Identification and expression of two types of chicken GnRH-II genes in mature hard-lipped barb, Osteochilus hasselti

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Identification and expression of two types of chicken GnRH-II genes in mature hard-lipped barb, *Osteochilus hasselti*

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Abstract. *Prayogo NA, Wijayanti GE, Sulistyo I, Sukardi P. 2016. Identification and expression of two types of chicken GnRH-II genes in mature hard-lipped barb,* Osteochilus hasselti. *Biodiversitas 17: xxx.* Gonadotropin-releasing hormone (GnRH) is synthesized in the brain and acts on the anterior pituitary to stimulate the release of gonadotropins in fishes as well as in other vertebrates. Genomic DNAs and cDNAs of two chicken-type GnRH-II genes of hard-lipped barb, namely cGnRH-II type 1 and type 2, were cloned. The length of cloned genomic DNA of cGnRH-II type 1 was 580 bp and cDNA was 206 bp. The length of cloned genomic DNA of cGnRH-II type 2 was 570 bp and cDNA was 206 bp. The cGnRH-II type 1 and type 2 cDNAs encode precursors of 68 and 63 amino acids, respectively. Those precursors consist of a signal peptide, cGnRH-II decapeptide and a GnRH-associated peptide (GAP) linked by a Gly-Lys-Arg proteolytic site. Using quantitative Real Time-PCR, expression levels of these two cGnRH-II genes were detected in the brain, liver and gonad of hard-lipped barb. Expression of the GnRH-II type 1 gene was found only in the brain and liver, on the other hand, expression of the cGnRH-II type 2 gene was found in the gonad, in addition to the brain and liver. The expression of the cGnRH-II genes outside the brain suggested that cGnRH-II might act as an autocrine or paracrine regulator.

Keywords: cGnRH-II, type 1 and 2, Real Time-PCR, amino acid

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a conserved neuro-decapeptide family, which plays a crucial role in regulating gonadal development and controlling the final sexual maturation in vertebrates (Gibson et al. 1997; Sayed et al. 2010; Gharaei et al. 2011). The GnRH decapeptide is synthesized by neuro-secretory cells in hypothalamus and secreted into portal vessels, transported to the pituitary gland where it stimulates secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gonadotrophs (Yaron et al. 1995).

The presence of either two or three forms of GnRH in teleost fishes has been well documented (Kah et al. 2007). The so-called GnRH-I system is regarded as a species specific form and includes mammalian GnRH (mGnRH), seabream GnRH (sbGnRH), chicken GnRH-I (cGnRH-I), and pejerrey GnRH (pjGnRH) (White and Fernald 1998; Morgan and Millar 2004; Kah et al. 2007; Sayed et al. 2010). The GnRH-I system is generally localized in the forebrain and is considered to exert the neuroendocrine control over LH secretion. Another form of GnRH designated as GnRH-II (Sherwood et al. 1993; Sealfon et al. 1997; Volkoff and Peter 1999) has been reported in all major vertebrate groups, including mammals and is mainly expressed in the midbrain (Sherwood et al. 1993). GnRH-II appears to have direct effects on sexual behavior in mammals, birds, and fish (Rissman et al. 1997; Muske 1998; Russell and Richard 1999; Troskie et al. 1998; Wang and Lin 1998), and this effect is believed to be its primary function. Finally, GnRH-III is represented by salmon GnRH (sGnRH) (Sherwood et al. 1983; Adam et al. 2002) and is found in the forebrain either alone or together with GnRH-I depending on the species (Adam et al. 2002; Morgan and Millar 2004). GnRH peptides are also reported in the ovary and testis of fish and in the ovary, testis, mammary gland and placenta of mammals (Sherwood et al. 1993). cGnRH-II exists in the brain tissues of all the fishes, in which cDNA sequences of GnRH have been characterized, and are distributed mainly in the midbrain. Both the function of cGnRH-II and the cycle variations of expression levels during gonad development are still controversial.

Hard-lipped barb (*Osteochilus hasselti* C.V.) species is an indigenous tropical fish and is synchronous batch spawner fish (Prayogo et al. 2008), which is capable of spawning several times during the peak of the spawning period. This fish, a familiar economical freshwater fish in Indonesia, is used as the model of endocrine regulation of freshwater fish (Prayogo et al. 2012). In our laboratory, cGnRH-II cDNAs have been cloned from hard-lipped barb brains for the first time, and all of them are encoded by two different gene loci. This study reports the isolation and identification of two differing cGnRH-II cDNAs and genes in the hard lipped barb. Expression levels of the cGnRH-II genes are assayed in the brain, liver and gonad by real time-PCR. The research results offer novel evidence for two types of cGnRH-II genes for understanding further the

function and regulation mechanism of cGnRH-II genes in the HPG axis in hard-lipped barb.

