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Characterization of *Oncorhynchus mykiss* 5-hydroxyisourate hydrolase/transthyretin superfamily: Evolutionary and functional analyses

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Abbreviations: *A*, absorbance; aa, amino acid; ANOVA, analysis of variance; BSA, bovine serum albumin; cDNA, DNA complementary to RNA; EST, expressed sequence tag; FPLC, fast protein liquid chromatography; 5-HIU, 5-hydroxyisourate hydrolase; HIUHase, 5-hydroxyisourate hydrolase; HPLC, high performance liquid chromatography; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kbp, kilobase pairs or 1000 bp; kDa, kilodalton(s); Myr, million years; nt, nucleotide(s); PA, polyacrylamide; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PTS2, peroxisomal targeting signal-2; re-, recombinant; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; T3, 3,3',5-triiodo-L-thyronine; T4, L-thyroxine; TH, thyroid hormone; TTR, transthyretin.

Abstract

Teleosts have highly diverged genomes that resulted from whole genome duplication, which lead to an extensive diversity of paralogous genes. Transthyretin (TTR), an extracellular thyroid hormone (TH) binding protein, is thought to have evolved from an ancestral 5-hydroxyisourate hydrolase (HIUHase) by gene duplication at some stage of chordate evolution. To characterize the functions of proteins that arose from duplicated genes in teleosts, we investigated the phylogenetic relationship of teleost HIUHase and TTR aa sequences, the expression levels of *Oncorhynchus mykiss* HIUHase and TTR mRNA in various tissues and the biological activities of the *O. mykiss* re-HIUHase and re-TTR. Phylogenetic analysis of the teleost aa sequences revealed the presence of two HIUHase subfamilies, HIUHase 1 (which has an N-terminal peroxisomal targeting signal-2 [PTS2]) and HIUHase 2 (which does not have an N-terminal PTS2), and one TTR family. The tissue distributions of HIUHase 1 and TTR mRNA were similar in juvenile *O. mykiss* and the mRNA levels were highest in the liver. The *O. mykiss* re-HIUHase and re-TTR proteins were both 40–50 kDa homotetramers consisting of 14–15 kDa subunits, with 30% identity. HIUHase had 5-hydroxyisourate (5-HIU) hydrolysis activity with Zn^{2+} sensitivity, whereas TTR had ligand binding activity with a preference for THs and several environmental chemicals, such as halogenated phenols. Our results suggest that *O. mykiss* HIUHase and TTR have diverged from a common ancestral HIHUase with no functional complementation.

Keywords: 5-hydroxyisourate hydrolase, transthyretin, paralogous gene, gene diversity, *Oncorhynchus mykiss*

1. Introduction

Teleosts are the largest and most genetically diverse group of vertebrates. The variety and range of genes in teleosts, which resulted from whole genome duplication, may contribute to their remarkable ability to adapt to various environments. Comparative analyses of chordate genomes indicate that the teleost ancestor experienced two rounds of whole genome duplication before the chondrichthyan lineage diverged 500 to 700 million years (Myr) ago, and a third round of whole genome duplication after the sarcopterygian and basal actinopterygian lineages diverged, but before the teleost lineages diversified, 320 to 400 Myr ago (Meyer and Van de Peer, 2005; Ravi and Venkatesh, 2008). Within the salmonid lineage, an additional whole genome duplication occurred 25 to 100 Myr ago (Ohno 1970; Allendorf and Thorgaard, 1984). Although redundant, the duplicated genomes in teleosts may enhance their genetic diversity through the non-functionalization, sub-functionalization, or neo-functionalization of duplicated genes (i.e. paralogous genes). Because of this, teleosts are ideal organisms to investigate the diversification and functional specialization of paralogous genes after whole genome duplication.

An example of paralogous genes with differently functioning proteins are the genes encoding 5-hydroxyisourate hydrolase (HIUHase), which catalyzes the hydrolysis of 5-hydroxyisourate (5-HIU) in the purine degradation pathway, and transthyretin (TTR), a thyroid hormone (TH) binding protein. The *TTR* gene is thought to be a duplicated copy of an ancestral *HIUHase* gene. This duplicated copy is thought to have appeared at some stage during chordate evolution, particularly given that *HIUHase* homologs, but not *TTR* homologs, have been detected in the genomes of invertebrates (sea urchin: *Strongylocentrotus purparats*, and lancelets: *Branchiostoma japonicum* and *B. floridae*) (Zanotti et al., 2006; Ramazzina et al., 2006). Genes for TTR-related proteins (Eneqvist et al., 2003) have been identified in a wide range of species including bacteria, plants, and animals. Biochemical analysis of the TTR-related protein in *Bacillus subtilis* showed that it was HIUHase (Lee et al., 2005). In addition,

the *HIUHase* and *TTR* genes in vertebrates have a similar exon and intron structure (Zanotti et al., 2006; Ramazzina et al., 2006; Cendron et al., 2011; Li et al, 2013). Interestingly, the conversion of HIUHase to TTR appears to have been experimentally initiated by two point mutations (Y116T and I16A) in zebrafish *Danio rerio* HIUHase (Cendron et al., 2011) and one mutation (Y156T) in *B. japonicum* HIUHase (Li et al, 2013). These changes in the aa sequence resulted in the loss of 5-HIU hydrolysis activity and acquisition of TH binding activity (Cendron et al., 2011). Although these changes in the HIUHase aa sequence were sufficient to alter its function, several other changes to the HIUHase aa sequence would have been necessary to result in TTR. These changes include the loss of the N-terminal peroxosomal targeting signal-2 (PTS2) (Swinkels et al., 1991; Glover et al., 1994) and appearance of the TTR N-terminal signal peptide, aa substitutions that influence TH binding specificity and affinity, and mutations in regulatory regions that determines TTR-specific expression. When these steps in the neo-functionalization of HIUHase to TTR occurred during chordate evolution is not known. In addition, there is a lack of information about the function of HIUHase and TTR in the same species of teleosts.

To understand the phylogenetic and functional relationships of paralogous genes in teleosts, we cloned cDNAs encoding HIUHase and TTR from the liver of the rainbow trout *Oncorhynchus mykiss* and performed bioinformatic analyses of the teleost HIUHase/TTR superfamily. Subsequently, we investigated the tissue distribution of the HIUHase and TTR mRNAs in *O. mikiss* and the 5-HIU hydrolysis activity and TH binding activity of the *O. mikiss* re-HIUHase and re-TTR.

2. Materials and Methods

2.1. Reagents

3,3',5-Triiodo-L-thyronine (T3), L-thyroxine (T4), 3,3',5-triiodo-D-thyronine, 3,3',5'-triiodo-L-thyronine, 3,3',5-triiodo-L-thyroacetic acid, 3,3',5,5'-tetraiodo-L- thyroacetic acid and

pentachlorophenol were obtained from Sigma (St. Louis, MI). Bisphenol A and 2,4,6-triiodophenol were purchased from Wako Pure Chemical Industries (Osaka, Japan), and diethylstilbestrol, 3,3',5,5'-tetrabromobisphenol A and 3,5-diiodo-L-thyronine were from Tokyo Chemical Industry (Tokyo, Japan). Ioxynil (3,5-diiodo-4-hydroxybezonitril, analytical standard) was obtained from Riedel-de Haën (Seeize, Germany). Ni-resins were from Bio-Rad (Ni-IMAC Profinity, Hercules, CA) and Invitrogen (Probond Nickel-Chelating Resin, Carlsbad, CA). Co-resin was obtained from Clontech (Talon Metal Affinity Resin, Mountain View, CA). All other chemicals used in this study were either chromatography grade or the highest grade available and were purchased from Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

All chemicals tested as endocrine disrupting chemicals were dissolved in dimethylsulfoxide to concentrations of 2–5 mM. These chemicals were then diluted with an appropriate buffer to give less than 1.0% (v/v) solvent. A blank assay without proteins with solvent alone at less than 1.0% (v/v) was done for ligand pull-down assay. The solvent did not affect the ligand pull-down assay.

2.2. Cloning of Oncorhynchus mykiss *HIUHase and TTR cDNAs*

Based on the cDNA sequences of *O. mykiss* HIUHase [BX313165] and TTR [CX256523] in the NCBI databases (http://www.ncbi.nlm.nih.gov/), primers were designed to clone the coding regions of the cDNAs to express mature proteins in *Escherichia coli*. Healthy *O. mykiss* were obtained commercially from Kaisaku Co. (Shizuoka, Japan). Total RNA was extracted from the liver of a juvenile fish (6 months old) using QIAamp RNA Blood Mini reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions. Subsequently, single stranded cDNAs were generated using a reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA) and oligo $(dT)_{16}$ primer. The PCR reaction was done using PrimeSTAR HS DNA polymerase (Takara, Otsu, Shiga, Japan) and10 pmoles of following primers, which contained a restriction site (underlined) for

HIUHase: sense 5'-ATTCATATGGCAGCTCCATACAGCCCCCTGACC-3' (142–165) and antisense 5'-GTTAGATCTCTAGCTCCCCCGGTAGGTAGT-3' (495–475); and for TTR: sense 5'-CGACATATGCCAGTGGACAGGCATGGTGAG-3' (108–128) and antisense 5'-GAAGGATCCTGGTCACTCGTGTGCTTTCAT3' (498–480), according to manufacturer's instructions. The ~0.4 kbp amplicons were purified and ligated into the *Eco*RV site of a pBS SK(-) vector. After confirming the nt sequences (accession nos. AB830920 for HIUHase cDNA and AB830919 for TTR cDNA), the insert cDNAs were excised (HIUHase: *Nde*I and *Bgl*II; TTR: *Nde*I and *Bam*HI) and subsequently cloned between the *Nde*I and *Bam*HI sites of a pET3a expression vector (Novagen, Madison, WI). We also constructed HIUHase and TTR cDNAs to express mature proteins with a N-terminal 3× histidine tag (double underlined) using the following sense primers for HIUHase: 5'-GGACATATGCATCACCATGCAGCTCCATACAGCCCCC-3'; and for TTR: 5'-GGACATATGCATCACCATCCAGTGGACAGGCATGGTGAGTC-3'.

2.3. Phylogenetic analysis

Protein and genomic sequences and gene location on chromosome were obtained from the following databases: NCBI (http://www.ncbi.nlm.nih.gov/), Ensembl (http://www.ensembl.org/index.html), ASalBase (http://www.asalbase.org/sal-bin/index) and SalmonDB (http://genomicasalmones.dim.uchile.cl/). 5-Hydroxyisourate hydrolase sequences and gene location were obtained from the following species: purple sea urchin *S. purpuratus* (XP_793771), acorn worm *Saccoglossus kowalevski*i (NM_001168202), Japanese lancelet *B. japonicum* (JX878390), Florida lancelet *B. floridae* (XP_002600822, NW_003101473**)**, Western clawed frog *Xenopus tropicals* (ENSXETG00000019849) and house mouse *Mus musculus* (NM_029821), West Indian Ocean coelacanth *Latimeria chalumnae* 1A and 1B (ENSLACG00000022372, ENSLACG00000022670), *D. rerio* 1 and 2 (EH487643, ENSDARG00000068644; BC090440, ENSDARG00000089331), channel

catfish *Ictalurus punctatus* 1 (JT415054), Atlantic salmon *Salmo salar* 1 and 2 (AGKD01085635, EG904021), *O. mikiss* 1 (BX313165, this study), Atlantic cod *Gadus morhua* 1 (ES471358), medaka *Oryzias latipes* 1 and 2 (DK047918, ENSORLG00000009326; ENSORLG00000000779), three-spined stickleback *Gasterosteus aculeatus* 1 and 2 (ENSGACG00000015936, ENSGACG00000008507), European seabass *Dicentrarchus labrax* 2 (FM000902), gilt-head seabream *Sparus aurata* 1 (AM952201), Japanese pufferfish *Takifugu rubripes*1 and 2 (XP_003970040, XP_003967321), and green spotted pufferfish *Tetraodon nigroviridis* 1 (ENSTNIG00000000957),. Transthyretin sequences and gene location were obtained from the following species: American brook lamprey *Lampetra appendix* (DQ855961), *X. tropicals* (NM_001103069), *M. musculus* (BC086926), *L. chalumnae* (ENSLACP00000023288), *D. rerio* (NM_001005598, ENSDARG00000037191), *I. punctatus* (FD017171), *S. salar* (CK888905), *O. mikiss* (CX256523, this study), *G. morhua* (ES240035, ENSGMOG00000014623), *D. labrax* (CV186278), *S. aurata* (AF047443), *T. rubripes* (XM_003978606, ENSTRUG00000006085), and *T. nigroviridis* (CR652101, ENSTNIG00000010473).

