Discovery of conventional prolactin from the holocephalan elephant fish, *Callorhinchus milii*.

Yoko Yamaguchi[1], Wataru Takagi[2], Shigehiro Kuraku[3], Shunsuke Moriyama[4], Justin D. Bell[5], Andre P. Seale[1], Darren T. Lerner[1, 6], E. Gordon Grau[1, 6] and Susumu Hyodo[2]

1. Hawai‘i Institute of Marine Biology, University of Hawai‘i, Kaneohe, HI 96744, USA
E-mail address: yoko7@hawaii.edu (Y. Yamaguchi), seale@hawaii.edu (A.P. Seale), lerner@hawaii.edu (D.T. Lerner), grau@hawaii.edu (E.G. Grau)

2. Laboratory of Physiology, Atmosphere and Ocean Research Institute, University of Tokyo, Kashiwa, Chiba 277-8564, Japan
E-mail address: wtakagi@aori.u-tokyo.ac.jp (W. Takagi), hyodo@aori.u-tokyo.ac.jp (S. Hyodo)

3. Phyloinformatics Unit, RIKEN Center for Life Science Technologies, Kobe, Hyogo 650-0047, Japan
E-mail address: shigehiro.kuraku@riken.jp (S. Kuraku)

4. School of Marine Biosciences, Kitasato University, Sagamihara, Kanagawa 252-0373, Japan
E-mail address: morisuke@kitasato-u.ac.jp (S. Moriyama)

5. Institute for Marine and Antarctic Studies, University of Tasmania, Taroona, TAS 7053, Australia
E-mail address: Justin.Bell@utas.edu.au (J.D. Bell)

6. Sea Grant College Program, University of Hawai‘i at Mānoa, Honolulu, HI 96822, USA
E-mail address: lerner@hawaii.edu (D.T. Lerner), grau@hawaii.edu (E.G. Grau)

*Corresponding author: Yoko Yamaguchi

Phone: +1-808-236-7447

Fax: +1-808-236-7453

E-mail address: yoko7@hawaii.edu

Abstract

The conventional prolactin (PRL), also known as PRL1, is an adenohypophysial hormone that critically regulates various physiological events in reproduction, metabolism, growth, osmoregulation, among others. PRL1 shares its evolutionary origin with PRL2, growth hormone (GH), somatolactin and placental lactogen, which together form the GH/PRL hormone family. Previously, several bioassays implied the existence of PRL1 in elasmobranch pituitaries. However, to date, all attempts to isolate PRL1 from chondrichthyans have been unsuccessful. Here, we cloned PRL1 from the pituitary of the holocephalan elephant fish, *Callorhinchus milii*, as the first report of chondrichthyan PRL1. The putative mature protein of elephant fish PRL1 (cmPRL1) consists of 198 amino acids, containing two conserved disulfide bonds. The orthologous relationship of cmPRL1 to known vertebrate PRL1s was confirmed by the analyses of molecular phylogeny and gene synteny. The *cmPRL1* gene was similar to teleost *PRL1* genes in gene synteny, but was distinct from amniote *PRL1* genes, which most likely arose in an early amphibian by duplication of the ancestral *PRL1* gene. The mRNA of cmPRL1 was predominantly expressed in the pituitary, but was considerably less abundant than has been previously reported for bony fish and tetrapod PRL1s; the copy number of cmPRL1 mRNA in the pituitary was less than 1% and 0.1% of that of GH and pro-opiomelanocortin mRNAs, respectively. The cells expressing cmPRL1 mRNA were sparsely distributed in the *rostral pars distalis*. Our findings provide a new insight into the studies on molecular and functional evolution of PRL1 in vertebrates.

Keywords: prolactin; GH/PRL family; cartilaginous fish; molecular evolution

1. Introduction

Prolactin (PRL) is an adenohypophysial hormone produced in the anterior pituitary gland and secreted into circulation in response to various physiological stimuli. While PRL was originally named for its ability to stimulate lactation in rabbit (Stricker and Grueter, 1928; Riddle et al., 1933), it is a versatile hormone, exerting over 300 biological functions in mammalian and non-mammalian vertebrates, as reviewed by Bole-Feysot et al. (1998). In teleosts, PRL plays a critical role in maintaining plasma homeostasis in freshwater by altering salt- and water permeability across epithelial cell membranes in the gill, gut and kidney (see Hirano, 1986; Manzon 2002; Breves et al., 2014; Takei et al., 2014).

PRL shares its structural features with growth hormone (GH), bony fish somatolactin (SL) and mammalian placental lactogen (PL); these hormones form a monophyletic hormone family called GH/PRL family (see Rand-Weaver and Kawauchi, 1993). The origin and evolution of this hormone family has attracted the attention of researchers because of their diverse and important activities in organisms. Among the members of the GH/PRL family, GH is the only molecule found in all vertebrate classes including sea lamprey, a jawless fish (Kawauchi et al., 2002), suggesting that the rest of this hormone group arose from the ancestral *GH* gene via multiple gene duplications and subsequent evolutionary diversification. Meanwhile, the timing of emergence of current GH/PRL family members from ancestral GH remains to be explored. In previous studies, extensive efforts have been made to isolate PRL from the lineages of bony fish (osteichthyans) and cartilaginous fish (chondrichthyans). However, this was only successful in the bony fish lineages; consequently, the existence of PRL in cartilaginous fish remains uncertain. The extract of cartilaginous fish pituitary showed positive activity in the red eft water-drive test, implying the presence of PRL (see Bern and Nicoll, 1968). In Atlantic stingray, *Dasyatis sabina*, lesion of the *rostral pars distalis* (RPD) caused a significant increase in plasma osmolality, as well as plasma urea

and sodium levels; these effects were reversed by the injection of ovine PRL (de Vlaming et al., 1975). Pituitary PRL activity in the stingray was also investigated using the *Gillichthys* xanthophore assay, where the putative activity of pituitary PRL was upregulated approximately 100-fold by 24 h following transfer of stingrays from seawater (SW) to brackish water (de Vlaming et al., 1975). However, neither immunoreactive signal nor cDNA fragment was obtained for chondrichthyan PRL, despite the use of various heterologous PRL antisera and primers designed for conserved sequences of already identified PRL mRNAs (see Kawauchi and Sower, 2006).

A breakthrough in chondrichthyan biology was achieved by Venkatesh and colleagues, who initiated whole genome sequencing for the holocephalan elephant fish (or elephant shark, *Callorhinchus milii*) (http://esharkgenome.imcb.a-star.edu.sg/) (Venkatesh et al., 2007). We have focused on this species as a model for molecular endocrinological and physiological studies of cartilaginous fish (Hyodo et al., 2007; Kakumura et al., 2009; Yamaguchi et al., 2012; Takagi et al., 2014). Recently, a PRL-like gene was found from the elephant fish genome and designated PRL2 (Huang et al., 2009). Orthologs of the PRL2 gene exists throughout non-mammalian vertebrates, and phylogenetically PRL2 is distinct from the conventional PRL, which was subsequently renamed PRL1. This finding implies that the duplication of ancestral GH/PRL family gene occurred before the chondichthyan-osteichthyan divergence, and offers the possibility that a gene encoding PRL1 also exists in cartilaginous fishes.

In January 2014, the genome project for the elephant fish led to more comprehensive genome sequences (Venkatesh et al., 2014). In the whole genome sequences, we finally identified the conventional PRL, PRL1, of the elephant fish and cloned its cDNA. The elephant fish PRL1 mRNA was predominantly detected in the RPD of the pituitary as reported in other vertebrates, while the number of cells expressing PRL1 mRNA was extremely small compared with that in pituitaries of bony

fishes and tetrapods. Our analyses on gene synteny and molecular phylogeny bring a new insight into the molecular evolution of vertebrate GH/PRL family.

2. Materials and methods

2-1. Animals

Elephant fish, *Callorhinchus milii*, were collected in Western Port Bay, Victoria, and in Pitt Water, Tasmania, Australia. They were kept in 2,000 to 10,000 L round tanks filled with running SW under a natural photoperiod. In tissue sampling, the fish were anesthetized with 0.1% ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, St Louis, MO, USA). Tissues for RNA extraction were immediately frozen in liquid nitrogen. For *in situ* hybridization, the whole brain was dissected and fixed in Bouin's solution without acetic acid at 4^{o}C for 2 days, and processed as described below.

All experiments were performed according to the Guideline for Care and Use of Animals approved by the committees of University of Tokyo, Deakin University, and University of Tasmania.

2-2. cDNA synthesis and Molecular cloning

The amino acid sequences of bony fish PRL and GH were used as BLAST queries to find candidate gene fragments in the Elephant Shark Genome Database (http://esharkgenome.imcb.a-star.edu.sg/), and the sets of specific primers were designed to amplify the transcripts of target genes. Total RNA was extracted from frozen tissue with Isogen (Nippon Gene, Tokyo, Japan) and treated by Turbo DNA-free kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. Two μg of RNA was used to synthesize the first-strand cDNA using High Capacity cDNA Reverse Transcription Kit (Life Technologies). The cDNAs encoding whole coding region of putative elephant fish PRL1 (cmPRL1) and partial coding region of

pro-opiomelanocortin (cmPOMC) were amplified with KAPATaq EXtra (Kapa Biosystems, Wilmington, MA, USA). For GH (cmGH), 5'- and 3'-RACE were performed to cover whole coding region using SMART cDNA library construction kit (Clontech, Mountain View, CA, USA). The amplified cDNA fragments were subcloned into pGEM T-easy (Promega, Madison, WI, USA) and their nucleotide sequences were determined by an automated DNA sequencer (3130xl Genetic Analyzer; Life Technologies). All primers used are listed in Table 1.

2-3. Molecular phylogenetic analysis

Protein sequences of PRL1, PRL2, GH and SL were retrieved from NCBI Genpept and Ensembl databases using aLeaves (Kuraku et al., 2013), and from coelacanth genome database (assembly ID; LatCha_J1.0, http://coelacanth.nig.ac.jp/) by BLAST search. The retrieved sequences were aligned with the alignment editor XCed in which the multiple sequence alignment algorithm is implemented (Katoh et al., 2013). Using amino acid sites unambiguously aligned, molecular phylogenetic trees were inferred preliminarily with the neighbor-joining (NJ) method using XCed and finally with the maximum-likelihood (ML) method using PhyML version 3.0 (Guindon et al., 2010). Among-site rate heterogeneity was taken into account by assuming the gamma distribution with the JTT model (JTT+Γ_4+I). In both NJ and ML methods, bootstrap resampling was performed with 100 replicates.

The GenBank accession IDs of sequences used in the analysis are listed in Supplementary Table 1. The amino acid sequences of putative PRL1s of Mexican salamander, *Ambystoma mexicanum*, were obtained by BLAST searches against their genome database at Sal-Site (assembly V3.0, http://www.ambyostoma.org/), using the sequences of *Xenopus laevis* proteins as queries.

2-4. Tissue distribution

Tissue distribution of cmPRL1 mRNA was examined by RT-PCR and quantitative real-time PCR (qPCR), using cDNAs prepared from each tissue as described above. RT-PCR was performed with KAPATaq EXtra (Kapa Biosystems) for 35 (cmPRL1) or 30 cycles (β-actin, as an internal control). Amplified PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide fluorescence.

The absolute quantification of target mRNAs was performed by qPCR with an ABI Prism 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA) and KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems), as previously described (Takagi et al., 2014). The results were shown either as normalized by β-actin or as the copy number of target mRNAs per gram of RNA. All primers used are listed in Table 1.

2-5. *in situ* hybridization

The fixed brain tissue was washed twice in 70% ethanol and a small block of tissue containing the hypothalamus and pituitary was dissected out. The pituitary block was then embedded in Paraplast (Kendall, Mansfield, MA, USA) and sagittally sectioned. The sections of 6 μm thickness were mounted onto MAS-GP-coated glass slides (Matsunami, Osaka, Japan)

The partial fragments of cmGH, cmPRL1 and cmPOMC transcripts were amplified using gene-specific primers listed in Table 1, and the PCR products were subcloned into pGEM-T easy (Promega). Digoxigenin (DIG)-labeled anti-sense cRNA probes were synthesized with DIG RNA Labeling Kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's protocols. The localization of target mRNAs in the brain was visualized by *in situ* hybridization with the cRNA probes mentioned above, as previously described (Takabe et al., 2012). Dual *in situ* hybridization was performed as described by Kanda et al. (2013), with minor

modifications, using the DIG-labeled probe for cmPRL1 and fluorescein-labeled probe for cmPOMC, which was synthesized using Fluorescein RNA Labeling Mix (Roche Applied Science). In brief, deparaffinized sections were digested by 5 μg/mL proteinase K (Wako Pure Chemical Industries, Osaka, Japan) and hybridized with 0.25 μg /mL probes at 58°C for 40h. Following immunohistochemical reactions with alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) and horseradish peroxidase-conjugated anti-fluorescein antibodies (PerkinElmer, Waltham, MA, USA) at 4°C overnight, hybridization signals were visualized using Fast Red Tablets (Roche Applied Science) and TSA Plus Fluorescein System (PerkinElmer) according to the manufacturer's instruction. To ascertain the specificity of the signals obtained, sense cRNA probes were used as negative controls.

