorhynchus nerka*: [D31868](#); *Oreochromis niloticus*: [AB101667](#); *Oryzias latipes*: [AB041332](#); *Pagrus major*: [D26108](#); *Porichthys notatus*: [U41669](#); *Rachycentron canadum*: [AY677173](#); *Rutilus rutilus*: [U60667](#); *Salmo salar*: [X79709](#); *Salmo trutta*: [X79713](#); *Salvelinus fontinalis*: [X79712](#); *Sciaenops ocellatus*: [AY677170](#); *Scleropages jardinii*: [AB047325](#); *Sparus aurata*: [U30311](#); *Verasper moseri*: [AB066358](#).

GnRH Jawless fish: *Geotria australis*: [AY307172](#); *Ichthyomyzon fossor*: [AY307174](#); *Ichthyomyzon unicuspis*: [AY307175](#); *Lampetra appendix*: [AY307176](#); *Lampetra richardsoni*: [AY307177](#); *Lampetra tridentatus*: [AY307178](#); *Mordacia mordax*: [AY307173](#); *Petromyzon marinus I*: [AF144479](#); *Petromyzon marinus III*: [AY052628](#).

References

- Abascal F, Zardoya R, Posada D (2005) ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21:2104–2105
- Adams BA, Vickers ED, Warby C, Park M, Fischer WH, Craig AG, Rivier JE, Sherwood NM (2002) Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. *Biol Reprod* 67:232–239
- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH (1998) Zebrafish hox clusters and vertebrate genome evolution. *Science* 282:1711–1714
- Benton MJ (1990) Phylogeny of the major tetrapod groups: morphological data and divergence dates. *J Mol Evol* 30:409–424
- Bogerd J, Li KW, Janssen-Dommerholt C, Goos H (1992) Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). *Biochem Biophys Res Commun* 187:127–134
- Bogerd J, Diepenbroek BD, Hund E, van Oosterhout F, Teves AC, Leurs R, Blomenröhr M (2002) Two gonadotropin-releasing hormone receptors in the African Catfish: no differences in ligand selectivity, but differences in tissue distribution. *Endocrinology* 143:4673–4682
- Carolsfeld J, Powell JF, Park M, Fischer WH, Craig AG, Chang JP, Rivier JE, Sherwood NM (2000) A novel form of gonadotropin-releasing hormone (GnRH) in herring sheds light on evolutionary pressures. *Endocrinology* 141:505–512
- Carroll RL (1988) Vertebrate paleontology and evolution. Freeman, New York
- Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B (2004) Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes. *Mol Biol Evol* 21:1146–1151
- Dubois EA, Zandbergen MA, Peute J, Goos HJ (2002) Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. *Brain Res Bull* 57:413–418
- Fernald RD, White RB (1999) Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. *Front Neuroendocrinol* 20:224–240
- González-Martínez D, Madigou T, Zmora N, Anglade I, Zanuy S, Zohar Y, Elizur A, Muñoz-Cueto JA, Kah O (2001) Differential expression of three different prepro-GnRH (gonadotropin-releasing hormone) messengers in the brain of the European bass (*Dicentrarchus labrax*). *J Comp Neurol* 429:144–155

- Gothilf Y, Muñoz-Cueto JA, Sagrillo CA, Selmanoff M, Chen TT, Kah O, Elizur A, Zohar Y (1996) Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. *Biol Reprod* 55:636–645
- Gray SL, Adams BA, Warby CM, von Schalburg KR, Sherwood NM (2002) Transcription and translation of the salmon Gonadotropin-Releasing Hormone genes in brain and gonads of sexually maturing rainbow trout (*Oncorhynchus mykiss*). *Biol Reprod* 67:1621–1627
- Grober MS, Myers TR, Marchaterre MA, Bass AH, Myers DA (1995) Structure, localization and molecular phylogeny of a GnRH cDNA from a paracanthopterygian fish, the plainfin midshipman (*Porichthys notatus*). *Gen Comp Endocrinol* 99:85–99
