Original Article

Molecular characterization of gonadotropin-releasing hormone (GnRH) in river catfish, *Hemibagrus nemurus* (Valenciennes 1840)

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Abstract: Gonadotropin-releasing hormone (GnRH) is the foremost neuroendocrine peptide required in the reproduction system. Characterization and the involvement of GnRH in fish reproduction, especially in fish species has been complicated by the discovery of multiple GnRH forms. In this paper, we determined the molecular characterization and phylogenetic analysis of GnRH1 and GnRH2 genes in a commercially cultured catfish, *Hemibagrus nemurus*. This species is a high-demand freshwater fish worldwide especially in the Asia Pacific regions due to its thick flesh and high nutritional value. Problems in their breeding restrict the production of this species in captivity. Therefore, a thorough study of the GnRH genes is important due to their critical role in stimulating the secretion of gonadotropins hormone, which leads to the release of steroid hormones and activates the reproduction system. A complete open reading frame (ORF) of GnRH1 and GnRH2 genes was obtained through PCR amplification and cloned into TOPO® TA Cloning® kit, following sequence assembly and phylogenetic analysis. Phylogenetic analysis revealed the GnRH1 and GnRH2 of *H. nemurus* were clustered with Siluriformes, consisting of mostly catfish species including *Pangasius nasutus* and *Pangasianodon hypopthalmus*. The cDNA of GnRH1 was 371 bp with an ORF of 262 bp encoding a highly variable 81 amino acids, while the cDNA of GnRH2 was 376 bp with an ORF of 260 bp encoding a highly conserved 87 amino acids. This study could offer an advanced idea to develop a new GnRH agonist for artificial breeding of *H. nemurus* and other catfish sp.

Introduction

The hypothalamus-pituitary-gonad (HPG) axis plays a fundamental role in the endocrine regulation of the reproductive system. The HPG axis begins with the alteration in environmental factors such as temperature, photoperiod, food, and stress through neurotransmitters and neuropeptides (Chaube et al., 2019). Gonadotropin-releasing hormone (GnRH) is the main neuroendocrine peptide required in the reproduction system of fish and other vertebrates. They are decapeptide hormones that stimulate the release of gonadotropin hormones (GTH) from the anterior pituitary, and later the GTH will act on the gonad to induce oogenesis and spermatogenesis by stimulating the production of sex steroids (Honji et al.,

2019). In addition, GnRH as a neuromodulator has also been regulated in feeding and reproductive behavior in many organisms, including teleost (Yamamoto et al., 1997; Volkoff and Peter, 1999; Xia et al., 2014).

There are up to 14 forms of GnRH in vertebrates classified into three forms GnRH1, GnRH2, and GnRH3 (Whitlock et al., 2019; Sahadan et al., 2022). The brain consists of two or three forms that are species-specific-type GnRH or seabream GnRH (GnRH1), chicken GnRH (GnRH2), and salmon GnRH (GnRH3) (Nyuji et al., 2020; Sahadan et al., 2022). It had been widely accepted that most bony fishes possess two forms of GnRHs but later works showed the presence of three forms of GnRH in

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perciforms (Weltzien et al., 2004). Catfish, however, do not have this third isoform, possessing only GnRH1 and GnRH2. They display two distinct populations of GnRH1 within the brain, in addition to GnRH2 in the midbrain (Dubois et al., 2001). GnRH1 is more directly involved in the coordination of reproduction and appetite in the catfish, while GnRH2 plays less of a role in nutrition and has more function as a neuromodulatory in the reproductive system (Okubo and Nagahama, 2008).

According to FAO (2022), though carp are still the most cultured fish worldwide, catfishes have surged past tilapia to take the number two spot globally in the past few years. Asian river catfish, *Hemibagrus nemurus*, is an important economic species for fisheries and aquaculture in Southeast Asian countries such as Malaysia, Vietnam, Thailand, Cambodia, and Indonesia (Adebiyi et al., 2013). The edible fillet of *H. nemurus* has high protein and nutritional values (Adebiyi et al., 2013; Othman et al., 2015; Hasan et al., 2022). Due to its high demand and market price (up to USD 5.46/kg) (Hasan et al., 2016), this species is suggested as a potential species for aquaculture (Kusmini et al., 2020). The production of *H. nemurus* has become significant in Malaysia after the success of its artificial breeding program and the development of grow-out feed (Kamarudin et al., 2011; Sahadan et al., 2022). Extensive research on different biological aspects of *H. nemurus* has been carried out including genetic variation and population (Yang et al., 2014; Syaifudin et al., 2017), nutrition (Farhana et al., 2015; Thongprajukaew and Rodjaroen, 2017; Hasan et al., 2019) and morphology (Aryani et al., 2013).

In 2021, the Department of Fisheries Malaysia (DOF) reported the annual production of *H. nemurus* was only 1.26% of the total aquaculture production. Most hatcheries faced problems in synchronizing the maturity of male and female brood stock, and its slow growth to fulfill the market demand (Ina-Salwany et al., 2019). There were limited studies on the reproductive physiology and biology of *H. nemurus*, which could provide solutions to improve the artificial reproduction in this species. Therefore, the study of the GnRH hormone of this species could help to better

understand solutions to improve artificial breeding programs to increase the productivity of *H. nemurus* in captivity.

There are previous studies that showed positive results of GnRH agonist (GnRHa) in fish reproduction. For example, the implantation of GnRHa with domperidone is more effective as it induces vitellogenesis and ovulation in the premature female *Pagrus major* (red seabream) (Kumakura et al., 2003). Besides, the study of the effect of GnRHa in *H. nemurus* on sex hormones showed the success of GnRHa treatment in stimulating steroid production through an optimum dose (Adebiyi et al., 2013). Hence, this study aimed to determine the GnRH genes of *H. nemurus*, and the findings could be further analyzed for the development of biologically active GnRH agonist for artificial spawning in fish, thus increasing *H. nemurus* production in the aquaculture. To better understand the phylogenetic diversity and evolution of GnRH hormone, we amplified and cloned the complete open reading frame (ORF) cDNAs of GnRH genes *H. nemurus*, aligned their amino acid sequences, and compared them to other fish species and other vertebrates.

Materials and Methods

Ethical Statement: The use of animals in this study was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia with the referral number UPM/IACUC/AUP-R026/2022.

Samples collection: A total of six adults *H. nemurus* (weight: 400-500 g; length: 30-40 cm) were obtained from Three Oceans Fish Pond and Trading, Rawang, Malaysia. The fish were euthanized using MS 222 (tricaine, methane, sulfonate; 500 mg L^{-1}) following decapitation by the guidelines of the institutional animal care and use committee, Universiti Putra Malaysia. The brain tissues were collected from each fish and kept in a 1.5 ml tube immersed with RNA*later*™ solution (Ambion, Austin, TX, USA). Then, the samples were stored at -80°C until further analysis.

RNA extraction and cDNA reverse-transcription:

Table 1. Oligonucleotide primers used for detection and full-length amplification of *Hemibagrus nemurus* GnRH genes.