Protein sequence alignment of the HIUHase/TTR superfamily in teleosts was done with two invertebrate (*S. kowalevskii* and *B. japonicum*) sequences using ClustalW ver. 1.83 in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/search/help/clustalwhelp-e.html). The data of aa sequences that may be functional were collected from the cDNA/expressed sequence tag (EST) databases within NCBI. The phylogenetic tree was created using the neighbor joining method (Saitou and Nei, 1987) and the MEGA5.1 program, where the primary sequences deduced from cDNA and genomic DNAs were included with four invertebrate sequences as the out-groups. All positions containing alignment gaps and missing data were eliminated. To verify the presence of two HIUHase subfamilies in teleosts, we examined the location of annotated genes surrounding the *HIUHase* and *TTR* genes in the Ensembl and NCBI databases.

2.4. Real-time PCR

Total RNA was extracted from the liver, kidney, intestine, stomach, heart, eye, gill and brain of one-year-old juvenile *O. mykiss* (males $n = 3$ and females $n = 3$) using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). To confirm its integrity, RNA (50 ng per lane) was electrophoresed in a 1% agarose gel containing 2.6 M formaldehyde, and 28S and 18S rRNA were visualized by ethidium bromide staining in an image analyzer (LAS-4000 miniEPUV, Fujifilm, Tokyo, Japan). The quantity of specific RNA species in each sample was estimated by real-time PCR using SYBR Green Master Mix and ABI Prism 7000 (Applied Biosystems, Foster City, CA) after the RNA samples had been treated with reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems), as previously described (Kudo and Yamauchi, 2005). Each PCR was run in duplicate to control for PCR variation. The thermocycler program included a step of denaturation at 95°C (10 min), and 40 cycles of amplification at 95°C (15 sec), 60°C (1 min), and 50°C (2 min). The endpoint used in real-time PCR quantification, Ct, was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold and is a function of the amount of target DNA present in the starting material. Quantification was determined by applying the 2^{-Ct} formula and calculating the average of the values obtained for each sample. To standardize each experiment, the amount of HIUHase or TTR transcript in each sample was divided by the amount of elongation factor 1α transcript in the same sample. The primer sequences used were as follows: HIUHase transcript (accession number: BX313165) sense 5'-GCTGCCCAGGGCTCATC-3' (nt numbers 290–306) and antisense 5'-CCCGTCTCAAAGCGTATCTTG-3' (336–356); TTR transcript (accession number: CX256523) sense 5'-GACAGGCATGGTGAGTCAGACA-3' (nt numbers 114–135) and antisense 5'-CCCTTCACCGCATCCAAA-3' (161–178); and elongation factor 1α transcript (accession number: NM_001124339) sense 5'-GGATTGCCACACTGCTCACA-3'

(1139–1158) and antisense 5'-GGAACGACGGTCGATCTTCTT-3' (1185–1205).

2.5. Protein expression and purification

Plasmids for expression were transformed into *E. coli* Rosetta 2(DE3)pLysS (Novagen). Bacteria were usually grown in 50 mL LB medium with 50 μg/mL ampicillin and 34 μg/ mL chloramphenicol at 37°C until the absorbance (*A*) at 600 nm reached 0.8. The temperature was lowered to 18°C, 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added, and incubation was continued for 16 h. Bacteria were pelleted by centrifugation (1,200 × *g*) and stored at -85°C until used.

After suspending the bacterial pellet with 5 mL of homogenization buffer (1 mM imidazole, 0.3 M NaCl, 50 mM sodium phosphate, pH 8.0, 1 mM benzamidine hydrochloride, 1 mM PMSF), the cells were disrupted by sonication 20 times for 10 s on ice at the range of 5 (UltraS homogenizer, VP-30S, TAITEC, Koshigaya, Saitama, Japan), and the lysate was centrifuged at 18,000 × *g* for 15 min at 4°C. Histidine-tagged recombinant proteins were isolated from the other proteins in the supernatant by nickel affinity chromatography (0.5 mL of the resin), by stepwise elution with various concentrations of imidazole (5, 20, 60, 150, and 250 mM) in 0.3 M NaCl, 50 mM sodium phosphate, pH 8.0. Peak fractions were immediately applied to a Cellulofine GCL-2000 sf column (1.5 × 93 cm, Seikagaku Co., Tokyo, Japan), which had been equilibrated with 20 mM Tris, pH 7.5. The column was calibrated with the following standards: γ-globulin (150 kDa), BSA (66 kDa), ovalbumin (45 kDa), and myoglobin (17 kDa).

For purification of non-tagged recombinant proteins, the cell lysate was fractionated by ammonium sulfate precipitation. Peak fractions (in 25–35% of saturated ammonium sulfate) were re-suspended in 10 mM sodium phosphate, pH 7.5, containing 0.5 M ammonium sulfate, and applied to a Phenyl-Cellulofine column (1.0 × 10 cm, Seikagaku Co.), which had been equilibrated with the same buffer. Elution was conducted under linear gradient conditions (from 10 mM sodium phosphate,

pH 7.5, containing 0.5 M ammonium sulfate to 10 mM sodium phosphate, pH 7.5, containing 50% ethylene glycol) using FPLC (GE Healthcare, Buckinghamshire, England). The proteins in the peak fraction were further fractionated by gel filtration chromatography on a Cellulofine GCL-2000 sf column, as described above. The yields of the recombinant proteins were 1.2–1.4 mg/50 mL bacterial culture.

2.6. Enzyme assay

Enzyme activity was measured at 25–27°C by following the decrease in *A* at 312 nm as 5-HIU was hydrolyzed (Lee et al., 2005). 5-Hydroxyisourate was generated *in situ* by the addition of 50 μL of 0.32 μM uricase (Wako, Tokyo, Japan) in 50 mM potassium phosphate buffer, pH 7.5, to 400 μL of 100 μM uric acid in the same buffer. When the amount of 5-HIU reached a maximum (3–4 min after starting the reaction), 10 μL of HIUHase (0.12 or 0.24 μM), TTR (0.12 or 0.24 μM) or the buffer alone was added to the mixture in a microcuvette. Absorbance at 312 nm was immediately monitored at 0.2 min intervals using a spectrophotometer (U-3210, Hitachi, Japan). As 5-HIU is not stable, the enzyme activity was expressed as the difference in *A* at 312 nm between the absence (spontaneous hydrolysis of 5-HIU) and the presence of proteins (spontaneous + enzymatic hydrolysis of 5-HIU). As TTR is known to have Zn^{2+} binding sites (Wilkinson-White and Easterbrook-Smith, 2007; Palmieri et al., 2010), the effects of metal ions on the 5-HIU hydrolysis activity of the HIUHase were also investigated.

2.7. Ligand pull-down assay using a metal chelate affinity resin

Histidine-tagged proteins (0.5 μM) were incubated with ligands (0.5 μM) in 500 μL of Tris-buffered saline, pH 7.5, for 1 h at 4°C, and the protein-ligand solution was further incubated with Co-resin (5 μL bed volume) in a 600-μL plastic tube using a rotator. After a brief centrifugation (800 × *g* for

1 min at 4°C), the protein-ligand complex bound to the resin in the pellet was recovered by the addition of 20 μL of elution buffer (500 mM imidazole, 0.3 M NaCl, 50 mM sodium phosphate, pH 8.0). The protein-ligand complex released from the resin was mixed with the same volume of 1% acetic acid in methanol to extract the ligand from the complex. The mixture was agitated then centrifuged at 12,000 × *g* for 10 min at 4°C. The ligand (15 μL) in the supernatant was analyzed by reverse phase- HPLC on a C_{18} analytical column (Mightysil RP-18 GP, 250 mm × 4.6 mm, 5 mm particle diameter, Kanto Chemical, Tokyo, Japan) at a flow rate of 1.0 mL/min at 40°C, under isocratic mobile phase (methanol:distilled water:acetic acid, 55:44:1) or gradient mobile phase conditions (from methanol:distilled water:acetic acid, 55:44:1, to methanol:acetic acid, 99:1). The HPLC system consisted of a pump and controller (model 600, Waters; Milford, MA), and a detector (Dual λ absorbance detector model 2487, Waters). Chromatographic elution was monitored at 254 nm. The amount of the ligand was quantified by comparison with the standards (4–100 pmoles).

2.8. Protein analyses

Protein concentration was measured by the micro-Lowry method (Jain et al., 2002) with BSA as the standard. Proteins was electrophoresed on an SDS-PA gel (15%) (Laemmli, 1970) with molecular markers: phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The gel was stained and proteins were visualized with Coomassie Brilliant Blue.

2.9. Statistics

The data are the mean ± standard error of the mean ($n \geq 3$), unless otherwise noted. Differences between two groups were analyzed by the Student's t-test. Differences between groups were analyzed by a one-way or a two-way analysis of variance (ANOVA), with the Fisher's

least-significant-difference test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Evolutionary analysis of HIUHase and TTR in teleosts

3.1.1. Phylogenetic analysis of HIUHase and TTR aa sequences

The cloned *O. mikiss* HIUHase and TTR aa sequences shared more than 65% and more than 58% aa identities with the other teleost HIUHase ($n = 10$) and TTR ($n = 8$) aa sequences, respectively (Fig. 1A). Thirty five residues were invariant among 11 HIUHase sequences whereas 57 residues were invariant among 9 TTR sequences. Thirteen aa residues (marked by * in Fig. 1A) were identical across the aligned HIUHase and TTR aa sequences. All invertebrate and teleost HIUHase sequences analyzed had a signature tetrapeptide (YRGS) at the C-terminal end (Eneqvist et al., 2003; Hennebry et al., 2006; Ramazzina et al., 2006) (Fig. 1A). All except three teleost HIUHase sequences and the *S. kowalevskii* HIUHase possessed a PTS2 (Swinkels et al., 1991; Glover et al., 1994) or PTS2-like signal at the N-terminal region (Fig. 1A).

Phylogenetic analysis of the teleost HIUHase and TTR aa sequences indicated that there are two HIUHase subfamilies (HIUHases 1 and 2) and one TTR family (Fig 1B). Of the three clades of the phylogenetic tree, the HIUHase 1 subfamily had a somewhat obscure tree topology due to the relative low bootstrap values. The *O. mikiss* HIUHase was assigned to the HIUHase 1 subfamily. For the two HIUHase subfamilies, analysis of the aligned HIUHase aa sequences showed that the percentage of aa identities shared between the HIUHase 1 and HIUHase 2 subfamilies was 56–71% (Fig 1A). The TTR family shared slightly more aa identities with the HIUHase 1 subfamily (24–37%) than with the HIUHase 2 subfamily (22–32%). The invertebrate HIUHases shared more aa identities

with the HIUHase 1 subfamily (46–57%) than with the HIUHase 2 subfamily (44–50%) or the TTR family (26–35%).

3.1.2. Synteny analysis of HIUHase genes

Our synteny analysis supports the presence of at least two HIUHase subfamilies in teleosts (Fig. 1C). There was a degree of shared synteny around the *HIUHase 1* gene in four teleosts and around the *HIUHase 2* gene in four teleosts. However, the synteny around the *HIUHase 1* gene and the *HIUHase 2* gene differed (Fig. 1C). The syntenies around the invertebrate (*S. purpuratus* and *B. floridae*) *HIUHase* genes were distinct from the synteny around the teleost *HIUHase 1* gene and the teleost *HIUHase 2* gene, whereas the synteny around the *L. chalumnae HIUHase 1A* gene had some similarity with that around the teleost *HIUHase 1* gene. The *L. chalumnae*, *D. rerio*, *O. latipes*, and *G. aculeatus HIUHase 1* genes were closely linked to the growth arrest-specific 8 (*GAS8*) gene, whereas the *D. rerio, O. latipes*, *G. aculeatus*, and *T. rubripes HIUHase 2* genes were closely linked to the ring finger and WD repeated domain 3 (*RFWD3*) gene. The synteny around the teleost *TTR* genes was somewhat similar with that around the *L. chalumnae TTR* gene, but was distinct to those around the teleost *HIUHase 1* genes and *HIUHase 2* genes (data not shown).

3.2. Characterization of O. mikiss *HIUHase and TTR mRNAs and proteins*

3.2.1. Tissue distribution of HIUHase and TTR mRNA

Both HIUHase and TTR mRNAs were abundant in the liver (Fig. 2). Although TTR mRNA levels were higher than HIUHase mRNA levels in the liver ($P < 0.01$; the Student's t-test), a two-way ANOVA showed no significant differences between HIUHase and TTR mRNA levels in the eight tissues ($P = 0.1928$). The tissue distribution pattern of both transcripts was similar: liver > kidney ~ intestine ~ brain ~ gill > eye ~ stomach ~ heart ($P < 0.01$; Fisher's least-significant-difference test).

There were no significant differences in mRNA levels in the eight tissues between males (n = 3) and females (n = 3) (P = 0.2344 for HIUHase transcripts and P = 0.1811 for TTR transcripts; two-way ANOVA).

