Middle-East Journal of Scientific Research 10 (3): 332-341, 2011
ISSN 1990-9233

Structure and Phylogenetic of Gnrh Genes of Hard-lipped Barb (*Osteochilus hasselti* C.V)

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Abstract: The gonadotropin-releasing hormone is known and named for its role as the final common signaling molecule used by the brain to regulate reproduction in all vertebrates. Two Genomic DNA GnRH and cDNAs of Hard-lipped barb carp, namely GnRH-II and GnRH-III, were firstly cloned from the brain reverse transcription-polymerase chain reaction (RT-PCR). The length of genomic DNA GnRH-II was 632 bp and cDNA GnRH-II was 253 bp, the length of genomic DNA GnRH-III was 486 bp and cDNA GnRH-II was 285 bp, respectively. The GnRH-II precursors encoded cDNA consisted of 84 amino acids and GnRH-III precursors encoded cDNA consisted of 95 amino acids, including a signal peptide, GnRH-II decapeptide and a GnRH-associated peptide (GAP) linked by a Gly-Lys-Arg proteolytic site. Recently, genes encoding two GnRH forms in hard-lipped barb have been discovered.

Key words: GnRH-II · GnRH-III · PCR · Amino Acid

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a conservative neurodecapeptide family, which plays a crucial role in regulating the gonad development and in controlling the final sexual maturation in vertebrate [1-3]. The GnRH decapeptide is synthesized by neurosecretory cells in the hypothalamus and secreted into portal vessels, to be transported to the pituitary gland where it stimulates secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gonadotrophs [4,5].

The presence of either two or three forms of GnRH in teleost fishes has been well documented [6]. 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), among others [2,6-9]. The GnRH-I system is generally localized in the forebrain and is considered to exert the neuroendocrine control over LH secretion. On the other hand, [His5 Trp7 Tyr8] GnRH (GnRH-II) also designated as GnRH-II [10-12] has been reported in all major vertebrate groups, including mammals and is mainly expressed in the midbrain [13]. GnRH-II appears to have direct effects on sexual behavior in mammals, birds and fish [14-18] and this effect is believed to be its primary function. Finally, GnRH-III is represented by salmon GnRH (sGnRH) [13,19] and is found in the forebrain either alone or together with GnRH-I depending on the species [8,19]. GnRH peptides also reported in ovary and testis of fish and in ovary, testis, mammary gland and placenta of mammals [13].

GnRH genes share the same basic structure. the GnRH preprohormone mRNA encodes GnRH and the GnRH-associated peptide (GAP), separated by a canonical cleavage site. The preprohormone mRNAs are encoded by four exons. Exon 1 encodes the 5??-UTR, Exon 2 encodes the signal peptide, GnRH decapeptide, the proteolytic cleavage site and the N-terminus of GAP, Exon 3 encodes the central portion of GAP and exon 4 encodes the C terminus of GAP along with the 3??-UTR [16].

In this study, Hard-lipped Barb (*Osteochilus hasselti* C.V an indigenous tropical fish [20] is a synchronous batch spawner fish [5] capable of spawning several time during the peak of the spawning period. GnRH-II and GnRH-III genes have been cloned from hard lipped barb brain tissue for the first time. In this study, the isolation and identification of two differing GnRH-II and GnRH-III cDNAs and genomic in the hard lipped barb are reported.

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MATERIALS AND METHODS

Brain Collection: Total RNA and genomic DNA were isolated from brain. Total 15 sexually mature female Hard-lipped Barb weighing of 100 g in average were purchased from local market in Indonesia. Fish brain were removed, frozen and stored at 150°C with liquid nitrogen until RNA and genomic extraction. Isolation, cloning and sequencing of GnRH-II and GnRH-III gene was measured at the Laboratory of Gene Function Animal, Nara Institute Science and Technology, Japan.

Genomic DNA Isolation: Total genomic DNA was extracted from whole brain using TNES, after that add RNAse and Proteinase K. After incubated 1 hour add phenol Cloroform. Sample were extraction based on Etanol-Phenol-chloroform extraction method [21]. The integrity of the DNA was verified in a denaturing agarose gel, stained with ethidium bromide.