Genes	Primers	Product length	Primer sequences (5'-3')	Reference
GnRH1	Forward	~344 bp	GCATCAGATTGTAGAGCTCA	In this study
	Reverse		GAAGCATTTTATTGAAGATC	
GnRH2	Forward	~388 bp	GACCTGCACATCTCAGAAGAT	In this study
	Reverse		CAGTGGGATGAGCGAAGAAA	
GnRH3	Forward	~308bp	TGGAAGTGAGCAGCAGAGTG	In this study
	Reverse		TCCTCCTGTACCCATCATCC	

Total RNA from each sample was homogenized and extracted using TRIzol reagent (Invitrogen, Carlsbad USA); a monophasic solution of phenol and guanine isothiocyanate, following manufacturer's procedures with some modifications. In brief, the sample was homogenized using 400 µl of TRIzol solution, and later 200 µl of chloroform was added to the sample, vortexed for 5-10 sec, and centrifuged at 10,000 g for 10 min at 10°C. Approximately, 600 µl isopropanol was then added to the homogenized sample and incubated for 30 min for RNA precipitation. The washing step was performed twice using 1 ml 75% and 100% ethanol following centrifugation at 10,000 g for 10 min at 4°C. The RNA pellet was resuspended in 20 µl nuclease-free water. The purified RNA was stored at 20°C until further use.

Next, QuantiTect® Reverse Transcription Kit (Qiagen, Germany) was used to convert the total RNA to cDNA following the manufacturer's protocols. First, 1 µl of total RNA was mixed with 2 µl of gDNA Removal Mix and nuclease-free water for a final volume of 14 µl. The samples were incubated at 42°C for 8 min using Mastercycler Gradient (Eppendorf, Germany). Later, the reverse-transcription process continued by adding 4 µl of 5X Quantiscript Reverse Transcript Buffer, 1 µl of RT primer, and 1 µl of Quantiscript Reverse Transcriptase Enzyme into the total RNA mixture from the previous reaction. The mixture was incubated at 42°C for 30 min, followed by Quantiscript inactivation at 95°C for 3 min by using the Mastercycler gradient, and stored at 20°C until further used.

PCR amplification of GnRH1, GnRH2 and GnRH3 genes in *H. nemurus*: Primers for full-length sequences of GnRH1 and GnRH2 were designed using OligoAnalyzer™ Tool available online (https://sg.idtdna.com/calc/analyzer) based on predicted *Pangasianodon hypophthalmus* GnRH1 and GnRH2 genes (Genbank accession numbers XM_026924976.1 for GnRH1 and XM_026929077.2 for GnRH2). For GnRH3, primers were designed based on conserved regions of other fish species, due to the unavailability of the GnRH3 sequence of predicted *P. hypophthalmus* in GenBank. For effective DNA amplification, the selected forward and reverse primers were also analysed to avoid any potential hairpin and primer-dimer interactions formed during PCR amplification. The gene-specific primers used for each PCR are shown in Table 1.

The PCR mixture for amplification of GnRH (GnRH1, GnRH2, and GnRH3) genes were performed in a final volume of 25 µl containing 10 ng of cDNA, 12.5 µl exTEN 2X PCR Master Mix (1xPCR buffer, 3 mM MgCl$_2$, 400 µM dNTP mix, 0.08 U/µl exTEN DNA Polymerase; 1st BASE, Singapore) and 25 µM of each forward and reverse primer (Table 1). The final volume was adjusted by adding nuclease-free water. The PCR cycles for amplification were as follows: 95°C for 7 min, 35 cycles of 95°C for 1 min, 55-60°C for 1 min, and 72°C for 1:30 min. The elongation step was extended at 72°C for 10 min.

Cloning, transformation, and screening of positive PCR clones of *H. nemurus*: The PCR products were resolved using 1.5% agarose gel electrophoresis, purified using GeneJET Gel Extraction kit (ThermoScientific, Waltham, MA) following manufacturer's protocols, and cloned into TOPO® TA Cloning kit (Invitrogen, Carlsbad, CA). The ligation process was performed by adding 3 µl of the purified PCR product into 1 µl of cloning vector, following

Figure 1. Gel electrophoresis for the detection and full-length amplification of GnRH1, GnRH2, and GnRH3. GnRH1 shows a clear band at ~344 bp and GnRH2 at ~388 bp as estimated length. GnRH3 and negative control shows no band.

protocols suggested by the manufacturer.

For the transformation process, 3 µl of the ligation mixture was added into a vial containing TOP 10 competent cells (Invitrogen, Carlsbad, CA). The mixture was incubated on ice for 5 min followed by heat shock for 30 sec at 42°C without shaking. The mixture was directly transferred into ice, followed by adding 250 µl of a pre-warmed Super Optimal Culture (SOC) medium into the vial. The vial was shaken horizontally for 1 h at 200 rpm at 37°C. Finally, 10-50 µl of the transformed cells were grown on a pre-warmed Luria-Bertani (LB) agar containing 50 µg/ml ampicillin and 40 µg/ml of X-gal and incubated overnight at 37°C.

Screening of the positive recombinant clones was performed by selecting white colonies grown on the LB agar (1st BASE, Singapore) supplemented with 50 µg/ml ampicillin and 40 µg/ml X-gal and amplified using specific primer pairs that hybridized the insert. The positive clones were then cultured in LB broth media (1st BASE, Singapore) containing 50 µg/ml ampicillin and incubated overnight at 37°C. Plasmid extraction was performed from the culture using GeneJET Plasmid Miniprep Kit Thermo Scientific™ following manufacturer instructions (Thermo-Fisher Scientific, Waltham, MA, USA). The plasmid was sent for DNA sequencing (1st BASE Laboratories, Malaysia) using universal primers, M13F (20) and M13R (20).

Sequence assembly and analysis: The DNA sequences were subjected to homology search using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) provided by the National Center for Biotechnology Information (NCBI) website. Later, the nucleotide sequences were translated into amino acids following multiple sequence alignment analysis using the Bioedit program (http://www.mbio.ncsu.edu/bioedit /bioedit.html). Comparison between GnRH1 and GnRH2 genes of *H. nemurus* to other fishes and vertebrates were performed by comparing amino acid sequences of each species obtained from the GenBank (http://www.ncbi.nlm.nih.gov). The distance matrix was performed to calculate GnRH gene percentage identities between species.

Phylogenetic analysis: The phylogenetic tree of GnRH genes of *H. nemurus* and other vertebrates was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 10, applying the Neighbor-Joining method. The robustness of the inferred nodes was evaluated using a bootstrapping procedure over 1000 replicates.

Results
DNA amplification of complete open reading frame (ORF) of GnRH1 and GnRH2 *H. nemurus* and BLASTn analysis: High band intensity of GnRH genes was detected using the brain samples, which was able to amplify the complete open reading frame (ORF) of GnRH1 and GnRH2 genes from *H. nemurus*. The PCR product of GnRH1 was detected at 344 bp, while GnRH2 was detected at 388 bp (Fig. 1). However, there was no band detected for GnRH3. Cloning of the GnRH genes was successfully ligated in the TOPO® vector (Invitrogen, Carlsbad, CA), and DNA sequencing was performed using the Sanger method.