2-6. Synteny analysis

Synteny analyses were performed using UCSC Genome Browser (http://genome.ucsc.edu/), Ensembl Genome Browser (http://www.ensembl.org/) and NCBI database (http://www.ncbi.nlm.nih.gov/), as previously described (Yamaguchi et al., 2012). Firstly, the loci of candidate genes encoding GH/PRL family molecules were determined in elephant fish (*C. milii*), stickleback (*Gasterosteus aculeatus*), zebrafish (*Danio rerio*), coelacanth (*Latimeria chalumnae*), frog (*X. tropicalis*), chicken (*Gallus gallus*), and human (*Homo sapiens*), using the genome browsers mentioned above. Subsequently, 3-27 genes adjacent to the each target gene were identified using the NCBI RefSeq database and Ensembl Genome Browser. For genes not registered in NCBI reference sequence database, the deduced amino acid sequences of encoded proteins were subjected to NCBI TBLASTN searches for verification. Non-protein coding genes and genes coding unknown proteins were excluded from further analysis. Then, the orthologs of the identified genes were comprehensively searched in all examined species using the genome browsers and verified by NCBI TBLASTN

searches, when needed, as mentioned above. Finally, information on the loci of all identified genes were sorted and organized in figures. The conserved syntenic gene blocks were manually detected and compared among target genes.

2-7. Presentation of data and statistical analyses

Quantitative data are presented as means ± SEM. The results of qPCR were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's HSD test, as described in a figure legend. The data were log-transformed to satisfy normality and homogeneity of variance requirements, when necessary. *P* values less than 0.05 were considered statistically significant. All statistics were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA).

3. Result

3-1. Cloning

A BLAST search against the updated elephant fish genome database yielded fragments homologous to bony fish GH and PRL1, in addition to the previously identified PRL2. Subsequently, the cDNAs encoding putative GH (cmGH, GenBank accession ID LC055147) and PRL1 (cmPRL1, GenBank accession ID LC055146) were cloned from the elephant fish pituitary and sequenced. Alignment of cloned cDNAs with elephant fish genome revealed that the coding regions of both cmGH and cmPRL1 consist of five exons (Fig. 1). The putative mature protein of cmPRL1 is composed of 198 amino acid residues, containing six cysteine residues (at positions 19, 60, 161, 173, 190 and 198). Four out of six Cys residues (Cys^{60}, Cys^{173}, Cys^{190} and Cys^{198}) were conserved among all GH/PRL family molecules, indicating that mature cmPRL1 forms two disulfide bridges (Fig. 1); on the other hand, the PRL1s of sturgeon, coelacanth, lungfish and tetrapods contain two more conserved Cys residues in amino-terminus

(N-terminus) forming an additional disulfide bond. A putative N-glycosylation site was detected at Asn^{62}. The carboxyl-terminal (C-terminal) region of cmPRL1 was highly homologous to known PRL1 proteins and also to other GH/PRL family members (Fig. 1). The Pro^{96} and Gly^{131} of mature cmPRL1 were residues found to be well conserved among all GH/PRL family members; these residues are considered to be important in the binding of molecules to their receptors (Goffin et al., 1996; Schenck et al., 2003).

The putative mature cmGH protein is composed of 184 amino acid residues, showing high similarity to blue shark GH (76% identity). Alignment of cmGH with known GHs revealed four conserved Cys residues forming two intramolecular disulfide bridges (Cys^{52}, Cys^{157}, Cys^{174} and Cys^{182}) in all GH proteins compared (Fig. 1). Regarding the conserved Pro and Gly residues described above, the Pro residue was replaced by the Ser^{88} in mature cmGH, whereas the Gly residue was found as Gly^{111}. The Thr-Val residues were extended in the C-terminal end after Cys^{182}, as another structural feature of GH molecules. The amino acid identity of cloned molecules to representative vertebrate PRL1s and GHs is shown in Table 2.

3-2. Molecular phylogenetic analysis

The phylogenetic relationship between the identified elephant fish genes and known GH/PRL family hormones was investigated by constructing a molecular phylogenetic tree. The tree was inferred with the maximum-likelihood method using 114 amino acid sites unambiguously aligned (Fig. 2). The resulting tree consisted of four separate monophyletic clades of PRL1, PRL2, GH and SL; cmPRL1 and cmGH were grouped into the PRL1 and GH clades, respectively. A notable finding was that at least two genes belonging to the PRL1 clade were detected in all examined amphibians. Phylogenetic relationships within the PRL1 clade were further investigated using 174 amino acid sites (Fig. 3). In *X. laevis*, three PRL proteins have been reported (GenBank accession IDs NP_001086486, AAH92151, and NP_001159915), and all of them were

grouped in the PRL1 clade, but not in the PRL2 clade (Figs. 2 and 3). For these PRLs of *X. laevis*, we follow the nomenclature suggested by Huang et al (2009): xlPRL1A for NP_001086486 and xlPRL1B for NP_001159915. Since a sister relationship was detected between NP_001086486 (xlPRL1A) and AAH92151, we propose to designate them as xlPRL1A1 and xlPRL1A2, respectively. The existence of multiple *PRL1* genes also became evident in *X. tropicalis* and urodele axolotl *A. mexicanum*. Two PRL proteins from *X. tropicalis*, one reported (GenBank accession ID AAI36078) and one predicted (GenBank accession ID XP_002938572), were orthologous to xlPRL1A and xlPRL1B, respectively (Fig. 3); the predicted amino acid sequence of XP_002938572 was identical to that of xlPRL1B. Similarly, in the genome of *A. mexicanum*, two putative genes encoding proteins orthologous to xlPRL1As and xlPRL1B were found on contig_62377 and contig_27042 (Sal-Site), respectively. These data suggest that the *PRL1* gene duplicated in an ancestral amphibian, generating *PRL1A* and *PRL1B* genes shared by both anurans and urodeles. In the phylogenetic tree, amphibian PRL1As form a sister group of amniote PRLs, while PRL1Bs branched between the PRL1s of actinopterygian and sarcopterygian fish (Fig. 3).

3-3. Synteny analysis

The orthologous relationships between elephant fish *GH* and *PRL* genes and known GH/PRL family genes were examined by comparing location of genes adjacent to target genes, among the representative species of different vertebrate classes. Consistent with the molecular phylogenetic tree (Figs. 2 and 3), four GH/PRL family members (PRL1, PRL2, GH and SL) were distinguishable by their unique patterns of conserved synteny (Fig. 4; Supplementary Figs. 1-3). For *GH* gene, the syntenic gene block was well conserved throughout vertebrates and was identified on chromosome 17 in human (Supplementary Fig. 1).

In the case of *PRL1* gene, the organization of its neighboring genes were

completely different between fishes and tetrapods; PRL1 genes of bony fishes (stickleback, zebrafish, and coelacanth) were adjacent to the orthologs of human genes on chromosome 19, while human *PRL1* gene locates on chromosome 6 showing a conserved syntenic pattern to tetrapod *PRL1* genes (Fig. 4). In *X. tropicalis*, two distinct *PRL1* genes identified in the molecular phylogenetic analysis, *PRL1A* and *PRL1B*, were found on separate genomic contigs. The gene blocks around *PRL1A* and *PRL1B* genes of *X. tropicalis* resembled those around amniote *PRL1* genes and bony fish *PRL1* genes, respectively. The elephant fish *cmPRL1* gene was identified on scaffold 350, where only three genes, *TRIM28*, *EMC10* and *ELF5*, were identified besides *cmPRL1* gene, due to a captured gap spanning nearly 60% of the scaffold length. Similar colocalization of *ELF5* gene with *PRL1* gene on the same chromosome was also detected in stickleback and zebrafish; this implies that the gene order around *cmPRL1* gene resembles that around teleost *PRL1* and *X. tropicalis PRL1B* genes, rather than that around *X. tropicalis PRL1A* and amniote *PRL1* genes.

Although *PRL2* gene was not detected in human and amphibians, its synteny pattern was well conserved from bony fish to tetrapods, being found on human chromosome 13 (Supplementary Fig. 2). In the elephant fish, unlike PRL2 genes of other species, *cmPRL2* gene was located together with the orthologs of human genes on chromosome 2 and 21. In the case of bony fish *SL* gene, the syntenic gene block was identified on human chromosome 11 (Supplementary Fig. 3). Despite the conserved gene synteny throughout the vertebrate species, *SL* gene was found only in bony fishes, but not in the elephant fish and tetrapods.

3-4. Tissue distribution of mRNAs

The tissue distribution of cmPRL1 mRNA was investigated by RT-PCR (Fig. 5). Among the examined tissues, cmPRL1 mRNA was predominantly expressed in the pituitary. Brain levels were considerably lower compared with those in the pituitary.

Subsequently, mRNA expression of cmPRL1, cmGH and cmPOMC in the pituitary and other brain tissues were further verified by qPCR (Fig. 6). Consistent with RT-PCR results, mRNA expression of PRL1, as well as that of cmGH and cmPOMC, was highest in the pituitary (Fig. 6A-C). The copy number of cmPRL1 mRNA in the pituitary was less than 1% of that of cmGH mRNA and 0.1% of that of cmPOMC mRNA (Fig. 6D).

3-5. Localization of mRNAs of cmPRL1, cmGH and cmPOMC in the pituitary

The spatial distribution of cmPRL1 mRNA in the pituitary was investigated by *in situ* hybridization (Fig. 7). Hybridization-positive cells for cmPRL1 mRNA were sparsely distributed in the RPD. A few positive cells were found in the *posterior pars distalis* (PPD) as well (Fig. 7A, B and C). Most RPD cells, as well as cells in the *pars intermedia* (PI), were intensely stained with the antisense cRNA probe for cmPOMC mRNA (Fig. 7A, B and D). Dual staining with the probes for cmPRL1 and cmPOMC showed that PRL1 mRNA and POMC mRNA are expressed in separate RPD cells (Fig. 8). The signals for cmPOMC mRNA were also detected in PPD and basal hypothalamic neurons at lower densities (Fig. 7A and C, Supplementary Fig. 4). On the other hand, cmGH mRNA was abundant in the PPD (Fig. 7A and C), while it was also expressed in some RPD cells (Fig. 7B). For all target mRNAs, hybridization with sense cRNA probes did not elicit any positive signal (data not shown), confirming the specificity of the signals obtained with antisense probes.

4. Discussion

4-1. Discovery of PRL1 from the elephant fish

In the present study, we identified and successfully cloned the cDNA of PRL1, the conventional PRL, from the elephant fish, as the first report of this hormone in

chondrichthyans. The orthologous relationship between *cmPRL1* gene and known *PRL1* genes was confirmed by molecular phylogenetic analysis and gene synteny analysis. Together with previously reported cmPRL2 and newly cloned cmGH, our findings revealed that the molecular diversification in GH/PRL family occurred at least, in the chondrichthyans, the earliest-diverged group of living gnathostomes.

The existence of PRL1 in chondrychtyans was implied by the red eft water-drive test using the pituitary extracts from cartilaginous fish (see Bern and Nicoll, 1968). In Atlantic stingray, plasma osmolality, sodium and urea concentrations were significantly upregulated following the lesion of RPD, while the effects of RPD-lesion were reversed by the injection of oPRL (de Vlaming et al., 1975). However, subsequent attempts over many years to isolate chondrichthyan PRL1 protein or PRL1 cDNA were unsuccessful. Consistent with the previous reports, cmPRL1 mRNA was predominantly expressed in the RPD of the elephant fish pituitary. Prior failures in searching for chondrychtyan PRL1 were most probably due to the considerably small population of PRL1-producing cells scattered in the RPD. Although we did not quantify the number of elephant fish RPD cells expressing cmPRL1 mRNA, they appeared to be less than 10% of cells expressing POMC mRNA. This observation was further supported by the qPCR analysis on whole pituitary; the quantity of cmPRL1 mRNA was less than 1% of cmGH mRNA and 0.1% of cmPOMC mRNA. Meanwhile, PRL1-producing cells account for 10-25% of human pituitary cells; this is the second largest cell population in the human pituitary after that of somatotrophs (40-50%) and comparable with that of corticotrophs (15-20%) (Nussey and Whitehead, 2001). In teleost fish pituitaries, PRL1-producing cells occupy a large homogenous mass in the RPD, making a clear contrast with the elephant fish pituitary (see Ball and Baker, 1969; Holmes and Ball, 1974).