RNA Isolation and RT-PCR: Total mRNA was extracted from whole brain using blue Sepasol R- RNA super 1 reagent (nacalai tesque), based on Etanol-phenol-chloroform extraction method. Sample were treatment with DNAse free RNAse (Takara). After that the quality and concentrations of total RNA were assayed by agarose gel electrophoresis and optical density reading at 260 and 280 nm, the RNA were loaded in batches and frozen at -70°C. Total mRNA sample (1,5 ng) was reverve transcript using cDNA synthezis kit (PrimeScript™ Reverse Transcriptase) from Takara and the cDNA was amplified by PCR.

cDNA Amplification: The primer pairs, Cyprinadae GnRH-II F contain *ecor*1 and Cyprinadae GnRH-II R contain *xho1*, were designed from cDNA *cyprinidae* like *cyprinus carpio* AY189961.1, *carrasius auratus*, U30386.1, *rutilus rutilus*, (U60668.1) and *ctenopharyngodon idella*, (EU981284.1). All sequence were aligment with multalin to found conserve region in ORF (Open Reading Frame) region. Primer were design using primer 3 software untill estimated the product to amplify the GnRH-II gene (Table 1).

The primers pairs, Cyprinadae GnRH-III F contain *ecor*1 and Cyprinadae GnRH-III R contain *xho1* were designed from *carrasius Auratus* (AB017271.1), *cyprinus carpio*, (AF521130.2), *rutilus rutilus*, (U60667.1), *ctenopharyngodon idella*, (EU981295.1) and *Danio Rerio*, (AY557019.1). All sequence were aligment with multalin to found conserve region in ORF (Open Reading Frame) region. Primer were design using primer 3 software untill estimated the product to amplify the GnRH-III gene. The genomic DNA also amplify with same primers to found genomic GnRH-II and GnRG-III gene in hard lipped-barb (Table 1).

Thirty cycles of PCR for hard lipped barb cGnRHs-II and GnRH-III were carried out using a thermal cycler (Robocycler, Stratagene) according to the step program of 95°C for 2 min, 35 cycles to 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, followed by a 5 min extension at 72°C [22]. 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 products amplified from genomic DNA and cDNA were separated by agarose gel electrophoresis and the incised gels were purified using the DNA gel extraction procedure [23]. The desired DNA fragments were subcloned into BSKS Eco R1/Xho 1 vector (10 ng) (Takara) and ligation with T4 ligase. Plasmid were transfection to E. coli and spread into LB medium [24]. The recombinant positive colonies were screened using ampicilin. Positive colonies were treatment with mini scale plasmid preparation for sequencing [23]. DNA sequences of these fragments were determined using the Big Dye version 3.1 sequencing method with specific primers. The data was automatically collected on the ABI PRISM 3100 Genetic Analyzer (PE Applied Bio-systems).

Sequence Analysis: Genomic DNA and cDNA sequences for GnRH-II gene and GnRH-III gene were checked using BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST/) were performed with default settings on the complete, non redundant GenBank database nucleotide sequences. After that genomic DNA and cDNA

Table 1: Primer were used to amplify the GnRH-II and GnRH-III

No	Primer code	Primers	PCR Product
1.	Cyprinidae GnRH-II F	GGA CCTAAG ATGGTGCACATCTGCAGGCT	253 bp
2.	Cyprinidae GnRH-II R	GGG CTCGAG TCTTTTGGAAATCCCGTATG	
3.	Cyprinidae GnRH-III F	GGA CCTAAG AGCATGGAGTGGAAAGGAAG	285 bp
4.	Cyprinidae GnRH-III R	GGG CTCGAG CACTCTTCCTCGTCTGTTGG	

sequences for GnRH-II gene and GnRH-III gene were aligned using CLUSTALW software to found intron and exon area.

Phylogenetic Analysis: For phylogenetic analyses, hard lipped barb cDNA GnRH-II was compared to cDNA GnRH-II sequences from nineteen fish species cDNA GnRH-III was compared to cDNA GnRH-III sequences from twentieth fish species. All sequences were retrieved from NCBI GenBank. The relationship was generated with CLUSTAL W with scoring method percent and and the unrooted tree was generated using Treeview version 1.5.2. [25].

RESULT

General Result: Amplification of genomic DNA and cDNA were successfully for GnRH-II and GnRH-III genes. For the genomic DNA the agarose gel electrophoresis showed a specific band, about 632 bp for GnRH-II and 476 bp for GnRH-III (Figure1). For the cDNA the agarose gel electrophoresis also showed a specific band, about 253 bp for GnRH-II and 285 bp for GnRH-III (Figure 1). The specific fragment was incised, reclaimed and subcloned into BSKS vector. Then, four positive colonies were sequenced.

