Relaxin gene family in teleosts: phylogeny, syntenic mapping, selective constraint, and expression analysis

Sara V Good-Avila*1, Sergey Yegorov1, Scott Harron2, Jan Bogerd3, Peter Glen2, James Ozon2 and Brian C Wilson*2,1

Address: 1Department of Biology, University of Winnipeg, Winnipeg, Manitoba, R3E 2H9, Canada, 2Department of Biology, Acadia University, Wolfville, Nova Scotia, B4P 2R6, Canada and 3Department of Biology, Utrecht University, Utrecht, 3584 CH, Netherlands

Email: Sara V Good-Avila* - s.good-avila@uwinnipeg.ca; Sergey Yegorov - yegorovsrg@gmail.com; Scott Harron - scott.harron@dal.ca; Jan Bogerd - j.bogerd@uu.nl; Peter Glen - peter_glen@hotmail.com; James Ozon - jamesozon@gmail.com; Brian C Wilson* - brian.wilson@acadiau.ca

* Corresponding authors

Abstract

Background: In recent years, the relaxin family of signaling molecules has been shown to play diverse roles in mammalian physiology, but little is known about its diversity or physiology in teleosts, an infraclass of the bony fishes comprising ~ 50% of all extant vertebrates. In this paper, 32 relaxin family sequences were obtained by searching genomic and cDNA databases from eight teleost species; phylogenetic, molecular evolutionary, and syntenic data analyses were conducted to understand the relationship and differential patterns of evolution of relaxin family genes in teleosts compared with mammals. Additionally, real-time quantitative PCR was used to confirm and assess the tissues of expression of five relaxin family genes in Danio rerio and in situ hybridization used to assess the site-specific expression of the insulin 3-like gene in D. rerio testis.

Results: Up to six relaxin family genes were identified in each teleost species. Comparative syntenic mapping revealed that fish possess two paralogous copies of human RLN3, which we call rln3a and rln3b, an orthologue of human RLN2, rln, two paralogous copies of human INSL5, insl5a and insl5b, and an orthologue of human INSL3, insl3. Molecular evolutionary analyses indicated that: rln3a, rln3b and rln are under strong evolutionary constraint, that insl3 has been subject to moderate rates of sequence evolution with two amino acids in insl3/INSL3 showing evidence of positively selection, and that insl5b exhibits a higher rate of sequence evolution than its parologue insl5a suggesting that it may have been neo-functionalized after the teleost whole genome duplication. Quantitative PCR analyses in D. rerio indicated that rln3a and rln3b are expressed in brain, insl3 is highly expressed in gonads, and that there was low expression of both insl5 genes in adult zebrafish. Finally, in situ hybridization of insl3 in D. rerio testes showed highly specific hybridization to interstitial Leydig cells.

Conclusions: Contrary to previous studies, we find convincing evidence that teleosts contain orthologues of four relaxin family peptides. Overall our analyses suggest that in teleosts: 1) rln3 exhibits a similar evolution and expression pattern to mammalian RLN3, 2) insl3 has been subject to positive selection like its mammalian counterpart and shows similar tissue-specific expression in Leydig cells, 3) insl5 genes are highly represented and have a relatively high rate of sequence evolution in teleost genomes, but they exhibited only low levels of expression in adult zebrafish, 4) rln is evolving under very different selective constraints from mammalian RLN. The results presented here should facilitate the development of hypothesis-driven experimental work on the specific roles of relaxin family genes in teleosts.
Background
The relaxin family of peptides belongs to the insulin superfamily and includes a group of signaling molecules that share similar gene and protein secondary structures. The genes have two exons that code for a prepropeptide consisting of a signal peptide, followed by B-, C-, and A-chains. Prohormone processing and activation occurs by removal of the C-chain by prohormone convertases that cleave at dibasic junctions [1]. In the mature peptide, six cysteine residues form three disulfide bonds that give this superfamily its distinctive secondary protein structure. In most mammals, the relaxin family consists of two relaxin peptides, RLN and RLN3, which share the receptor binding domain RXXXRXXI/V and four insulin-like peptides, INSL3, INSL4, INSL5, and INSL6, which have a less conserved motif [2]. Additionally, relaxin family peptides activate G protein-coupled receptors (GPCR) while other members of the insulin superfamily signal via tyrosine kinases [3].

The hormone RLN was the first member of the family to be studied in detail [2]. Originally characterized as a reproductive hormone [4], RLN is now implicated in diverse physiological processes, via its role in stimulating the production of matrix metalloproteinases (MMPs) which degrade extracellular matrix proteins and cause tissue remodeling [2]. By this action, the hormone is involved in parturition where it softens the connective tissues of the reproductive tract and prepares the mammary glands for lactation [2]; RLN has also been found to be involved in diverse processes involving tissue remodeling such as wound healing, angiogenesis and tumor formation [5,6]. In mammals, the RLN gene tandemly duplicated to give rise to two additional members of the family, INSL4 and INSL6, both of which are poorly understood, but which are both predominantly expressed in placenta and testis [7]. A more recent duplication of the RLN gene, specific to humans and anthropoid apes, resulted in primates having two copies of RLN, called RLN1 and RLN2, with RLN2 being functionally equivalent to the RLN in other mammals [2]. More recently, other members of the relaxin family have been identified: RLN3 was found to be expressed in the brain and testis of rodents and to exhibit high sequence conservation across mammalian species [8,9]. This led to predictions that RLN3 may function as a neuropeptide [8], which has received some empirical support because the peptide has been shown to be involved in the modulation of feeding activities, body weight regulation and in stress coordination, learning and memory [10,11].

Another member of the relaxin family, insulin-like peptide 3 (INSL3), attracted the attention of andrologists after it was discovered to play a crucial role in testicular descent in young males of human and mice [12]. There is also evidence that INSL3 is a survival factor for male and female germ cells in mammals [12,13]; it is expressed in significant amounts in testicular Leydig cells, while in females the distribution of INSL3 producing sites is less specifically localized, detected mainly in ovarian follicular theca cells [12]. However, the receptor for INSL3, RXFP2, has been identified in a broad range of tissues: brain, kidney, muscle, testis, thyroid, uterus, lymphocytes and bone marrow [12]. One of the least understood members of the relaxin family is INSL5, which was originally identified from analyses of expressed sequence tags in the human genome [14], and its expression has been detected in fetal brain, pituitary and colon as well as in the cortex of the thymus gland [15,16]. The receptor for INSL5, RXFP4, is broadly distributed in the human body, but the colon appears to be the most prominent site of RXFP4 mRNA expression. This is consistent with current hypotheses that it is involved in gut contractility and neuroendocrine signaling [15,16]. Thus, collectively, the relaxin family is revealing itself to be a group of peptides primarily involved in reproductive processes in mammals, and at the same time plays a broader role in other aspects of mammalian physiology.