4-2. Evolution of GH/PRL family

382 Comparison of the deduced amino acid sequences of elephant fish proteins
383 with those of the known PRLs, GHs and SLs revealed some structural features. First,
384 the C-terminal region, in particular the residues between Cys^{173} and Cys^{190} (positions in
385 mature cmPRL1) is well conserved among all examined GH/PRL family molecules. In
386 cmPRL1, Pro^{96} and Gly^{131}, the residues which significantly contribute to the receptor
387 binding ability of the molecules (Goffin et al., 1996; Schenck et al., 2003), were
388 conserved between known PRL1s. Although the C-termini of cmPRL1 and known
389 PRL1 proteins terminate with the conserved Cys residue, the cmGH has the extension
390 of two amino acid residues after Cys^{182} in its C-terminal end; such an extension of
391 C-terminal residues is a feature of known GHs and SLs.
392 The most prominent structural characteristic of GH/PRL family is the
393 conserved intramolecular disulfide bonds. All reported SLs, PRL2s (except for
394 cmPRL2) and tetrapod PRL1s contain three disulfide bonds, while GHs and teleost
395 PRL1s have only two disulfide bonds, lacking one in the N-terminus and a subsequent
396 small loop structure (see Manzon, 2002; Rand-Weaver and Kawauchi, 1993; Sinha,
397 1995). These disulfide bonds are important clues to understand the evolutionary history
398 of the GH/PRL family. Previous studies of primitive actinopterygians have provided
399 valuable information. The sturgeon PRL1, as well as the sarcopterygian (coelacanth,
400 lungfish and tetrapods) PRL1s, contains three disulfide bonds (Noso et al., 1993).
401 Meanwhile, the PRL1 of holostean bowfin (*Amia calva*) does not have the disulfide
402 bond in the N-terminus; the analysis of N-terminus of bowfin PRL1 and GH suggested
403 that these proteins are more closely related to teleost PRLs and GHs than to those of
404 tetrapods (Dores et al., 1993). Although basal actinopterygian relationships are still
405 somewhat controversial (Inoue et al., 2003), molecular phylogenetic analyses using
406 multiple nuclear gene sequences showed that holosteans (bowfin and gar) and teleosts
407 share a common ancestor, forming a sister group of the order Acipenseriformes
408 (sturgeons and paddlefishes) (Kikugawa et al., 2004; Near et al., 2012). This is

consistent with the currently accepted taxonomic classification, in which both holostean and teleost fish are grouped into the subclass Neopterygii (see Nelson, 2006). Based on these findings, Kawauchi and colleagues drew a schematic diagram of the molecular evolution of the GH/PRL family, postulating that an ancestor of the ray-finned fishes possessed PRL with three disulfide bonds, and that the N-terminal bond was lost throughout the evolution of teleosts (Dores et al., 1993; Kawauchi and Sower, 2006; Noso et al., 1993). In this context, it is of great interest that cmPRL1, as well as previously reported cmPRL2, lacks the N-terminal disulfide bond; so far, the N-terminal disulfide bond has not been found in any of GH/PRL family proteins in cyclostomes and cartilaginous fish. Further studies are required to clarify when the N-terminal intramolecular disulfide bond was acquired and lost along the divergence of the GH/PRL family.

The present synteny analyses offered a new insight into the evolution of *PRL1* gene. In the case of *GH* gene, the gene synteny around it was well conserved throughout vertebrates, from elephant fish to human. On the other hand, the gene order around *PRL1* gene was completely different between fishes and tetrapods. For bony fish PRL1, the orthologous gene block was found on human chromosome 19, while that for tetrapod PRL1 was identified on human chromosome 6. This discrepancy was solved by the existence of two *PRL1* genes in amphibians, designated as *PRL1A* and *PRL1B*, respectively. The *X. tropicalis PRL1B* gene showed a conserved synteny with fish *PRL1* genes, while conservation of gene order was identified between the regions containing *X. tropicalis PRL1A* gene and amniote *PRL1* genes. The observed gene synteny patterns, together with the molecular phylogenetic trees, suggest that the ancestral *PRL1* gene duplicated in an early amphibian to generate these two *PRL1* genes. After the duplication of *PRL1* gene in amphibians, the original *PRL1B* gene was lost and the newly arisen *PRL1A* gene was inherited by amniotes. Similar to the distinct gene syntenic patterns of fish and tetrapod *PRL1* genes, the gene blocks around *PRL2* gene

were not conserved between elephant fish and other species. A putative gene duplication of ancestral *PRL2* gene, which supposedly took place before the divergence of teleost lineages, may account for this inconsistency in gene synteny. Further studies in primitive vertebrates are required to test this hypothesis.

Among GH/PRL family molecules, SL has been reported to be unique to bony fish lineages (Amemiya et al., 1999; Ono et al., 1990; Rand-Weaver et al., 1991). Consistent with this observation, we could not find *SL* gene in the genome of either elephant fish or tetrapods, while the gene block around *SL* gene is extremely well conserved throughout vertebrates. In our molecular phylogenetic tree, PRL1 and SL were branched as sister groups of PRL2 and GH, respectively, suggesting that the current GH/PRL family was established via at least two gene (or genome) duplication events; in the first event, an ancestral gene, possibly *GH* gene, was duplicated into two lineages of PRL1/PRL2 and GH/SL, and subsequent gene duplications in each linage formed the four distinct clades. Since three of the four members of the GH/PRL family were found in the elephant fish (cmPRL1, cmPRL2 and cmGH), at least the first duplication event and the subsequent diversification between PRL1 and PRL2 clades were completed before the chondrichthyan-osteichthyan split. So far, we do not have definitive evidence to conclude the exact timing of *SL* gene occurrence; it may have diverged at a very early stage of vertebrate evolution together with other GH/PRL family members, and secondarily lost in both chondrichthyans and tetrapods. Alternatively, the *SL* gene may have been acquired in the evolution of bony fishes, and then lost in the tetrapods.

4-3. Possible function of PRL1 in cartilaginous fish

Currently, the function of PRL1 in cartilaginous fish remains unclear. As seen with PRL1 in other vertebrates, the transcript of cmPRL1 was predominantly expressed in the RPD. The involvement of PRL1 in body fluid homeostasis has been well

established throughout vertebrates (Bole-Feysot et al., 1998). In teleost lineages, PRL1 is known as a key hormone in freshwater adaptation (see Manzon 2002). Hypophysectomized euryhaline killifish, *Fundulus heteroclitus*, fail to survive in fresh water; their ability to survive is restored by administration of ovine PRL (Pickford and Phillips, 1959). Similarly, the *in vivo* injection of oPRL to RPD-lesioned Atlantic stingray reversed the effects of surgery, in terms of plasma parameters (de Vlaming et al., 1975). We previously demonstrated, however, that the elephant fish can only survive in a relatively narrow window of salinities (Hyodo et al., 2007), suggesting that the major roles of cmPRL1 may not involve osmoregulation. This idea is further supported by our finding that PRL1 is produced in extremely small numbers of RPD cells in the stenohaline elephant fish pituitary. In euryhaline Mozambique tilapia, *Oreochromis mossambicus*, several PRL-related parameters are greater in fish acclimated to fresh water than that reared in seawater, such as the size of RPD and PRL1-producing cells (Dharmamba and Nishioka, 1968), pituitary content of PRL1 mRNA (Magdeldin et al., 2007) and protein (Nicoll et al., 1981), and circulating PRL1 level (Nicoll et al., 1981). Thus, it is of great interest to examine the dynamics of chondrichthyan PRL1 in the context of their distinct salinity tolerances; while most species of the class Chondricthyes inhabit marine environments, stingrays of the family Potamotrygonidae spend all their lives in freshwater, and some species travel from the ocean to rivers, such as the bull shark and Atlantic stingray (see Ballantyne and Fraser, 2013; Martin, 2005). In Atlantic stingray, the putative PRL activity measured by *Gillichthys* xanthophore assay increased 100-fold following the transfer of stingrays from seawater to brackish water (de Vlaming et al., 1975). Future investigations on the levels of PRL1 gene expression and protein in the pituitary and circulation of cartilaginous fishes under a variety of salinity and other physiological conditions will help elucidate the functional evolution of PRL.

In addition to the aspect as an osmoregulatory factor, a putative contribution of

PRL1 in chondrichthyan reproduction is also of interest. PRL1 is indispensable in vertebrate reproduction by triggering numerous events such as mammalian lactation, avian crop sac growth (see Horseman and Buntin, 1995), hepatic vitellogenin synthesis in amphibians (Carnevali at al., 1993), steroidogenesis and gonadogenesis in teleosts (see Whittington and Wilson 2013), and parental behaviors in all these species (see Bole-Feysot et al., 1998; Horseman and Buntin, 1995; Polzonetti-Magni et al., 1995; Whittington and Wilson 2013). Cartilaginous fish are well known for their diverged reproductive strategies ranging from oviparity to various modes of viviparity (see Compagno, 1990; Wourms, 1977). The rays of the order Myliobatiformes, for example, adopt a form of reproduction called histotrophy, where "uterine milk" is secreted from the uterus for embryonic growth (see Compagno, 1990; Wourms, 1977). The endocrine regulation of their reproductive status, however, is largely unknown (see Awruch, 2003).

For understanding the function of PRL1 in cartilaginous fish, further attempts to determine and characterize its target receptor (PRLR) are indispensable. The PRLR exhibits a single chain structure with one transmembrane domain, and dimerizes upon binding to PRL1. While our preliminary search in the elephant fish genome sequences failed to find a gene potentially orthologous to known *PRLR* genes, the gene encoding putative GH receptor (GHR) was identified on scaffold 89 (GenBank accession ID XM_007901587). It is noteworthy that GHR belongs to the same receptor superfamily as PRLR and these receptors show considerable similarity both in structure and in signal transduction (see Freeman et al., 2000; Kopchick and Andry, 2000). The similarities between GH and PRL1 proteins and between their receptors allow GH to bind and signal via PRLR, and vice versa. Human GH is known to interact with both GHR and PRLR (Cunningham et al., 1990; Somers et al., 1994), and in Mozambique tilapia, one of two isoforms of PRL1 (PRL_{177}) was suggested to elicit somatotropic effect via GHR (Shepherd et al., 1997). While our observation does not necessarily preclude PRLR in the elephant fish, the binding of cmPRL1 to GHR is worth exploring. Further

characterization of the GH/PRL molecules and their receptors in other chondrichthyans and cyclostomes will shed light into the missing links underling the evolutionary history of this classical hormone family.

Acknowledgements

We thank Dr. Hiroshi Miyanishi of Keio University, Japan, for expertise in dual fluorescent *in situ* hybridization, and Prof. Byrappa Venkatesh of A*STAR, Singapore, for kind help in conducting *in silico* analysis using elephant fish genome database.

Role of the funding source

This study was supported by Grant-in-Aid for Scientific Research (B) (grant no. 26291065) and for Challenging Exploratory Research (grant no. 26650110), and a Japan-Australia Research Cooperative Program (reference nos 10034011-000062 and 13039901-000237) from the Japan Society for the Promotion of Science to S.H. This work was also funded by grants from the National Science Foundation (IOS-1119693) to E.G.G. and D.T.L, and National Oceanic and Atmospheric Administration (NA14OAR4170071) which is sponsored by the University of Hawaii Sea Grant College Program, SOEST (Project R/SS-12) to A.P.S.

The views expressed herein are those of the authors and do not necessarily reflect the views of the aforementioned granting agencies.
UNIHI-SEAGRANT-JC-14-38.