- Guilgur LG, Moncaut NP, Canário AVM, Somoza GM (2006) Evolution of GnRH ligands and receptors in Gnathostomata. *Comp Biochem Physiol* 144A:272–283
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
- Guindon S, Lethiec F, Duroux P, Gascuel O. (2005) PHYML Online—a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res* 1:33 (Web Server issue): W557–W559
- Hedges SB, Kumar S (2003) Genomic clocks and evolutionary timescales. *Trends Genet* 19:200–206
- Hoegg S, Brinkmann H, Taylor JS, Meyer A (2004) Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J Mol Evol* 59:190–203
- Ikemoto T, Park MK (2003) Identification and characterization of the reptilian GnRH-II gene in the leopard gecko, *Eublepharis macularius*, and its evolutionary considerations. *Gene* 316:157–165
- Jaillon O, Aury JM, Brunet F, et al. (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature* 431:946–957
- Janvier P (1999) Catching the first fish. *Nature* 402:21–22
- Jobb G (2005) TREEFINDER version of October 2005 Munich, Germany. Distributed by the author at <http://www.treefinder.de>
- Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *CABIOS* 8:275–282
- Kakar SS, Jennes L (1995) Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor mRNAs in various non-reproductive human tissues. *Cancer Lett* 98:57–62
- Kim MH, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, Aida K (1995) Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. *J Comp Neurol* 356:72–82
- King JA, Dufour S, Fontaine YA, Millar RP (1990) Chromatographic and immunological evidence for mammalian GnRH and chicken GnRH-II in eel (*Anguilla anguilla*) brain and pituitary. *Peptides* 11:507–514
- Kudo H, Hyodo S, Ueda H, Hiroi O, Aida K, Hany I, Hasegawa Y, Miyamoto K (1996) Cytophysiology of gonadotropin-releasing hormone neurons in chum salmon (*Oncorhynchus keta*) forebrain before and after upstream migration. *Cell Tissue Res* 284:261–267
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5:150–163
- Kuo MW, Lou SW, Postlethwait J, Chung BC (2005) Chromosomal organization, evolutionary relationship, and expression of zebrafish GnRH family members. *J Biomed Sci* 12:629–639
- Lethimonier C, Madigou T, Munoz-Cueto JA, Lareyre JJ, Kah O (2004) Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. *Gen Comp Endocrinol* 135:1–16
- Lin XW, Peter RE (1996) Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. *Gen Comp Endocrinol* 101:282–296
- Lynch M, Connery JS (2000) The evolutionary fate and consequences of duplicated genes. *Science* 290:1151–1155
- Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. *Genetics* 154:459–473
- Mohamed JS, Thomas P, Khan IA (2005) Isolation, cloning, and expression of three prepro-GnRH mRNAs in Atlantic croaker brain and pituitary. *J Comp Neurol* 488:384–395
- Moncaut NP, Somoza GM, Power DM, Canário AVM (2005) Colocalization of GnRH ligands and their receptors in the European sea bass, *Dicentrarchus labrax* Presented at the 15th International Congress on Comparative Endocrinology, Boston, MA, pp 23–28
- Montaner AD, Park M, Fischer WH, Craig AG, Chang JP, Somoza GM, Rivier JE, Sherwood NM (2001) Primary structure of a novel gonadotropin-releasing hormone (GnRH) variant in the brain of pejerrey (*Odontesthes bonariensis*). *Endocrinology* 142:1453–1460
- Muske LE (1993) Evolution of gonadotropin releasing hormone (GnRH) neuronal systems. *Brain Behav Evol* 42:215–230
- Muske LE (1997) Ontogeny, phylogeny and anatomical organization of multiple molecular forms of GnRH. In: Parhar IS, Sakuma Y (eds) GnRH neurons. Gene to behavior. Brain Shuppan, Tokyo, pp 145–180