Through BLASTn analysis of the GnRH1 gene, *H. nemurus* shared the highest similarity up to 100% with *P. hypophthalmus* (Iridescent shark catfish), 83% with *Ictalurus punctatus* (Channel catfish), 93% with *Silurus meridionalis* (Southern catfish) and 89% with *Tachysurus fulvidraco* (yellowhead catfish) (Table 2). Meanwhile, for GnRH2, *H. nemurus* shared the
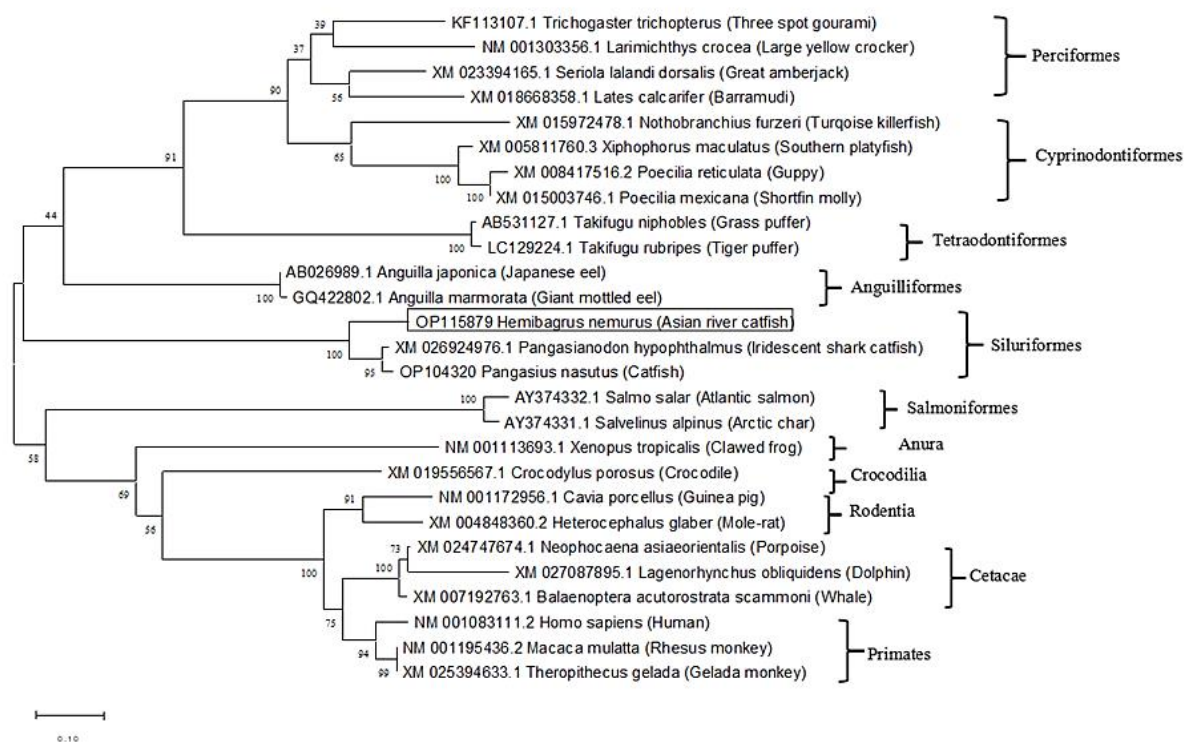
Figure 2. Phylogenetic tree for the GnRH1 gene. Bootstrap analysis was performed using the Neighbor-Joining method. The bootstrap value proportions are shown at the forks.

highest homology up to 98% with *T. fulvidraco,* 95% with *Heteropneustes fossillis* (Asian stinging catfish), 81% with *Clarias gariepinus* (African sharptooth catfish) and 78% with *Astyanax altiparanae* (Yellowtail tetrafish) (Table 3).

Multiple sequence alignment of GnRH1 and GnRH2 genes of *H. nemurus* and other vertebrates: The full-length cDNA of GnRH1 was 371 bp; starting with signal peptide from 1-79 bp, an open reading frame (ORF) at 80-246 bp, and stop codon at 247 bp, which encoded for 81 amino acids (aa) (Table 5; GenBank accession no. OP115879). The translation of aa consisting of 21 signal peptides, GnRH1 decapeptide sequences, QHWSHGLNPG from 31 to 40 (Table 5), 3-amino acid signal processing site, GKR, and 47 aa GnRH-associated peptides.

The full-length cDNA of GnRH2 was 388 bp; starting with signal peptide from 1-57 bp, an open reading frame (ORF) at 58-324 bp, and stop codon at 325 bp, which encoded a highly conserved protein of 87 amino acids (aa) (Fig. 3; GenBank accession no.

OP115878). The translation of aa consisting of the 24-amino acid signal peptide, GnRH2 decapeptide sequences, QHWSHGWYPG from 25 to 34, 3-amino acid signal processing site, GKR, and 50 aa GnRH-associated peptides.

The multiple sequence alignment of proteins GnRH1 and GnRH2 (Tables 7, 8) showed the evolutionary relationships between species and their relationship between genes. For GnRH1, there was a conserved aa position at 39-43 bp for all aligned fishes and vertebrates. The aligned GnRH1 decapeptide between the catfish family species: *H. nemurus* (Asian river catfish)*, P. hypopthalmus* (Striped catfish), and *Pangasius nasutus* (Silver catfish) were similar from aa position 31 to 44 including the signal processing site, [31]QHWSHGLNPGGKR[44]. There were aa differences at position 36 following their order, where histidine (H) was found in all Siluriformes, whereas for Salmoniformes and other fish orders were replaced with either tyrosine (Y) or phenylalanine (F). There is also aa substitution at positions 38 and 39 identified between the catfish family in Siluriformes with other

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Table 2. BLASTn sequence analysis showed four fish species have similarities with GnRH1 in *Hemibagrus nemurus*. The highest percentage was 100% with *Pangasionodon hypophthalmus* and the lowest percentage among the four species was 82% with *Ictalurus punctatus*.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E Value	%Identity	Acc. Len	Accession
1	Predicted: *Pangasianodon hypophthalmus* progonadoliberin-1 (LOC113533115), mRNA	*Pangasionodon hypophthalmus*	784	784	100%	0.0	100.00%	424	XM_026924976
2	Predicted: *Ictalurus punctatus* progonadoliberin- 1 (LOC108265734), transcript variant 1, mRNA	*Ictalurus punctatus*	520	520	83%	9e-143	92.82%	362	XM_047155459
3	Predicted: *Ictalurus punctatus* progonadoliberin-1 (LOC108265734), transcript variant X2, mRNA	*Ictalurus punctatus*	492	492	82%	2e-134	91.69%	361	XM_017468372
4	Predicted: *Silurus meridionalis* progonadoliberin-1-like (LOC124400249), transcript variant X2, mRNA	*Silurus meridionalis*	468	468	93%	3e-127	87.93%	493	XM_046871947
5	Predicted: *Tachysurus fulvidraco* progonadoliberin-1 (LOC113656283), transcript variant X1, mRNA	*Tachysurus fulvidraco*	399	399	89%	1e-106	86.08%	540	XM_027167480.2