Cloning of Genomic and cDNA GnRH-II in Hard Lipped Barb: Amplification of GnRH-II genes in hard lipped barb showed cDNA fragment contained the open reading frame of GnRH-II. The same result was obtained by sequencing multiple single clones and sequencing repeatedly, until consistent. Genomic GnRH-II show exclusive fragment, with the length of about 632 bp (JN867722) (Figure 1A). The cDNA fragment contained 3 exon (coding region) and 2 intron (non coding region) (Figure.2A). All intron-exon boundary sequences conform to the GT-AG rule. The cDNA including complete coding sequences, 253 bp in length (Figure 1A), was gotten, which was called GnRH-II cDNA (GenBank accession JN867720.). The corresponding mRNA and genomic DNA sequences were called GnRH-II, respectively.

Cloning of GnRH-III in Hard Lipped Barb: Sequence analysis showed that every cDNA fragment contained the open reading frame of GnRH-III. The same result was obtained by sequencing multiple single clones and sequencing repeatedly, until consistent. Genomic GnRH-III show exclusive fragment, with the length of about 476 bp (JN867723) (Figure 1B). The cDNA fragment contained 3 exon (coding region) and 2 intron (non coding region) (Figure 2B). All intron-exon boundary sequences conform to the GT-AG rule. The cDNA including complete coding sequences, 285 bp in length (Figure 1B) was gotten, which was called GnRH-III cDNA (GenBank accession JN867721). The corresponding mRNA and genomic DNA sequences were called GnRH-III.

Gene Structure: All GnRH genes (GnRH-II and GnRH-III) share the same basic structure. The GnRH-II preprohormone mRNA encodes GnRH and the GnRH-associated peptide (GAP), separated by a canonical cleavage site. The preprohormone mRNAs are encoded

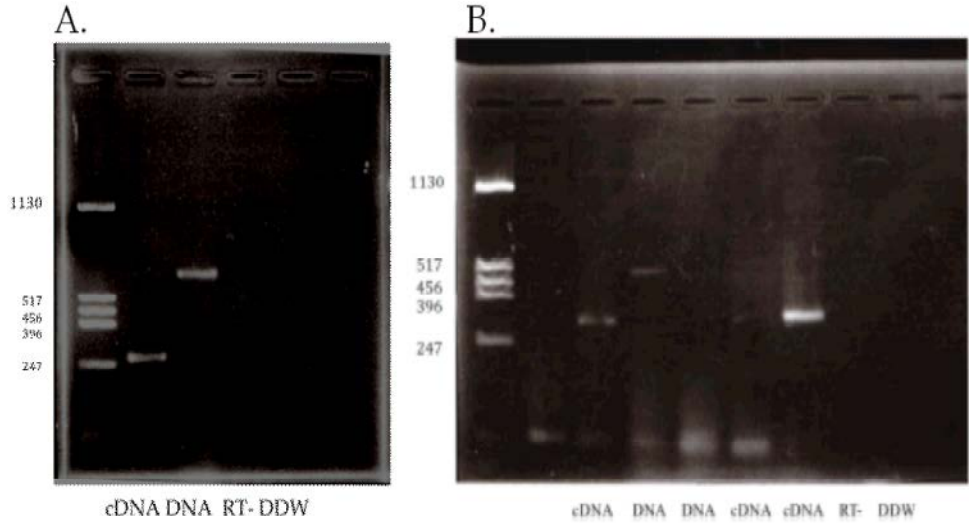

Fig. 1: PCR product amplification mRNA and genomic DNA ORF gene GnRH in Hard lipped-barb (Osteochillus Hasseltii, C.V), (A= GnRH-II, B=GnRH-III).