MATERIALS AND METHODS

Brain, liver and gonad collection

Total RNA and genomic DNA were isolated from brain, liver and gonad. Total of 30 sexually mature female Hard-lipped Barb weighing of 100 g in average were purchased from local market in Banyumas District, Central Java, Indonesia. Fish brains were removed, snapped frozen, and stored at-150°C with liquid nitrogen until the time for RNA and genomic extraction. Isolation, cloning, and sequencing of two cGnRH-II genes were conducted at the Laboratory of Molecular Biologi, Universitas Jenderal Soedirman, Purwokerto, Banyumas, Indonesia.

Genomic DNA isolation

Total genomic DNA was extracted from whole brain, liver and gonad. The tissue was mixed with 400 µL TNES (Tris, NaCl, EDTA, and SDS), and 0.5 µL RNAse and 3 µL Proteinase K were added to the sample. The sample was incubated in 37^{0}C for 2 hour and then centrifuged for 15 minutes. Then the sample was extracted with phenol chloroform followed by centrifugation for 5 minutes. DNA in the water phase was precipitated with ethanol. The integrity of the DNAs was verified by agarose gel electrophoresis and staining with ethidium bromide.

RNA isolation and RT-PCR

Total mRNA was extracted from whole brain, liver and gonad using Blue Sepasol R-RNA super 1 reagent (nacalaitesque) based on ethanol-phenol-chloroform extraction method. The prepared RNA was treated with RNase-free DNase (Takara). The quality and concentration of RNA were assayed by denaturing agarose gel electrophoresis and optical density reading at 260 and 280 nm. The RNA was aliquoted in batches and frozen at-70^0 C. Total RNA samples (1.5 ng each) were reversely transcribed using cDNA synthesis kit (PrimeScript™ Reverse Transcriptase) from Takara.

Amplification of GnRH-II genomic DNA and cDNA

The primer pairs, Cyprinidae cGnRH-II Type 1F containing an *EcoR*1 site and Cyprinidae cGnRH-II T1 R containing a *Xho1* site were designed based on cGnRH-II cDNA sequences of Cyprinidae (*Cyprinus carpio* AY189961.1) and *Carassius auratus*, U30386.1. The primer pairs, Cyprinidae cGnRH-II Type 2 F containing an *EcoR1* site and Cyprinidae cGnRH-II T2 R containing a *Xho1*site were designed from cGnRH-II cDNA sequences of *Carassius auratus* (AB017271.1), and *Cyprinus carpio*, (AF521130.2). The sequences were aligned with MultAlin to identify the conserved sequences in the ORF region. The primers to amplify the cGnRH-II type 1 and type 2 cDNAs were designed using Primer 3 software (Table 1). The same primer pairs were used to amplify cGnRH-II type 1 and type 2 genes of hard lipped-barb (Table 1).

PCR for both genomic DNA and cDNA was carried out using a thermal cycler (Robocycler, Stratagene) according to the following cycle; 95°C for 2 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by a 5 min extension at 72°C. After amplification, the PCR products was electrophoretically separated on a 1.5% agarose gel and stained with ethidium bromide.

Cloning and Sequencing of PCR Products

PCR amplified fragments of genomic DNA and cDNA were separated by agarose gel electrophoresis. DNA was extracted from the incised gels using the DNA gel extraction procedure (Green and Sambrook 2012.). The desired DNA fragments were subcloned into BSKS Eco R1/Xho 1 vector (10 kb) (Takara) using ligation with T4 ligase. The plasmid was transfected into *E. coli* and the bacteria were spread on LB medium plates (Mohamed et al. 2008). The recombinant positive colonies were screened using ampicillin. Plasmid DNAs were purified from positive colonies with mini scale plasmid preparation. DNA sequences were determined using the Big Dye version 3.1 sequencing method with specific primers. Primers used for sequencing of cGnRH-II type 1 were *Cyprinidae F cGnRH-II T1* and *Cyprinidae R cGnRH-II T1*, and those for cGnRH-II type 2 were *Cyprinidae F cGnRH-II* F2 and *Cyprinidae F cGnRH-II R2* (Table 1). The sequence data were automatically collected on the ABI PRISM 3100 Genetic Analyzer (PE Applied Bio-systems).