Table 3. The sequence analysis shows four fish species have similarities with GnRH2 in *Hemibagrus nemurus*. The highest percentage was 98% with *Tachysurus fulvidraco* and the lowest percentage among the four species was 78% with *Astyanax altiparanae*.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E Value	% Identity	Acc. Len	Accession
1	Predicted: *Tachysurus fulvidraco* gonadotropin-releasing hormone 2 (GnRH2)	*Tachysurus fulvidraco*	459	459	98%	2e-124	91.37%	1522	XM_027133341.2
2	*Tachysurus fulvidraco* progonadoliberin2 precursor (GnRH2), mRNA, complete cds	*Tachysurus fulvidraco*	420	420	90%	8e-113	91.26%	347	KJ396290.1
3	*Heteropneustes fossillis* gonadotropin-releasing hormone 2(GnRH2), mRNA, complete cds	*Heteropneustes fossillis*	377	377	95%	5e-100	87.69%	537	MF166829.1
4	*Clarias gariepinus* pre-pro-chicen gonadotropin-releasing hormone	*Clarias gariepinus*	375	375	81%	2e-99	91.30%	546	X78047.1
5	*Astyanax altiparanae* voucher MZUSP 113746 g onadotropin-releasing hormone GnRH2 preproprotein	*Astyanax altiparanae*	255	255	78%	2e-63	84.15%	612	KP863468.1

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Table 4. Distance matrix of mature proteins between *Hemibagrus nemurus* (Asian river catfish) with other vertebrates and GenBank accession numbers of GnRH1 and GnRH2 used for sequences identities and phylogenetic tree.

Order	GnRH1 identities (%)	GnRH2 identities (%)	GenBank Accession numbers GnRH1	GenBank Accession numbers GnRH2
Siluriformes				
Hemibagrus nemurus (Asian river catfish)	-	-	OP115879	OP115878
Pangasianodon hypophthalmus (Iriscedent shark)	99.76	99.79	XM_026924976.1	XM_026929077.2
Pangasius nasutus, (Catfish)	99.71	99.77	OP104320	OP104321
Salmoniformes				
Salmo salar (Atlantic salmon)	75.0	91.29	AY374332.1	AY374332.1
Salvelinus alpinus, (Arctic char)	79.88	91.29	AY374331.1	AY374334.1
Tetraodontiformes				
Takifugu niphobles (Grass puffer)	93.93	93.09	AB531127.1	AB531128.1
Takifugu rubripes (Tiger puffer)	94.0	93.08	LC129224.1	LC129225.1
Anguilliformes				
Anguilla japonica (Japanese eel)	93.80	99.18	AB026989.1	AB026990.1
Anguilla marmorata (Giant mottled eel)	93.80	99.15	GQ422802.1	GQ422803.1
Perciformes				
Trichogaster trichopterus (Three spot gourami)	78.10	92.69	KF113107.1	KF113108.1
Larimichthys crocea (Large yellow crocker)	77.18	91.34	NM_001303356.1	XM_010743322.3
Seriola lalandi dorsalis (Great amberjack)	93.08	91.94	XM_023394165.1	XM_023428006.1
Lates calcarifer (Barramudi)	94.37	91.83	XM_018668358.1	XM_018700270.1
Cyprinodontiformes				
Xiphophorus maculatus (Southern platyfish)	92.83	91.46	XM_005811760.3	XM_005808463.2
Nothobranchius furzeri (Turqoise killerfish)	92.14	87.41	XM_015972478.1	XM_015959339.1
Poecilia reticulata (Guppy)	93.16	91.46	XM_008417516.2	XM_008409509.2
Poecilia mexicana (Shortfin molly)	93.17	91.40	XM_015003746.1	XM_015002583.1
Cetacae				
Neophocaena asiaeorientalis (Porpoise)	76.59	91.61	XM_024747674.1	XM_024743041.1
Lagenorhynchus obliquidens (Dolphin)	67.98	92.77	XM_027087895.1	XM_027078754.1
Balaenoptera acutorostrata scammoni (Whale)	54.24	89.97	XM_007192763.1	XM_007195899.1
Rodentia				
Cavia porcellus (Guinea pig)	92.06	96.52	NM_001172956.1	XM_023560164.1
Heterocephalus glaber mole-rat	93.01	94.06	XM_004848360.2	XM_004840693.3
Anura				
Xenopus tropicalis (Clawed frog)	90.81	98.01	NM_001113693.1	NM_001114078.1
Crocodilia				
Crocodylus porosus, (Crocodile)	91.50	94.39	XM_019556567.1	XM_019553429.1
Primates				
Homo sapiens (Human)	87.80	93.58	NM_001083111.2	NM_001501.2
Macaca mulatta (Rhesus monkey)	86.99	97.20	NM_001195436.2	NM_001034202.1
Theropithecus gelada (Gelada monkey)	54.227	93.91	XM_025394633.1	XM_025400095.1

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Table 5. Nucleotide sequences of GnRH1 gene from *Hemibagrus nemurus*. This sequence has been deposited in the GenBank nucleotide database, under accession no. OP115879.

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1    TGCATCAGATTGTAGAGCTCATCGCTGTGTGAAGAGCAGCAGAAGCGGGCGTCTGTAGTGCGACTGATCGAAGCAGGTGTTCAGG                              79

80   ATG  AGT  GTG  AAG  CGA  GCG  CTC  TGG  TGG  ATG  GTG  GTG  TGT  GTG  GTG  GTG   128
1     M    S    V    K    R    A    L    W    W    M    V    V    C    V    V    V    16

129  CTG  CAG  GTG  AGC  GCT  CAG  CAC  TGG  TCT  CAT  GGC  CTG  AAT  CCG  GGA  GGA   176
17    L    Q    V    S    A   *Q*  *H*  *W*  *S*  *H*  *G*  *L*  *N*  *P*  *G*  _G_   32

177  AAG  CGT  GCC  ATG  GCA  GAA  GAG  CCT  CGG  AAT  GCA  GAT  TCA  GCA  GCC  TCC   225
33   _K_  _R_   A    M    A    E    E    P    R    N    A    D    S    A    A    S    48

226  GGC  TAC  GTG  TGC  GAT  GCA  TAT  GAC  CGG  AAT  AAA  CGA  GAA  ACT  ATC  TAC   274
49    G    Y    V    C    D    A    Y    D    R    N    K    R    E    T    I    Y    64

275  AGA  CTG  AAG  GAT  AAG  GTT  GCT  GTT  CGT  GTT  GAA  CGA  GAA  GAA  GGA  CAA   323
65    R    L    K    D    K    V    A    V    R    V    E    R    E    E    G    Q    80

324  TAA                                                                               
81    *

327  CAAAATTGGTCAAGATCTTCAATAAAATGCTTCAGGGGCTGCTAGGTCA                                 371
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Table 6. Nucleotide sequences of GnRH2 gene from *Hemibagrus nemurus*. This sequence has been deposited in GenBank nucleotide database, under accession no. OP115878.