3.2.2. Molecular properties of re-HIUHase and re-TTR proteins

Re-HIUHase and re-TTR could be expressed as soluble forms in *E. coli.* The molecular mass under native conditions was estimated to be ~40 kDa for HIUHase (Fig. 3A) and ~50 kDa for TTR by gel filtration column chromatography (Fig. 3B). The molecular masses for HIUHase (14 kDa) and TTR (15 kDa) polypeptides on SDS-PAGE were in agreement with those predicted from the HIUHase and TTR cDNAs.

3.3. Functional analyses of O. mikiss *re-HIUHase and re-TTR*

3.3.1. 5-HIU hydrolysis activity of HIUHase

Hydrolysis of 5-HIU by HIUHase was detected at HIUHase concentrations of 0.06–0.30 μM (Fig.4A). Activity peaked at 2.4–4.0 min and decreased thereafter. Transthyretin (0.12 μM) did not hydrolyze 5-HIU (Fig. 4B). Hydrolysis of 5-HIU by HIUHase was significantly inhibited by Zn^{2+} (50 μM), but not by Cr^{3+}, Mg^{2+}, or Ca^{2+} (300 or 1000 μM for all elements) (Figs 4C to 4F).

3.3.2. TH binding activity of TTR

3,3',5-Triiodo-L-thyronine bound to TTR then extracted with methanol was eluted at the same retention time (11.46 min) as the T3 standard (Fig. 5A). The peak of T3 was negligible when the same ligand pull-down assay was done without TTR (blank). Binding activity for T3 was detected in a TTR-dependent manner (Fig. 5B). However, HIUHase, at the same concentrations (0.05-0.5 μM) as TTR, showed no T3-binding activity. Transthyretin exhibited high binding activity for natural ligands

or their analogs, with the following order of affinity: T3 ~ 3,3',5-triiodo-L-thyroacetic acid ~ 3,3',5-triiodo-D-thyronine > T4 ~ 3,3',5,5'-tetraiodo-L- thyroacetic acid ~ 3,3',5'-triiodo-L-thyronine ≥ 3,5-diiodo-L-thyronine. With regards to environmental chemicals, TTR showed binding activity for triiodophenol, pentachlorophenol, diethylstilbestrol, and ioxynil, and the binding activity for triiodophenol was comparable with that for T3 and for T4. The binding of bisphenol A and tetrabromobisphenol A was negligible.

4. Discussion

The findings from our study of teleosts suggest the sub-functionalization and neo-functionalization of duplicated *HIUHase* genes that resulted from whole genome duplication. Using phylogenetic analysis, we detected two HIUHase subfamilies (HIUHase 1 and 2) and one TTR family in the teleost sequences analyzed. The presence of the two HIUHase subfamilies in teleosts was supported by the shared synteny around the *HIUHase 1* gene from four teleost species and around the *HIUHase 2* gene from four teleost species. These findings suggest the divergence of HIUHase 2 from HIUHase 1 (e.g., sub-functionalization) at some point during teleost evolution. The appearance of TTR may be the result of the neo-functionalization of HIUHase. The tissue-specific pattern of *O. mikiss* HIUHase and TTR mRNA was similar. In addition, the *O. mikiss* re-HIUHase (a member of the HIUHase 1 subfamily) and re-TTR proteins showed similarities in molecular sizes and subunit structure, but were shown to have distinct functions with no functional complementation.

4.1. Evolution of teleost HIUHase/TTR superfamily

We identified a HIUHase/TTR superfamily in teleosts. The presence of two HIUHase subfamilies was supported by the following findings: (1) a high percent identity among primary aa sequences within each subfamily, which resulted in two distinct clades in our phylogenetic tree; (2) an N-terminal PTS2

in HIUHase 1, but not HIUHase 2; and (3) distinct syntenies around the *HIUHase 1* gene and the *HIUHase 2* gene. As the synteny around the teleost *HIUHase 1* gene, but not the teleost *HIUHase 2* gene, was similar to the synteny around the *L. chalumnae HIUHase 1A* gene, the HIUHase 2 subfamily might have arisen from a teleost-specific third round whole genome duplication event. As salmonids experienced a fourth round of whole genome duplication (Ohno, 1970; Allendorf and Thorgaard, 1984), it was expected that *O. mikiss* has two paralogous genes encoding TTR, HIUHase 1 and HIUHase 2. However, the populations of *O. mikiss* HIUHase and TTR cDNAs collected from the NIBI and SalmonDB databases did not reveal significant nt divergence showing the presence of the paralogous genes resulting from the salmonid-specific whole genome duplication.

4.1.1. Non-functionalization, sub-functionalization and neo-functionalization of duplicated genes in the teleost HIUHase/TTR superfamily

The distribution of the members of the *HIUHase/TTR* gene superfamily in teleosts is widespread (Fig. 6). Some of the nt sequences were collected from cDNA/EST databases and may be functional, whereas others were collected from genome databases and may, or may not, be functional because their expression has not been determined. Interestingly, the distribution of HIUHase family among the teleost species analyzed was not related to their phylogenetic relationships (see Fig. 6), suggesting that the random inactivation or loss of the *HIUHase 1* and/or *2* gene may have occurred in a species-dependent manner. We also found several *HIUHase* genes that may be progressing toward inactivation. This is because aa substitutions or deletions were detected at the C-terminal signature sequence YRGS in the HIUHase 2 sequences predicted from the *O. latipes*, *G. aculeatus* and *T. rubripes* genes.

The presence or absence of PTS2 in HIUHase aa sequences and the presence of a signal peptide in TTR determine their subcellular and extracellular localization, respectively. The acquisition

of a different signal sequence or the loss of PTS2 might have precipitated the evolution of HIUHase to TTR (neo-functionalization) or HIUHase 2 (sub-functionalization), depending on the prevailing selection pressures. Differing selection pressures between the duplicated genes would also affect their expression pattern. Most of the teleost species investigated expressed both HIUHase mRNA (either HIUHase 1 or HIUHase 2) and TTR mRNA. This expression pattern suggests TTR functions regardless of the expression of HIUHase. Tranthyretin in *S. aurata* (Santos and Power, 1999; Funkenstein et al., 1999) and *O. mikiss* (this study) has been shown to be biochemically functional, and these species also express HIUHase 1. In contrast, HIUHases 1 and 2 may have a similar function but different subcellular localization, resulting in sub-functionalization. All aa residues of HIUHase that are potentially relevant for catalysis (52H, 91D, 93R, 146H and 162S; residue numbering of Fig. 1A) (Zanotti et al., 2006) are well conserved between the teleost HIUHases 1 and 2. However, there are several residues that are conserved in the teleost HIUHase 1 sequences only (85T and 101E) and HIUHase 2 sequences only (D58, R64, 85V, 107M and 144R). *Danio rerio* (Cendron et al., 2011) and *O. mikiss* (this study) HIUHase 1 proteins have been shown to be biochemically functional. Mouse *Mus musculus HIUHase* gene (*Urah*) transcribed two alternative mRNAs, with and without part of exon 1 that contains a PTS2-coding region (Stevenson et al., 2010). Their translated products were localized predominantly in the cytoplasm of the hepatocytes. To explain this cellular distribution of the HIUHases independent of PTS2, the authors postulated the presence of a regulatory mechanism by which the *M. musculus* HIHUase with PTS2 is translocated into peroxisome under some conditions. Whether the teleost HIUHases 1 and 2 have distinct enzymatic properties and whether they have distinct subcellular localizations remains to be elucidated.

4.1.2. Evolutionary story of the teleost HIUHase/TTR superfamily

Evolutionarily, the expression of TTR is restricted to vertebrates including the agnatha lampreys

(Manzon et al., 2007), whereas the expression of HIUHase is broad: from eubacteria to eukaryotes including protista, fungi, plants, and animals (Eneqvist et al., 2003; Hennebry et al., 2006). Genome and EST projects in deuterostomes other than vertebrates have identified HIUHase transcripts in *S. kowalevskii and B. japonicum,* and *HIUHase* genes in *S. purpuratus and B. floridae*, but neither TTR transcripts nor genes (Fig. 6). This distribution of the HIUHase/TTR superfamily, the results of phylogenetic analysis from previous (Zanotti et al., 2006; Hennebry et al., 2006) and our studies, the synteny analysis of the HIUHase/TTR gene superfamily, and the same intron and exon structures within this superfamily (Ramazzina et al., 2006; Li et al., 2013) suggest that *TTR* gene may have originated from an ancestral *HIUHase* gene by gene duplication during early chordate evolution or after the separation of the earliest deuterostomes about 500 Myr ago, sometime during round 1 and 2 of whole genome duplication.

The TTR tree (Fig. 1B) corresponded well to the teleost species tree published elsewhere (Santini et al., 2009). However, such a clear relationship was not obtained in the HIUHase tree with low bootstrap values. Although we made the phylogetic trees of the HIUHase/TTR superfamily using the maximum-parsimony, maximum likelihood and minimum-evolution methods, besides the neighbor-joining method. The four methods gave the same tree topology in the TTR tree, but not in the HIUHase three even in the nodes with >50% bootstrap values. The neighbor-joining HIUHase three, which showed the same topology as the minimum-evolution HIUHase tree, corresponded closely to the teleost species tree, compared with the maximum-parsimony and maximum likelihood HIUHase trees (data not shown).

Our phylogenetic tree of HIUHase and TTR has raised the question of why the TTR lineage is farther away from the HIUHase lineage. One possible explanation is that the rate of evolution in the TTR lineage was more rapid than that in the HIUHase lineage after the TTR ancestor separated from the HIUHase lineage. Asymmetric evolutionary rates between duplicated gene pairs have been

reported for other teleost genes (Brunet et al., 2006; Steinke et al., 2006; Douard et al., 2008). The acquisition of a signal peptide for secretion early in the TTR lineage would have been a prominent evolutionary driving force toward neo-functionalization, a likely scenario in the teleost TTR family. Alternatively, the TTR ancestor may have diverged from the ancestral HIUHase by a local gene duplication event earlier than expected, e.g., before round 1 of whole genome duplication. Our synteny analysis around *HIUHase* genes did not assist with establishing the origin of the *TTR* gene. More bioinformatic data about the *HIUHase/TTR* gene superfamily in invertebrates and primitive vertebrates may help answer this question.

4.2. Characterization of O. mikiss *HIUHase and TTR gene expression and their translational products*

4.2.1. Common features in transcript levels and proteins of O. mikiss HIUHase and TTR

We detected a similar tissue-dependent expression pattern between *HIUHase* and *TTR* genes, with predominant expression in the liver. This similar expression pattern may reflect the expression of a common ancestral gene. The predominant expression in the liver was found in *M. musculus* HIUHase (Stevenson et al., 2010) and TTRs from some teleosts (Santos and Power, 1999; Funkenstein et al., 1999; Kawakami et al., 2006) and other vertebrates (Schreiber and Richardson, 1997; Richardson, 2007). However, TTR transcripts were one order of magnitude more abundant than HIUHase transcripts in the liver of *O. mikiss*. The neo-functionalization of HIUHase from HIUHase functioning within a restricted space in liver cells to TTR circulating and functioning in extracellular fluid might have influenced and enhanced the level of TTR transcription in the liver.

Recombinant *O. mikiss* HIUHase and TTR were 40–50 kDa homotetramer proteins consisting of 14–15 kDa polypeptides. However, the molecular masses of the native proteins were somewhat smaller than those of the expected tetramers. Therefore, both *O. mikiss* HIUHase and TTR proteins are likely to be compact homotetramers. Similar compactness was found in the *Salmonella*

dublin HIUHase (Hennebry et al., 2006). A possible common feature of HIUHase and TTR may be Zn^{2+} binding. Human TTR has three Zn^{2+} binding sites per monomer and a fourth Zn^{2+} binding site when the tetramer forms (Palmieri et al., 2010). We found that Zn^{2+} (50 μM) inhibited *O. mikiss* HIUHase activity. The protein structure of *E. coli* HIUHase demonstrated the presence of zinc ions bound to the functional sites (Lundberg et al., 2006). Some zinc ions are coordinated to the side chains of residues at the putative ligand binding site. These findings suggest that Zn^{2+} may bind to the HIUHase catalytic site, blocking enzyme activity, or may bind elsewhere on HIUHase, causing a conformational change that indirectly affects the HIUHase catalytic site.

4.2.2. Physiology of ligand-binding to O. mikiss TTR

Oncorhynchus mikiss TTR may play an important role in the delivery of TH to tissues during smoltification of juvenile anadromous salmonids. During smoltification, which precedes migration from freshwater streams to the sea, plasma TH concentrations increase (Young et al., 1989; Dickhoff et al., 1990). Smoltification is analogous to amphibian metamorphosis, in which plasma TH levels reach a peak and THs act as a trigger of metamorphosis. In our study, *O. mikiss* TTR may act as a T3/T4 binder in plasma from juvenile, with slightly higher affinity for T3 than for T4. Similar TH binding specificity of TTR was detected in the allied species *O. masuo* (Yamauchi et al., 1999) and other teleosts such as the Pacific bluefin tuna *Thunnus orientalis* (Kawakami et al., 2006) and *S. aurata* (Santos and Power, 1999).