References

544 Amemiya, Y., Sogabe, Y., Nozaki, M., Takahashi, A., Kawauchi, H., 1999. Somatolactin
545 in the white sturgeon and African lungfish and its evolutionary significance.
546 Gen. Comp. Endocrinol. 114, 181-190.
547 Awruch, C.A., 2013. Reproductive endocrinology in chondrichthyans: the present and
548 the future. Gen. Comp. Endocrinol. 192, 60-70.
549 Ball, J.N., Baker, B.I., 1969. The pituitary gland: anatomy and histophysiology, in: Hoar,
550 W.S., Randall, D.J. (Eds.), Fish Physiology, Vol. 2. Academic Press, New York,
551 pp. 1-110.
552 Ballantyne, J.S., Fraser, D.I., 2013. Euryhaline elasmobranchs, in: McCormick, S.D.,
553 Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, Vol. 32. Academic Press,
554 Amsterdam, pp. 125-198.
555 Bern, H.A., Nicoll, C.S., 1968. The comparative endocrinology of prolactin. Recent
556 Prog. Horm. Res. 24, 681-720.
557 Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A., 1998. Prolactin (PRL)
558 and its receptor: actions, signal transduction pathways and phenotypes
559 observed in PRL receptor knockout mice. Endocr. Rev. 19, 225-268.
560 Breves, J.P., McCormick, S.D., Karlstrom, R.O., 2014. Prolactin and teleost ionocytes:
561 new insights into cellular and molecular targets of prolactin in vertebrate
562 epithelia. Gen. Comp. Endocrinol. 203, 21-28.
563 Carnevali, O., Mosconi, G., Yamamoto, K., Kobayashi, T., Kikuyama, S.,
564 Polzonetti-Magni, A.M., 1993. In-vitro effects of mammalian and amphibian
565 prolactins on hepatic vitellogenin synthesis in *Rana esculenta*. J. Endocrinol.
566 137, 383-389.
567 Compagno, L.J.V., 1990. Alternative life-history styles of cartilaginous fishes in time
568 and space. Environ. Biol. Fish. 28, 33-75.
569 Cunningham, B.C., Bass, S., Fuh, G., Wells, J.A., 1990. Zinc mediation of the binding
570 of human growth hormone to the human prolactin receptor. Science 250,

1709-1712.

de Vlaming, V.L., Sage, M., Beitz, B., 1975. Pituitary, adrenal and thyroid influences on osmoregulation in the euryhaline elasmobranch, Dasyatis sabina. Comp. Biochem. Physiol. A Comp. Physiol. 52, 505-513.

Dharmamba, M., Nishioka, R.S., 1968. Response of "prolactin-secreting" cells of *Tilapia mossambica* to environmental salinity. Gen. Comp. Endocrinol. 10, 409-420.

Dores, R.M., Noso, T., Rand-Weaver, M., Kawauchi, H., 1993. Isolation of prolactin and growth hormone from the pituitary of the holostean fish *Amia calva*. Gen. Comp. Endocrinol. 90, 346-354.

Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. Physiol. Rev. 80, 1523-1631.

Goffin, V., Shiverick, K.T., Kelly, P.A., Martial, J.A., 1996. Sequence-function relationships within the expanding family of prolactin, growth hormone, placental lactogen, and related proteins in mammals. Endocr. Rev. 17, 385-410.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307-321.

Hirano, T., 1986. The spectrum of prolactin action in teleosts, in: Ralph, C.L. (Ed.), Comparative Endocrinology: Developments and Directions. Alan R. Liss, Inc., New York, pp. 53-74.

Holmes, R.L., Ball, J.N., 1974. The Pituitary Gland: A Comparative Account. Cambridge University Press, Cambridge.

Horseman, N.D., Buntin, J.D., 1995. Regulation of pigeon cropmilk secretion and parental behaviors by prolactin. Annu. Rev. Nutr. 15, 213-238.

Huang, X., Hui, M.N., Liu, Y., Yuen, D.S., Zhang, Y., Chan, W.Y., Lin, H.R., Cheng, S.H., Cheng, C.H., 2009. Discovery of a novel prolactin in non-mammalian

598 vertebrates: evolutionary perspectives and its involvement in teleost retina
599 development. PLoS One 4, e6163.
600 Hyodo, S., Bell, J.D., Healy, J.M., Kaneko, T., Hasegawa, S., Takei, Y., Donald, J.A.,
601 Toop, T., 2007. Osmoregulation in elephant fish *Callorhinchus milii*
602 (Holocephali), with special reference to the rectal gland. J. Exp. Biol. 210,
603 1303-1310.
604 Inoue, J.G., Miya, M., Tsukamoto, K., Nishida, M., 2003. Basal actinopterygian
605 relationships: a mitogenomic perspective on the phylogeny of the "ancient fish".
606 Mol. Phylogenet. Evol. 26, 110-120.
607 Kakumura, K., Watanabe, S., Bell, J.D., Donald, J.A., Toop, T., Kaneko, T., Hyodo, S.,
608 2009. Multiple urea transporter proteins in the kidney of holocephalan elephant
609 fish (*Callorhinchus milii*). Comp. Biochem. Physiol. B Biochem. Mol. Biol.
610 154, 239-247.
611 Kanda, S., Akazome, Y., Mitani, Y., Okubo, K., Oka, Y., 2013. Neuroanatomical
612 evidence that kisspeptin directly regulates isotocin and vasotocin neurons.
613 PLoS One. 8, e62776.
614 Katoh, K., Standley, D. M. 2013. MAFFT multiple sequence alignment software version
615 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772-780.
616 Kawauchi, H., Sower, S.A., 2006. The dawn and evolution of hormones in the
617 adenohypophysis. Gen. Comp. Endocrinol. 148, 3-14.
618 Kawauchi, H., Suzuki, K., Yamazaki, T., Moriyama, S., Nozaki, M., Yamaguchi, K.,
619 Takahashi, A., Youson, J., Sower, S.A., 2002. Identification of growth hormone
620 in the sea lamprey, an extant representative of a group of the most ancient
621 vertebrates. Endocrinology 143, 4916-4921.
622 Kikugawa, K., Katoh, K., Kuraku, S., Sakurai, H., Ishida, O., Iwabe, N., Miyata, T.,
623 2004. Basal jawed vertebrate phylogeny inferred from multiple nuclear
624 DNA-coded genes. BMC Biol. 2, 3.

625 Kopchick, J.J., Andry, J.M., 2000. Growth hormone (GH), GH receptor, and signal
626 transduction. Mol. Genet. Metab. 71, 293-314.
627 Kuraku, S., Zmasek, C. M., Nishimura, O., Katoh, K. 2013. aLeaves facilitates
628 on-demand exploration of metazoan gene family trees on MAFFT sequence
629 alignment server with enhanced interactivity. Nuc. Acids Res. 41, W22-28.
630 Magdeldin, S., Uchida, K., Hirano, T., Grau, E.G., Abdelfattah, A., Nozaki, M., 2007.
631 Effects of environmental salinity on somatic growth and growth
632 hormone/insulin-like growth factor-I axis in juvenile tilapia *Oreochromis*
633 *mossambicus*. Fish. Sci. 73, 1025-1034.
634 Manzon, L.A., 2002. The role of prolactin in fish osmoregulation: a review. Gen. Comp.
635 Endocrinol. 125, 291-310.
636 Martin, R.A., 2005. Conservation of freshwater and euryhaline elasmobranchs: a review.
637 J. Mar. Biol. Ass. U. K. 85, 1049-1073.
638 Near, T.J., Eytan, R.I., Dornburg, A., Kuhn, K.L., Moore, J.A., Davis, M.P., Wainwright,
639 P.C., Friedman, M., Smith, W.L., 2012. Resolution of ray-finned fish
640 phylogeny and timing of diversification. Proc. Natl. Acad. Sci. U. S. A. 109,
641 13698-13703.
642 Nelson, J.S., 2006. Fishes of the World, fourth ed. John Wiley & Sons, Inc., New Jersey.
643 Nicoll, C.S., Wilson, S.W., Nishioka, R., Bern, H.A., 1981. Blood and pituitary
644 prolactin levels in tilapia (*Sarotherodon mossambicus*; Teleostei) from different
645 salinities as measured by a homologous radioimmunoassay. Gen. Comp.
646 Endocrinol. 44, 365-373.
647 Noso, T., Nicoll, C.S., Polenov, A.L., Kawauchi, H., 1993. The primary structure of
648 sturgeon prolactin: phylogenetic implication. Gen. Comp. Endocrinol. 91,
649 90-95.
650 Nussey, S.S., Whitehead, S.A., 2001. Endocrinology: An Integrated Approach. BIOS
651 Scientific Publishers, Oxford.

652 Ono, M., Takayama, Y., Rand-Weaver, M., Sakata, S., Yasunaga, T., Noso, T., Kawauchi,
653 H., 1990. cDNA cloning of somatolactin, a pituitary protein related to growth
654 hormone and prolactin. Proc. Natl. Acad. Sci. U. S. A. 87, 4330-4334.
655 Pickford, G.E., Phillips, J.G., 1959. Prolactin, a factor in promoting survival of
656 hypophysectomized killifish in fresh water. Science 130, 454-455.
657 Polzonetti-Magni, A., Carnevali, O., Yamamoto, K., Kikuyama, S., 1995. Growth
658 hormone and prolactin in amphibian reproduction. Zool. Sci. 12, 683-694.
659 Rand-Weaver, M., Kawauchi, H., 1993. Growth hormone, prolactin and somatolactin; a
660 structural overview, in: Hochachka, P.W., Mommsen, T.P. (Eds.), The
661 Biochemistry and Molecular Biology of Fishes, Vol. 2. Elsevier, Amsterdam,
662 pp. 39-56.
663 Rand-Weaver, M., Noso, T., Muramoto, K., Kawauchi, H., 1991. Isolation and
664 characterization of somatolactin, a new protein related to growth hormone and
665 prolactin from Atlantic cod (*Gadus morhua*) pituitary glands. Biochemistry 30,
666 1509–1515.
667 Riddle, O., Bates,R.W., Dykshorn, S.W., 1933. The preparation, identification and assay
668 of prolactin--a hormone of the anterior pituitary. Am. J. Physiol. 105, 191-216.
669 Schenck, E.J., Canfield, J.M., Brooks, C.L., 2003. Functional relationship of serine 90
670 phosphorylation and the surrounding putative salt bridge in bovine prolactin.
671 Mol. Cell. Endocrinol. 204, 117-125.
672 Shepherd, B.S., Sakamoto, T., Nishioka, R.S., Richman, N.H., Mori, I., Madsen, S.S.,
673 Chen, T.T., Hirano, T., Bern, H.A., Grau, E.G., 1997. Somatotropic actions of
674 the homologous growth hormone and prolactins in the euryhaline teleost, the
675 tilapia, *Oreochromis mossambicus*. Proc. Natl. Acad. Sci. U. S. A. 94,
676 2068-2072.
677 Sinha, Y.N., 1995. Structural variants of prolactin: occurrence and physiological
678 significance. Endocr. Rev. 16, 354-369.

679 Somers, W., Ultsch, M., De Vos, A.M., Kossiakoff, A.A., 1994. The X-ray structure of a
680 growth hormone-prolactin receptor complex. Nature 372, 478–481.
681 Stricker, P., Grueter, P., 1928. Action du lobe antérieur de l'hypophyse sur la montée
682 laiteuse. C. R. Seances Soc. Biol. Fil. 99, 1978-1980.
683 Takabe, S., Teranishi, K., Takaki, S., Kusakabe, M., Hirose, S., Kaneko, T., Hyodo, S.,
684 2012. Morphological and functional characterization of a novel
685 Na^+/K^+-ATPase-immunoreactive, follicle-like structure on the gill septum of
686 Japanese banded houndshark, *Triakis scyllium*. Cell Tissue Res. 348, 141-153.
687 Takagi, W., Kajimura, M., Tanaka, H., Hasegawa, K., Bell, J.D., Toop, T., Donald, J.A.,
688 Hyodo, S., 2014. Urea-based osmoregulation in the developing embryo of
689 oviparous cartilaginous fish (*Callorhinchus milii*): contribution of the
690 extraembryonic yolk sac during the early developmental period. J. Exp. Biol.
691 217, 1353-1362.
692 Takei, Y., Hiroi, J., Takahashi, H., Sakamoto, T., 2014. Diverse mechanisms for body
693 fluid regulation in teleost fishes. Am. J. Physiol. Regul. Integr. Comp. Physiol.
694 307, 778-792.
695 Venkatesh, B., Kirkness, E.F., Loh, Y.H., Halpern, A.L., Lee, A.P., Johnson, J., Dandona,
696 N., Viswanathan, L.D., Tay, A., Venter, J.C., Strausberg, R.L., Brenner, S., 2007.
697 Survey sequencing and comparative analysis of the elephant shark
698 (*Callorhinchus milii*) genome. PLoS Biol. 5, e101.
699 Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., Ohta, Y.,
700 Flajnik, M.F., Sutoh, Y., Kasahara, M., Hoon, S., Gangu, V., Roy, S.W., Irimia,
701 M., Korzh, V., Kondrychyn, I., Lim, Z.W., Tay, B.H., Tohari, S., Kong, K.W.,
702 Ho, S., Lorente-Galdos, B., Quilez, J., Marques-Bonet, T., Raney, B.J., Ingham,
703 P.W., Tay, A., Hillier, L.W., Minx, P., Boehm, T., Wilson, R.K., Brenner, S.,
704 Warren, W.C., 2014. Elephant shark genome provides unique insights into
705 gnathostome evolution. Nature 505, 174-179.