Sequence analysis

The genomic and cDNA sequences for two cGnRH-II genes were analyzed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) with default settings on the complete, non-redundant GenBank database nucleotide sequences. The genomic and cDNA sequences were aligned using CLUSTALW software to identify introns and exons.

Table 1. The primers used to amplify the two cGnRH-II genes and cDNAs and to sequence their PCR products.

Primer	Code	Sequences	Tm	PCR Product
F cGnRH-II T1	F2	TGGGGATGTTGCTGTGTCTA	64.18	580 bp
R cGnRH-II T1	R2	TCTTTTGGAAATCCCGTATG	57.55	
F cGnRH-II T2	F3	GGTGATGGGGATGTTGATGT	59.28	580bp
R cGnRH-II T2	R3	TCTTTTGGAAATCCCGTATG	58.43	

Phylogenetic analysis

For phylogenetic analyses, hard-lipped barb cDNAs of cGnRH-II type 1 and type 2 were compared to cDNA sequences of cGnRH-II from nineteen fish species. All sequences were retrieved from NCBI GenBank (Appendix 1). The relationship between hard-lipped barb GnRH and other teleost GnRH was generated with CLUSTAL W with scoring method percent, and the unrooted tree was generated using Treeview version 1.5.2. (Magdy et al. 2007).

Quantitative Real Time analysis

The primers were designed using the Primer 3.0 software. The used primers were as follows: type 1 cGnRH-II forward, 5-TGGGGATGTTGCTGTGTCTA-3; type 1 cGnRH-II reverse, 5-TCTTTTGGAAATCCCGTATG-3; type 2 cGnRH-II forward 5-GGTGATGGGGATGTTGATGT-3; type 2 cGnRH-II reverse, 5-TCTTTTGGAAATCCCGTATG-3. Goldfish actin (GenBank accession number AB039726.2), used as endogenous control, was amplified using the following primers: actin forward, 5-GAGCTATGAGCTCCCTGACGG-3; actin reverse, 5-AAACGCTCATTGCCAATGGT-3, and were used to normalize variations in RNA. After optimization, PCR was performed in a 10 µL solution containing 2 µL cDNA, 5 µL SYBR mix (Applied Biosystem), 0.3 µL forward primer, 0.3 µL reverse primer and 2.4 µL DDW using the following conditions: 95°C for 45 s, 45 cycles of 95°C for 15 s and 60°C for 1min, then 95°C for 15s, 60°C for 15s and 95°C for 15s. The results were analyzed using the standard curve mode, according to the manufacturer's recommendations (Applied Biosystems).

Data analysis

The mRNA levels for each sample were expressed as the ratio of cGnRH-II mRNA to actin mRNA. The data were subjected to ANOVA followed by Turkey's multiple-comparisons tests. Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

Cloning of Genomic DNA and cDNA of two cGnRH-II genes in Hard Lipped Barb

The two types of cGnRH-II genes of the hard-lipped barb were successfully amplified from genomic DNA and cDNA. The agarose gel electrophoresis of PCR products from the two types of cGnRH-II genomic DNAs showed specific bands, approximately 580bp in size, which were designated as cGnRH-II type 1 (JN867722) and cGnRH-II type 2 (GenBank accession 1697609) (Figure 1). The genomic sequences of the two cGnRH-II genes were analyzed with BLAST and we found that they were different from each other and also different from GnRH genes of other species. The nucleotide sequence identity of cGnRH-II type 1 cDNAs was 92% with cGnRH-II of carp (*Cyprinus* carpio, AY189961.1), 90% with goldfish (*Carassius auratus*, U30386.1), 92% with roach (*Rutilus rutilus*, U60668.1), and 90% with grass carp (*Ctenopharyng odonidella*, EU981284.1).

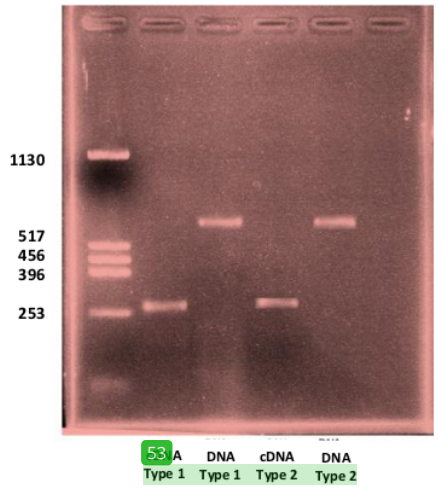

Figure 1. PCR products amplified from genomic DNA using the primer sets designed for each of the two cGnRH-II genes of hard-lipped barb (*Osteochilus hasselti* C.V).