Salmon and trout are sentinel species for monitoring the aquatic environment. Nevertheless, little is known about the non-natural ligands of teleost TTRs, including agricultural, industrial, and pharmaceutical chemicals, which may displace THs from TTR and act as endocrine disruptors. Findings from our study were similar to those from a study of *O. masuo* (Ishihara et al., 2003) and suggested that *O. mikiss* TTR has an affinity for diethylstilbestrol, pentachlorophenol, triiodophenol

and ioxynil, which was comparable with that for TH, but not bisphenol A and tetrabromobisphenol A. Thus, these chemicals may interfere with T3 binding to *O. mikiss* TTR in plasma *in vivo*. However, the binding specificity of TTR for phenolic compounds may vary among teleosts (Morgado et al., 2007).

In conclusion, findings from our study suggest the presence of at least two HIUHase subfamilies and one TTR family in teleosts. Both *O. mikiss* HIUHase and TTR proteins were compact homotetramers consisting of 14–15 kDa polypeptides, with 30% identity at the aa level. *Oncorhynchus mikiss* HIUHase and TTR exhibited 5-HIU hydrolysis and TH binding activities, respectively, but not functional complementation each other, in agreement with the concept that paralogous genes persisted in the genome have usually distinct function (Ohno, 1970).

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Figure legends

Fig. 1. Comparison of 5-hydroxyisourate hydrolase (*HIUHase*) and transthyretin (*TTR*) genes and aa sequecnes in teleosts. (*A*) Alignment of HIUHases and TTRs aa sequences. All TTRs and HIUHase aa sequences were from nt sequences of cDNAs that may be functional. Nine teleost HIUHase sequences, and two invertebrate (hemichordate *Saccoglossus kowalevskii* and protochordate *Branchiostoma japonicum*) HIUHase sequences, have the HIUHase-specific tetrapeptide, YRGS (boxed), at the C-terminal region. Teleost HIUHase 1 and the two invertebrate sequences have a peroxisomal targeting signal 2 (PTS2) (Swinkels et al., 1991; Glover et al., 1994) or PTS2-like signals at N-terminal region (boxed), whereas teleost HIUHase 2 does not. Marks underneath the sequence alignment indicate positions where aa residues are invariant among the 20 HIUHase and TTR sequences (*), among the 11 HIUHase sequences (+), and among the 9 TTR sequences (.), and where the aa residues invariant in the 11 HIUHase sequences are distinct from those that are invariant in the 9 TTR sequences (:) (*B*) Phylogenetic tree of fish HIUHases and TTRs. The unrooted tree was constructed with the neighbor joining method using MEGA 5.1 from 29 fish and 4 tetrapod sequences with 4 invertebrate sequences as an outer group. Node values represent the percent bootstrap confidence derived from 1,000 replicates. Bootstrap values lower than 50% are not shown. Data from genomic sequences are marked by asterisks (*). (*C*) Conserved synteny with the chromosomal location of fish duplicated *HIUHase* genes and corresponding genes in some invertebrates. The position of *HIUHase* genes is boxed. Genetic loci linked with the *HIUHase 1* and *2* genes are marked in blue and red, respectively. Only loci that are annotated are shown.

Fig. 2. 5-Hydroxyisourate hydrolase (HIUHase) and transthyretin (TTR) mRNA expression in eight different tissues of *Oncorhynchus mykiss.* Total RNA was prepared from tissues of one-year-old juvenile males (n = 3) and females (*n* = 3) *O. mykiss* then reverse-transcribed using random primers

and amplified using real-time PCR. The expression levels for HIUHase and TTR are shown as arbitrary units that were normalized against the expression level of elongation factor 1α. Each value is the mean ± standard error of the mean. As there were no significant differences in the expression levels between male ($n = 3$) and female tissues ($n = 3$), the data are shown as $n = 6$. Difference letters denote means that are significantly different ($P < 0.05$, two-way ANOVA with Fisher's least-significant-difference-test for multiple comparisons). **, $P < 0.05$ (the Student's t-test).

Fig. 3. Gel chromatography of recombinant *Oncorhynchus mykiss* 5-hydroxyisourate hydrolase (HIUHase) and transthyretin (TTR) on a Cellulofine GCL-2000 sf column. (*A*) Recombinant HIUHase was purified from the bacterial extract by ammonium sulfate precipitation, hydrophobic interaction chromatography on Phenyl-Cellulofine, and then by gel filtration chromatography on Cellulofine GCL-2000 sf. (*B*) Histidine-tagged TTR was purified by metal chelate affinity chromatography on a Ni-resin (Ni-IMAC Profinity) and gel filtration chromatography on Cellulofine GCL-2000 sf (γ-globulin. 150 kDa; BSA, 68 kDa; ovalbumin, 45 kDa; and myoglobin, 17 kDa). *Insets*, SDS-PAGE under reduced conditions of the peak fraction of each gel chromatography.

Fig. 4. Time course of enzyme-dependent 5-hydroxyisourate (5-HIU) hydrolysis by (*A*) recombinant *Oncorhynchus mykiss* 5-hydroxyisourate hydrolase (re-HIUHase), (*B*) recombinant *O. mykiss* transthyretin (re-TTR) (0.12 μM), and *O. mykiss* re-HIUHase in the presence of (*C*) Zn^{2+}, (*D*) Cr^{3+}, (*E*) Mg^{2+}, and (*F*) Ca^{2+}. The hydrolysis of 5-HIU was monitored spectrophotometrically at 312 nm as described in the Materials and Methods. The vertical axis represents the difference in *A* at 312 nm (ΔA_{312}) between the samples in the absence (spontaneous degradation of 5-HIU) and the presence of re-HIUHase (spontaneous + enzymatic degradation of 5-HIU). These experiments were repeated at least three times, with similar results.

Fig. 5. Ligand-binding to histidine-tagged recombinant *Oncorhynchus mykiss* transthyretin (TTR). (*A*) Ligand pull-down assay using metal chelate affinity resin. After incubating TTR (0.5 μM) with ligand (0.5μM) at 4°C, the TTR-ligand solution was further incubated with Co-resin (5 μL of bed volume). The TTR-ligand complex bound to the Co-resin was recovered in the supernatant by the addition of 500 mM imidazole, and the ligand was then analyzed by reverse phase-HPLC as described in the Materials and methods. The amount of the ligand was quantified by comparison with the standard (15 pmoles 3,3',5-triiodo-L-thyronine [T3]). Blank data were obtained from samples in the absence of TTR. (*B*) TTR-dependent T3 binding activity. Binding activity for T3 was measured in the presence of TTR or 5-hydroxyisourate hydrolase (HIUHase) (0.05 to 0.5 μM) by the ligand pull-down assay. (*C*): Binding specificity of thyroid hormones (THs) or their analogs to TTR. Ligands used were T3, L-thyroxine (T4), 3,3',5-triiodo-L-thyroacetic acid (Triac), 3,3',5,5'-tetraiodo-L- thyroacetic acid (Tetrac), 3,3',5'-triiodo-L-thyronine (rT3), 3,5-diiodo-L-thyronine (T2) or 3,3',5-triiodo-D-thyronine (D-T3), (each 0.5 μM). (*D*): Binding of environmental chemicals to TTR. Ligands used were bisphenol A (BPA), tetrabromobisphenol A (BR4BPA), diethylstilbestrol (DES), pentachlorophenol (PCP), triiodophenol (TIP), or ioxynil (each 0.5μM). Each point represents the mean ± standard error of the mean (n = 6–9). Different letters indicate significance between two groups ($P < 0.05$, one-way ANOVA with Fisher's least-significant-difference-test for multiple comparisons).

Fig. 6. Distribution and evolution of 5-hydroxyisourate hydrolase/transthyretin (HIUHase/TTR) superfamily in fish and some invertebrates. Distribution of the HIUHase/TTR superfamily is overlaid on an accepted phylogenetic tree of fish (Santini et al., 2009), protochordate (*Branchiostoma floridae*), hemichordate (*Saccoglossus kowalevskii*) and echinoderm (*Strongylocentrotus purpuratus*) (Ravi and Venkatesh, 2008; Santini et al., 2009), suggesting that this superfamily arose by repeated gene

duplication events at early stage of chordate evolution or during deuterostome evolution. Filled stars, three rounds of whole genome duplication (1R to 3R) and salmonid-specific whole genome duplication; *, species where synteny analysis was conducted; ○, sequences sourced from cDNA/EST databases; □, sequences sourced from an EST database, but is un-processed (may be non-functional); ●, sequences found in genome databases alone; ‡, HIUHase with a peroxisomal targeting signal 2 (PTS2); #, the presence of two genes for HIUHase 1 (HIUHase 1A has PTS2 but HIUHase 1B has not); -, not detected in available genomic databases; ?, unclear whether this family is present or absent because of limited cDNA/EST data.

Fig. 1A

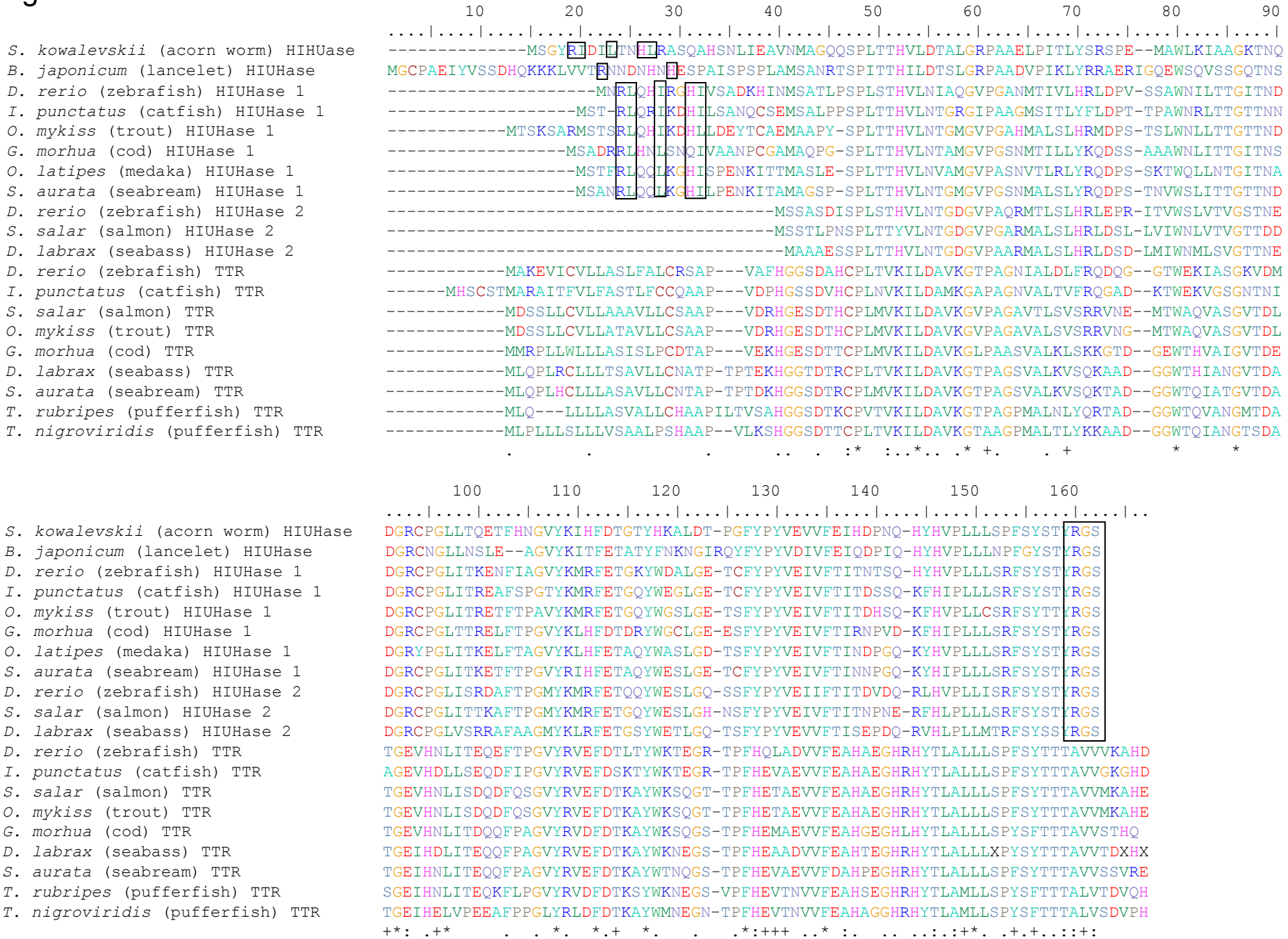

10 20 30 40 50 60 70 80 90
S. kowalevskii (acorn worm) HIHUase
B. japonicum (lancelet) HIUHase
D. rerio (zebrafish) HIUHase 1
I. punctatus (catfish) HIUHase 1
O. mykiss (trout) HIUHase 1
G. morhua (cod) HIUHase 1
O. latipes (medaka) HIUHase 1
S. aurata (seabream) HIUHase 1
D. rerio (zebrafish) HIUHase 2
S. salar (salmon) HIUHase 2
D. labrax (seabass) HIUHase 2
D. rerio (zebrafish) TTR
I. punctatus (catfish) TTR
S. salar (salmon) TTR
O. mykiss (trout) TTR
G. morhua (cod) TTR
D. labrax (seabass) TTR
S. aurata (seabream) TTR
T. rubripes (pufferfish) TTR
T. nigroviridis (pufferfish) TTR
100 110 120 130 140 150 160
S. kowalevskii (acorn worm) HIUHase
B. japonicum (lancelet) HIUHase
D. rerio (zebrafish) HIUHase 1
I. punctatus (catfish) HIUHase 1
O. mykiss (trout) HIUHase 1
G. morhua (cod) HIUHase 1
O. latipes (medaka) HIUHase 1
S. aurata (seabream) HIUHase 1
D. rerio (zebrafish) HIUHase 2
S. salar (salmon) HIUHase 2
D. labrax (seabass) HIUHase 2
D. rerio (zebrafish) TTR
I. punctatus (catfish) TTR
S. salar (salmon) TTR
O. mykiss (trout) TTR
G. morhua (cod) TTR
D. labrax (seabass) TTR
S. aurata (seabream) TTR
T. rubripes (pufferfish) TTR
T. nigroviridis (pufferfish) TTR