706 Whittington, C.M., Wilson, A.B., 2013. The role of prolactin in fish reproduction. Gen.
707 Comp. Endocrinol. 191, 123-136.
708 Wourms, J.P. 1977. Reproduction and development in chondrichthyan fishes. Amer.
709 Zool. 17, 379-410.
710 Yamaguchi, Y., Kaiya, H., Konno, N., Iwata, E., Miyazato, M., Uchiyama, M., Bell, J.D.,
711 Toop, T., Donald, J.A., Brenner, S., Venkatesh, B., Hyodo, S., 2012. The fifth
712 neurohypophysial hormone receptor is structurally related to the V2-type
713 receptor but functionally similar to V1-type receptors. Gen. Comp. Endocrinol.
714 178, 519-528.
715
716
717 **Figure legends**
718 Figure 1. Multiple alignment of amino acid sequences of PRL/GH/SL. The alignment
719 was constructed with the MAFFT algorithm (see Methods) and manually modified for
720 improved demonstration of conserved residues as well as structural features unique to
721 each hormone. Arrows; the positions of the introns of cmPRL1 (black) or cmGH (white),
722 black circles; conserved cysteine residues, triangles; putative *N*-glycosylation site of
723 cmPRL1 (black) or conserved proline and glycine residues important in receptor
724 binding (white). Abbreviations: ag, *Acipenser gueldenstaedtii* (sturgeon); cm,
725 *Callorhinchus milii* (elephant fish); dr, *Danio rerio* (medaka); gg, *Gallus gallus*
726 (chicken); hs, *Homo sapiens* (human); lo, *Lepisosteus oculatus* (gar); paSL, *Protopterus*
727 *annectens* (African lungfish) SL; paPRL1, *Protopterus aethiopicus* (Marbled lungfish)
728 PRL1; xl, *Xenopus laevis* (frog). See Supplementary Table 1 for accession IDs of these
729 sequences.
730
731 Figure 2. Molecular phylogeny of the entire PRL/GH family inferred using 114 amino
732 acid sites (shape parameter of the gamma distribution $\alpha = 2.2$). The elephant fish

sequences identified in this study are shown in white letters with a black background. Individual gene names are given when they do not follow the basic subgrouping on right (PRL1, PRL2, GH, and SL). At nodes, bootstrap probabilities from the maximum-likelihood method and neighbor-joining method are shown in order. The arrow indicates the position of the branch leading to the sequence of *Petromyzon marinus* GH (GenBank accession ID, AB081461) in a preliminary phylogenetic analysis as reference.

Figure 3. Molecular phylogeny of the jawed vertebrate PRL1 inferred using 165 amino acid sites (shape parameter of the gamma distribution α=1.7). At nodes, bootstrap probabilities the maximum-likelihood method and neighbor-joining method are shown in order. The gray diamond indicates gene duplication that gave rise to the two *PRL1* genes (*PRL1A* and *PRL1B*) in an early sarcopterygian. The white diamond indicates gene duplication unique to the Xenopus lineage. Note that the predicted amino acid sequence of *X. tropicalis* PRL1B was identical to that of *X. laevis*.

Figure 4. Gene synteny around *PRL1* genes of elephant fish (*C. milii*), stickleback (*G. aculeatus*), zebrafish (*D. rerio*), coelacanth (*L. chalumnae*), frog (*X. tropicalis*), chicken (*G. Gallus*), and human (*H. sapiens*). Gene loci on a chromosome (chr.) and scaffold (scf.) are shown by ellipses serially numbered in alphabetical order. The color of the ellipses represents the human chromosome on which corresponding human orthologs are found.

Figure 5. Gel images showing the tissue distribution of cmPRL1 and β-actin as an internal control. For non-gonadal tissues, left and right lanes represent the results of female and male, respectively. Abbreviations: Bra, brain; Pit, pituitary; Gil, gill; Int, intestine; Kid, kidney; Liv, Liver; RG, rectal gland; U, uterus; T, testis; M, molecular

760 weight marker.

761

762 Figure 6. (A-C) Quantification of mRNA of cmPRL1 (A), cmGH (B) and cmPOMC (C)
763 in the elephant fish brain (Bra) and pituitary (Pit). The results are shown as normalized
764 values by β-actin. ***Significantly different from brain at $P < 0.001$ (Student's *t*-test).
765 (D) Absolute copy number of mRNA in the pituitary was compared among the above
766 three targets. Note that Y axis is in logarithmic scale. Means not sharing the same letter
767 are significantly different at $P < 0.01$ (Tukey's HSD test following one-way ANOVA).

768

769 Figure 7. Images of sagittally-sectioned elephant fish pituitary subjected to either
770 hematoxylin-eosin (HE) staining or hybridization with cRNA probes for cmPRL1,
771 cmPOMC or cmGH. (A) Whole pituitary images. Scale bar represents 1 mm. (B-C)
772 Magnified images of RPD (B), PPD (C) and PI (D). Positive hybridization signals for
773 probes of cmPRL1 in RPD (B) and PPD (C) and of cmPOMC in PPD (C) are indicated
774 by arrowheads. Scale bars represent 0.5 mm.

775

776 Figure 8. Dual fluorescent *in situ* hybrydization of the elephant fish pituitary for
777 mRNAs of cmPRL1 (magenta) and cmPOMC (green). Areas enclosed by white line in
778 top row are magnified in bottom row. Scale bars represent either 100 μm (top row) or 50
779 μm (bottom row), respectively. No overlap was observed between signals of cmPRL1
780 and cmPOMC mRNAs.

Table 1

Table 1. List of primers used in the present study. *For cmGH, forward and reverse primers were used for 3'- and 5'-RACE, respectively.

Cloning		Forward primer (5'-3')	Reverse primer (5'-3')
	cmPRL1	gtcggacgagccgtggacact	cagaggggtgtcgagagatgc
	cmGH*	acctcttgtccaacgctgttcac	cttcttaggtttccttcgaaccg
	cmPOMC	agaccctgactgtcacctcaa	attcctgaagagggtgagcagt
RT-PCR			
	cmPRL1	(same with the cloning primer)	tcagcaggacaccgcgtgagg
	β-actin	aagacatcagggtgtgatggttg	ggagcaatgatcttgatcttcatgg
qPCR			
	cmPRL1	ctctgtccaacgacatcttcaa	acgggttcgtgccaatac
	cmGH	ccggaagagaagcgtaactatc	agtacgcaagaagctctgtatc
	cmPOMC	gcagaagaaggatgggaagt	gaacatgggaagcagatgtttag
in situ hybridization			
	cmPRL1	(same with the cloning primer)	(same with the cloning primer)
	cmGH	caggattgtcgctgtgttct	ggtaggtttcaactttgtgcatatc
	cmPOMC	(same with the cloning primer)	(same with the cloning primer)

Table 2

Table 2. Amino acid identity of cmPRL1 and cmGH to representative vertebrate PRL1s and GHs.

PRL1

Species (GenBank accession ID)	% identity to cmPRL1
Sturgeon (AAB28396)	35%
Trout (M24738)	32%
Lungfish (AAB27569)	35%
Frog (CAA34199)	37%
Chicken (BAB18728)	37%
Human (NP_000939)	35%

GH

Species (GenBank accession ID)	% identity to cmGH
Sea lamprey (BAC15763)	27%
Shark (P34006)	76%
Sturgeon (AAX36064)	59%
Tilapia (M26916)	34%
Lungfish (AAC16496)	53%
Newt (CAB55428)	53%
Chicken (AEZ51530)	54%
Human (NP_00205)	42%