Investigation on relaxin family peptides outside mammals has been limited. Relaxin-like peptides have been found in the testis, ovary, and/or alkaline glands of three species of sharks [17-21] and in bird testis [22]. However, there are only a few physiological studies on the expression of relaxin in teleosts [23,24], which are an infraclass of the bony fishes, and comprise 96% of the 26,000 ray-finned fish species and ~ half of all vertebrates on the planet [25].

Molecular estimates indicate that the common ancestor of teleosts and tetrapods existed ~ 450 million years ago (mya) [25,26]. A whole genome duplication (WGD), that occurred early in teleost evolution, ~ 350 mya, is hypothesized to have contributed to the rapid divergence of the group in part because of the opportunities that WGD’s offer for acquisition of new gene functions [27,28]. After gene duplication, newly derived paralogous sequences are assumed to share similar functions to the ancestral gene. However, over time, the genes may be non-, sub- or neo-functionalized [29,30]. Because the teleost WGD event is ancient, examination of the proportional frequency and consequences of non-, sub- and neo-functionalization in teleosts have provided important insights into the role of gene duplication in vertebrates [31-35].

There have been two previous bioinformatics studies on the relaxin family [9,36]. Neither study focused on the molecular evolution nor expression of the family in teleosts, but they both included sequences of relaxin family genes from teleosts. Additionally, Park et al. [36] per-
formed a syntenic data analysis of relaxin family genes in vertebrates and found that the common ancestor of teleosts and tetrapods harboured three independent relaxin family loci (RFL): RFLA - INSL5-like genes, RFLB - RLN-like genes and RFLC - RLN3-like genes. In this paper we expand upon these previous studies by including 32 relaxin family gene sequences from eight teleost species and by focusing our analyses on understanding the specific forces influencing orthologous and paralogous gene copy evolution of relaxins in teleosts. To this end, detailed analyses of teleost relaxin family genes were performed to assess the number of orthologous and paralogous sequences of relaxins in teleosts, their syntenic relationship to human relaxin family genes, the strength of purifying versus diversifying selection, the role of positive selection at the codon level, the relative expression of fying versus diversifying selection, the role of positive sequences of relaxins in teleosts, their syntenic relationship to human relaxin family genes (Figure 3). For the Bayesian trees, the GTR + Γ model of sequence evolution was employed, and partitioning and unlinking the three codon positions revealed that the rate of change was approximately four times higher at the third compared to first and second codon positions (0.51, 0.40 and 2.00 respectively) and that the gamma parameter was 0.98 for the first two positions, but 5.06 for the third position. Hierarchical likelihood tests of the amino acid models included in ProTest v. 1.0.6 and Bayesian methods both strongly supported the WAG + Γ (a = 0.60) model of amino acid sequence evolution which was used to reconstruct the phylogenetic relationship among amino acid sequences (Additional File 2: Figure S1). Although the topology of the Bayesian partitioned DNA sequence tree was similar to the Bayesian tree based on amino acid data, the saturation of the third base position lowered confidence in the Bayesian Posterior Probabilities (BPP) and led to some problems of long-branch attraction: the two trees shown are the distance (minimum evolution) tree based on DNA (Figure 4) and the Bayesian topology based on the amino acid sequences using the WAG + Γ model of sequence evolution (Additional File 2: Figure S1).

The phylogenetic tree reconstructed from the DNA sequence data support the presence of four relaxin family groups in teleosts with reasonably high bootstrap support 1) rln3a and rln3b (81%) 2) rln (91%) 3) insl5a and insl5b (86%) and 4) insl3 (50%). All teleost relaxin family genes cluster with their mammalian orthologues as identified through the syntenic data analyses except teleost rln, which is sister clade to rln3 with high bootstrap support (91%) and exhibits, overall, a closer resemblance to these sequences (Figure 4) than to its true orthologue, mammals RLN. The three frog sequences cluster with their orthologue basal to the teleost clade: in particular the X. tropicalis rln sequence shows greater similarity to teleost rln than to mammalian RLN. The BPP support for the Bayesian tree reconstructed with amino acid sequences gave similar results and statistical support (Additional File 2: Figure S1) to that based on DNA sequence data, with the main exception that teleost insl3 sequences have higher BPP support (74%) than based on the DNA sequence but they do not group with mammalian INSL3.

Teleost relaxin family genes are subject to different levels of purifying selection

The two-cluster test was performed on the topology shown in Figure 5 and identified the following groups as having differential rates of evolution 1) teleost rln3a and rln3b exhibited accelerated evolution compared to mammalian RLN3, 2) teleost insl5 and insl3 independently exhibited accelerated evolution compared to teleost rln3a, rln3b, rln and mammalian RLN3 and 3) teleost insl5b showed accelerated evolution relative to insl5a.
Figure 1 (see legend on next page)
The average value of $d_\alpha/d_\gamma$ within each relaxin family gene or within the B- and A-chains ranged from 0.05 ($rln3b$, A-chain) to 0.48 ($insl5b$, A-chain) in teleosts and from 0.04 ($RLN3$, B-chain) to 0.78 ($INSL6$, B-chain) in mammals (Table 1). Values were generally lower in the B- compared to the A-chain. No gene was found to exhibit, overall, evidence of positive selection in which $d_\alpha/d_\gamma > 1$. In general, teleost $rln3a$, $rln3b$ and $rln$ had few non-synonymous changes in both the B- and A-chains; their $d_\alpha/d_\gamma$ ratios were less than 0.10 indicating that they are under strong purifying selection comparable to mammalian $RLN3$ which had a $d_\alpha/d_\gamma$ ratio of 0.08. Teleost $insl5a$ exhibited similar and moderate levels of purifying selection to mammalian $INSL5$, with the $d_\alpha/d_\gamma$ ratio in teleosts (0.25) being similar to that in mammals (0.23). On the other hand, teleost $insl5b$ exhibited weaker purifying selection with an overall $d_\alpha/d_\gamma$ ratio of 0.40, and having $d_\alpha/d_\gamma$ ratios more than twice that for $insl5a$ in the B- (0.34) and A-chains (0.48). Teleost $insl3$ exhibited similar (0.37) overall sequence divergence to $insl5b$. Although $insl5b$ and $insl3$ are the teleost genes under the weakest evolutionary constraint (a result also supported by the molecular clock analyses), they are under stronger purifying selection than mammalian $RLN$ and $INSL6$, which have $d_\alpha/d_\gamma$ ratios of 0.64 and 0.46 respectively, with the B-chain of mammalian $INSL6$ exhibiting the highest rate of $d_\alpha/d_\gamma$ at 0.78.