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1    TTCTGACCTGCACATCTCAG AAGATTTAAT TCCGTCTTCAAAAACATCGTACAGAGTG                       57

58   ATG  GTC  AGT  GTG  TGC  AGA  CTG  TTG  CTG  CTG  GCC  TTG  CTG  TGT  CTG  TGT   105
1     M    V    S    V    C    R    L    L    L    L    A    L    L    C    L    C    16

106  TTA  CAT  GCA  CAG  CTG  GTC  TCT  CAG  CAC  TGG  TCT  CAT  GGC  TGG  TAC  TAC   153
17    L    H    A    Q    L    V    S   *Q*  *H*  *W*  *S*  *H*  *G*  *W*  *Y*  *Y*   32

154  CCT  GGA  GAG  ATC  GAC  TCT  TAC  AGC  TCT  CCA  GAG  ATA  TCT  CCA  AAG  CCA   201
33   _P_  _G_   E    I    D    S    Y    S    S    P    E    I    S    P    K    P    48

202  GGA  GAG  ATT  AAA  CTG  AAC  CTG  AAG  AGC  GGA  GCA  TGC  TAT  CTG  AAG  CCA   249
49    G    E    I    K    L    N    L    K    S    G    A    C    Y    L    K    P    64

250  CTG  AGA  ACC  AAC  ATC  CTG  AAG  AGC  ATC  CTG  GAC  GCT  CTT  GCA  GCT  AGG   297
65    L    R    T    N    I    L    K    S    I    L    D    A    L    A    A    R    80

298  GAA  TTC  CAA  AAG  AGG  AAG  TGA                                                 318
81    E    F    Q    K    R    K    *                                                   87

319  CCTCGCCAAATCATGCCACAGTATCATAGCCTCTTTCTTCGCTCATCCCACTGATCAG                        376
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Table 7. Sequence alignment of the *Hemibagrus nemurus* GnRH1 amino acid with other vertebrates, respectively. Identical sequences to *H. nemurus* GnRH1 are in dots. Gaps (-) were inserted to obtain maximum homology.

SPECIES	
	`....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|`
	`10        20        30        40        50        60        70        80        90        100`
OP115879 *Hemibagrus nemurus*	`-------MSVKRALWWMVVCVVVLQVS--AQHWSHGLNPGGKR------AAMQETVEEMPRSSG--YVCDYADA-SPRNKIYRLKDLLSRVAERETGQ--------`
OP104320 *Pangasius nasutus*	`-------C.......C..............--S...................L..A...T.-...V.V-..L.....TS.....I..................`
XM_026924976.1 *Pangasianodon hypopthalmus*	`-------C....................--S.....................L.........T.-...V.V-..L.....TS.....I..................`
AY374332.1 *Salmo salar*	`-------.DLSSKTVVQ.MLALIAQVTFS....Y.WL....SVGELE.TIRRMDTG-GVMVLPEETGAHIPE-RLRP--------------------------`
AY374331.1 *Salvelinus alpinus*	`-------.DLSSKTVVQ.MLALIAQVTLS....Y.WL....SVAELE.TIRRMDTG-GAMALPEETGAHIPE--RLRP-------------------------`
AB531127.1 *Takifugu niphobles*	`-------.ATASFSL.LLLLGSLMSQL-CC....Y..S.....DVDHLSDSVDTV.VSV.HAETSSALLGC.ENVAAFSR.FGI.EI.NSRTD.RAKRFQTSNK-----`
LC129224.1 *Takifugu rubripes*	`-------.ATASFSL.LLLVGSLMSQL-CC....Y..S.....DVDHLSDSVDTV.VSV.HAETSSALLGC.ENAAAFSR.FGI.EI.NSRTD.RAKRFQTSNK-----`
AB026989.1 *Anguilla japonica*	`-------.AD.S..L.LGLA.ALVCQG-CC....Y..R.....GADSLQDTL.DII..LQKLDT-SSLPSCN.L-..HITLSS..EI.ANL.D....R-KNI-------`
GQ422802.1 *Anguilla mamorata*	`-------.AD.S..L.LGLA.ALVCQG-CC....Y..R.....GADSLQDML.DII..LQKLDT-SSLPSCN.L-..HITLSS..EI.ANL.D....R-KNI-------`
KF113107.1 *Trichogaster trichopterus*	`-------.A..LLAL--LLLGTLVPQH-FC....Y..S.....GLDSISDTLDNM..F..VDTPCS.VSC.E-...SA....M.AFFD--TD.GN.R-RTYKKR----`
NM_001303356.1 *Larimichthys crocea*	`-------.APQTSAMLLLLVGS..WCHGCC....Y..S.....DLDGRSDT---M.DSF.HVDSPCS.LSCVED-F.-.....M.GF.DG.TD..N.R-RTFKK-----`
XM_023394165.1 *Seriola lalandi*	`-------.GM.TLSL.LLLVGML.PQH-RC....Y..S.....ELDSLSDTLANI..GA.HVETPCRMLGCSEE--.FAR..M.GF.GSATD..N.H-RTYKK-----`
XM_018668358.1 *Lates calcarifer*	`----MHRR.A..TLAL.ILLVATLVPQH-CC....Y..S.....ELDSLPDTLGNV..GF.HEDTQCS.LG..EE--.FARV..M.GVFGS.TD..N.H-RTYKK----`
XM_005811760.3 *Xiphophorus maculatus*	`-------.A.GTLTL.LLLVGT..SLG-SG....F..S.....ELDGFPNTLDSIY.GFAHMAAPCT.LGC.EE-.LPA....M.G..GS.S...H..-QPFEK----`
XM_015972478.1 *Nothobranchius furzeri*	`-------.AL.TLVLRLLLVGA.VPLV-CC....F..S.....ELDGFQSTLDNI..GFSHMDAPCT.LRCEED-.LA....M.GF.GS.SDGGN.H-KVYKK----`
XM_008417516.2 *Poecilia reticulata*	`-------.A.RTLTL.LLLVGT..SLG-SG....F..S.....ELDGYPNTLDSIF.GFAHMAAPRN.LGC.EE-.LPA....M.G..GSMS...HRH-QPFEK----`
XM_015003746.1 *Poecilia mexicana*	`-------.A.RTLTL.LLLVGT..SLG-SG....F..S.....ELDGFPNTLDSIF.GFAHMAAPRN.LGC.EE-.LPA...M.G..GSMS...H.H-QPFEK----`
XM_024747674.1 *Neophocaena asiaeorientalis*	`MVALMFLR.EPSPK.LAGLILLTLCV.GCSS....Y..R.....NAAENVLDSF..IAK.VDQPAEPQRFECTIHQ-R.SPLGD..GA.ESL-IE.ETGQKM--------`
XM_027087895.1 *Lagenorhynchus obliquidens*	`MVALMFLR.EPSPK.LAGLILLTLCV.GCSS....Y..R.....NAENVLDSF..IAK.VDQPA.PQRFECTVHQ--RHSPLRD..GA.VSYRVNMHGPWAPPLWSST`
XM_007192763.1 *Balaenoptera acutrstrata*	`---MFLR.EPIPK.LAGLILLTLCV.GCSS....Y..R.....NAENVLDSF..IAK.VDQPAEPQRFECTIHQ-R.SPLRD..GA.ETL-IE.ETGQKM-------`
NM_001172956.1 *Cavia porcellus*	`-------.GLIPK.LAGL.LLTLCVENGSG.Y..Y.VR.....NIEPLVDSF..MAK.IDQLAEPQHFECTLHQ--.SPLRD..GA.ESL-ME.ETGQKKI------`
XM_004848360.2 *Heterocephalus glaber*	`-------.GPIPK.LAGFILLTLCVENISG....Y..R.....NAETLVDSF..IAK.IDQLAEPQHSECALHQ-APSPLSD.RGA.ESL-IE.ETGQKKI------`
NM_001113693.1 *Xenopus tropic*	`-------.KAISTYALLLLVLLFSAHVGH....Y..R.....DAESLQDMYP..PN.V.LFPELERLECSVPQ----SRLNV.RGA.MSW-LDGENRKKI-----`
XM_019556567.1 *Crocodylus porosus*	`-------.QKT.KVFVSLLLLILSVDICL....Y..Q.....NAENLVESF..MAND.EKFGEMQQFECSGPH--QPS.LSD..EA.ANL-MEGETGRKTT-----`
NM_001083111.2 *Homo sapiens*	`-------.KPIQK.LAGLILLLTWCVEGCSS....Y..R.....DAENLIDSF..I.K.VGQLAETQRFECTTHQ--.SPLRD..GA.ESL-IE.ETGQKKI-----`
NM_001195436.2 *Macaca mulatta*	`-------.EPIPK.LAGLILLTLCVEGCSS....Y..R.....DAENLMDSF..I.K.VGQLAETQHFECTMHQ--.SPLQD..GA.ESL-IE.ETGQKKI-----`
XM_025394633.1 *Theropithecus gelada*	`----MCLR.EPIPK.LAGLILLTLCVEGCSS....Y..R.....DAENLMDSF..I.K.VGQLAETQHFECTMHQ--.SPLQD..GA.ESL-IE.ETGQKKI------`