Figure 1

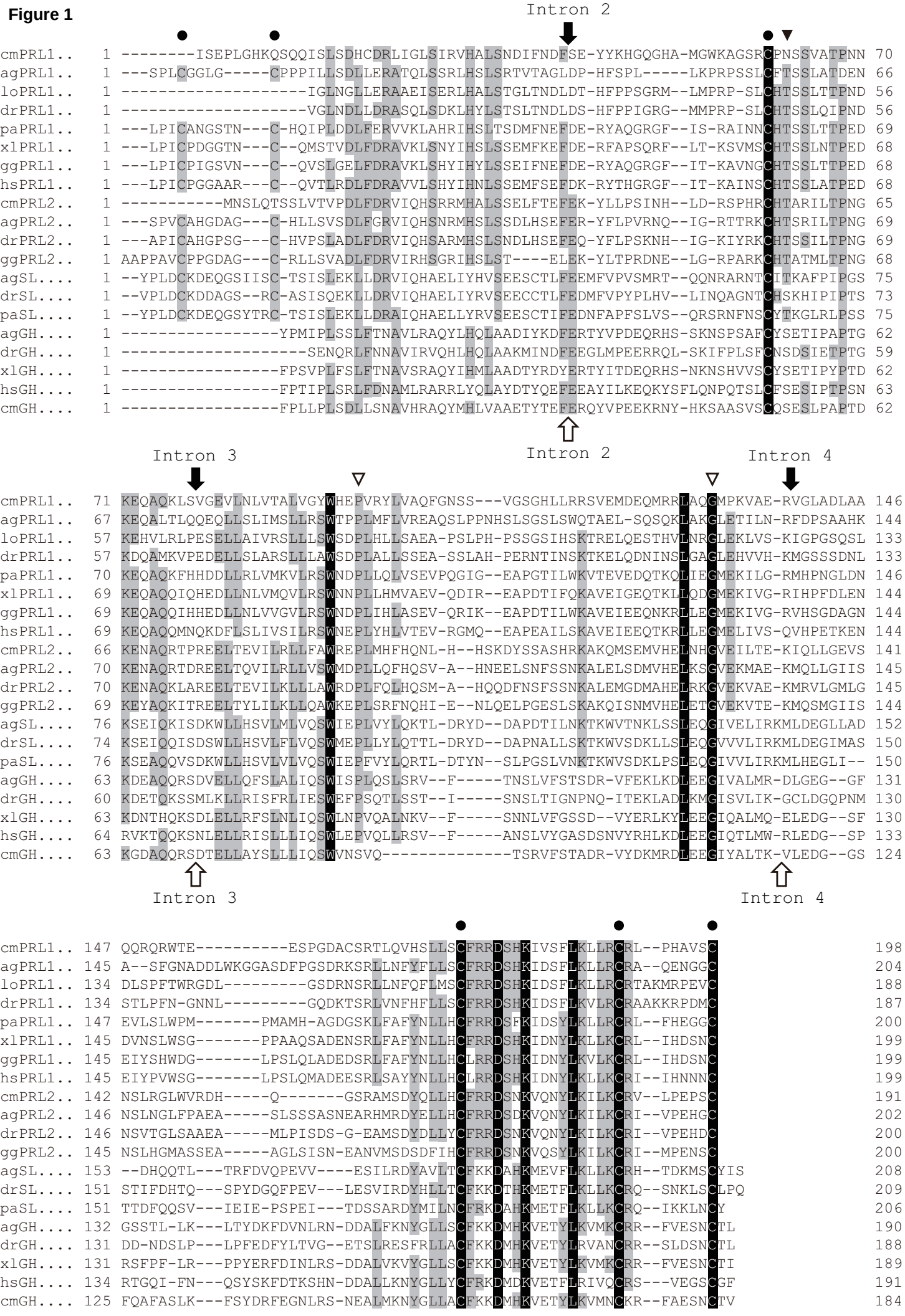
Intron 2
Intron 3
Intron 4
cmPRL1.. 1 --------ISEPLGHKQSQQISLSDHCDRLIGLSIRVHALSNDIFNDFSE-YYKHGQGHA-MGWKAGSRCPNSSVATPNN 70
agPRL1.. 1 ---SPLCGGLG-----CPPPILLSDLLERATQLSSRLHSLSRTVTAGLDP-HFSPL-----LKPRPSSLCFTSSLATDEN 66
loPRL1.. 1 --------------------IGLNGLLERAAEISERLHALSTGLTNDLDT-HFPPSGRM--LMPRP-SLCHTSSLTTPND 56
drPRL1.. 1 --------------------VGLNDLLDRASQLSDKLHYLSTSLTNDLDS-HFPPIGRG--MMPRP-SLCHTSSLQIPND 56
paPRL1.. 1 ---LPICANGSTN---C-HQIPLDDLFERVVKLAHRIHSLTSDMFNEFDE-RYAQGRGF--IS-RAINNCHTSSLTTPED 69
xlPRL1.. 1 ---LPICPDGGTN---C--QMSTVDLFDRAVKLSNYIHSLSSEMFKEFDE-RFAPSQRF--LT-KSVMSCHTSSLNTPED 68
ggPRL1.. 1 ---LPICPIGSVN---C--QVSLGELFDRAVKLSHYIHYLSSEIFNEFDE-RYAQGRGF--IT-KAVNGCHTSSLTTPED 68
hsPRL1.. 1 ---LPICPGGAAR---C--QVTLRDLFDRAVVLSHYIHNLSSEMFSEFDK-RYTHGRGF--IT-KAINSCHTSSLATPED 68
cmPRL2.. 1 -----------MNSLQTSSLVTVPDLFDRVIQHSRRMHALSSELFTEFEK-YLLPSINH--LD-RSPHRCHTARILTPNG 65
agPRL2.. 1 ---SPVCAHGDAG---C-HLLSVSDLFGRVIQHSNRMHSLSSDLHSEFER-YFLPVRNQ--IG-RTTRKCHTSRILTPNG 69
drPRL2.. 1 ---APICAHGPSG---C-HVPSLADLFDRVIQHSARMHSLSNDLHSEFEQ-YFLPSKNH--IG-KIYRKCHTSSILTPNG 69
ggPRL2.. 1 AAPPAVCPPGDAG---C-RLLSVADLFDRVIRHSGRIHSLST----ELEK-YLTPRDNE--LG-RPARKCHTATMLTPNG 68
agSL.... 1 --YPLDCKDEQGSIISC-TSISLEKLLDRVIQHAELIYHVSEESCTLFEEMFVPVSMRT--QQNRARNTCITKAFPIPGS 75
drSL.... 1 --VPLDCKDDAGS--RC-ASISQEKLLDRVIQHAELIYRVSEECCTLFEDMFVPYPLHV--LINQAGNTCHSKHIPIPTS 73
paSL.... 1 --YPLDCKDEQGSYTRC-TSISLEKLLDRAIQHAELLYRVSEESCTIFEDNFAPFSLVS--QRSRNFNSCYTKGLRLPSS 75
agGH.... 1 ------------------YPMIPLSSLFTNAVLRAQYLHQLAADIYKDFERTYVPDEQRHS-SKNSPSAFCYSETIPAPTG 62
drGH.... 1 ----------------------SENQRLFNNAVIRVQHLHQLAAKMINDFEEGLMPEERRQL-SKIFPLSFCNSDSIETPTG 59
xlGH.... 1 ------------------FPSVPLFSLFTNAVSRAQYIHMLAADTYRDYERTYITDEQRHS-NKNSHVVSCYSETIPYPTD 62
hsGH.... 1 ------------------FPTIPLSRLFDNAMLRARRLYQLAYDTYQEFEEAYILKEQKYSFLQNPQTSLCFSESIPTPSN 63
cmGH.... 1 ------------------FPLLPLSDLLSNAVHRAQYMHLVAAETYTEFERQYVPEEKRNY-HKSAASVSCQSESLPAPTD 62
Intron 3
Intron 2
Intron 4
cmPRL1.. 71 KEQAQKLSVGEVLNLVTALVGYWHEPVRYLVAQFGNSS---VGSGHLLRRSVEMDEQMRRLAQGMPKVAE-RVGLADLAA 146
agPRL1.. 67 KEQALTLQQEQLLSLIMSLLRSWTPPLMFLVREAQSLPPNHSLSGSLSWQTAEL-SQSQKLAKGLETILN-RFDPSAAHK 144
loPRL1.. 57 KEHVLRLPESELLAIVRSLLLSWSDPLHLLSAEA-PSLPH-PSSGSIHSKTRELQESTHVLNRGLEKLVS-KIGPGSQSL 133
drPRL1.. 57 KDQAMKVPEDELLSLARSLLLAWSDPLALLSSEA-SSLAH-PERNTINSKTKELQDNINSLGAGLEHVVH-KMGSSSDNL 133
paPRL1.. 70 KEQAQKFHHDDLLRLVMKVLRSWNDPLLQLVSEVPQGIG--EAPGTILWKVTEVEDQTKQLIEGMEKILG-RMHPNGLDN 146
xlPRL1.. 69 KEQAQQIQHEDLLNLVMQVLRSWNNPLLHMVAEV-QDIR--EAPDTIFQKAVEIGEQTKLLQDGMEKIVG-RIHPFDLEN 144
ggPRL1.. 69 KEQAQQIHHEDLLNLVVGVLRSWNDPLIHLASEV-QRIK--EAPDTILWKAVEIEEQNKRLLEGMEKIVG-RVHSGDAGN 144
hsPRL1.. 69 KEQAQQMNQKDFLSLIVSILRSWNEPLYHLVTEV-RGMQ--EAPEAILSKAVEIEEQTKRLLEGMELIVS-QVHPETKEN 144
cmPRL2.. 66 KENAQRTPREELTEVILRLLFAWREPLMHFHQNL-H--HSKDYSSASHRKAKQMSEMVHELNHGVEILTE-KIQLLGEVS 141
agPRL2.. 70 KENAQRTDREELTQVILRLLVSWMDPLLQFHQSV-A--HNEELSNFSSNKALELSDMVHELKSGVEKMAE-KMQLLGIIS 145
drPRL2.. 70 KENAQKLAREELTEVILKLLLAWRDPLFQLHQSM-A--HQQDFNSFSSNKALEMGDMAHELRKGVEKVAE-KMRVLGMLG 145
ggPRL2.. 69 KEYAQKITREELTYLILKLLQAWKEPLSRFNQHI-E--NLQELPGESLSKAKQISNMVHELETGVEKVTE-KMQSMGIIS 144
agSL.... 76 KSEIQKISDKWLLHSVLMLVQSWIEPLVYLQKTL-DRYD--DAPDTILNKTKWVTNKLSSLEQGIVELIRKMLDEGLLAD 152
drSL.... 74 KSEIQQISDSWLLHSVLFLVQSWMEPLLYLQTTL-DRYD--DAPNALLSKTKWVSDKLLSLEQGVVVLIRKMLDEGIMAS 150
paSL.... 76 KSEAQQVSDKWLLHSVLVLVQSWIEPFVYLQRTL-DTYN--SLPGSLVNKTKWVSDKLPSLEQGIVVLIRKMLHEGLI-- 150
agGH.... 63 KDEAQQRSDVELLQFSLALIQSWISPLQSLSRV--F-----TNSLVFSTSDR-VFEKLKDLEEGIVALMR-DLGEG--GF 131
drGH.... 60 KDETQKSSMLKLLRISFRLIESWEFPSQTLSST--I-----SNSLTIGNPNQ-ITEKLADLKMGISVLIK-GCLDGQPNM 130
xlGH.... 63 KDNTHQKSDLELLRFSLNLIQSWLNPVQALNKV--F-----SNNLVFGSSD--VYERLKYLEEGIQALMQ-ELEDG--SF 130
hsGH.... 64 RVKTQQKSNLELLRISLLLIQSWLEPVQLLRSV--F-----ANSLVYGASDSNVYRHLKDLEEGIQTLMW-RLEDG--SP 133
cmGH.... 63 KGDAQQRSDTELLAYSLLLIQSWVNSVQ--------------TSRVFSTADR-VYDKMRDLEEGIYALTK-VLEDG--GS 124
Intron 3
Intron 4
cmPRL1.. 147 QQRQRWTE----------ESPGDACSRTLQVHSLLSCFRRDSHKIVSFLKLLRCRL--PHAVSC 198
agPRL1.. 145 A--SFGNADDLWKGGASDFPGSDRKSRLLNFYFLLSCFRRDSHKIDSFLKLLRCRA--QENGGC 204
loPRL1.. 134 DLSPFTWRGDL---------GSDRNSRLLNFQFLMSCFRRDSHKIDSFLKLLRCRTAKMRPEVC 188
drPRL1.. 134 STLPFN-GNNL---------GQDKTSRLVNFHFLLSCFRRDSHKIDSFLKVLRCRAAKKRPDMC 187
paPRL1.. 147 EVLSLWPM--------PMAMH-AGDGSKLFAFYNLLHCFRRDSFKIDSYLKLLRCRL--FHEGGC 200
xlPRL1.. 145 DVNSLWSG--------PPAAQSADENSRLFAFYNLLHCFRRDSHKIDNYLKLLKCRL--IHDSNC 199
ggPRL1.. 145 EIYSHWDG--------LPSLQLADEDSRLFAFYNLLHCLRRDSHKIDNYLKVLKCRL--IHDSNC 199
hsPRL1.. 145 EIYPVWSG--------LPSLQMADEESRLSAYYNLLHCLRRDSHKIDNYLKLLKCRI--IHNNNC 199
cmPRL2.. 142 NSLRGLWVRDH-----Q--------GSRAMSDYQLLHCFRRDSNKVQNYLKILKCRV--LPEPSC 191
agPRL2.. 146 NSLNGLFPAEA-----SLSSSASNEARHMRDYELLHCFRRDSDKVQNYLKILKCRI--VPEHGC 202
drPRL2.. 146 NSVTGLSAAEA-----MLPISDS-G-EAMSDYDLLYCFRRDSNKVQNYLKILKCRI--VPEHDC 200
ggPRL2.. 145 NSLHGMASSEA-----AGLSISN-EANVMSDSDFIHCFRRDSNKVQSYLKILKCRI--MPENSC 200
agSL.... 153 --DHQQTL---TRFDVQPEVV----ESILRDYAVLTCFKKDAHKMEVFLKLLKCRH--TDKMSCYIS 208
drSL.... 151 STIFDHTQ---SPYDGQFPEV---LESVIRDYHLLTCFKKDTHKMETFLKLLKCRQ--SNKLSCLPQ 209
paSL.... 151 TTDFQQSV---IEIE-PSPEI---TDSSARDYMILNCFRKDAHKMETFLKLLKCRQ--IKKLNCY 206
agGH.... 132 GSSTL-LK---LTYDKFDVNLRN-DDALFKNYGLLSCFKKDMHKVETYLKVMKCRR--FVESNCTL 190
drGH.... 131 DD-NDSLP---LPFEDFYLTVG--ETSLRESFRLLACFKKDMHKVETYLRVANCRR--SLDSNCTL 188
xlGH.... 131 RSFPF-LR---PPYERFDINLRS-DDALVKVYGLLSCFKKDMHKVETYLKVMKCRR--FVESNCTI 189
hsGH.... 134 RTGQI-FN---QSYSKFDTKSHN-DDALLKNYGLLYCFRKDMDKVETFLRIVQCRS---VEGSCGF 191
cmGH.... 125 FQAFASLK---FSYDRFEGNLRS-NEALMKNYGLLACFKKDMHKVETYLKVMNCKR--FAESNCTV 184