The branch-site model A analyses indicated that only $insl3$ exhibited evidence of codon-specific selection within teleosts or mammals. The null model was rejected when both teleosts and mammals were used as the foreground branch and two amino acids were identified as being subject to positive selection with Bayes Empirical Bayes (BEB) values of > 0.95. Using teleosts as the foreground lineage, site 27 in the B/C cut site, which is valine/leucine in teleosts but tryptophan in mammals, shows evidence of positive selection. Setting mammalian $INSL3$ to the foreground branch, identified site 21 in the B-chain, which is fixed as valine in mammals but to serine in teleosts, is also under positive selection (Table 2). An additional 10 sites were identified by the branch-site model as being potential sites of selection but none had BEB values $>$0.95. However, it is interesting to note that of the 12 sites identified by the model, 3 are in the B/C cut site (Table 2, Figure 4).

**Quantitative PCR analysis showed evidence of significant expression of rln3a/rln3b and insl3 genes in the gonads and brain of zebrafish**

DNA sequencing of the products amplified using qPCR confirmed the identity of all $D. rerio$ amplification products (not shown). The results of the expression analyses of relaxin family genes in $D. rerio$ indicated that $rln3a$ and $rln3b$ are predominantly expressed in the brain, although $rln3b$ was also expressed in the gonads, while $rln3a$ was not (Figure 6). Additionally, the data strongly support a role for $insl3$ in both ovary and testes with additional expression of $insl3$ in the brain and gill: the expression of $insl3$ was not significantly lower than that of the housekeeping gene, b2m, in ovary, and was only marginally lower than b2m in testis (data not shown). Lastly, the results showed little evidence of expression of either $insl5a$ or $insl5b$ in any tissue; $insl5a$ was expressed in most tissues (except heart) at low levels while $insl5b$ showed uniformly low, essentially negligible, expression (Figure 6).

**In situ hybridization identified expression of insl3 in zebrafish testis in Leydig cells**

A strong and specific signal of $insl3$ mRNA was observed in the interstitial area in the Leydig cells (Figure 7A). Higher magnification showed that the cytoplasm of these cells was strongly labeled while the nucleus remained
Figure 2 (see legend on next page)
unstained (Figure 7B). Positive cells formed clusters that were often arranged around blood vessels. There were no apparent (rostro-caudal or dorso-ventral) gradients in staining intensity in an adult testis, and all Leydig cells appeared to be labeled strongly. However, although not properly quantified yet, it is possible that the size of the Leydig cell clusters is larger in the periphery than in central areas of the testis. The intratubular area, containing Sertoli and germ cells at different stages of spermatogenesis, remained unlabeled. Taken together, these findings suggest that \textit{insl3} is a reliable Leydig cell marker in zebrafish testis tissue. No signal was observed with the sense cRNA \textit{insl3} probe (data not shown), indicating the specificity of the antisense probe generated against the sequence of zebrafish \textit{insl3} mRNA.

**Discussion**

Reconstructing the evolutionary relationship among relaxin family genes in teleosts and tetrapods has highlighted the difficulties of determining orthologous and paralogous relationships in ancient gene families using phylogenetic data alone, in particular for small, relatively quickly evolving genes [25,26]. Previous phylogenetic studies on relaxin family genes [9,36] have also found a poor resolution of relaxin family genes, particularly in teleosts: by including more teleost species we find that only teleost \textit{rln} failed to group with its mammalian orthologue and this is evidently due to very different selective pressures operating on the gene in the two groups (see discussion below). Using synteny mapping data, we identify that the six relaxin family genes in teleosts are orthologous to four mammalian genes: \textit{RLN3}, \textit{RLN}, \textit{INSL5} and \textit{INSL3} with two of the genes, \textit{INSL5} and \textit{RLN3}, containing paralogous copies in teleosts, \textit{insl5a/insl5b} and \textit{rln3a/rln3b} (Figure 3). These results are similar to those presented by Park \textit{et al.} [36] with the exception that we find evidence that teleosts possess an orthologue to \textit{INSL3}, while they argue that \textit{INSL3} arose via duplication from \textit{RLN3} after the divergence of teleosts from tetrapods. We therefore propose that the relaxin family genes that were first identified as \textit{RLX3a-3f} by Wilkinson \textit{et al.} [9] be named based on their orthology to the mammalian counterparts: \textit{rln3a/rln3b}, \textit{rln}, \textit{insl5a/insl5b}, and \textit{insl3} respectively (Table 3). We encourage the adoption of this nomenclature, since there is currently considerable confusion regarding the identity of relaxin family peptides in teleosts on publicly available databases.

In teleosts, these relaxin family genes are subject to strong or moderate purifying selection: \textit{rln3a}, \textit{rln3b} and \textit{rln} are all similar in sequence and highly conserved, \textit{insl5a} exhibits a slightly faster rate of molecular evolution, and \textit{insl3} and \textit{insl5b} exhibit the highest levels of molecular evolution in teleosts, the latter having a significantly faster rate of evolution than its parologue \textit{insl5a}. Using the branch-site model A test, we find evidence that one codon in teleost \textit{insl3} and another in mammalian \textit{INSL3} have been subject to positive selection. Lastly, we find evidence that five of the six relaxin family genes present in the model organism \textit{Danio rerio} are expressed in one or multiple tissues, especially brain and gonads and that \textit{insl3} is specifically expressed in interstitial Leydig cells in zebrafish testis. The significance of these results will be discussed first within the context of the comparative analysis of orthologous relaxin family genes in teleosts and mammals and then with respect to the evolution and expression of paralogous relaxin family genes in teleosts.
Relaxin family genes: teleosts versus mammals

Teleost rln is more similar in sequence to rln3 than to its mammalian orthologue RLN