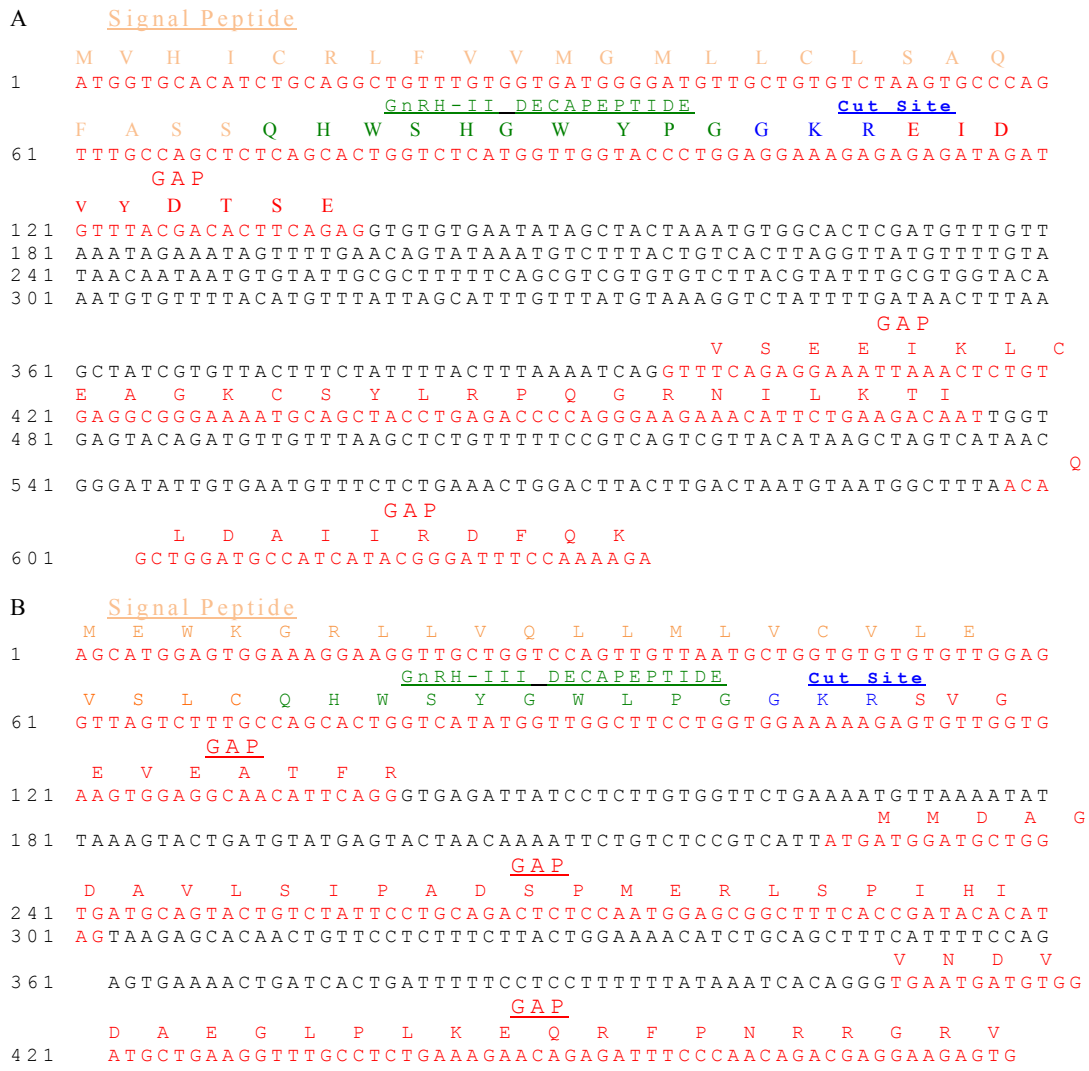

Fig. 2: Nucleotide sequences of GnRH-II and GnRH-III in hard lipped barb. A. Sequences of GnRH-II, B. Sequences of GnRH-III, C. Sequences of genomic GnRH-II and D. Sequences of genomic GnRH-III (Red color: exon, black color: intron).

by three exons (Figure. 2A). Exon 1 encodes the signal peptide, GnRH decapeptide, the proteolytic cleavage site and the N-terminus of GAP. Exon 2 encodes the central portion of GAP and exon 3 encodes the C terminus of GAP. Similar with GnRH-II preprohormone mRNA, the GnRH-III prehormone encodes GnRH and the GnRH-associated peptide (GAP), separated by a canonical cleavage site. The preprohormone mRNAs are encoded by three exons (Figure. 2B). Exon 1 encodes the signal peptide, GnRH decapeptide, the proteolytic cleavage site and the N-terminus of GAP. Exon 2 encodes the central portion of GAP and exon 3 encodes the C terminus of GAP.