Figure 2

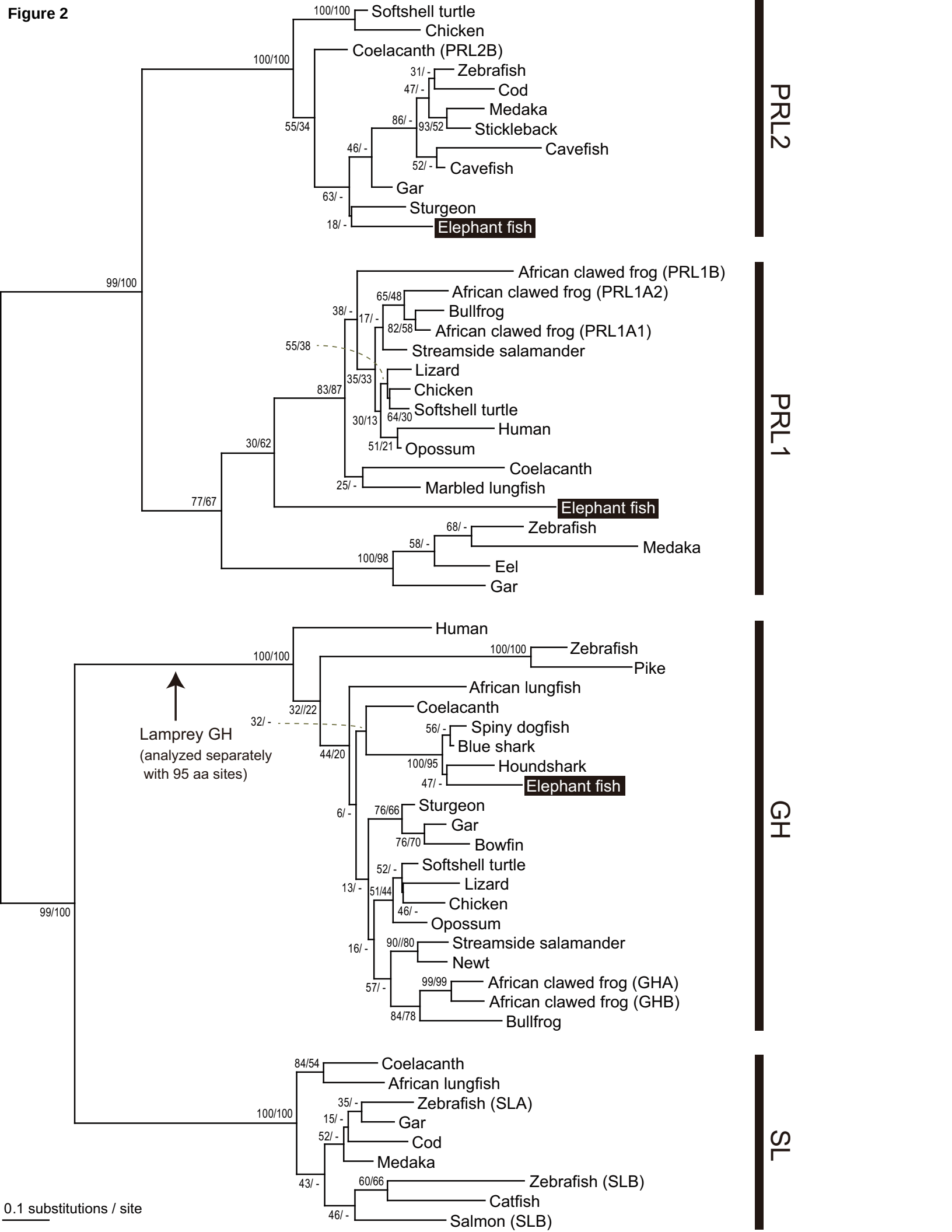
PRL2
Softshell turtle
Chicken
Coelacanth (PRL2B)
Zebrafish
Cod
Medaka
Stickleback
Cavefish
Cavefish
Gar
Sturgeon
Elephant fish
PRL1
African clawed frog (PRL1B)
African clawed frog (PRL1A2)
Bullfrog
African clawed frog (PRL1A1)
Streamside salamander
Lizard
Chicken
Softshell turtle
Human
Opossum
Coelacanth
Marbled lungfish
Elephant fish
Zebrafish
Medaka
Eel
Gar
GH
Human
Zebrafish
Pike
African lungfish
Coelacanth
Spiny dogfish
Blue shark
Houndshark
Elephant fish
Sturgeon
Gar
Bowfin
Softshell turtle
Lizard
Chicken
Opossum
Streamside salamander
Newt
African clawed frog (GHA)
African clawed frog (GHB)
Bullfrog
Lamprey GH
(analyzed separately
with 95 aa sites)
SL
Coelacanth
African lungfish
Zebrafish (SLA)
Gar
Cod
Medaka
Zebrafish (SLB)
Catfish
Salmon (SLB)
0.1 substitutions / site

Figure 3

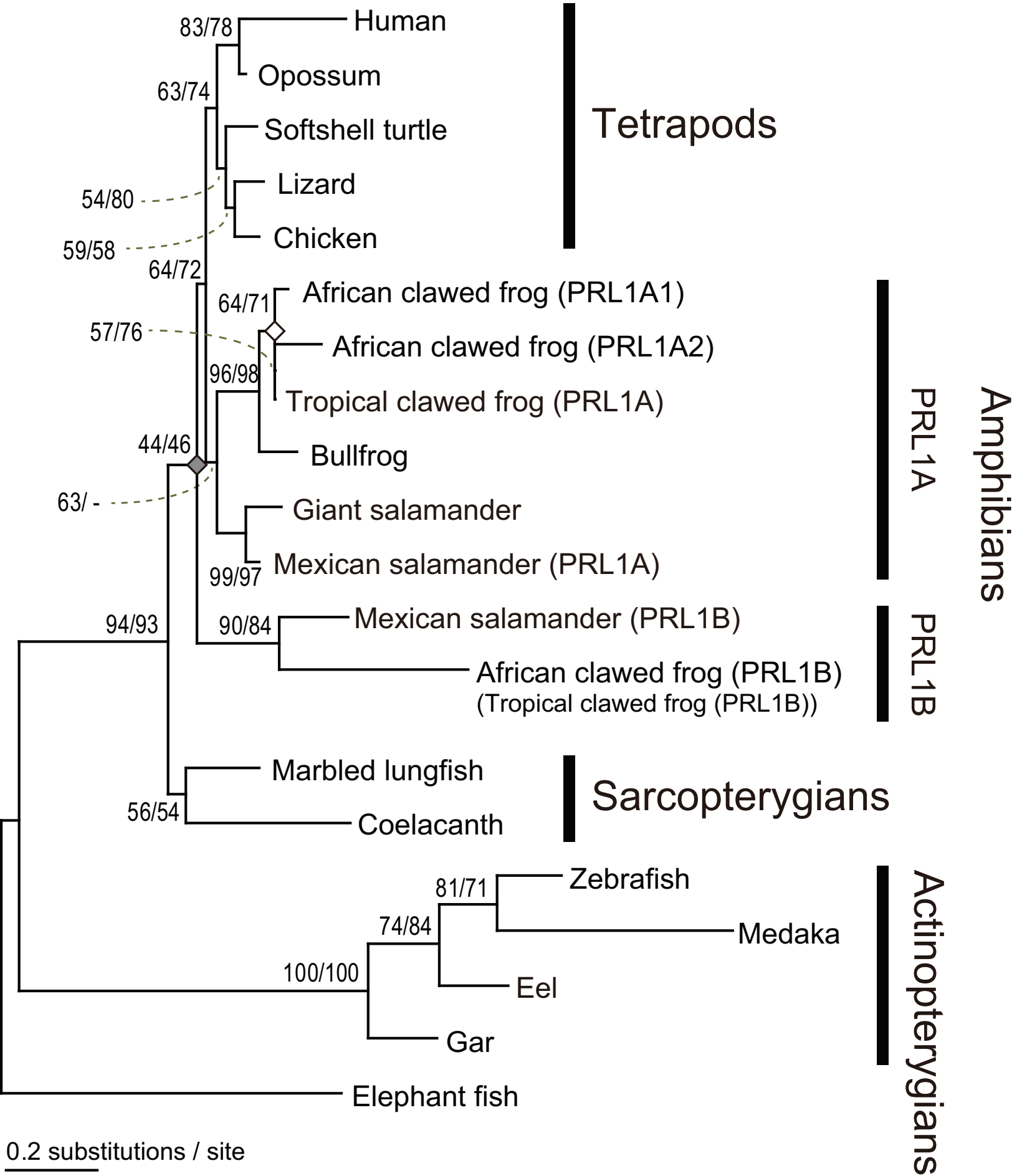
Human
Opossum
Softshell turtle
Lizard
Chicken
Tetrapods
African clawed frog (PRL1A1)
African clawed frog (PRL1A2)
Tropical clawed frog (PRL1A)
Bullfrog
Giant salamander
Mexican salamander (PRL1A)
PRL1A
Mexican salamander (PRL1B)
African clawed frog (PRL1B)
(Tropical clawed frog (PRL1B))
PRL1B
Amphibians
Marbled lungfish
Coelacanth
Sarcopterygians
Zebrafish
Medaka
Eel
Gar
Elephant fish
Actinopterygians
83/78
63/74
54/80
59/58
64/72
64/71
57/76
96/98
44/46
63/ -
99/97
94/93
90/84
56/54
81/71
74/84
100/100
0.2 substitutions / site

Figure 4

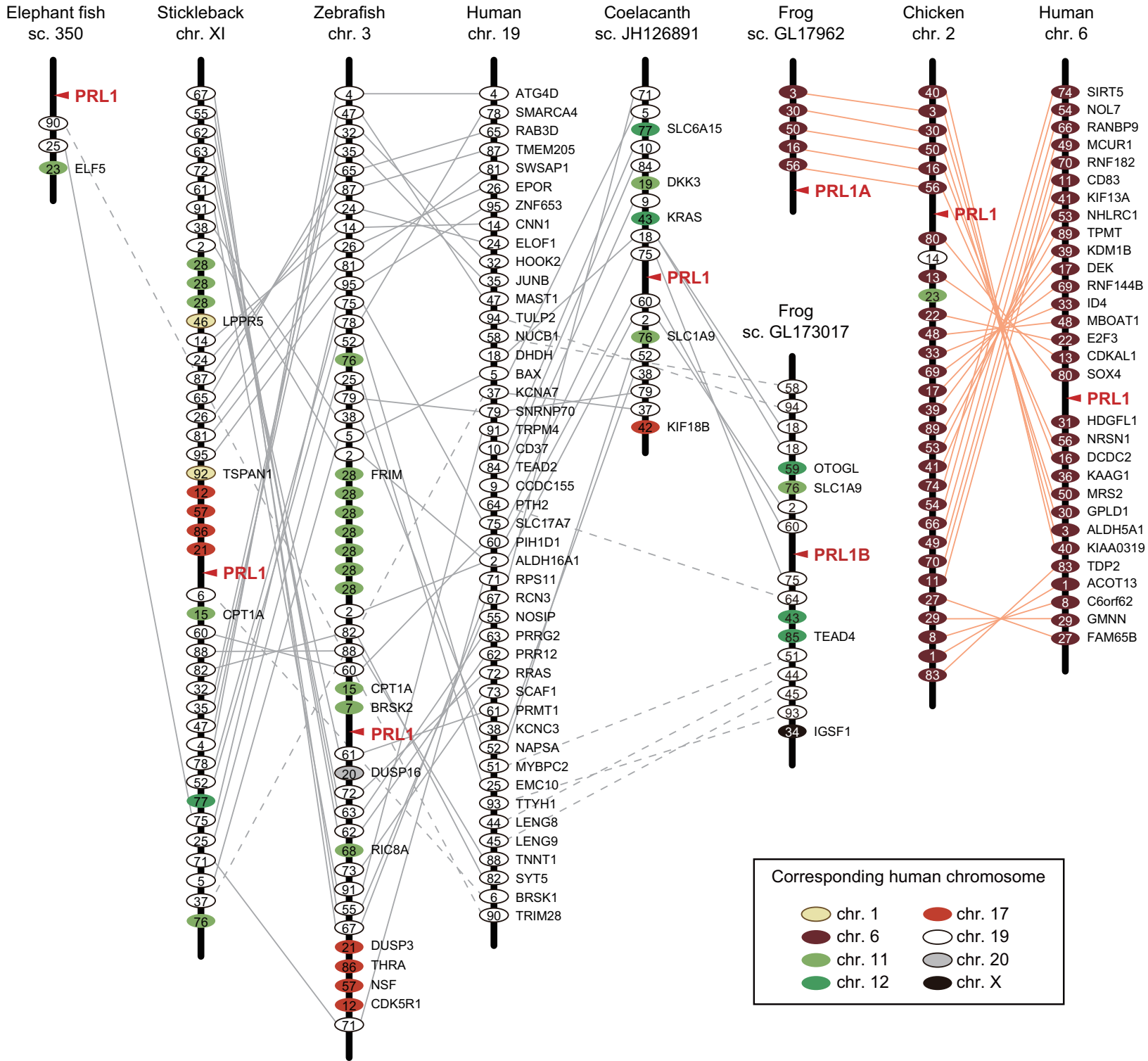
Elephant fish
sc. 350
Stickleback
chr. XI
Zebrafish
chr. 3
Human
chr. 19
Coelacanth
sc. JH126891
Frog
sc. GL17962
Frog
sc. GL173017
Chicken
chr. 2
Human
chr. 6
PRL1
ELF5
LPPR5
TSPAN1
PRL1
CPT1A
FRIM
CPT1A
BRSK2
PRL1
DUSP16
RIC8A
DUSP3
THRA
NSF
CDK5R1
ATG4D
SMARCA4
RAB3D
TMEM205
SWSAP1
EPOR
ZNF653
CNN1
ELOF1
HOOK2
JUNB
MAST1
TULP2
NUCB1
DHDH
BAX
KCNA7
SNRNP70
TRPM4
CD37
TEAD2
CCDC155
PTH2
SLC17A7
PIH1D1
ALDH16A1
RPS11
RCN3
NOSIP
PRRG2
PRR12
RRAS
SCAF1
PRMT1
KCNC3
NAPSA
MYBPC2
EMC10
TTYH1
LENG8
LENG9
TNNT1
SYT5
BRSK1
TRIM28
SLC6A15
DKK3
KRAS
PRL1
SLC1A9
KIF18B
PRL1A
OTOGL
SLC1A9
PRL1B
TEAD4
IGSF1
PRL1
SIRT5
NOL7
RANBP9
MCUR1
RNF182
CD83
KIF13A
NHLRC1
TPMT
KDM1B
DEK
RNF144B
ID4
MBOAT1
E2F3
CDKAL1
SOX4
PRL1
HDGFL1
NRSN1
DCDC2
KAAG1
MRS2
GPLD1
ALDH5A1
KIAA0319
TDP2
ACOT13
C6orf62
GMNN
FAM65B
Corresponding human chromosome
chr. 1
chr. 6
chr. 11
chr. 12
chr. 17
chr. 19
chr. 20
chr. X