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Table 8. Sequence alignment of the *Hemibagrus nemurus* GnRH2 amino acid with other vertebrates, respectively. Identical sequences to *H. nemurus* GnRH2 are in dots. Gaps (-) were inserted to obtain maximum homology.

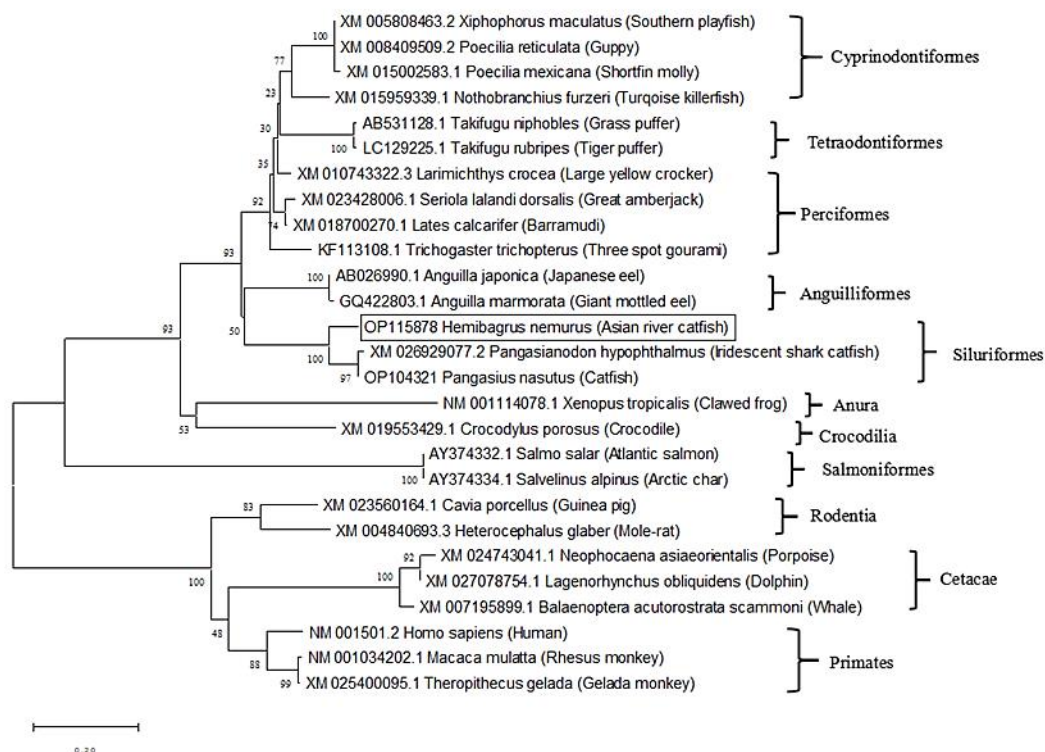| Species	Sequence
OP115878 *Hemibagrus nemurus*	MVSVCRLL-------LFAALLCLHAQLSVSQHWSHG-----WYPGGKREIDS-------------YSSPEISGEIK-------LCEAGECSYLKPLRTNILKSILI--DALA-REFQ-KRK-
OP104321 *Pangasius nasutus*	............-------......V.......QS...I...-----..................................R...R....V.......-.....-K.....-
XM_026929077.2 *Pangasianodon hypopthalmus*	............-------......V.......QS...I...-----........L...................................R...R....V.......-.....-K.....-
AY374332.1 *Salmo salar*	-MDLSNRT-------------VVQVVV.A.V..VTL......Y.......L.....SVG-----.LEAT............-------MMDT.GVVA..PEETSAHVSER.R----P---------
AY374334.1 *Salvelinus alpinus*	-MDLSNRT-------------VVQVVV.A.V..VTL......Y.......L.....SVG-----.LEAT............-------MMDT.GVVA..PEETSAHVSER.R----P---------
AB531128.1 *Takifugu niphobles*	-MR.PG..-------...LLG...AG.H..NG............L.P............-----FNPS...E.................R.Q.R.V.RNF.L----...T-.L...-
LC129225.1 *Takifugu rubripes*	-MR.PG..-------...LLG...TG.H..NG............L.P............-----FNPS...E.................R.Q.R.V.RNF.L----...T-.L...-
AB026990.1 *Anguilla japonica*	..NTG..V-------...IIGV...G....LC............L..............-----L.TA.VLE...............DG........R.Q.KSL..N..L----....R...-
GQ422803.1 *Anguilla marmorata*	..NTG..V-------...IIGV...G....LC............L..............-----LTTA.VLE...........-.DG........R.Q.KSL..N..L----....R...-
KF113108.1 *Trichogaster trichopterus*	-MC.A..V-------...LLG...IGV.M.SA............L..............-----FGTS...E...................R.Q.RSL..T..L----....L...-
XM_010743322.3 *Larimichthys crocea*	-MC.S..V-------...LLG....VG....NA............L..............-----FGTS...E.................R.Q.RGV.R....L----....L...-.-
XM_023428006.1 *Seriola lalandi*	-MC.S..V-------...LLG....VG....NA............L..............-----F.TS...E.................R.Q.RS...N..L----...I-.L...-
XM_018700270.1 *Lates calcarifer*	-MC.S..V-------...LLG....VG....NA............L..............-----F.TS...E.................R.Q.RS...RN.L----....L...-.-
XM_005808463.2 *Xiphophorus maculatus*	---MS..V-------...LLIV..M.VS...DA............L..............-----FGAS.M.E.................R.Q.R.T.RN.VL----....L...-.-
XM_015959339.1 *Nothobranchius furzeri*	---MS..V---------...LLG....YVG...HA............L..............-----FGVS...E...................R.Q.R.V.RD.FL------....GL...-.-
XM_008409509.2 *Poecilia reticulata*	---MS..V---------...LLIV..M.VS...DA............L..............-----FGAS.M.E.................R.Q.T.RN.VL----...-.L...-.-
XM_015002583.1 *Poecilia mexica*	---MS..I---------...LLIV..M.VS...DA............L..............-----FGAS.M.E.................R.Q.R.T.RN.VL----...-.L...-.-
XM_024743041.1 *Neophocaena asiaeorientalis*	-MASFG.G--------...LLIV...TT.PEP.KA..C..S-----.H.E..QASS.-----LHD.QHTPGPP-------AHSPGQIVPN.PSNVLAPPEDSVPWESRTM.WWLLHR.QHL
XM_027078754.1 *Lagenorhynchus obliquidens*	-MASFG.G--------...LLIV...TT.PEP.KA..C..S-----.H.E..QASS.-----LHD.QHTPGPPGRVLGTAAHSPGQIVPN.PSDVLAPPEDSVPWESRTM.RWLLHR.QHL