Fig. 1B

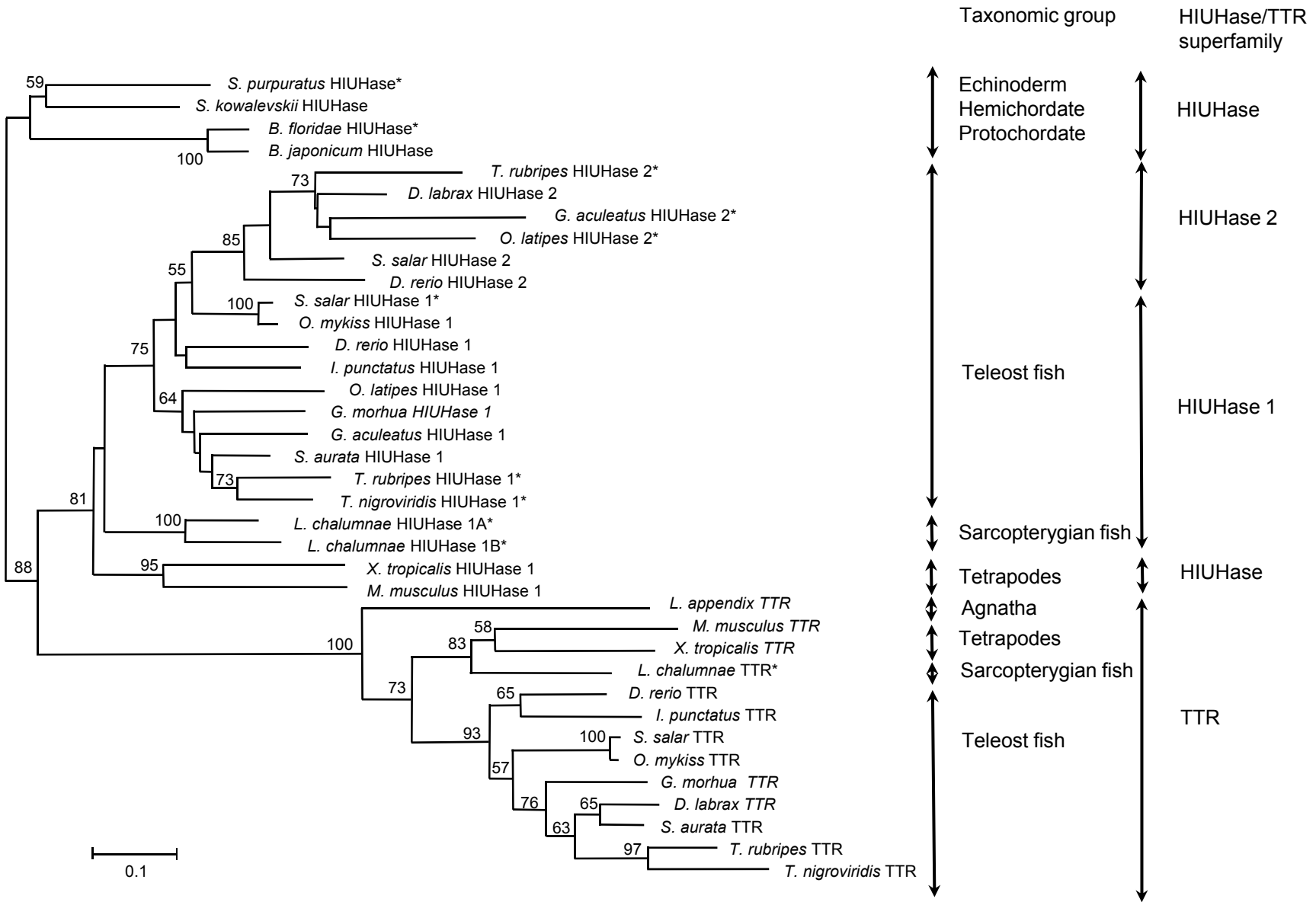

Taxonomic group
HIUHase/TTR superfamily
S. purpuratus HIUHase*
S. kowalevskii HIUHase
B. floridae HIUHase*
B. japonicum HIUHase
T. rubripes HIUHase 2*
D. labrax HIUHase 2
G. aculeatus HIUHase 2*
O. latipes HIUHase 2*
S. salar HIUHase 2
D. rerio HIUHase 2
S. salar HIUHase 1*
O. mykiss HIUHase 1
D. rerio HIUHase 1
I. punctatus HIUHase 1
O. latipes HIUHase 1
G. morhua HIUHase 1
G. aculeatus HIUHase 1
S. aurata HIUHase 1
T. rubripes HIUHase 1*
T. nigroviridis HIUHase 1*
L. chalumnae HIUHase 1A*
L. chalumnae HIUHase 1B*
X. tropicalis HIUHase 1
M. musculus HIUHase 1
L. appendix TTR
M. musculus TTR
X. tropicalis TTR
L. chalumnae TTR*
D. rerio TTR
I. punctatus TTR
S. salar TTR
O. mykiss TTR
G. morhua TTR
D. labrax TTR
S. aurata TTR
T. rubripes TTR
T. nigroviridis TTR
0.1
Echinoderm
Hemichordate
Protochordate
HIUHase
HIUHase 2
Teleost fish
HIUHase 1
Sarcopterygian fish
Tetrapodes
HIUHase
Agnatha
Tetrapodes
Sarcopterygian fish
Teleost fish
TTR

Fig. 1C

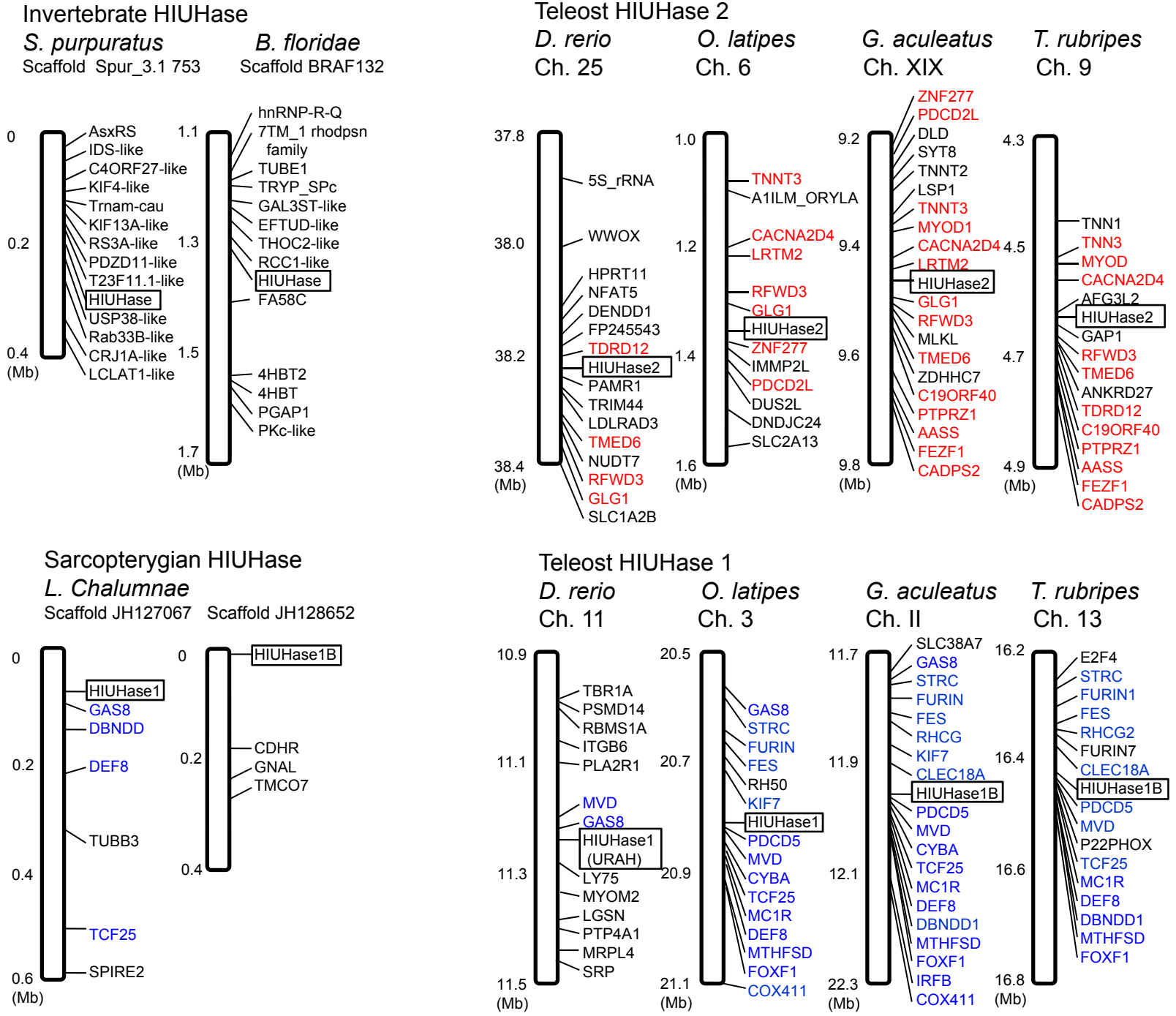

Invertebrate HIUHase
S. purpuratus
Scaffold Spur_3.1 753
B. floridae
Scaffold BRAF132
0
0.2
0.4
(Mb)
AsxRS
IDS-like
C4ORF27-like
KIF4-like
Trnam-cau
KIF13A-like
RS3A-like
PDZD11-like
T23F11.1-like
HIUHase
USP38-like
Rab33B-like
CRJ1A-like
LCLAT1-like
1.1
1.3
1.5
1.7
(Mb)
hnRNP-R-Q
7TM_1 rhodpsn family
TUBE1
TRYP_SPc
GAL3ST-like
EFTUD-like
THOC2-like
RCC1-like
HIUHase
FA58C
4HBT2
4HBT
PGAP1
PKc-like
Teleost HIUHase 2
D. rerio
Ch. 25
O. latipes
Ch. 6
G. aculeatus
Ch. XIX
T. rubripes
Ch. 9
37.8
38.0
38.2
38.4
(Mb)
5S_rRNA
WWOX
HPRT11
NFAT5
DENDD1
FP245543
TDRD12
HIUHase2
PAMR1
TRIM44
LDLRAD3
TMED6
NUDT7
RFWD3
GLG1
SLC1A2B
1.0
1.2
1.4
1.6
(Mb)
TNNT3
A1ILM_ORYLA
CACNA2D4
LRTM2
RFWD3
GLG1
HIUHase2
ZNF277
IMMP2L
PDCD2L
DUS2L
DNDJC24
SLC2A13
9.2
9.4
9.6
9.8
(Mb)
ZNF277
PDCD2L
DLD
SYT8
TNNT2
LSP1
TNNT3
MYOD1
CACNA2D4
LRTM2
HIUHase2
GLG1
RFWD3
MLKL
TMED6
ZDHHC7
C19ORF40
PTPRZ1
AASS
FEZF1
CADPS2
4.3
4.5
4.7
4.9
(Mb)
TNN1
TNN3
MYOD
CACNA2D4
AFG3L2
HIUHase2
GAP1
RFWD3
TMED6
ANKRD27
TDRD12
C19ORF40
PTPRZ1
AASS
FEZF1
CADPS2
Sarcopterygian HIUHase
L. Chalumnae
Scaffold JH127067
Scaffold JH128652
0
0.2
0.4
0.6
(Mb)
HIUHase1
GAS8
DBNDD
DEF8
TUBB3
TCF25
SPIRE2
0
0.2
0.4
HIUHase1B
CDHR
GNAL
TMCO7
Teleost HIUHase 1
D. rerio
Ch. 11
O. latipes
Ch. 3
G. aculeatus
Ch. II
T. rubripes
Ch. 13
10.9
11.1
11.3
11.5
(Mb)
TBR1A
PSMD14
RBMS1A
ITGB6
PLA2R1
MVD
GAS8
HIUHase1 (URAH)
LY75
MYOM2
LGSN
PTP4A1
MRPL4
SRP
20.5
20.7
20.9
21.1
(Mb)
GAS8
STRC
FURIN
FES
RH50
KIF7
HIUHase1
PDCD5
MVD
CYBA
TCF25
MC1R
DEF8
MTHFSD
FOXF1
COX411
11.7
11.9
12.1
22.3
(Mb)
SLC38A7
GAS8
STRC
FURIN
FES
RHCG
KIF7
CLEC18A
HIUHase1B
PDCD5
MVD
CYBA
TCF25
MC1R
DEF8
DBNDD1
MTHFSD
FOXF1
IRFB
COX411
16.2
16.4
16.6
16.8
(Mb)
E2F4
STRC
FURIN1
FES
RHCG2
FURIN7
CLEC18A
HIUHase1B
PDCD5
MVD
P22PHOX
TCF25
MC1R
DEF8
DBNDD1
MTHFSD
FOXF1