Figure 5

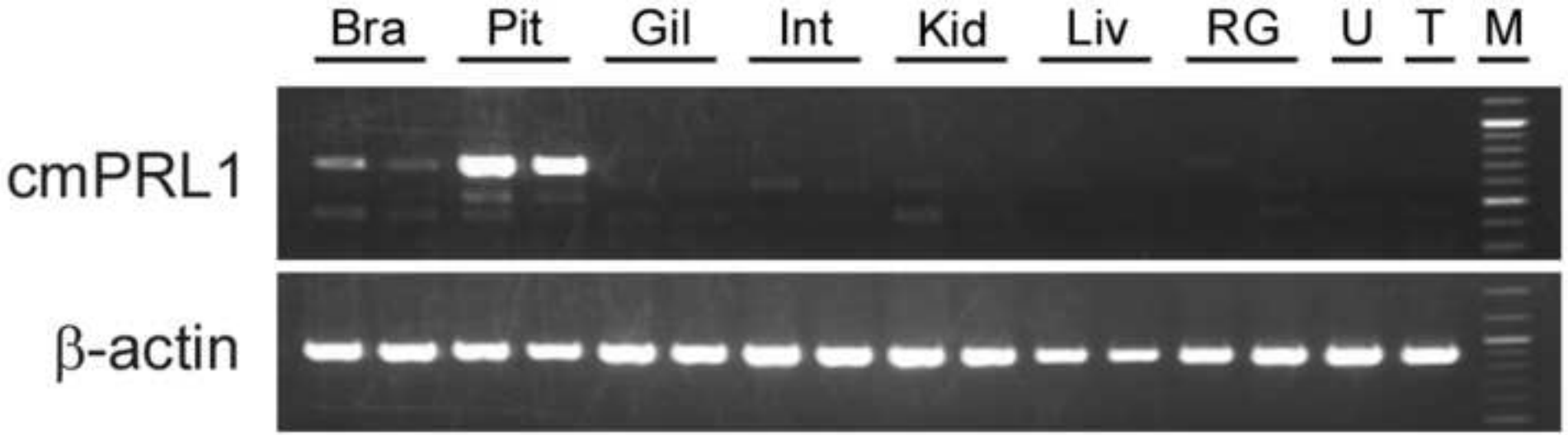

Figure 6

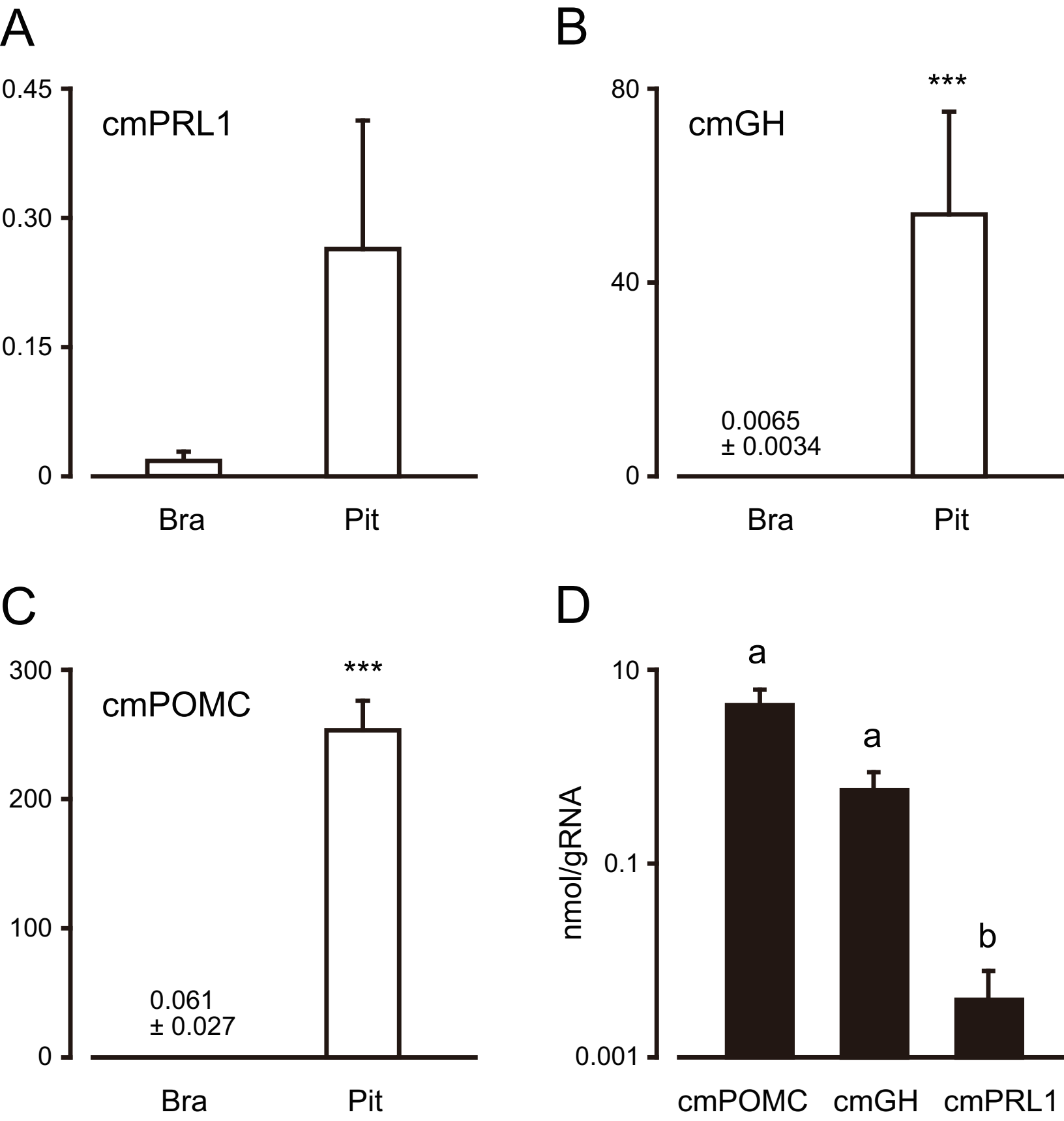
A
0.45
0.30
0.15
0
cmPRL1
Bra
Pit
B
80
40
0
***
cmGH
0.0065
± 0.0034
Bra
Pit
C
300
200
100
0
***
cmPOMC
0.061
± 0.027
Bra
Pit
D
10
0.1
0.001
nmol/gRNA
a
a
b
cmPOMC
cmGH
cmPRL1

Figure 7

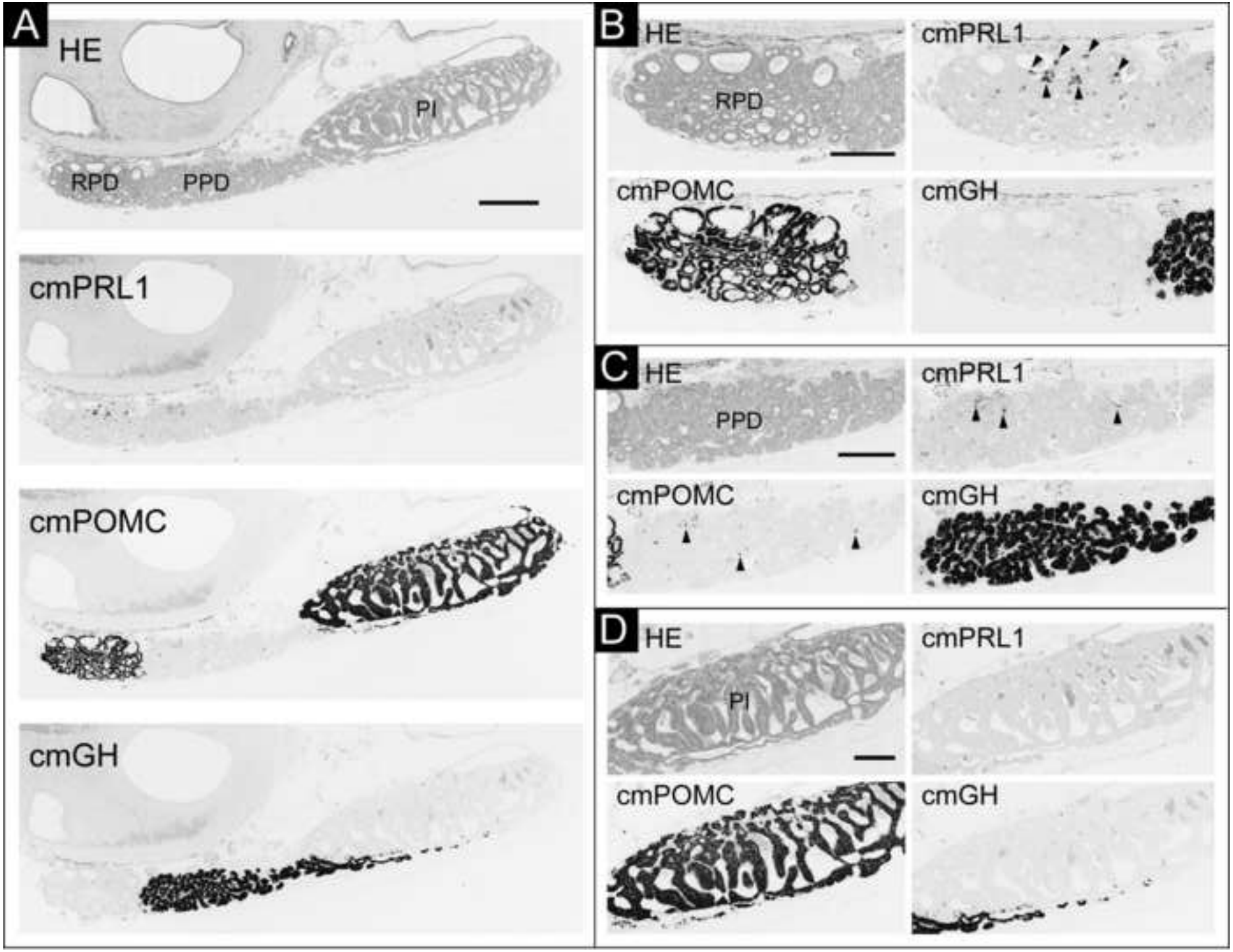
A
HE
PI
RPD
PPD
cmPRL1
cmPOMC
cmGH
B
HE
RPD
cmPRL1
cmPOMC
cmGH
C
HE
PPD
cmPRL1
cmPOMC
cmGH
D
HE
PI
cmPRL1
cmPOMC
cmGH

Figure 8