XM_007195899.1 *Balaenoptera acutorostrata*	-MASFG.G--------...LLIV...TT.PEP.KA....S-----LH.E..QASS.-----LHD.QHTPGPP-------AHSPGQIVPN.PSDVLAPH.DSVPRESRTM.RWLLCR.QHL
XM_023560164.1 *Cavia porcellus*	-MASS..G--------L.LLL.R.TTCPGPLRA...............ASG.-----PQDSRTIPRLPGSVLGPAAGSL.--AHR.PNDDLAPSEDRAPWKGRSM.XWTLHR.QHQ
XM_004840693.3 *Heterocephalus glaber*	-MASS..SLLLLLLLL.LLL...TTYPGPLRA.Y................AYS.-----SQDSQNVPRLSGRILGTAAGSL.QAAHR.PSKDLAPSEDTVPWEGRSMFRWTLHR.QHL
NM_001114078.1 *Xenopus tropicalis*	.ACQGH.V-------...LLIV.FAFSTH..NA................QL.T-----R.F...D.L..-----P..GES.D..PMN-EMS...GL.T---RF.FP..R.--..-
XM_019553429.1 *Crocodylus porosus*	.ACPRS..-------...LLIV...AIGVP.ARA.............L.L-----SRA..QA.E..--------I.DGE..A..RSP.M.VV.TL.A---M..-.QL-.K..-
NM_001501.2 *Homo sapiens*	-MASS.RG-------...LLLL..TA.LGP.EA............ALS.-----AQD.QNALRPPGRALDTAAGSPVQTAHG.PSDALAP.DDSMPWEGRTT.QWSLHR..HL
NM_001034202.1 *Macaca mulatta*	-MASS.RGL------...LLLM..TA.PGP.EA............ALS.-----AQD.QNALRPP-------AGSP.QATYG.PSDALAH.EDSMPWEGRTM.WWSLRR..YL
XM_025400095.1 *Theropithecus gelada*	-MASS.RG-------...LLLM..TA.PGP.EA............ALS.-----AQD.QNALRPPGRALGTAAGSP.QATYG.PSDALAH.EDSMPWEGRTM.WWSLRR..HL

Figure 3. Phylogenetic trees for the GnRH2 gene. Bootstrap analysis was performed using the Neighbor-Joining method. The bootstrap value proportions are shown at the forks.

vertebrates.

The identical sequences of protein across species were observed in various positions of the GnRH2 gene. The amino acid at positions 32-35 (^{32}QHWS^{35}) was conserved for all species except for Cetacea and one from Rodentia. A similar trend of aa was observed at position 44-49 (^{44}WYPGGK^{49}), in which only Cetacea (*Neophocaena asiaeorientalis*, *Lageno-rhynchus obliquidens*, and *Balaenoptera acutorostrata scammoni*) and Salmoniformes (*Salmo salar* and *Salvelinus*) did not shared identical aa as other species. The substitution in tyrosine (Y) and glycine (G) is either replaced with lysine (L), histidine (H) or glutamic acid (E).

Distance matrix and phylogenetic analysis: In the GnRH1 gene, *H. nemurus* shared 99.76% homology to *P. hypophthalmus* and 99.71% to *P. nasutus* (Table 4). Compared to other fish orders, *H. nemurus* shared 92 to 99% similarity with Anguilliformes, Tetraodontiformes, Perciformes, and Cyprinodonti-formes. Species in the order of Anura and Rodentia shared the percentage identity within 90-93%, and

they were clustered in different clades in the phylogenetic tree. *Hemibagrus nemurus* shared the lowest similarities to Primates including *Homo sapiens* (87.80%), *Macaca mulatta* (86.99%), and *Theropithecus gelada* (54.22%).

In GnRH2, *H. nemurus* shared 99.79% homology with *P. hypophthalmus* and 99.77% with *P. nasutus*. Compared to the Anguilliformes, *H. nemurus* shared a high similarity of 99.15 and 99.18% with *Anguilla japonica* and *A. marmorata*, respectively. Compared to other fish orders, *H. nemurus* shared between 89-98% with Salmoniformes, Tetraodontiformes, Perciformes, Anura, Rodentia, Cetacea, and Primates. Comparison between the distance matrix of GnRH1 and GnRH2 revealed that GnRH1 consists of higher amino acid variations between vertebrates compared to GnRH2, which contained slight differences in their amino acids even between fishes and mammals.