Fig. 2

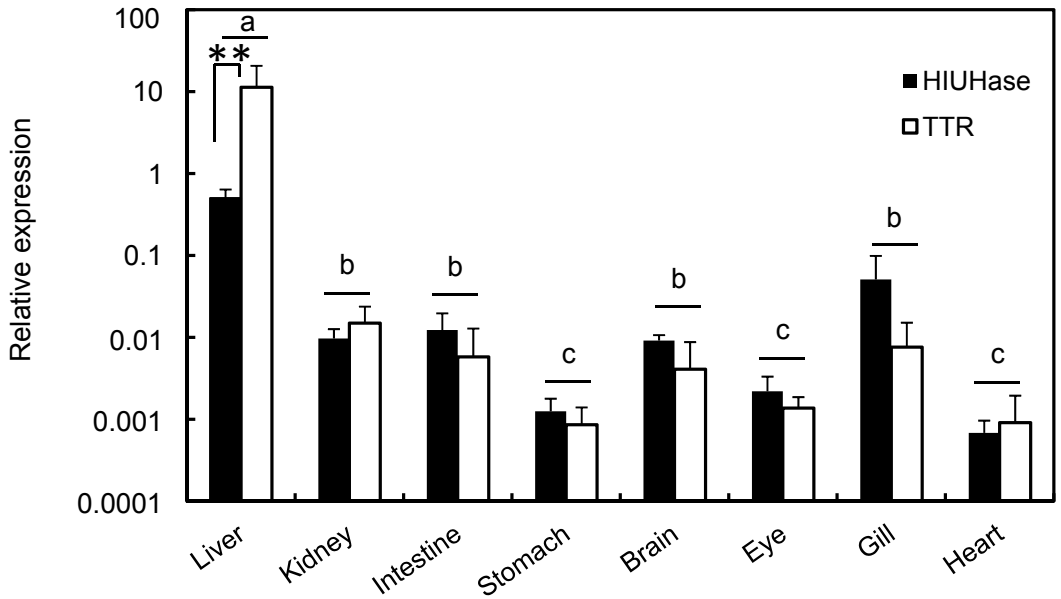
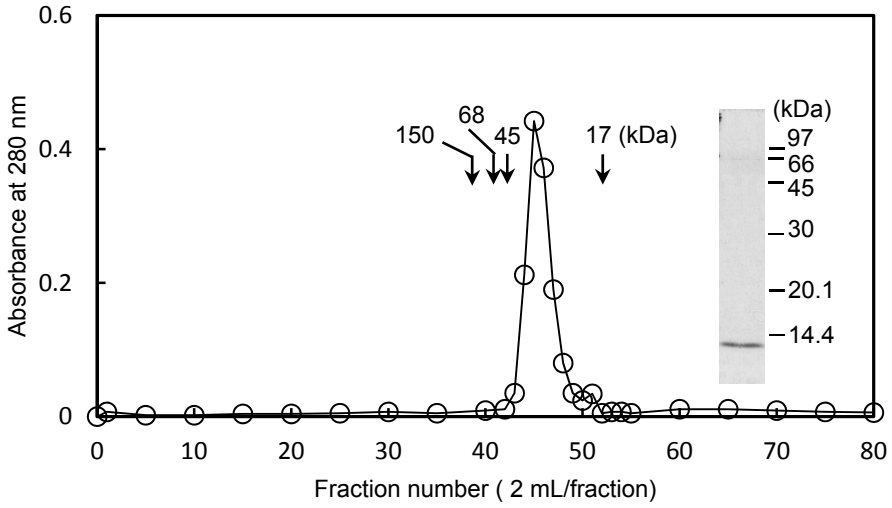

100
10
1
0.1
0.01
0.001
0.0001
Relative expression
**
a
b
b
c
b
c
b
c
HIUHase
TTR
Liver
Kidney
Intestine
Stomach
Brain
Eye
Gill
Heart

Fig. 3

A


0.6
0.4
0.2
0
Absorbance at 280 nm
150
68
45
17 (kDa)
(kDa)
97
66
45
30
20.1
14.4
0
10
20
30
40
50
60
70
80
Fraction number (2 mL/fraction)

B

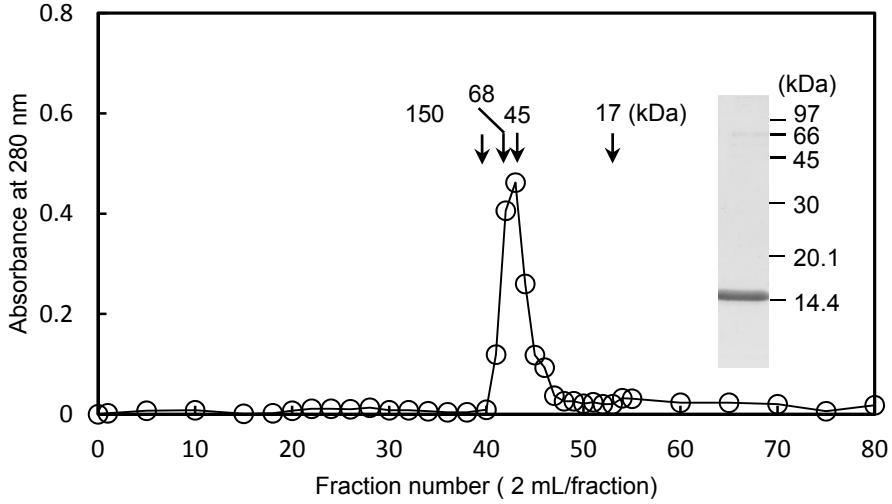

0.8
0.6
0.4
0.2
0
Absorbance at 280 nm
150
68
45
17 (kDa)
(kDa)
97
66
45
30
20.1
14.4
0
10
20
30
40
50
60
70
80
Fraction number (2 mL/fraction)

Fig. 4

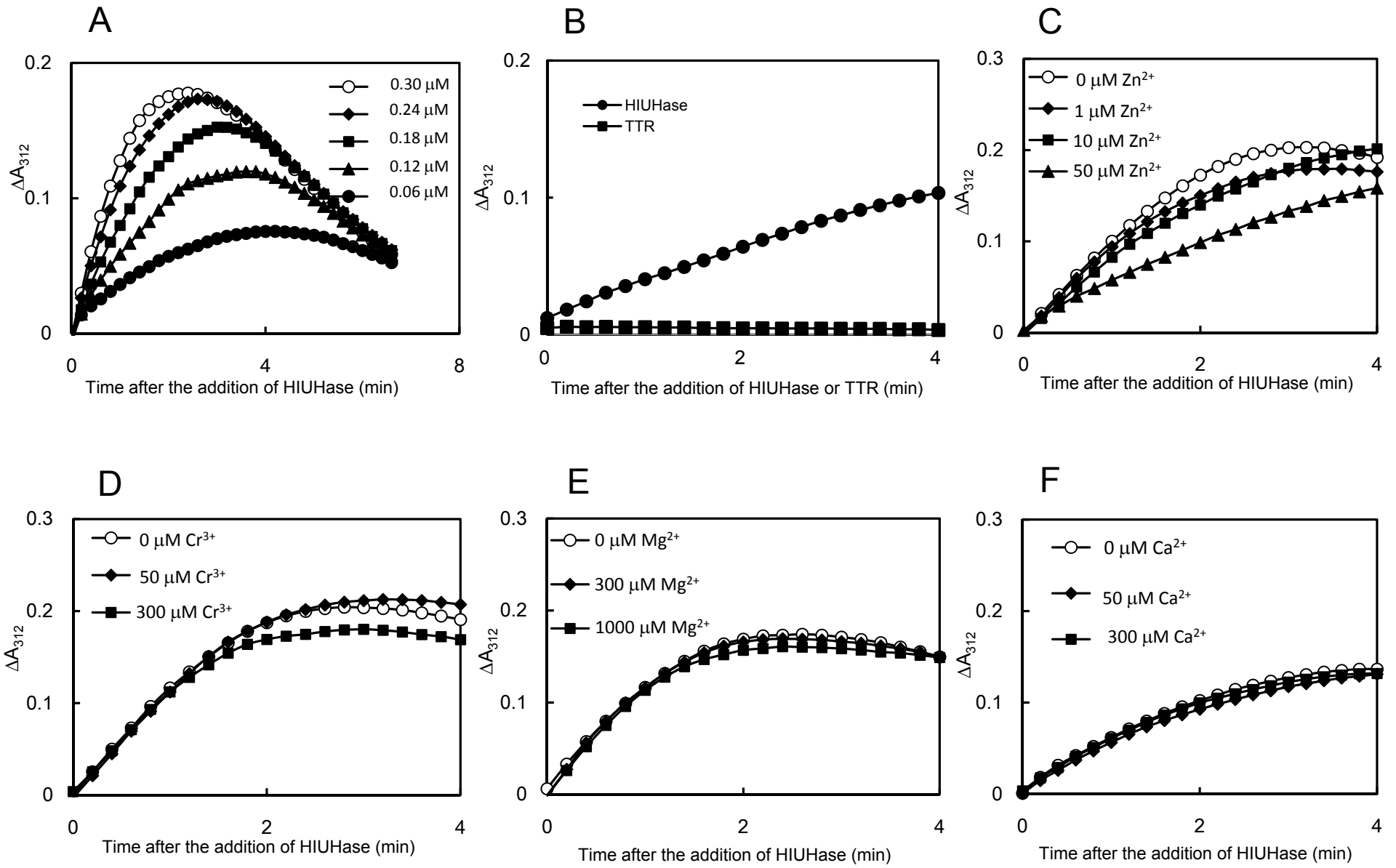

A
0.30 μM
0.24 μM
0.18 μM
0.12 μM
0.06 μM
ΔA312
Time after the addition of HIUHase (min)
B
HIUHase
TTR
ΔA312
Time after the addition of HIUHase or TTR (min)
C
0 μM Zn2+
1 μM Zn2+
10 μM Zn2+
50 μM Zn2+
ΔA312
Time after the addition of HIUHase (min)
D
0 μM Cr3+
50 μM Cr3+
300 μM Cr3+
ΔA312
Time after the addition of HIUHase (min)
E
0 μM Mg2+
300 μM Mg2+
1000 μM Mg2+
ΔA312
Time after the addition of HIUHase (min)
F
0 μM Ca2+
50 μM Ca2+
300 μM Ca2+
ΔA312
Time after the addition of HIUHase (min)