Structure for GnRH-II and GnRH-III had most similar in length for exons 1 and 2, but the intron sizes for GnRH-II compared with GnRH-III are the most different. The level of similarity in the coding sequences can be read from the phylogenetic tree distances (Figure.7). The greatest differences within the preprohormone are within the GAP coding sequences. The striking contrast between conservation of the GnRH coding sequence and lack thereof in the GAP coding sequence is evidence of differential selective pressure within the gene. This is evident in cases where the identity and similarity of GnRH and GAP coding sequences have been compared for mRNAs of different GnRH genes within a species [9,16].

Phylogenetic Analyses: Phylogenetic analyses were performed to establish an evolutionary context for the GnRH-II and GnRH-III gene. Genetic distances (measured as substitutions per site) showed moderate low values and the topology was well-supported by strong bootstrap values. As expected, GnRH-II and GnRH-III in hard lipped barb was included within a sub-cluster of the *carp* (*cyprinus carpio, carrasius auratus*) with high bootstrap values (Figure 7).

DISCUSSION

This paper reports the clone of two differing cDNAs encoding the GnRH-II and GnRH-III from brain tissues of hard lipped barb for the first time. Comparison the GnRH-II gene structure with previously reported gene structures of other fish species shows a high conservation of exon size (Figure 4). The nucleotide sequence identity of GnRH-II cDNAs was 96% with carp (*cyprinus carpio* AY189961.1), 95 % with goldfish (*carrasius auratus*, U30386.1), 94 % with roach (*rutilus rutilus*, U60668.1) and 94% with grass carp (*ctenopharyngodon idella*, EU981284.1). The GnRH-II precursor encoded by cDNAs contained 84 amino acid residues. The GnRH-II precursor was composed of a 24 amino acids signal peptide, GnRH-II decapeptide and a 47 amino acids GAP linked by the processing site (Gly-Lys-Arg) (Figure. 2A).

The nucleotide sequence identity of GnRH-III cDNAs was 95% with goldfish(*carrasius Auratus*, AB017271.1), 94 % with carp (*cyprinus carpio*, AF521130.2) 92 % with roach (*rutilus rutilus*, U60667.1), 92% with grass carp (*ctenopharyngodon idella*, EU981295.1) and 91% with zebrafish (*Danio Rerio*, AY557019.1) (Figure.5). The GnRH-III precursor encoded by cDNAs contained 95 amino acid residues. The GnRH-III precursor was composed of a 23 amino acids signal peptide, GnRH-III decapeptide and a 59 amino acids GAP linked by the processing site (Gly-Lys-Arg) (Figure 2B). The structure characteristics of GnRH-II and GnRH-III precursors in Hard Lipped Barb are similar with the other GnRH variants. The results showed that GnRH-II gene and other GnRH genes might evolve from a common ancestral molecule.