Teleost rln was found in 4 of the 5 species for which the whole genome was available but it is, surprisingly, absent from the D. rerio genome. The close identity of teleost rln to rln3, and yet its striking difference from mammalian RLN, suggest that the gene has been subject to different evolutionary pressures in the two groups. Several processes could potentially have caused this such as: 1) teleost rln has retained the ancestral function of the gene while mammalian RLN has diversified in function or 2) teleost rln has undergone convergent evolution with rln3. Certainly there is support for the hypothesis that mammalian RLN has diversified in function: it exhibits the highest rate of molecular evolution of any of the relaxin family genes except INSL6 and it has duplicated giving rise to four fast-evolving paralogues in humans and anthropoid apes - RLN1, RLN2, INSL4 and INSL6 (Figure 1B), all of which are produced by human reproductive tissues [37,38]. Thus, the clustering of mammalian RLN with its linked paralogues, rather than with teleost rln, arises in part because the clade has a higher rate of evolution and more recent common ancestry than the clade harbouring teleost rln. This hypothesis is also supported by the phylogenetic clustering of frog and teleost rln sequences (Figure 5). However, the identity of the B-domain of teleost rln with rln3 suggests that some factor such as shared receptor binding domains may have selected for them to retain the same sequence. Until the receptors of teleost relaxin family genes are known and the physiological role of teleost rln understood, it will be difficult to assess this hypothesis. Although the physiological role of relaxin in mammals is primarily associated with the reconstruction of connective
Figure 4
Alignment of the deduced amino acid relaxin sequences from mammals and teleost species used for the phylogenetic analysis. Conserved residues are boxed. Location of the relaxin receptor binding motif residues (RXXXRXXI/V), B-chain, A-chain, and twin dibasic junctions (B/C and C/A) are shown. Amino acids that are underlined are those identified as potential candidates of codon-specific positive selection using the branch-site model A analyses (see text for details), but only the two that are in bold and underlined were found to have a significant probability of being subject to positive selection with a BEB probability >0.95.
Figure 5
Phylogenetic reconstruction of the relationship among relaxin family DNA sequences. Phylogenetic tree reconstructed using the minimum evolution algorithm (a distance method) and including only the first two positions of each codon and employing the Tamura-3-parameter + $\Gamma$ model of sequence evolution. Numbers at each node indicate the bootstrap values. Genes located at each of the four relaxin family loci, insl5 (RFLA), rln (RFLB), rln3 (RFLCII) and insl3 (RFLCII), are shown in the same colour. Paralogous copies of insl5 (insl5a and insl5b) and rln3 (rln3a and rln3b) that arose after the teleost WGD are indicated. Mammalian INSL6 is a tandemly duplicated member of the relaxin family that is linked and paralogous to mammalian RLN.
Table 1: Results of the analyses using the branch-site model A of Yang and Nielsen (2002) on relaxin family orthologues in teleosts.

| Gene | Model | Foreground branch | Parameter | df | 2 Δ L | Positively selected sites |
|------|-------|-------------------|-----------|----|-------|-------------------------|
| insl5 | A (alt) | teleost insl5b | p0 = .55, p1 = .3, p2 = .14, ω2 = 1.0 | 3 | 0.0 | -- |
|      | A (ω2 = 1) | teleost insl5b | p0 = .53, p1 = .28, p2 = .17 | 2 | -- | N/A |
|      | A (alt) | teleost insl5a | p0 = .52, p1 = .28, p2 = .2, ω2 = 1.04 | 3 | 1.0 | -- |
|      | A (ω2 = 1) | teleost insl5a | p0 = .51, p1 = .27, p2 = .22 | 2 | N/A | |
| rln  | A (alt) | teleost rln | p0 = .43, p1 = .56, p2 = 0.0, ω2 | 3 | 0.0 | -- |
|      | A (ω2 = 1) | teleost rln | p0 = .43, p1 = .56, p2 = 0.0 | 2 | N/A | |
| insl3 | A (alt) | teleost insl3 | p0 = 0, p1 = 0, p2 = 1.0, ω2 | 3 | 4.1 | 6R(.54), 24S(.83), 27V(.97), 50M(.87), 54I(.91), 55Q(.64) |
|      | A (ω2 = 1) | teleost insl3 | p0 = 0, p1 = 0, p2 = 1.0 | 2 | - | N/A |
|      | A (alt) | mammalian INSL3 | p0 = .56, p1 = .21, p2 = .23, ω2 = 41.9 | 3 | 4.0 | 7V(.75), 13E(.87), 21S(.95), 28K(.92), 29R(.9) |
|      | A (ω2 = 1) | mammalian INSL3 | p0 = .6, p1 = .22, p2 = .17 | 2 | - | (61.41G,.79) |
| rln3 | A (alt) | teleost rln3 | p0 = 0, p2 = 1.0, ω2 = 999 | 3 | 0.48 | All sites selected* |
|      | A (ω2 = 1) | teleost rln3 | p0 = 0, p1 = 0, p2 = 1.0 | 2 | -- | |

For each gene, either teleosts or mammals were used as the foreground branch on which the alternate (alt) hypothesis of positive selection was compared to the null model (ω = 1, fixed). The proportion of sites subject to purifying (p0), nearly neutral (p1) and positive selection (p2) and the estimate of ω (ω2) in the free model are all given as is the Likelihood (L) of the model. The codon positions (using D. rerio as the reference sequence) of the sites estimated to be subject to positive selection are indicated where significant. The null and alternative models are significantly different when 2 Δ L > 3.841. *For rln3, the models were not statistically different.

Table 2: Proposed nomenclature for relaxin family loci (RFL) in teleosts.

| Teleost gene name | Human | Park et al. | Wilkinson et al. | T. rubripes | D. rerio |
|-------------------|-------|-------------|-----------------|-------------|---------|
| insl5a            | INSL5 | RFLA1       | TrRLX3d         | DrRLX3b     |
| insl5b            | --    | RFLA2       | TrRLX3e         | DrRLX3d     |
| rln               | RLN   | RFLB1       | TrRLX3c         | --          |
| rln3a             | RLN3  | RFLC2       | TrRLX3a         | DrRLX3a     |
| rln3b             | --    | RFLC1       | TrRLX3b         | --          |
| insl3             | INSL3 | RFLCII*     | TrRLX3f         | DrRLX3c     |

The proposed names for the six teleost RFL are given along with the nomenclature used for the orthologous genes in humans, and the nomenclature proposed by Park et al. (2008) and Wilkinson et al. (2005) in Takifugu rubripes and Danio rerio for teleost relaxin family genes. Park et al. (2008) propose that teleosts do not have an insl3 orthologue. They suggest that insl3 adopted its role in mammals after the divergence of Amphibia, and refer to the ancestral insl3 locus in amphibians as RFLCII.
tissue during reproduction [2], the gene is involved in several pathways not specific to reproduction, e.g. metalloproteinase activation, wound healing and reduction of fibrosis in non-reproductive tissues, that may reflect the ancestral role of the gene and its potential action in teleosts.

Teleost insl3 shows a similar spatial pattern of expression to mammalian INSL3
Since the descent of testicles from the abdominal cavity is solely specific to therian mammals, insl3 is postulated to have adopted this specialized role prior to the emergence of marsupials [36]. Park et al. [36] propose that the duplication of RFLCI, that gave rise to RFLCII harbouring INSL3, occurred prior to the divergence of amphibians and mammals but after the divergence of teleosts from tetrapods. They base this conclusion on the putative absence of an INSL3-like orthologue in fish. However, by studying more fish species, we find convincing syntenic evidence that the duplication of RFLCI and RFLCII occurred prior to the divergence of fish and tetrapods (Figure 3). Indeed, our qPCR results show that insl3 was the most abundantly expressed relaxin in D. rerio and that it was highly expressed in both ovary and testis, exhibiting only marginally lower expression levels than the housekeeping gene. The in situ hybridization results additionally showed that insl3 is expressed in the interstitial area of D. rerio testis (i.e. in Leydig cells) but is completely absent from the intratubular section (containing Sertoli and germ cells). This pattern of Leydig cell-specific staining has also been identified for Cyp17a1 [39] and for 3βHSD [40], both Leydig cell proteins involved in zebrafish germ cell sexual differentiation.