- Nabissi M, Soverchia L, Polzonetti-Magni AM, Habibi HR (2000) Differential splicing of three gonadotropin-releasing hormone transcript in the ovary of the sea bream *Sparus aurata*. *Biol Reprod* 62:1329–1334
- Ngamvongchon S, Lovejoy DA, Fischer WH, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM (1992) Primary structures of two forms of gonadotropin-releasing hormone, one distinct and one conserved, from catfish brain. *Mol Cell Neurosci* 3:7–22
- Ohno S (1970) Evolution by gene duplication. Springer-Verlag, New York
- Okubo K, Aida K (2001) Gonadotropin-releasing hormones (GnRHs) in a primitive teleost, the arowana: phylogenetic evidence that three paralogous lineages of GnRH occurred prior to the emergence of teleosts. *Gen Comp Endocrinol* 124:125–133
- Okubo K, Suetake H, Aida K (1999) Expression of two gonadotropin-releasing hormone (GnRH) precursor genes in various tissues of the Japanese eel and evolution of GnRH. *Zool Sci* 16:471–478
- Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K (2000) A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* 276:298–303
- Okubo K, Suetake H, Aida K (2002) Three mRNA species for mammalian-type gonadotropin-releasing hormone in the brain of the eel *Anguilla japonica*. *Mol Cell Endocrinol* 192:17–25
- Okuzawa K, Granneman J, Bogerd J, Goos HJTh, Zohar Y, Kagawa H (1997) Distinct expression of GnRH genes in the red seabream brain. *Fish Physiol Biochem* 17:71–79
- O'Neill DF, Powell JF, Standen EM, Youson JH, Warby CM, Sherwood NM (1998) Gonadotropin-releasing hormone (GnRH) in ancient teleosts, the bonytongue fishes: putative origin of salmon GnRH. *Gen Comp Endocrinol* 112:415–425
- Pandolfi M, Muñoz-Cueto JA, Lo Nostro FL, Downs JD, Paz DA, Maggese MC, Urbanski HF (2005) GnRH systems of *Cichlasoma dimerus* (Perciformes, Cichlidae) revisited: a localization

- study with antibodies and riboprobes to GnRH-associated peptides. *Cell Tissue Res* 321:219–232
- Parhar IS, Koibuchi N, Saki M, Iwata M, Yamoaka S (1994) Gonadotropin-releasing hormone (GnRH): expression during salmon migration. *Neurosci Lett* 172:15–18
- Parhar IS, Soga T, Ishikawa Y, Nagahama Y, Sakuma Y (1998) Neurons synthesizing gonadotropin-releasing hormone mRNA subtypes have multiple developmental origins in the medaka. *J Comp Neurol* 401:217–226
- Pati D, Habibi HR (1998) Presence of salmon gonadotropin-releasing hormone GnRH and compounds with GnRH-like activity in the ovary of goldfish. *Endocrinology* 139:2015–2024
- Pedersen AG, Nielsen H (1997) Neural network prediction of translation initiation sites in eukaryotes: perspectives for EST and genome analysis. *ISMB* 5:226–233
- Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818
- Powell JFF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA, Sherwood NM (1994) Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc Natl Acad Sci USA* 91:12081–12085
- Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Mol Biol Evol* 19:101–109
- Sanderson MJ (2003) r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. *Bioinformatics* 19:301–302
- Sanderson M (2004) r8s version 170: analysis of rates (“r8s”) of evolution. Section of Evolution and Ecology, UC Davis, Davis; available at: <http://ginger.ucdavis.edu/r8s>
- Sherwood NM, Eiden L, Brownstein M, Spiess J, Rivier J, Vale W (1983) Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci USA* 80:2794–2798
- Shimodaira H, Hasegawa M (1999) Multiple comparisons of log likelihoods with applications to phylogenetic inference. *Mol Biol Evol* 16:1114–1116