The nucleotide sequence identity of cGnRH-II type 2 cDNAs was 94% with cGnRH-II of carp (*Cyprinus carpio* AY189961.1), 92% with goldfish (*Carassius auratus*, U30386.1), 94% with roach (*Rutilus rutilus*, U60668.1), and 92% with grass carp (*Ctenopharyng odonidella*, EU981284.1). These results indicate the presence of two different genes and cDNAs encoding cGnRH-II in the brain of hard-lipped barb for the first time.

The nucleotide sequence identity of cGnRH-II type 2 cDNAs was 94% with cGnRH-II of carp (*Cyprinus carpio* AY189961.1), 92% with goldfish (*Carassius auratus*, U30386.1), 94% with roach (*Rutilus rutilus*, U60668.1), and 92% with grass carp (*Ctenopharyng odonidella*, EU981284.1). These results indicate the presence of two different genes and cDNAs encoding cGnRH-II in the brain of hard-lipped barb for the first time.

Gene Structure of cGnRH-II

The two cGnRH-II genes share the same basic structure. The genomic DNA fragments both contained 3 exons (coding region) and 2 introns (non coding region). The first exon encoded a signal peptide (17 amino acids for type 1 and 13 amino acids for type 2), GnRH-II decapeptide, the proteolytic cleavage recognition site (3 amino acids for both types) and N-terminus of GnRH-associated peptide (GAP) (first 9 amino acids for both types). Exon 2 encoded the central portion of GAP and exon 3 encoded the C terminus of GAP (Figure 2). All intron-exon boundary sequences conformed to the GT-AG rule.

Structures for two types of cGnRH-II had a high similarity in length for exon 1 and 2, but the intron sizes of cGnRH-II type 1 were different from cGnRH-II type 2 (Figure 2). The level of similarity in the coding sequences can be seen as the distance at the phylogenetic tree (Figure 6). The greatest differences within the preprohormone are within the GAP coding sequences. The striking contrast

between the conservation of the GnRH coding sequences and the lack thereof in the GAP coding sequences is the evidence of differential selective pressure within the gene (Figure 5). This is evident in cases where the identity and similarity of the GnRH and GAP coding sequences have been compared for mRNAs of GnRH-II genes from different species (Figure 4) (White and Fernald 1998; Russell and Richard 1999).

Phylogenetic analyses

Phylogenetic analyses were performed to establish an evolutionary context for the two cGnRH-II genes. Genetic distances (measured as substitutions per site) showed moderate low values, and the topology was well supported by strong bootstrap values. As expected, two types of cGnRH-II in hard-lipped barb were included within a sub-cluster of the *carp* (*Cyprinus carpio*, *Carassius auratus*) with high bootstrap values (Figure 6).