Park et al. [36] show how specific changes to the receptors for mammalian RGN3 and INSL3, RXFP1 and RXFP2...
respectively, during early therian evolution allowed for INSL3 to adopt its specific role in testicular descent in mammals: they further show that the gene products of RFLCI (rln3a and rln3b) activate both rxfp1 and rxfp2 in teleosts. A role for codon-specific positive selection in the evolution of the insl3 gene was also found in this study: insl3 was the only relaxin family gene which exhibited evidence of lineage and site-specific selection in teleosts and mammals. Two amino acids were found to show evidence of positive selection at the 95% significance level when teleosts or mammals were used as the foreground lineage, although a total of twelve sites were included in the most probable posterior model. Interestingly, three of these twelve sites were in the B/C dibasic junction. Prohormone convertases activate hormones by cleaving dibasic chain junctions; our results suggest that different expression patterns between mammalian and teleost relaxin family genes may be mirrored by these convertases [41,42].

Table 3: Average pairwise dn/ds values of relaxin family genes in teleost and mammals.

| Relaxin family Gene | B + A - chain | B-chain | A-chain |
|---------------------|---------------|---------|---------|
|                     | d_s/d_a       | d_s/d_a| d_s/d_a|
| Teleost rln3a       | 0.08 (0.60, 0.05) | 0.09 (0.57, 0.05) | 0.07 (0.62, 0.04) |
| Teleost rln3b       | 0.07 (0.58, 0.04) | 0.10 (0.57, 0.06) | 0.05 (0.60, 0.03) |
| Teleost rln         | 0.09 (0.46, 0.04) | 0.04 (0.45, 0.02) | 0.11 (0.52, 0.06) |
| Teleost insl5a      | 0.25 (0.51, 0.13) | 0.13 (0.53, 0.07) | 0.20 (0.49, 0.10) |
| Teleost insl5b      | 0.40 (0.57, 0.23) | 0.34 (0.58, 0.20) | 0.48 (0.52, 0.25) |
| Teleost insl3       | 0.37 (0.63, 0.23) | 0.41 (0.64, 0.26) | 0.45 (0.53, 0.24) |
| Mammalian RLN       | 0.64 (0.50, 0.32) | 0.59 (0.56, 0.33) | 0.60 (0.50, 0.30) |
| Mammalian RLN3      | 0.08 (0.51, 0.04) | 0.04 (0.45, 0.02) | 0.11 (0.56, 0.06) |
| Mammalian INSL3     | 0.36 (0.51, 0.18) | 0.20 (0.50, 0.10) | 0.40 (0.61, 0.24) |
| Mammalian INSL5     | 0.23 (0.79, 0.17) | 0.19 (0.61, 0.12) | 0.21 (0.98, 0.21) |
| Mammalian INSL6     | 0.46 (0.46, 0.20) | 0.78 (0.32, 0.25) * | 0.24 (0.58, 0.14) |

Only the five teleost and four mammalian species for which complete genomic information was available were included in the analyses. Z-tests of whether d_s/d_a > 1 for any gene was performed using bootstrapping to compute the variance (+ p < 0.10, * p < 0.05). Average d_s/d_a < 0.1 represent strongly purifying selection, 0.11-0.5 represent weakly purifying selection, and >1 represents recent positive selection. See text for details.
Overall, our data support Park et al.’s [36] conclusion that the co-evolution of INSL3-RXFP2 may have allowed INSL3 to adopt its particular role in mammalian testicular descent, but we also show that INSL3-like genes are present in teleosts and that they are also involved in Leydig cell differentiation.

**Teleost rln3 paralogues show similar gene sequence and expression to mammalian RLN3**

Examining the spatial and temporal expression of rln3 paralogues, Donizetti et al. [23] recently found evidence for the expression of both genes in adult zebrafish brain. Additionally, they found expression during larval stages for rln3a in the nucleus incertus and for rln3b in the peri-aqueductal gray (PAG) matter, the latter being implicated in vocal communication in fish. Our qPCR results in adult zebrafish are consistent with theirs, and the putative role of RLN3 as a neuropeptide involved in feeding, body weight regulation, stress coordination, learning and memory [10,11]. Interestingly, while we find significant expression of rln3b in the ovary, Donizetti et al. [23] showed evidence of expression of rln3b in the testis but not the ovary of adult zebrafish. Even though the difference between the expression of rln3b in the two sexes deserves further attention, the hypothesis that rln3 performs a dual function in teleosts is supported by the work of McGowan et al. [43]. They found evidence for the involvement of RLN3 in the hypothalamic-pituitary-gonadal axis in mice, suggesting that it may be a signal linking nutritional status and reproductive function. Collectively, these data suggest that rln3 (RLN3) may play similar roles in teleosts and mammals, which is further supported by its high degree of sequence conservation between the two groups [9, this study].

**Both teleost insl5 paralogues are well represented but, as in mammals, their role(s) are unclear**

Seven of the eight examined species of teleosts harboured insl5a genes, and all the species for which the whole genome had been sequenced, additionally contained the paralogous sequence insl5b. Despite its presence in the genome, the qPCR data in *D. rerio* were more ambivalent. While some expression of insl5a was found in several tissues, particularly brain and gill, only very low expression of insl5b was found in the examined adult zebrafish tissues.

**Evolution and expression of paralogous genes, rln3 and insl5, in teleosts**

Our data show that rln3a/b and insl5a/b arose by duplication after the tetrapod-teleost divergence. It has been proposed that duplicate gene copies may 1) accumulate nonsense mutations in regulatory or gene elements and become non-functionalized, 2) diverge in the tissue or timing of expression compared to the ancestral copy and become sub-functionalized, or 3) acquire new functions and be neo-functionalized [30]. Theory suggests that duplicated genes are most likely to be lost or sub-functionalized [44], and genome wide scans indicate that about 80%-85% of teleost genes were non-functionalized after the WGD [29,45]. The relative rates of sub- versus neo-functionalization are difficult to determine, in part because sub-functionalization may lead to neo-functionalization over long evolutionary timescales [46]. Additionally, changes associated with sub-functionalization often occur in promoter regions that regulate timing or control of gene expression and studies that examine the rate or pattern of molecular evolution in the protein coding region alone may not detect sub-functionalization [44].