Fig. 5

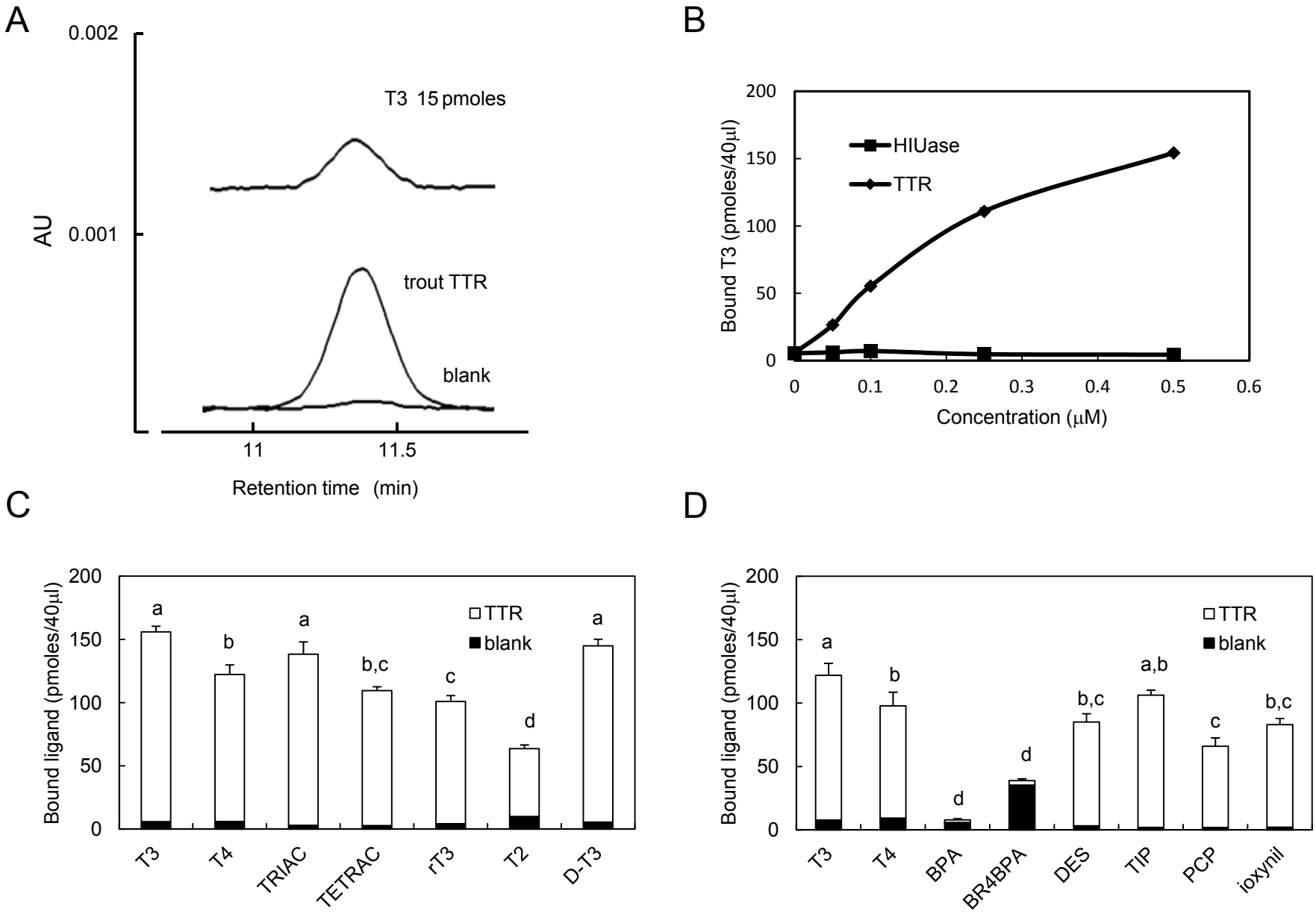
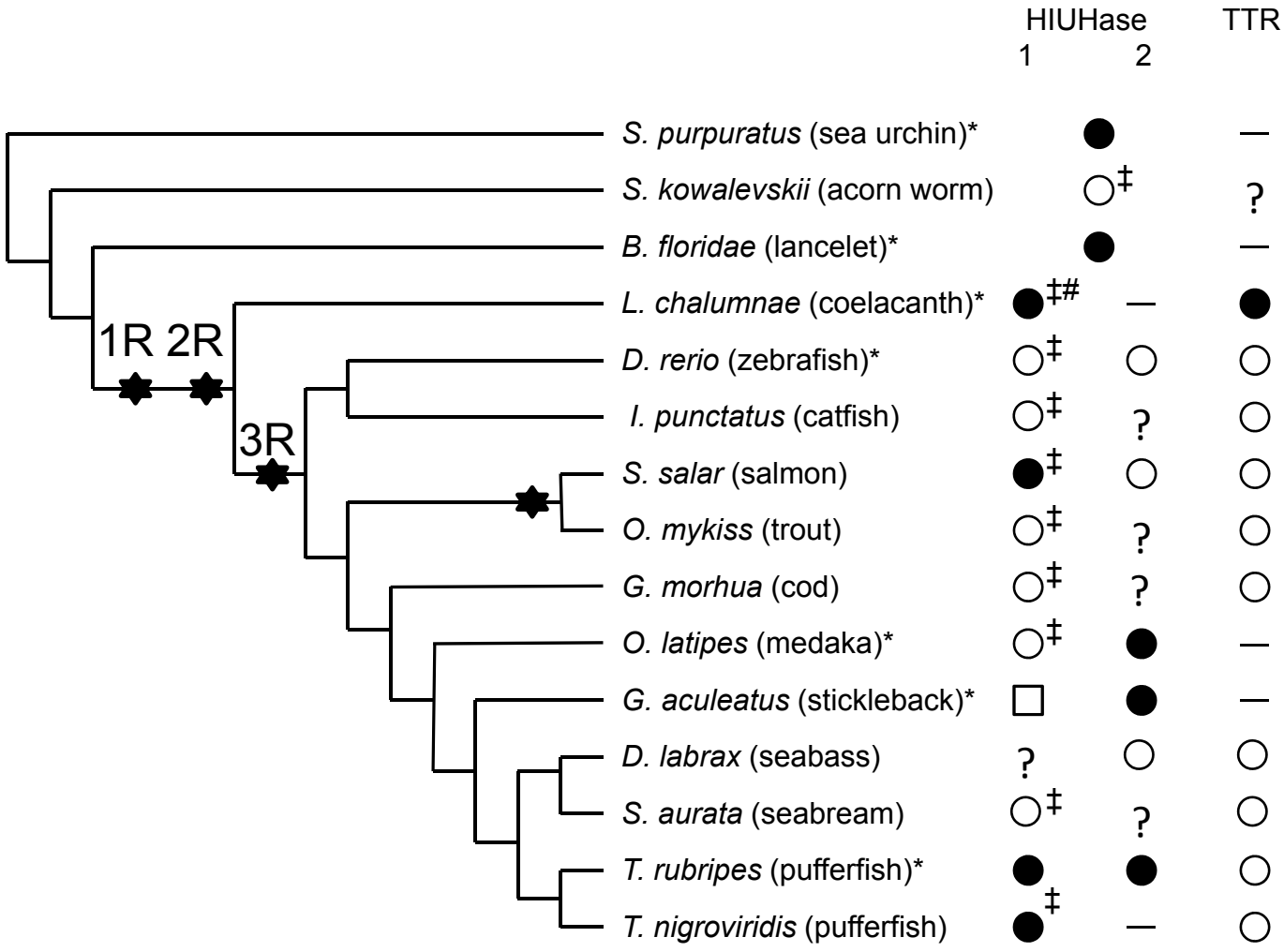
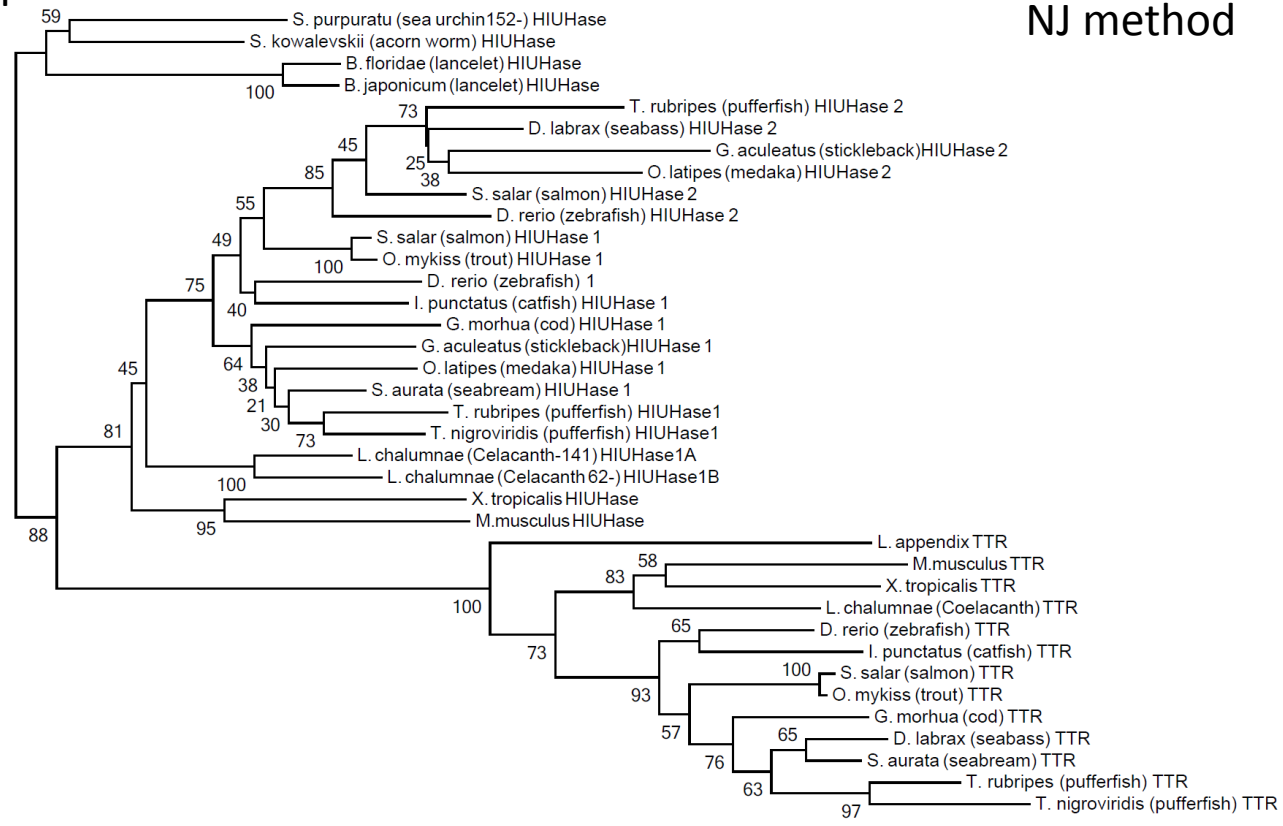

A
0.002
0.001
AU
T3 15 pmoles
trout TTR
blank
11
11.5
Retention time (min)
B
Bound T3 (pmoles/40μl)
HIUase
TTR
Concentration (μM)
C
Bound ligand (pmoles/40μl)
TTR
blank
T3
T4
TRIAC
TETRAC
rT3
T2
D-T3
D
Bound ligand (pmoles/40μl)
TTR
blank
T3
T4
BPA
BR4BPA
DES
TIP
PCP
ioxynil