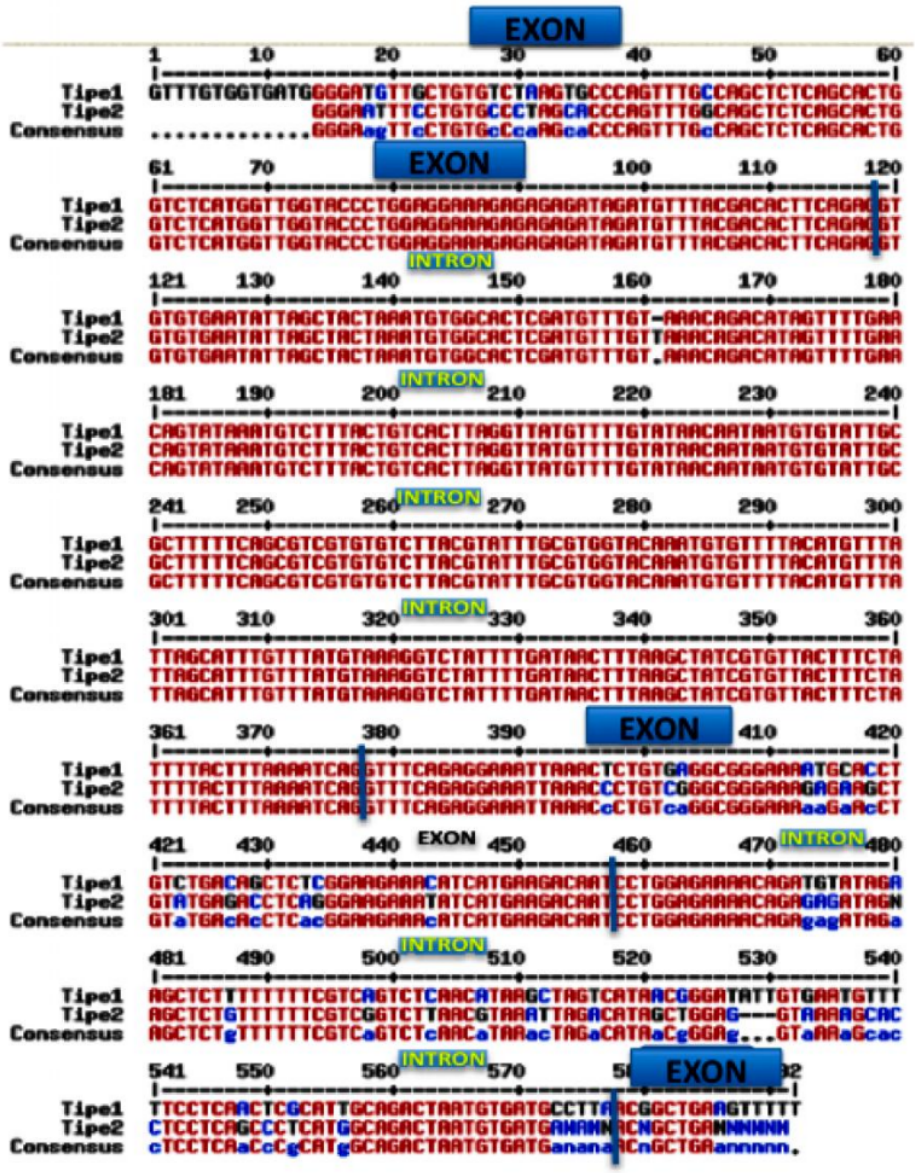

Figure 2. Nucleotide sequences and exon/intron structure of two cGnRH-II genes in hard-lipped barb

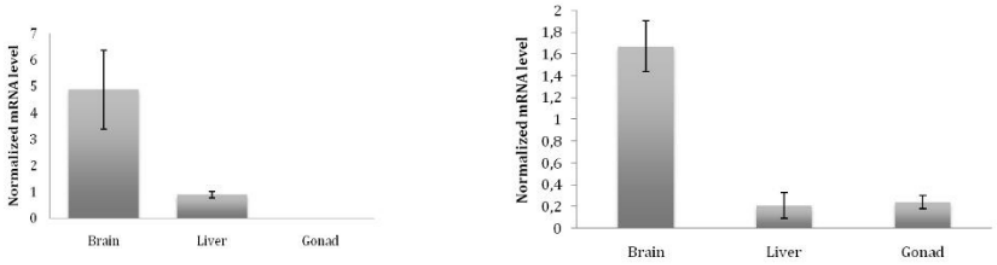

Figure 3. Expression of two types of cGnRH-II mRNA in the brain, liver and gonad of mature female hard-lipped barb. (A) Expression of cGnRH-II type 1 mRNA. (B) Expression of cGnRH-II type 2 mRNA

Expression of two cGnRH-II genes in the brain, liver and gonad of mature fish

Total RNA was isolated from the brain, liver, and gonads of mature female hard lipped barb, and the reverse transcription products of total RNA were amplified by primers F2 and R2 for cGnRH-II type 1, and primers F3 and R3 for cGnRH-II type 2 (Figure 3). The results of qRT-PCR analysis showed that two types of cGnRH-II genes were coexpressed in the brain of mature hard-lipped barb. cGnRH-II type 2 mRNA was expressed in the liver and gonad, and type 1 mRNA was expressed in the liver, but not in the gonad. The expression levels of the two types of cGnRH-II mRNA in the brain were much higher than those in the liver and gonad. It is also reported that two types of cGnRH-II mRNAs were expressed in the liver of female common carp, but only cGnRH-II type 2 mRNA is expressed in the gonad (Lin et al. 2003).