The data presented here suggest that the paralogous copies of rln3 and insl5 may have been subjected to different forces of “functionalization” post-duplication. The paralogues rln3a and rln3b, exhibit similar patterns of molecular evolution consistent with sub-functionalization of the duplicated copy. Our qPCR data indicate that rln3b is expressed in the brain and ovary while rln3a is expressed only in the adult zebrafish brain. This result is consistent with the findings of Donizetti et al. [23] although they additionally find evidence of distinct differential expression of these two paralogues during zebrafish embryogenesis. Seemingly, the pattern observed in adult zebrafish for rln3 paralogues differs somewhat from the classical definition of sub-functionalization because sub-functionalized copies are expected to diverge in temporal or spatial expression but collectively span the ancestral expression patterns, although our results are consistent with other data on the expression of paralogous genes [45,46]. On the other hand, the duplicated copies of insl5 appear to be subject to different selective pressures. The molecular clock analyses revealed that insl5b has had an accelerated rate of evolution compared to insl5a, and the average value of $d_{ab}/d_{aa}$ was more than twice as high in the B- and A-chains of insl5b compared to insl5a, a pattern identified for other duplicated teleost genes believed to have undergone neo-functionalization [45]. Support for the contention that insl5a, rather than insl5b, has retained the ancestral function is further given by the syntentic data analyses in which many of the genes linked to INSL5 in humans are preferentially linked to insl5a (Figure 1A). The low levels of expression for insl5a and even lower levels for insl5b suggest that either 1) we have not identified the main tissues of expression for these genes and/or 2) they are expressed at developmental stages not included in this preliminary analysis (adult male and female zebrafish): in the future this will be explored using more detailed qPCR studies.
Conclusions
We find that teleosts harbour orthologues of four mammalian relaxin family genes: RLN, RLN3, INSL3 and INSL5. Two of the orthologues exist as paralogous duplicates in teleosts (rln3a/rln3b and insl5a/insl5b) probably as a result of the WGD event that occurred early in the evolution of teleosts. By combining the bioinformatics and expression analyses performed in this study we can draw the following conclusions about each teleost relaxin gene: 1) both rln3 paralogues exhibit similar evolution and expression to mammalian RLN3 and the paralogous copies appear to have been sub-functionalized, 2) teleost insl3 has evolved moderately quickly like its mammalian counterpart and shows similar tissue-specific expression in Leydig cells, has undergone site-specific codon selection in both teleosts and mammals, and additionally exhibited high expression in the ovary of teleosts, 3) insl5 genes are well represented in teleosts, insl5a exhibits similar rates of evolution to insl3, while insl5b shows accelerated evolution compared to insl5a and may have been neo-functionalized, 4) molecular evolutionary analyses indicate that teleost rln is operating under very different selective constraints from mammalian RLN, and appears to mimic rln3 in its sequence evolution. Taken together, these results underscore the diverse roles that relaxin family peptides must play in teleosts: further experimental work is needed to shed light on the similarities and differences of their physiological roles in teleosts.

Methods
Nomenclature of teleost relaxin family genes
Wilkinson et al. (2005) identified six copies of relaxin in Takifugu rubripes and called them RLX3a through RLX3f. Recently, using both syntenic and phylogenetic data, Park et al. (2008) estimated that the ancestor of tetrapods and teleosts harboured three relaxin family loci (RFL): RFLA - hosting INSL5-like genes, RFLB - containing RLN-like genes and RFLC - including RLN3-like genes; they suggest that the duplication of RFLCII that gave rise to INSL3 occurred just prior to or after the divergence of Amphibia. These previous studies included 11 [9] and 14 [36] teleost sequences, and focused on resolving the phylogenetic and molecular evolutionary patterns of the relaxin family in tetrapods. Here, by searching the genomic databases of five completed teleost genomes, and including 32 teleost sequences, our results generally support the conclusions of Park et al. [36], except that we find that teleosts harbour an orthologue to human INSL3, indicating that the duplication of RFLC occurred prior to the divergence of teleosts and tetrapods. The phylogenetic and syntenic data analyses presented below indicate that the genes originally called RLX3a-3f by Wilkinson et al. [9] pertain to four relaxin family loci and are more accurately named rln3a, rln3b, rln, insl5a, insl5b, and insl3 respectively. The orthologous relationship of these genes to human relaxin family genes and their equivalent in the nomenclature of Wilkinson et al. [9] and Park et al. [36] is provided (Table 3).

Sequence identification and syntenic relationship of relaxin family genes in teleosts
Publicly available databases were searched for relaxin family homologues in the five teleost species for which a significant region of the genome has been sequenced: Tetraodon nigroviridis version 7 (Jaillon et al., 2004, http://www.ensembl.org/Tetraodon_nigroviridis/Info/Index), Takifugu rubripes version 4 (Aparicio et al. 2002, http://www.ensembl.org/Takifugu_rubripes/Info/Index), Danio rerio version 6 (The Wellcome Trust Sanger Institute, http://www.sanger.ac.uk/Projects/D_rerio and as available at Ensembl, http://www.ensembl.org). Oryzias latipes version 1 (Medaka Genome Project, http://dolphin.lab.nig.ac.jp/medaka) and Gasterosteus aculeatus version 1 http://www.ensembl.org/Gasterosteus_aculeatus/Info/Index. The sequences were first identified by using the algorithms BLASTP and TBLASTN to search for the following D. rerio B-chain protein sequences: YGVKLCGRE-FIRAVIDTCCGSRW (rln3b), RTVKLCGREFIRAVIDYTCGGSRW (insl5a), and VRVKL-CGREFVRTVVASCSCFVR (insl3). High-scoring hits (> 65% sequence identity over the entire region) were identified and then the upstream and downstream regions of the candidate relaxin family genes were searched for complete open-reading frames and other relaxin motifs, such as the conserved A-chain structural motif (CXXXCX8C), the B/C and C/A dibasic junctions, and the B-chain relaxin receptor binding motif RXXXRXXV, as well as general gene structure before being included in the data analyses. In total 26 genes were identified from these five genomes, 23 of the genes are annotated and identified as belonging to the RLN/INSL family in Ensembl (release 54); all of the relaxin family genes exhibited the expected gene structure for the family, complete open reading frames even through the post-translationally cleaved C-peptide. The Ensembl gene ID of these 23 genes as well as the location of all 26 genes is given (Release 54, Appendix Table two).