- Silver MR, Kawauchi H, Nozaki M, Sower AS (2004) Cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey representing the three families of Petromyzoniformes. *Gen Comp Endocrinol* 139:85–94
- Somoza GM, Miranda LA, Strobl-Mazzulla P, Guilgur LG (2002a) Gonadotropin-releasing hormone (GnRH): From fish to mammalian brains. *Cell Mol Neurobiol* 22:589–609
- Somoza GM, Lescheid DW, Miranda LA, Lo Nostro FL, Magliulo-Cepriano L, Montaner AD, Schreiber MP, Rivier JE, Sherwood NM (2002b) Expression of pejerrey gonadotropin-releasing hormone (pjGnRH) in three orders of fish. *Biol Reprod* 67:1864–1871
- Spring J (1997) Vertebrate evolution by interspecific hybridization—Are we polyploidy? *FEBS Lett* 400:2–8
- Stefano AV, Aldana-Marcos HJ, Affanni JM, Somoza GM (2000) Gonadotropin-releasing hormone (GnRH) neuronal systems in the pejerrey *Odontesthes bonariensis* (Atheriniformes). *Fish Physiol Biochem* 23:215–223
- Strüssmann CA (1989) Basic studies on seed production of pejerrey *Odontesthes bonariensis*. Doctoral thesis. Tokyo University of Fisheries, Tokyo
- Suzuki K, Gamble RL, Sower SA (2000) Multiple transcripts encoding lamprey gonadotropin-releasing hormone-I precursors. *J Mol Endocrinol* 24:365–376
- Swofford DL (2002) PAUP. Phylogenetic analysis using parsimony (and other methods). Version 4. Sinauer Associates, Sunderland, MA
- Taylor JS, Braasch I, Frickey T, Meyer A, Van de Peer Y (2003) Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome Res* 13:382–390
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Torgersen J, Nourizadeh-Lillabadi R, Husebye H, Aleström P (2002) *In silico* and *in situ* characterization of the zebrafish (*Danio rerio*) GnRH3 (sGnRH) gene. *BMC Genom* 3:25–36
- Uzbekova S, Lareyre JJ, Guiguen Y, Ferriere F, Bailhache T, Breton B (2001) Expression of sGnRH mRNA in gonads during rainbow trout gametogenesis. *Comp Biochem Physiol* 129B:457–465
- Uzbekova S, Lareyre JJ, Madigou T, Davail B, Jalabert B, Breton B (2002) Expression of prepro-GnRH and GnRH receptor messengers in rainbow trout ovary depends on the stage of ovarian follicular development. *Mol Reprod Devel* 62:47–56
- Van de Peer Y, Taylor JS, Meyer A (2003) Are all fishes ancient polyploids? *J Struct Funct Genom* 3:65–73
- Vandepoele K, De Vos W, Taylor JS, Meyer A, Van de Peer Y (2004) Major events in the genome evolution of vertebrates: paranome age and size differ considerably between ray-finned fishes and land vertebrates. *Proc Natl Acad Sci USA* 101:1638–1643
- Vickers ED, Laberge F, Adams BA, Hara TJ, Sherwood NM (2004) Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, *Coregonus clupeaformis*. *Biol Reprod* 70:1136–1146
- von Schalburg KR, Warby CM, Sherwood NM (1999) Evidence for gonadotropin-releasing hormone peptides in the ovary and testis of rainbow trout. *Biol Reprod* 60:1338–1344
- White RB, Eisen JA, Kasten TL, Fernald RD (1998) Second gene for gonadotropin-releasing hormone in humans. *Proc Natl Acad Sci USA* 95:305–309
- White RB, Fernald RD (1998) Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. *Gen Comp Endocrinol* 112:17–25
- Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J Mol Evol* 39:306–314
- Yu KL, Sherwood NM, Peter RE (1988) Differential distribution of two molecular forms of gonadotropin-releasing hormone in discrete brain areas of goldfish (*Carassius auratus*). *Peptides* 9:625–630
- Zhu M, Yu X, Janvier P (1999) A primitive fossil fish sheds light on the origin of bony fishes. *Nature* 397:607–610