Discussion

This paper reports for the first time that hard-lipped barb had two forms of cGnRH-II namely cGnRH-II type 1 and cGnRH-II type 2, similar to *Cyprinus carpio* (Lin and Lin 1994; Wang and Lin 1998), and goldfish (Kim et al. 1995; Lin and Peter 1996; Chik et al. 1997; Yu et al. 1998). The two cGnRH-II genes and cDNAs cloned in this study are missing 5' and 3' sequences, due to the design of the PCR primers. Our analyses hence have limitation, but still give deep insights into the evolution and physiological functions of these genes. The newly identified hard-lipped barb type 1and type 2 genes show a high conservation with other GnRH-II genes previously reported (Figure 4). The nucleotide sequence of cGnRH-II type 1 cDNA shows, based on BLAST search, 96, 95, 94, 94% similarity to cGnRH-II cDNAs of carp (*Cyprinus carpio*AY189961.1), goldfish (*Carassius auratus*, U30386.1), roach (*Rutilus rutilus*, U60668.1), and grass carp (*Ctenopharyng odonidella*, EU981284.1), respectively. The nucleotide sequence of cGnRH-II type 2 cDNA is also very similar to other cGnRH-II cDNAs, 95, 94, 92, 92, 91% similarity to cGnRH-II of goldfish (*Carassius auratus*, AB017271.1), carp (*Cyprinus carpio*, AF521130.2), roach (*Rutilus rutilus*, U60667.1), grass carp (*Ctenopharyng odonidella*, EU981295.1) and zebrafish (*Danio rerio*, AY557019.1), respectively (Figure 5). The structural characteristics of GnRH-II type1 and type 2 genomic loci in hard lipped barb are similar with GnRH genes in other species. The results show that GnRH-II genes might evolve from a common ancestral molecule.

The hard-lipped barb cGnRH-II type 1 and type 2 precursor peptides are composed of, as predicted from the partial cDNA sequence, at least 68 and 64 amino acid residues, respectively, which consist of a signal peptide, cGnRH-II decapeptide, and a GAP linked by the highly conserved processing site (Gly-Lys-Arg) (Figure 5). The signal peptides and GAP are only partially cloned. The amino acid sequences of the precursors were compared with previously identified fish GnRH-II precursors including roach (*Rutilus rutilus*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), grass carp (*Ctenopharyng odonidella*), zebrafish (*Danio rerio*) (Table 2). The results show that the amino acid homology of GnRH-II type 1 precursors within Cyprinoids is 85-92%, but only 50-71% among other teleosts. The amino acid homology of the cGnRH-II type 2 precursor within Cyprinoids was 83-96%, but only 50-64% among other teleosts (Table 2).

The decapeptide is the minimal structural requirement for gonadothropin releasing activity (Raymond et al. 1986). The processing site (Gly-Lys-Arg) is essential for releasing GAP. The decapeptides and processing sites of the two hard-lipped barb GnRH-II precursors were entirely conserved in vertebrate evolution. However, signal peptides that direct the transport of proteins and GAP are diverged between the two cGnRH-II precursors as well as among other teleosts. The amino acid divergence in the signal peptides and GAP was much higher between the two types of cGnRH-II than between those of neighboring species. It is presumed therefore that the cGnRH-II type 1 and type 2 precursors could have different functions obtained through adapting to natural selections during evolution.

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cfathead        ATGGTGCACATCTGCAGGCTGCTTGTGCTGATGGGGATGTTGCTGTGTTTAAGTGCCCAG 60
cPimephales     ATGGTGCACATCTGCAGGCTGCTTGTACTGATGGGGATGTTGCTGTGTTTAAGTGCCCAG 60
croach          ATGGTGCACATCTGCAGGCTGCTTGTGCTGATGGGGATGTTGCTGTGTCTAAGTGCCCAG 60
cgrass          ATGGTGCACATCTGCAGGCTGCTTGTGCTGATGGGGATGTTGCTGTGTCTAGGTGCCCAG 60
cred            ATGGTGCACATCTGCAGGCTGTTTGTGGTGATGGGGATGTTGCTGTGTCTAAGTGCCCAG 60
cgoldfish       ATGGTGCACATCTGCAGGCTGTTTGTGGTGATGGGGATGTTGCTGTGTCTAAGTGCCCAG 60
cCyprinus       ATGGTGCACATCTGCAGGCTGTTTGTGGTGATGGGGATGTTGCTGTGTCTAAGTGCCCAG 60
cZebrafish      ATGGTGCTGGTCTGCAGGCTGCTGTTGGTCATGGGGCTGATGCTGTGTCTGAGTGCTCAG 60
type1           --------------------GTTTGTGGTGATGGGGATGTTGCTGTGTCTAAGTGCCCAG 40
type2           ---------------------------------GGGAATTTCCTGTGCCCTAGCACCCAG 27
                                                ***.: :* *****    .* .* ***