All known mammalian relaxin sequence were obtained from Homo sapiens RLN1, RLN2, RLN3, INSL3, INSL 5 and INSL 6; Mus musculus RLN, RLN3, INSL3, INSL5 and INSL6; Rattus norvegicus RLN, RLN3, INSL3 and INSL6; and Canis familiaris RLN, RLN3, INSL3 and INSL6 from GenBank http://www.ncbi.nlm.nih.gov/, and all known relaxin sequences from Xenopus tropicalis were included. Additionally, six relaxin family genes were identified from other teleost species from cDNA or EST databases at NCBI: Oncorhynchus mykiss rln3a and insl5b; Pimephales promelas rln3b and insl3; and Salmo salar rln3a and insl5a (Appendix Table two) such that a total of 32 teleost relaxin family genes were included. Mammalian sequences for INSL4
were not included in the analysis because the gene contains a large insertion, is present only in mammals, and was the subject of a previous bioinformatic analysis [9].

A syntenic analysis of the relationship between teleost and mammalian relaxin family genes was performed by identifying the position of up to ten genes both up- and downstream of the focal genes in D. rerio, T. nigroviridis, T. rubripes, O. latipes and G. aculeatus. Syntenic maps were constructed based on the information regarding the location of genes available from Ensembl’s BioMart data mining tool http://www.ensembl.org/multi/martview and, as needed, verified using the UCSC Genome Bioinformatics web server http://genome.ucsc.edu.

Phylogenetic analyses
For all sequences, the location of the signal peptide was determined using SignalP 3.0 [47] using default settings and then the sequence was removed. Sequence alignment was accomplished by manually aligning the translated B- and A-chain conserved motifs and twin dibasic motifs, the latter correspond to the protease cleavage sites between the B/C and C/A chain (Figure 4). The sequence between the two twin dibasic motifs was removed before alignment, because it contained the non-conserved intron and C-chain, but the entire B- and A-domains plus the four amino acids at the B/C and C/A protease cleavage sites were included since the latter could be potential targets of selection (Figure 4). Relaxin family members have classically been distinguished by the presence of the receptor binding RXXXRXXI/V motif on the B-chain; however, this is not specific enough to identify individual teleost relaxin family genes. Therefore, sequence motifs for the B-chain and dibasic cut sites were identified to characterize potentially important structural and functional residues and to aid in distinguishing teleost relaxin family genes (Additional File 1: Table S3). Teleost rln3a, rln3b and rln share an identical strongly conserved B-chain motif that is also shared by mammalian RLN3 but teleost rln differs from teleost and mammalian RLN3 in its C/A dibasic motif. Teleost insl5a and insl5b are less conserved than rln3a, rln3b and rln and contain unique but related B-chain and dibasic motifs. Finally, insl3 contains the least conserved B-chain but has specific dibasic processing sites that distinguish it from the remaining relaxin family genes (Additional File 1: Table S3).

The most appropriate model of nucleotide sequence evolution was identified using likelihood ratio tests as implemented in Model Test [48]. The phylogenetic relationship among nucleotide sequences was reconstructed using the optimality criteria of both minimum evolution and Bayesian methods as implemented in MEGA 3.1 [49] and MrBayes 3.12 [50] respectively. Preliminary analyses indicated that variation at the third position was saturated and confounded resolution at deep internal nodes. Therefore, trees based on nucleotide data were reconstructed in MrBayes by partitioning the data into first, second and third codon position, and allowing each partition to evolve at its own rate with its own shape (gamma) parameter, or by including only the first two positions when minimum evolution was the optimality criteria. The relationship among amino acid sequences was also reconstructed using Bayesian methods available in MrBayes 3.12 [50] and the appropriate model of amino acid change was determined using hierarchical likelihood tests as implemented in ProTest version 1.0.6 [51] or by Bayesian methods. For the Bayesian analyses, the model of amino acid change was examined by allowing the parameter space explored by the MCMC algorithm in MrBayes to include eight different amino acid models (prset = mixed) and then choosing the model with the highest posterior probability as the best available model. For the Bayesian analyses of both the amino acid and nucleotide data, the MCMC algorithm was run with four simultaneous chains that sampled from the posterior distribution every 300 generations; trees sampled before the cold chain reached stationarity based on plots of the maximum likelihood scores were discarded as “burnin” while sampling continued until convergence was achieved based on the average standard deviation of the split frequencies and the potential scale reduction factor (PSRF) as given in MrBayes. Statistical confidence in the deduced evolutionary trees was assessed by examining the Bayesian Posterior Probabilities (BPP) on the majority-rule consensus tree containing branch lengths for the Bayesian analyses or by bootstrapping the sequences for 1000 generations for the minimum evolution analyses.

Evidence for selection at the gene and codon level
To test whether the rates of molecular evolution were homogeneous across gene families, the two-cluster test was employed [32]. This test identifies those clades/genes that have significantly different rates of substitution based on an a priori hypothesis about which clades should be examined. Here, the rate of evolution was compared in nine clades: teleost rln3a, rln3b, rln, insl5a, insl5b and insl3 and mammalian RLN3, INSL3 and INSL5, while mammalian RLN and INSL6 were used as outgroups. The two-cluster test was conducted on the phylogenetic tree generated using minimum evolution using the program TPCV in LINTREE and only those comparisons with Z-scores high enough to give a p < 0.01 were taken as significant.

To assess the strength of purifying selection among genes, we calculated the average proportion of mutations leading to synonymous (dS) versus non-synonymous (dN) changes for all orthologous relaxin family sequences separately in teleosts and mammals. The ratio of dN/dS was calculated in MEGA 3.1 using a Nei-Gojobori model of nucleotide sub-
sition [49]. Pairwise comparisons between teleost relaxin family genes were performed and average \( d_{s}/d_{i} \) values calculated across the entire gene, or for only the B-chain and A-chains; additionally the codon-based Z-test was used to determine if \( d_{s}/d_{i} \) within each gene or gene region was significantly different from 1.0 using bootstrapping to estimate the variances [49]. Because \( d_{s}/d_{i} \) values are calculated pairwise and the average value from all pairwise comparisons reported, the same five teleost species (those for which the whole genomes were available) and four mammalian sequences were included in these analyses.

Positive selection is often restricted to specific lineages and a few amino acid sites, therefore, we further employed the branch-site model A [53] to look for evidence of codon-specific positive selection on orthologous gene families in teleosts versus mammals. The application of this model requires that the user specify \textit{a priori} which branch is being tested for evidence of positive selection, the so-called foreground branch, while the remaining groups are defined as background branches. Tests of positive selection were made by comparing the branch-site model A in which \( (d_{s}/d_{i}) \geq 1 \) (alternative hypothesis) to the model A in which \( \omega = 1 \) fixed (null hypothesis) and setting the foreground branch to the base of the clade containing the relaxin family orthologue in teleosts and the background branch was set to the same orthologue in mammals or vice versa [54]. Analysis of the branch-site model A was done using CODEML from the PAML package (PAML v. 4.2); models were compared using the Likelihood Ratio Test with 1 degree of freedom and, where significant, the posterior probability that a codon was under positive selection was estimated using the Bayes empirical Bayes (BEB) procedure [55].