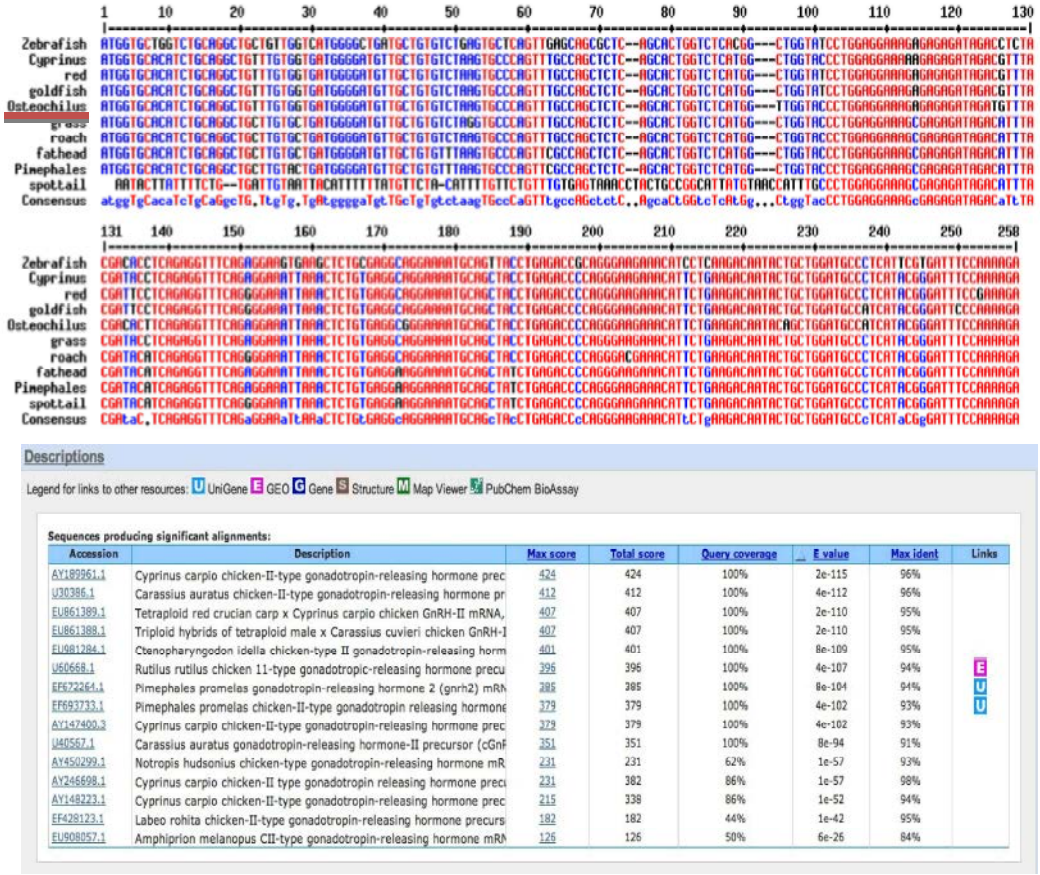

Fig. 4: Nucleotides alignment and BLAST of GnRH-II cDNA in hard lipped barb with another teleost.

Fig. 5: Nucleotides alignment and BLAST of GnRH-III cDNA in hard lipped barb with another teleost.

From this result showed that Hard-lipped barb had a high similarity with carp and goldfish. For the GnRH-II maximum identity were *cyprinus carpio* (96%) and for the GnRH-III maximum identity were *carrasius auratus* (95%). From this result we suggest that hard lipped barb only had two molecule form of GnRH like GnRH-II and GnRH-III same with carp [18,26,27] and with goldfish [28-31].

The amino acid sequences of common carp GnRH-II precursors encoded by cDNA were compared with that of some identified GnRH-II precursors (table 6A), such as the precursors of teleost fishes roach (*Rutilus rutilus*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), grass carp (*ctenopharyngodon idella*), Zebrafish (*danio rerio*)..The result showed that the amino acid homology of GnRH-II precursors between cyprinoids was 84-97%. However, when comparing with some other teleosts GnRH-II precursors, the amino acid homology of GnRH-II precursors in teleosts was only 40-67%.

The amino acid sequences of common carp GnRH-III precursors encoded by cDNA were compared with that of some identified GnRH-II precursors (table 6B), such as the precursors of teleost fishes roach (*Rutilus rutilus*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), Zebrafish (*danio rerio*)..The result showed that the amino acid homology of GnRH-III precursors between cyprinoids was 83-94%. However, when comparing with some other teleosts GnRH-III precursors, the amino acid homology of GnRH-III precursors in teleosts was only 35-64%.

The comparison results of amino acid sequences of GnRH-II and GnRH-III precursors from different vertebrates showed that the GnRH-II and GnRH-III decapeptide and adjacent processing site (Gly-Lys-Arg) were very conservative. A signal peptide is a peptide chain that directs the transport of a protein. So, both the encoding characteristic of GnRH-II and