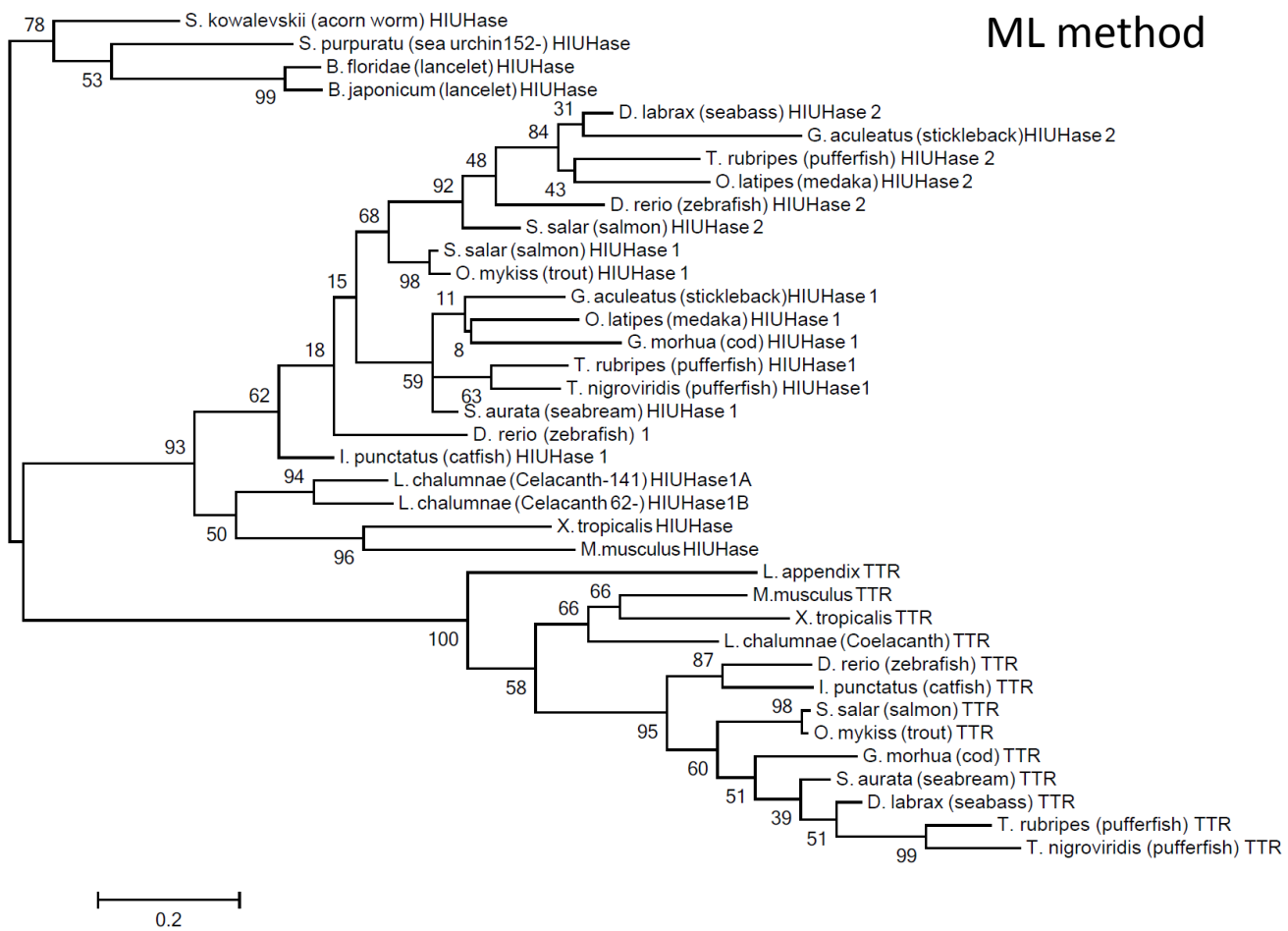
Fig. 6


HIUHase
1
2
TTR
S. purpuratus (sea urchin)*
S. kowalevskii (acorn worm)
B. floridae (lancelet)*
L. chalumnae (coelacanth)*
D. rerio (zebrafish)*
I. punctatus (catfish)
S. salar (salmon)
O. mykiss (trout)
G. morhua (cod)
O. latipes (medaka)*
G. aculeatus (stickleback)*
D. labrax (seabass)
S. aurata (seabream)
T. rubripes (pufferfish)*
T. nigroviridis (pufferfish)
1R 2R
3R

Suppl. 1

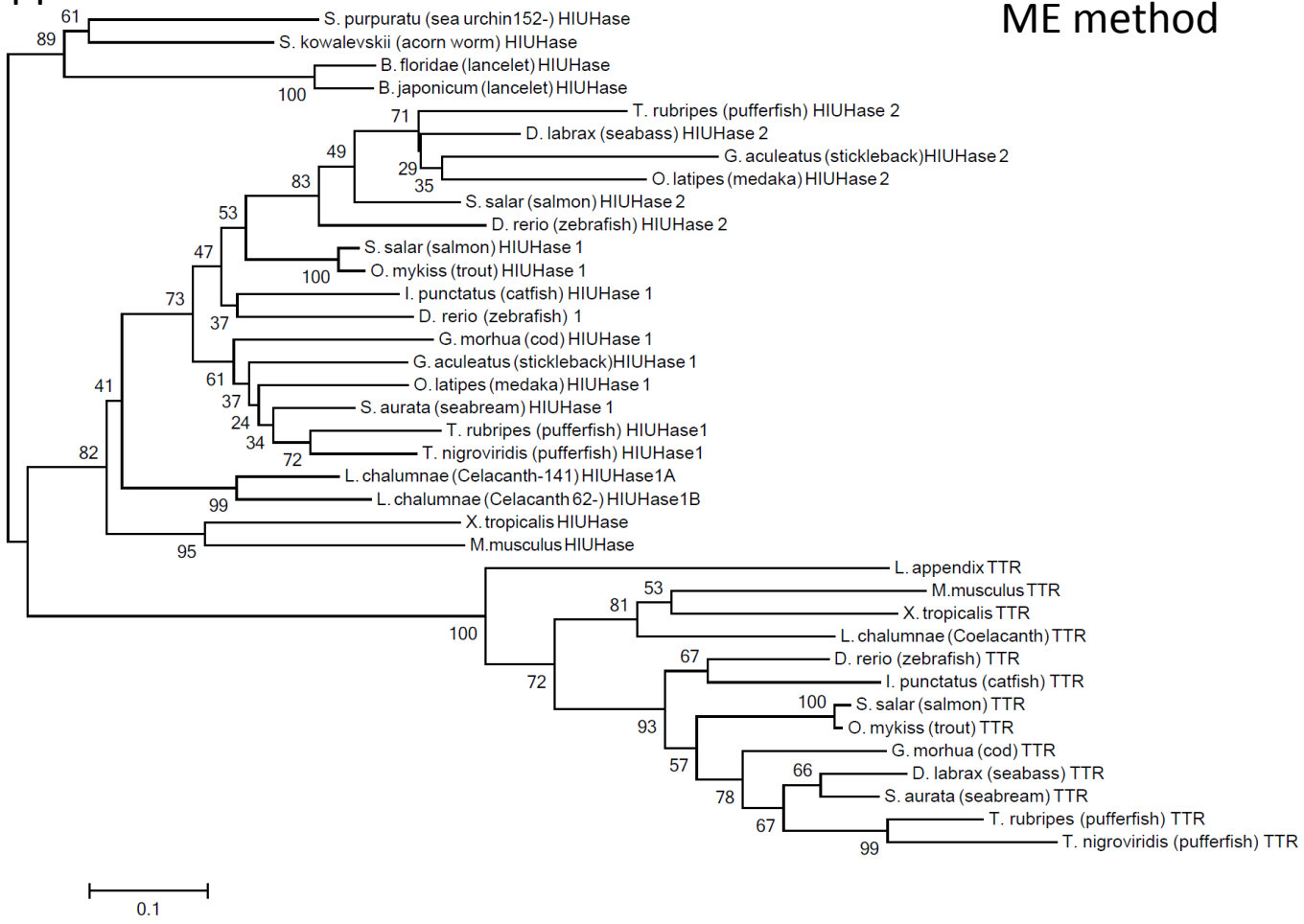


Suppl. 2



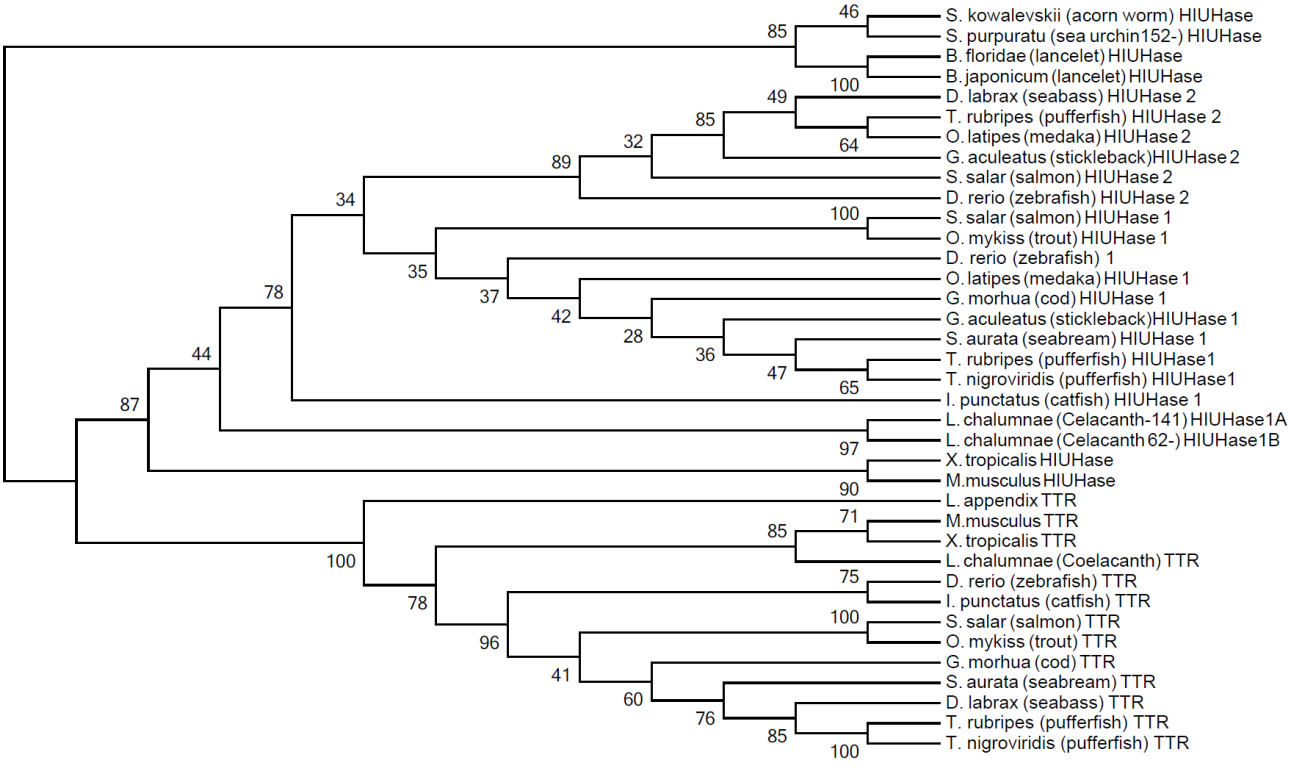
Suppl. 3

ME method



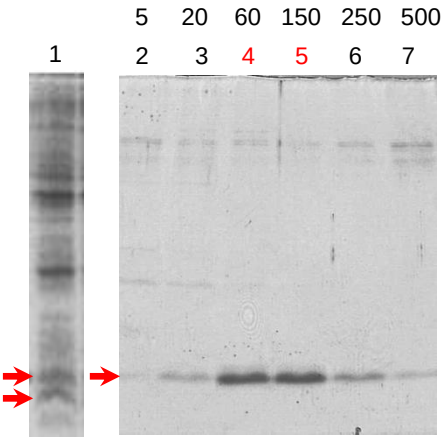
Suppl. 4

MP method



Suppl. 5

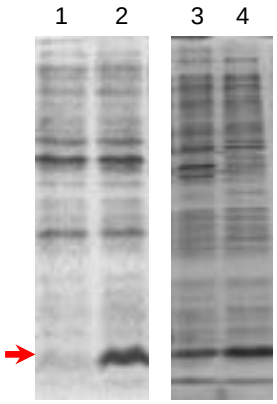
SDS-PAGE of re-TTR



1. whole cells (+ IPTG) containing re-TTR without the His tag
2-7. Ni-resin affinity chromatography of re-TTR with 3xHis tag

Suppl. 6

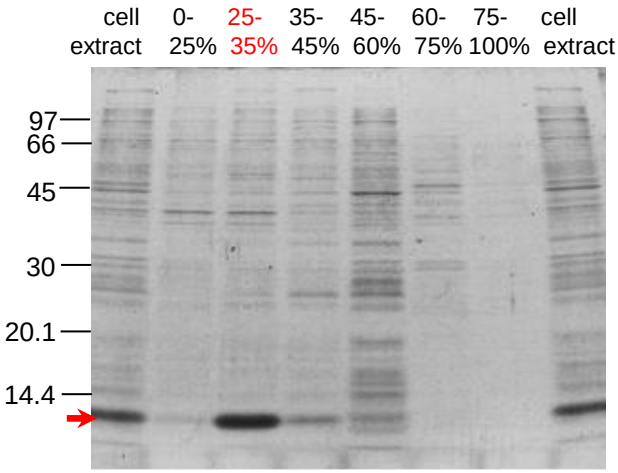
SDS-PAGE of re-HIUHase



1. whole cells (- IPTG)
2. whole cells (+IPTG)
3. cell pellets (+IPTG) after sonication
4. cell extract (+IPTG) after sonication

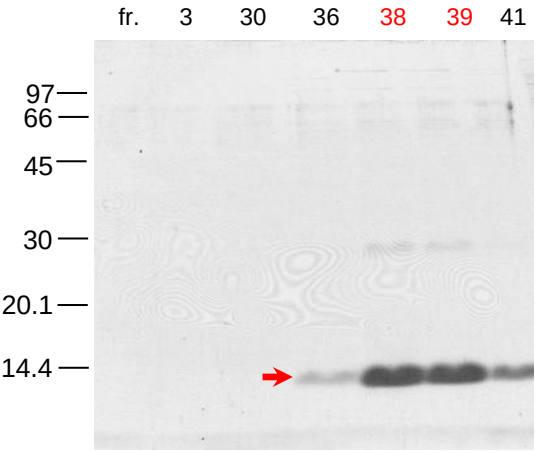
Suppl. 7

Fractionation of re-HIUHase by ammonium sulfate precipitation



Suppl. 8

Fractionation of re-HIUHase by hydrophobic interaction chromatography



Suppl. 9

Hydrophobic interaction column chromatography on phenyl–cellulofine of re-HIUHase after ammonium sulfate precipitation