**Expression of relaxin family genes in zebrafish using real-time, quantitative PCR**

We tested for the expression of \( rln3a, rln3b, \) insl5a, \( \) insl5b and \( \) insl3 in \( D. \) rerio, which lacks \( \) rln (Appendix, Table two), using real-time, quantitative PCR (qPCR). Total RNA was extracted from the brain, heart, gut, ovary, testis, and eye of adult zebrafish using the Aurum total RNA mini kit (BioRad) and first strand cDNA synthesized from 5 \( \mu \)g of total RNA with oligo dT and random hexamer priming (iScript Select cDNA Synthesis Kit, BioRad).

The relative transcript abundance of the five relaxin family genes in \( D. \) rerio across tissues was then calculated via qPCR using a MiniOpticon Real-Time detection system (BioRad). Oligonucleotide primers for \( rln3a, \) insl5a and \( \) insl3 were taken from Wilson \textit{et al.} (2008), while those for \( rln3b \) and \( \) insl5b were designed using PRIMER3 software [56]. The primers selected for \( rln3b \) were: F: 5'-CGGCTCTCGTATGTGCTGCTG-3' and R: 5'-CCCTGTTCACCCTGTGCAGT-3' and for \( \) insl5b were: F: 5'GCACGAGAAACAGCTC-3' and R: 5'-GCTGGAGTCCTGTGCTCTTC-3'. The iQ™SYBR® Green Supermix kit was used according to the manufacturer's suggested protocols (BioRad). Standard curves were generated for all of the used primers to compute the amplification efficiency values for each primer set. The insignificant difference observed among the calculated efficiency values permitted us to calibrate Ct values of the target relaxin family genes in each tissue relative to their expression in eye (low abundance transcript, used as the calibrator in the equation below), and normalize them to a reference, housekeeping gene (\( b2m \)), previously shown to exhibit consistent expression across sexes, tissues and developmental stages in \( D. \) rerio [57]. This further allowed us to determine the relative fold increase of each relaxin gene relative to the housekeeping gene according to the formula: \( 2^{\Delta Ct, \text{target} - \Delta Ct, \text{ref}} \) [58]. Each gene was tested three times and standard errors were calculated so that comparisons could be made across genes and tissues using the coefficient of variation (CV) where:

\[
CV = \left( \frac{\sqrt{\text{CV}_{\text{test}, \text{target}} + \text{CV}_{\text{test}, \text{ref}}}}{100} \right)
\]

**In situ hybridization using insl3 on zebrafish testis**

A zebrafish-specific \( \) insl3-specific PCR product was generated with primer 2126 (5'-GGCCGGGTGTATTAAACCCTACACTAAAGGGAGTTGAG-TGGTCGCGTGAAGC-3'); containing the \( T3 \) RNA polymerase promoter sequence [underlined]) and primer 2127 (5'-CCGCTGGTTGTAATACGCTATAGGGTTCTGAAATCTGTTCA-3'); containing the \( T7 \) RNA polymerase promoter sequence (underlined). The \( \approx 450 \) bp PCR product was gel purified, and served as a template for digoxigenin-labeled cRNA probe synthesis. For digoxigenin-RNA labeling by \textit{in vitro} transcription, 500 ng PCR product was incubated at 37°C for 2.5 h in a 20 \( \mu \)l reaction volume containing 4 \( \mu \l 5 \times T3/T7 RNA buffer (Invitrogen), 2 \( \mu \l 0.1 \) M DTT, 1 \( \mu \l \) (29.7 units/\( \mu \)l) RNAguard (GE Healthcare, Fairfield, CT, USA) and 2 \( \mu \l 10 \times \) DIG RNA labeling mix (Roche), and either 2 \( \mu \l \) (50 units/\( \mu \)l) \( T3 \) RNA polymerase (Epiconcept; for sense cRNA probe synthesis) or 2 \( \mu \l \) (50 units/\( \mu \)l) \( T7 \) RNA polymerase (Epiconcept; for antisense cRNA probe synthesis).

To visualize cellular expression sites of \( \) insl3 mRNA in zebrafish testis, whole mount \textit{in situ} hybridization was performed on zebrafish testicular tissue, fixed in 4% paraformaldehyde in PBS (pH 7.4), as described by Wester-
field (2000) http://zfin.org/zf_info/zfbook/chapt9/9.8.2.html with some modifications to the protocol. Briefly, tissue was treated with 20 μg/ml proteinase K (Sigma-Aldrich) at 37 °C for 20 min. Moreover, after post-fixation and before pre-hybridization, an acetic anhydride (0.25% in 0.1 M triethanolamine; Merck) treatment was included to reduce background. After termination of NBT/BCIP (Sigma-Aldrich) staining with 3 consecutive PBS washings, tissue was examined with a binocular connected to a digital camera.

Authors’ contributions
SVG-A. Oversaw the project, performed the phylogenetic, dS/dN, molecular clock, synteny, and codon selection analyses and wrote the bulk of the final paper. SY performed the qPCR laboratory work and analyses, helped with the synteny analyses, drew the synteny figures and helped with the literature search and writing and editing of the manuscript. SH helped collect the sequences, trimmed and aligned them, and devised the teleost gene motifs and helped write a previous version of the paper. JB performed the in situ hybridization analyses and helped with revising the manuscript, PG and JO both helped collect the original sequences and JO managed most of the information in Appendix 1, BW helped edit the manuscript and initiated the study. All authors have read and agreed to the final version of the manuscript.

Additional material

Additional file 1
Tables describing the map positions of genes covered in the synteny analyses, sequence accession numbers for all relaxin family genes included in the paper and additionally the map positions of those in teleosts, and finally B-chain and dibasic junction sequence motifs of teleost relaxin family genes. Table S1 covers the chromosomal map position of all genes used in the synteny analyses in each of the five teleost genomes on which syntenic mapping was conducted. Table S2 covers the NCBI or Ensemble accession number for each of the relaxin family loci presented, and, for the teleost genes, additionally the map position, intron and C-domain length. Table S3 presents the conserved B-chain motifs and B/C and C/A dibasic junctions for each of the teleost relaxin family loci. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2148-9-293-S1.DOC]

Additional file 2
Bayesian phylogenetic tree based on relaxin family protein sequences. The figure legend and Bayesian phylogenetic tree (in colour) based on amino acid sequences of relaxin family sequences from teleosts and mammals in pdf format. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2148-9-293-S2.DOC]

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