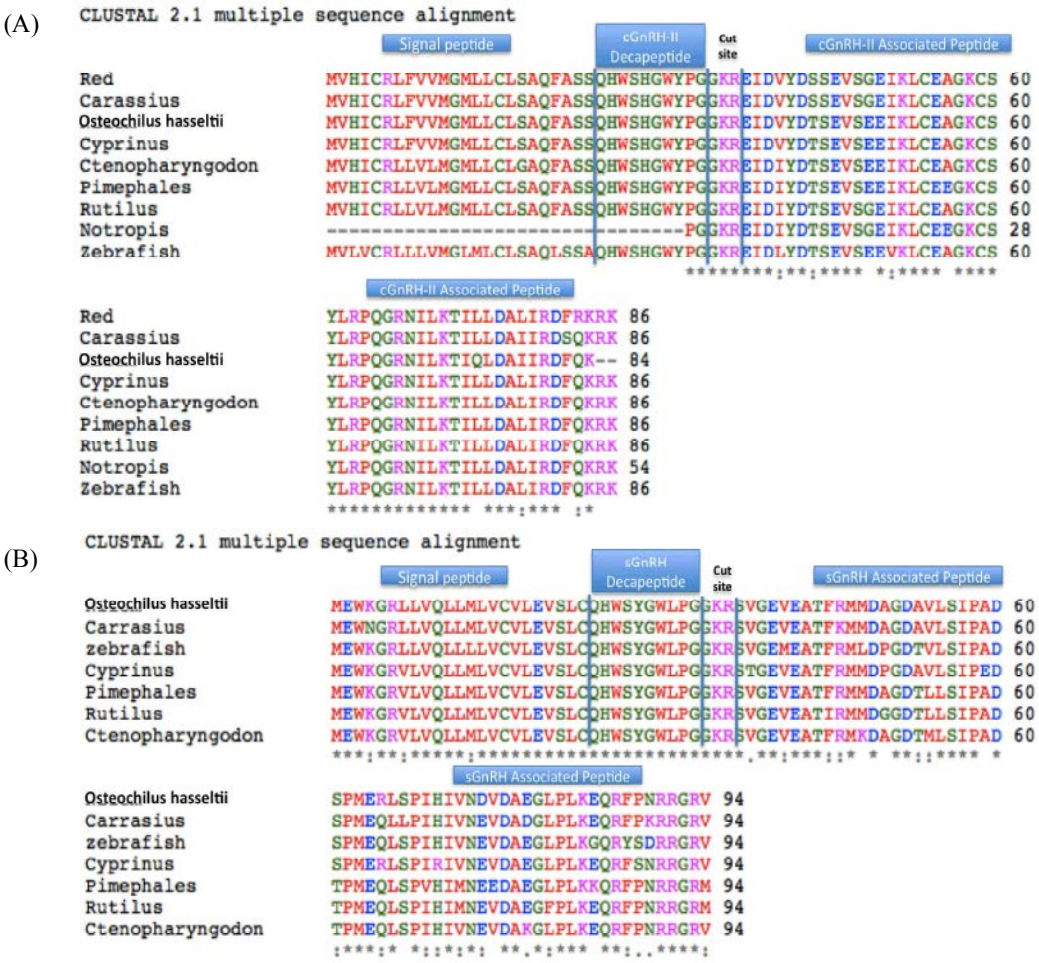

Fig. 6: Amino acids alignment of GnRH cDNA in hard lipped barb with another teleost. A: Amino acids alignment of GnRH-II cDNA and B: Amino acids alignment of GnRH-III cDNA.

GnRH-III cDNA decapeptide and processing site were entirely conservative in vertebrate evolution. However, the amino acid divergence of GnRH-II and GnRH-III signal peptide and GAP between vertebrates from different evolution lineages was much higher than that between neighboring species. Then, it was presumed that the function of GnRH-II and GnRH-III peptide might change in different evolution lineages for adapting the natural selection during evolution.

The present study is the first description of GnRH-II and GnRH-III genes in hard lipped barb, providing new evolutionary information on this gene family in the brain. GnRH-II and GnRH-III in hard lipped barb is grouped together with other teleost in the phylogenetic tree, suggesting a common ancestor for both groups of genes. Phylogenetic analysis shows that GnRH-II can be separated into 3 major groups. Subgroup I contains GnRH-II from goldfish (*carrasius auratus*), carp (*cyprinus carpio*), sub group II from nila (*orechromus nilaticus*) until *thunus thunus*, and third subgroup were lates (Figure.7). This phylogenetic trees means that hard lipped barb were evolution from carp (*cyprinus carpio*).

Phylogenetic analysis shows that GnRH-III can be separated into 3 major groups. Subgroup I contains GnRH-III from goldfish (*carrasius auratus*), carp (*cyprinus carpio*), sub group II from nila (*orechromis nilaticus*) and third subgroup were thunnus thunnus (Fig.7). This phylogenetic trees means that hard lipped barb were evolution from the other teleost such as goldfish (*carrasius auratus*) and carp (*cyprinus carpio*). For GnRH-II and GnRH-III are similar to teleost GnRH, indicated they also have some same features and function, for example with pejjerey [7] and carp [27].