A phylogenetic tree of the GnRH genes, GnRH1 and GnRH2 was constructed using amino acids homology of *H. nemurus*, other fishes, and vertebrates (Figs. 2, 3). The results revealed that GnRH1 and

GnRH2 of *H. nemurus* were sub-clustered with other catfish species in Siluriformes, including *P. nasutus* and *P. hypophthalmus*. Both GnRH1 and GnRH2 genes of *H. nemurus* were also grouped with orders, including Anguilliformes, Cypriniformes, Tetraodontiformes, and Perciformes. For GnRH1, fish orders of Siluriformes, Anguilliformes, Cypriniformes, Tetraodontiformes, and Perciformes clustered together except for Salmoniformes (*Salmo salar* and *Salvelinus*), which clustered with other vertebrates, including Anura, Rodentia, Crocodilia, Cetacea, and Primates. For the GnRH2 phylogenetic tree, species from the orders of Rodentia, Cetacea, and Primates were clustered together, while Anura and Crocodilia clustered together with fish orders, including Siluriformes, Anguilliformes, Cypriniformes, Tetraodontiformes and Perciformes.

Discussions

This study encompassed the full-length cloning and sequence analysis of gonadotropin-releasing hormone, GnRH1 and GnRH2 of *H. nemurus*, a commercially cultured catfish in the Asian region. The PCR amplification of GnRH1 and GnRH2 in *H. nemurus* has been successfully detected in the brain sample. Previous reports have shown GnRH expression is mostly detected in the fish brain, and could also be detected in other fish organs such as the pituitary, skeletal muscle, heart, liver, kidney, spleen, placenta, mammary gland, ovary, and testis (Kakar and Jennes, 1995; White and Fernald, 1998; Nabissi et al., 2000; Uzbekova et al., 2001; Ikemoto and Park, 2003; Guilgur et al., 2007). For example, in *H. fossilis*, the GnRH2 gene was expressed highly in the brain, followed by the gonads (Chaube et al., 2019). The expression of GnRH genes might be related to species-specific analysis due to different distributions of GnRH genes in the brain and pituitary (Shahi et al., 2017).

Generally, at least two forms of GnRH were identified in all types of fish (Chehade et al., 2020). The GnRH1 has a crucial role in the onset of maturation in the reproductive system of teleost that controls the release of sex steroids (Mamta et al.,

2020). Meanwhile, the role of GnRH2 is in reproduction, growth, oocyte quality, spawning behavior, and feeding behavior (Marvel et al., 2019). GnRH3 is a teleost-specific GnRH isoform located in the olfactory bulb and it can regulate sexual behavior and spawning migration (Sukhan et al., 2022). In *Clarias macrocephalus* (Thai catfish) and *C. gariepinus* (African catfish), two forms of GnRH were characterized by protein purification methods, a catfish-specific GnRH1 and GnRH2 (Bogerd et al., 1992; Ngamvongchon et al., 1992; Chaube et al., 2019). Similar to the findings, this study also showed *H. nemurus* consists of GnRH1 and GnRH2, and GnRH3 was not detected by the PCR amplification. It was suggested that the eels and catfish appeared to have lost the GnRH3 gene, however, GnRH1-expressing neurons have been discovered in the olfactory bulbs and terminal nerve, which appear to substitute the function of the GnRH3 (Munoz-Cueto et al., 2020).

The cDNA of GnRH1 is 371 bp long with an open reading frame (ORF) of 262 bp that encodes 81 amino acids, while the cDNA of GnRH2 is 376 bp long with an ORF of 260 bp with a highly conserved protein of 87 amino acids (NCBI accession no. GnRH1: OP115879; GnRH2: OP115878). The length of the DNA sequences was similar to other catfish families in Siluriformes, such as *P. nasutus*, where a full-length cDNA of GnRH1 gene consists of 355 bp with 242 ORF and 80 amino acids (NCBI accession No: OP104320), and GnRH2 gene consist of 362 bp, 260 ORF and a highly conserved protein of 86 amino acids (NCBI accession No: OP104321). In *H. fossilis*, the GnRH1 protein also encoded 80 aa with a cDNA of 351 bp including 240 bp of ORF, and the GnRH2 protein encoded 86 aa with a cDNA of 546 bp including 258 bp of ORF, respectively (Chaube et al., 2019). These show that the length of DNA sequences was almost similar within Siluriformes, with high sequence identities and similarities in their amino acids.

The pairwise sequence comparison showed that the amino acid sequences of both GnRH1 and GnRH2 genes of *H. nemurus* were highly similar to homologs

from Siluriformes, Cypriniformes, Tetraodonti-formes, and Anguilliformes, and became less similar when compared to Salmoniformes, Primates, Cetacea, Rodentia, Anura, and Crocodilia. A previous study reported sequence comparison of the deduced amino acid sequences of the GnRH2 gene from *H. fossilis* was conserved to other teleosts and vertebrates (Chaube et al., 2019). The GnRH2 is the most ancient GnRH and has been highly conserved from bony fish to mammals (Desaulniers et al., 2017).

Comparison of the GnRH genes based on their amino acid regions revealed a high homology identified in their decapeptides, but less similar in the signal peptide. The variable of the amino acid sequences in the signal peptide indicated the evolution occurred within this region, whereas the conserved GnRH decapeptides indicated functional importance, resulting in having a low tolerance to structural changes during species evolution (Okubo and Nagahama, 2008; Chehade et al., 2020). The GnRH1 evolved 350 million years ago, and its sequence varies abundantly among vertebrates (Millar et al., 2008; Desaulniers et al., 2017). Meanwhile, the GnRH2 gene has been preserved even though its evolution has reached more than 500 million years (Fernald and White, 1999; Desaulniers et al., 2017), reflecting that it might be the most ancient form of GnRH (Millar et al., 2004).

Phylogenetic tree analysis based on GnRH1 and GnRH2 genes showed *H. nemurus* clustered together with other catfish species from Siluriformes; *P. nasutus* and *P. hypophthalmus*. Previous studies using phylograms illustrated the catfish species also clustered together under the same clades and subclades especially Siluriformes, as compared with outgroups consisting of Primates (Tello et al., 2008). The GnRH2 gene from *H. fossilis* was found to be clustered closely with *C. gariepinus* and *I. punctatus* (Chaube et al., 2019). Other studies involving GnRH genes of teleost fish showed Cypriniformes (goldfish, and zebrafish) and Perciformes (Nile tilapia) also found to have a close relationship with Siluriformes, indicating that they shared a common ancestor (London and Volkoff, 2018).

Conclusion

We successfully cloned and characterized the full-length cDNA sequences of the gonadotropin-releasing hormone (GnRH) genes, GnRH1 and GnRH2, of *H. nemurus*. In the phylogenetic tree of GnRH1 and GnRH2 genes, *H. nemurus* was sub-clustered with other catfish species including *P. nasutus* and *P. hypophthalmus*. The present study could offer advanced research to develop a new GnRH implant delivery system for the artificial breeding of *H. nemurus* and other catfishes in captivity, thus improving the reproduction system of cultured catfish for sustainable aquaculture.

Acknowledgments

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