cfathead        TTCGCCAGCTCTCAGCACTGGTCTCATGGCTGGTACCCTGGAGGAAAGCGAGAGATAGAC 120
cPimephales     TTCGCCAGCTCTCAGCACTGGTCTCATGGCTGGTACCCTGGAGGAAAGCGAGAGATAGAC 120
croach          TTTGCCAGCTCTCAGCACTGGTCTCATGGCTGGTACCCTGGAGGAAAGCGAGAGATAGAC 120
cgrass          TTTGCCAGCTCTCAGCACTGGTCTCATGGCTGGTACCCTGGAGGAAAGCGAGAGATAGAC 120
cred            TTTGCCAGCTCTCAGCACTGGTCTCATGGCTGGTATCCTGGAGGAAAGAGAGAGATAGAC 120
cgoldfish       TTTGCCAGCTCTCAGCACTGGTCTCATGGCTGGTATCCTGGAGGAAAGAGAGAGATAGAC 120
cCyprinus       TTTGCCAGCTCTCAGCACTGGTCTCATGGCTGGTACCCTGGAGGAAAAAGAGAGATAGAC 120
cZebrafish      TTGAGCAGCGCTCAGCACTGGTCTCACGGCTGGTATCCTGGAGGAAAGAGAGAGATAGAC 120
type1           TTTGCCAGCTCTCAGCACTGGTCTCATGGTTGGTACCCTGGAGGAAAGAGAGAGATAGAT 100
type2           TTTGGCAGCTCTCAGCACTGGTCTCATGGTTGGTACCCTGGAGGAAAGAGAGAGATAGAT 87
                ** . **** ***************** ** ***** ***********..**********

cfathead        ATTTACGATACATCAGAGGTTTCAGAGGAAATTAAACTCTGTGAGGAAGGAAAATGCAGC 180
cPimephales     ATTTACGATACATCAGAGGTTTCAGAGGAAATTAAACTCTGTGAGGAAGGAAAATGCAGC 180
croach          ATTTACGATACATCAGAGGTTTCAGGGGAAATTAAACTCTGTGAGGCAGGAAAATGCAGC 180
cgrass          ATTTACGATACCTCAGAGGTTTCAGAGGAAATTAAACTCTGTGAGGCAGGAAAATGCAGC 180
cred            GTTTACGATTCCTCAGAGGTTTCAGGGGAAATTAAACTCTGTGAGGCAGGAAAATGCAGC 180
cgoldfish       GTTTACGATTCCTCAGAGGTTTCAGGGGAAATTAAACTCTGTGAGGCAGGAAAATGCAGC 180
cCyprinus       GTTTACGATACCTCAGAGGTTTCAGAGGAAATTAAACTCTGTGAGGCAGGAAAATGCAGC 180
cZebrafish      CTCTACGACACCTCAGAGGTTTCAGAGGAAGTGAAGCTCTGCGAGGCAGGAAAATGCAGT 180
type1           GTTTACGACACTTCAGAGGTTTCAGAGGAAATTAAACTCTGTGAGGCGGGAAAATGCACC 160
type2           GTTTACGACACTTCAGAGGTTTCAGAGGAAATTAAACCCTGTCGGGCGGGAAAGAGAAGC 147
                 * ***** :* **************.****.* **.* ***  .**..*****.:*.*

cfathead        TATCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
cPimephales     TATCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
croach          TACCTGAGACCCCAGGGACGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
cgrass          TACCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
cred            TACCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
cgoldfish       TACCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCATCATACGG 240
cCyprinus       TACCTGAGACCCCAGGGAAGAAACATTCTGAAGACAATACTGCTGGATGCCCTCATACGG 240
cZebrafish      TACCTGAGACCGCAGGGAAGAAACATCCTCAAGACAATACTGCTGGATGCCCTCATTCGT 240
type1           TGTCTGACAGCTCTCGGAAGAAACATCATGAAGACAATACGGCTGA-------------- 206
type2           TGTATGAGACCTCAGGGAAGAAATATCATGAAGACAATACGGCTGA-------------- 193
                *. .*** * * *: ***.**** ** .* ********** ****.
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Figure 4. Nucleotides alignment of two types of cGnRH-II cDNA of hard-lipped barb with those of other teleosts

Figure 5. Amino acids alignment of cGnRH-II type 1 and type 2 of hard-lipped barb.