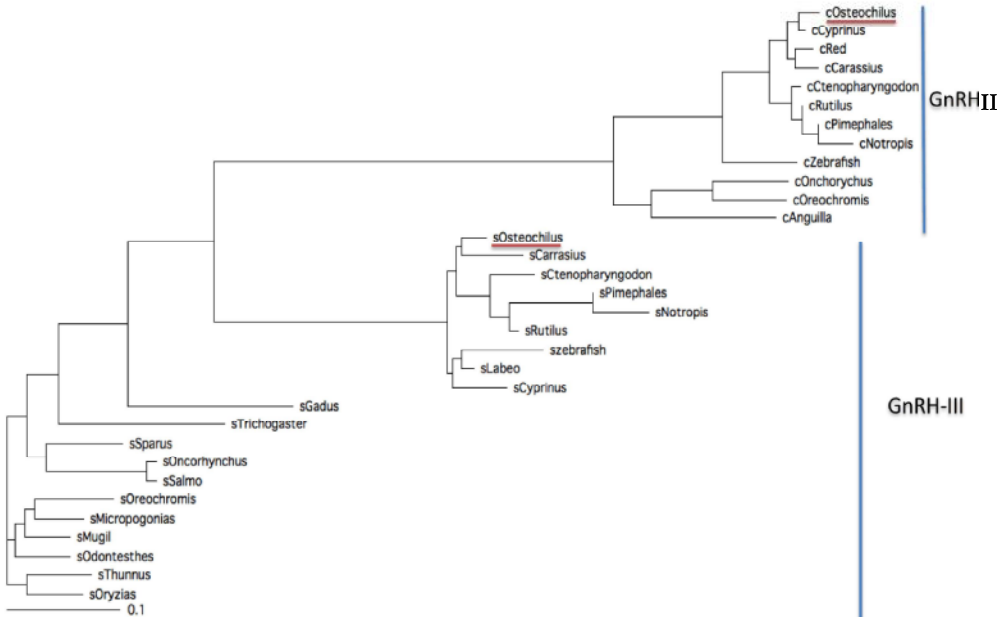

Fig. 7: Phylogenetic relationship of precursors derived from known amino acid encoding gonadotropin-releasing hormone (GnRH). The relationship was generated with CLUSTAL W and the unrooted tree was generated using Treeview version 1.5.2. The scale bar represents the estimated evolutionary distance as 0.1 amino acid substitutions per site.

In summary, the present work has reported for the first time the genomic DNA and cDNA sequence of two GnRH variants in an Hard-lipped barb, the phylogenetic results presented in this work support the idea that all GnRH genes share the same basic structure. That was meaning GnRH-II and GnRH-III in Hard lipped barb very conserve, that assumed had a same function with another teleost. We also suggest that hard lipped barb only had two molecule form of GnRH type like GnRH-II and GnRH-III.

ACKNOWLEDGEMENTS

This work was supported by the Sandwich Grant from Gadjah Mada University Indonesia in colaboration with Nara Institute Science and Technology Japan. Also Thanks to Dr. Chio Oka and dr. Supandji from their assistance and knowledge.

APPENDIX 1

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

GnRH II clade: Anguilla japonica: AB026990; Carassius auratus: U30386; Clarias gariepinus: X78047; Coregonus clupeaformis:
AY245102; Cyprinus carpio: AY147400; Danio rerio: AF511531; Dicentrarchus labrax: AF224281; Macaca mulatta: AF097356; Micropogo- nias undulatus: AY324669; Monopterus albus: AY786183; Morone saxatilis: AF056313; Mugil cephalus: AY373451; Odontesthes bonariensis: AY744687; Oncorhynchus mykiss: AF125973; Oreochromis niloticus: AB101666; Oryzias latipes: AB041330; Rutilus rutilus: U60668; Sciaenops ocellatus: AY677171; Sparus aurata: U30325; Suncus murinus: AF107315; Trichosurus vulpecula: AF193516; Tupaia belangeri: U63327; Typhlonectes natans: AF167558; Verasper moseri: AB066359.

GnRH III clade: Carassius auratus: AB017271; Coregonus clupeaformis: AY245103; Cyprinus carpio: AY189960; Danio rerio: NM_182887; Dicentrarchus labrax: AF224280; Micropogonias undulatus: AY324670; Monopterus albus: AY858055; Mugil cephalus: AY373449; dontesthes bonariensis: AY744688; Oncorhynchus mykiss: AF232212; Oreochromis niloticus: AB101667; Oryzias latipes: AB041332; Pagrus major: D26108; Porichthys notatus: U41669; Rutilus rutilus: U60667; Salmo salar: X79709; Salmo trutta: X79713: AB047325; Sparus aurata: U30311.

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