Table 2. Amino acid homology of hard-lipped barb cGnRH-II type 1 and type 2 with those of other species

Species	Type 1		Type 2	
	Accession no.	Homology (%)	Accession no.	Homology (%)
Cyprinus carpio	AY189961.1	92	AAO39975.2	94
Carassius auratus	U30386.1	91	BAB18904.1	92
Ctenopharyng odonidella	EU981284.1	90	ACH78254.1	91
Pimephales promelas	EF672264.1	90	ABV45418.1	88
Rutilus rutilus	U60668.1	90	AAR18405.1	83
Danio rerio	AY094357.1	85	NP_878307.2	90
Oncorhynchus mykiss	AF125973.1	71	AAK82957.1	58
Coregonus clupeaformis	AY245102.1	71	ABP04042.1	96
Anguilla japonica	AB026990.1	61	AAR20401.1	64
Anguilla marmorata	GQ422803.1	61	ACN88548.1	56
Thunnus thynnus	EU239502.1	68	ABX10868.1	50
Oreochromis niloticus	AB101666.1	69	AAD02425.1	51
Mugil cephalus	AY373451.2	50	AAQ83268.1	54
Gadus morhua	GU332294.1	59	ADD92007.1	62

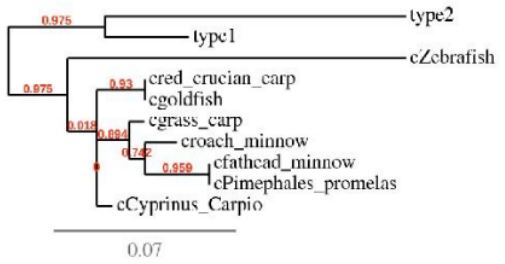

Figure 6. Phylogenetic relationship of hard-lipped barb cGnRH-II type 1 and type 2 precursors with known GnRH. The relationship was generated with CLUSTAL W and the unrooted tree was generated using T view version 1.5.2. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site

The present study is the first to describe the two types of cGnRH-II genes in hard-lipped barb, and provides new evolutionary information on this gene family. The cGnRH-II type 1 and type 2 genes in hard-lipped barb can be grouped together with other teleost cGnRH-II genes in the phylogenetic tree, suggesting a common ancestor for both groups of genes. Phylogenetic analysis showed that the cGnRH-II type 1 gene is highly homologous to cGnRH-II genes of goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), red carp, and the cGnRH-II type 2 is highly homologous to that of zebrafish (*Danio rerio*) (Figure 6).

The distribution of cGnRH-II peptides and the expression pattern of cGnRH-II genes in brain regions of teleost fishes has indicated that cGnRH-II mainly acts as a neurotransmitter and/or neuromodulator. mRNAs of two goldfish cGnRH-II genes are detected not only in brain regions, but also in the ovary and testis (Lin and Peter 1996; Y al. 1998). In hard lipped barb, the two cGnRH-II genes are expressed in the brain and liver. The cGnRH-II Type 2 gene, but not the type 1 gene, is expressed in the ovary, although at much lower levels than in the brain. cGnRH-II should mainly work as the neurotransmitter and neuromodulator and herewith, operate in the regulation of the GnRH release. The expression of the cGnRH-II genes in the liver and gonad suggests that cGnRH-II stimulate the release of er hormones, such as estradiols and testosterone, in an autocrine or paracrine manner

In summary, the present study has revealed the genomic and cDNA sequences of two cGnRH-II variants namely cGnRH-II type 1 and cGnRH-II type 2 in hard-lipped barb. The phylogenetic analyses support the idea that the two cGnRH genes share the same basic structure with other teleost cGnRH-II genes. It means that the two cGnRH-II genes of hard-lipped barb are conserved, assuming a similar function with other teleost cGnRH-II genes.

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APPENDIX 1

Accession numbers of the GnRH sequences from teleost fishes, downloaded from GenBank.

GnRH II clade: Anguilla japonica: AB026990; Carassius auratus: U30386; Clariasgariepinus: X78047; Coregonusclupeaformis: AY245102; *Cyprinus carpio*: AY147400; Danio rerio: AF511531; Dicentrarchuslabrax: AF224281; Macacamulatta: AF097356; Micropogoniasundulatus: AY324669; Monopterusalbus: AY786183; Moronesaxatilis: AF056313; Mugilcephalus: AY373451; Odontesthesbonariensis: AY744687; Oncorhynchusmykiss: AF125973; Oreochromisniloticus: AB101666; Oryziaslatipes: AB041330; Rutilus rutilus: U60668; Sci-aenopsocellatus: AY677171; Sparus aurata: U30325; Suncusmurinus: AF107315; Trichosurusvulpecula: AF193516; Tupaiabelangeri: U63327; Typhlonectesnatans: AF167558; Veraspermoseri: AB066359.

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