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The cloning and expression of the two types of kisspeptin gene in hard-lipped barb (*Osteochilus vittatus*)

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Abstract. *Prayogo NA, Rahayu SC, Sugito PPA, Meyanadewi SB, Sukardi P, Rositas RE, Bessho Y. 2023. The cloning and expression of the two types of kisspeptin gene in hard-lipped barb* (Osteochilus vittatus). *Biodiversitas 24: 2797-2804.* Hard-lipped barb (*Osteochillus vittatus* Valenciennes, 1842) is a native Indonesian species with many benefits for economic, aquaculture, and environmental sustainability. Several studies showed that the species had high reproductive potential, with kisspeptin gene playing a role in reproductive hormone production and GnRH secretion. Kisspetin gene is also important for gonad maturation in fish. Therefore, this study aimed to determine the structure of Kiss-1 and Kiss-2 genes, as well as the expression of the kisspeptin gene in the hypothalamus and gonads in each phase of female hard-lipped barb development. The study procedure involved several stages, including organ preparation, isolation, sequence identification, and measurement of cGnRH gene expression. The structure of Kiss-1 gene in hard-lipped barb was obtained, and the nucleotide sequence had a product length of 213, coding for 89 amino acids. Meanwhile, Kiss-2 consisted of 230 nucleotides, coding for 76 amino acids, and the sequences observed had 70-80% with other fish species. The results showed that the expression values of Kiss-1 and Kiss-2 genes in the hypothalamus were higher compared to the gonads, with the lowest in the adult phase and the highest in the Juvenile phase. This information will lead us to use the kisspeptin gene to increase hard-lipped barb reproduction.

Keywords: FSH, gene identification, GnRH, LH, reproduction

INTRODUCTION

One freshwater fish species from the Cyprinidae family that has spread in Southeast Asia is the hard-lipped barb (*Osteochilus vittatus* Valenciennes, 1842). Several studies found that *O. vittatus* was a native fish in Indochina, Thailand, and Indonesia (Hasan et al. 2019). Hard-lipped barb has several advantages as a fishery commodity, both from the economic, cultivation, and environmental sustainability aspects. However, overfishing this species due to high demand can threaten its sustainability and affect the continuity of stocks in nature (HLPE-FSN 2014). That indicates preserving hard-lipped barbs through cultivation activities is necessary by considering their reproductive aspect (Syamsuri 2018).

Reproduction in fish is periodic phenomenon controlled by the environment. Environmental signals the hypothalamus often receives cause the secretion of two hormones: Gonadotropin Release Hormone (GnRH) and Gonadotropin Release Inhibitory Factor (GnRIF). Furthermore, GnRH is known to stimulate the anterior pituitary to secrete gonadotropin (GtH) hormones, which are divided into two types, namely Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), with the final role in gonad maturation and fish spawning (Elakkanai et al. 2015). One of the genes that play an important role in the secretion of reproductive hormones and stimulate GnRH is the kisspeptin gene.

Kisspeptin is believed to be a precursor to reproductive maturity and sex steroid production through the Hypothalamus Pituitary Gonad (HPG) axis (Simanjuntak 2017). Based on previous studies, it is classified into various types of vertebrate peptides and is known to influence gonadal development and interaction with metabolic pathways (Clarke et al. 2015).

In several groups of fish, kisspeptin is encoded by two types of genes, Kiss-1 and Kiss-2 (Biran et al. 2008). Although the sequences of these genes differ, they still share some similarities at the amino acid level, with the smallest kisspeptin decapeptide KP-10 sharing 60-80% similarity (Mitani et al. 2010). The difference in the amino acids effectively generates Kiss receptors (Zohar et al. 2010). Kiss-1 is a recently discovered neuropeptide that controls gonadal maturation and stimulates the release of GnRH-releasing hormone from the Pre-Optic Area (POA) (Soga et al. 2016). Meanwhile, Kiss-2 acts through two pathways to directly modulate gonadotrophic function. Apart from acting as a hypophysiotropic neuropeptide, it also serves as an autocrine/paracrine factor that affects gonadotrophic activity (Espigares et al. 2015).

Some groups of fish only express one of the two types of kisspeptin gene, but some can express both. Freshwater fish, such as Zebrafish (*Danio rerio* Hamilton, 1822), Medaka (*Oryzias latipes* Temminck & Schlegel, 1846), and Goldfish (*Carassius auratus* Linnaeus, 1758), as well as seawater fish, including seabass (*Dicentrarchus labrax*

Linnaeus, 1758), can express Kiss-1 and Kiss-2. According to Zhao et al. (2014), Kiss-1 is present in zebrafish, while Fugu (*Fugu rubripes* Temminck & Schlegel, 1850), the green spotted puffer fish (*Tetraodon nigroviridis* Marion de Procé, 1822), Stickleback (*Gasterosteus aculeatus* Linnaeus, 1758), and Tilapia (*Oreochromis niloticus* Linnaeus, 1758) only express Kiss-2 (Park et al. 2016).

Previous reports show limited studies on the kisspeptin gene, particularly in hard-lipped barb (Prayogo et al. 2020). That indicates it is important to identify kisspeptin and determine the species' reproduction maturity level as conservation. Therefore, this study aims to determine the structure of Kiss-1 and Kiss-2 genes as well as the expression of kisspeptin gene in the hypothalamus and gonads in each phase of female hard-lipped barb development.

MATERIALS AND METHODS

Tissue collection

A total of 18 hard-lipped barb female head consisting of six juvenile (2 weeks), six adult (2 months), and six mature (4 months) was cut from the tip of the mouth to the back of the operculum. The bones near the nostrils in the head of the fish were then cut horizontally, and the hypothalamus $\pm$0.5 g was collected using an earpick, followed by the gonads $\pm$1 g. Subsequently, the obtained samples were weighed, stored in a tube, and placed in a freezer at -80°C until the organ sample was isolated.

RNA isolation and reverse transcriptase Polymerase Chain Reaction

The total mRNA was extracted from the brain using Blue Sepasol R-RNA super-1 reagent (Nacalaitesque) with ethanol-phenol-chloroform extraction. The prepared RNA was then treated with RNase-free DNase (Takara). Furthermore, the quality and concentration of the samples were assayed using denaturing agarose gel electrophoresis and optical density reading at 260 and 280 nm. The RNA was then aliquoted in batches and frozen at -70°C for storage. Finally, the RNA samples weighing 1.5 ng were reverse transcribed using the cDNA synthesis kit (PrimeScriptTM Reverse Transcriptase) from Takara.

Identification of Kiss-1 and Kiss-2

cDNA amplification

The PCR reaction was carried out using the 12.5 Kappa PCR master mix, with nine templates, 7 ddH_2O sterile, and two forward and reverse primers each for Kiss-1 and Kiss-2. The initial stage of PCR was performed with heating at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, and annealing at 55°C for 30 seconds. The last step was an extension at 72°C for 60 seconds and post-extension at 72°C for 5 minutes, totaling 35 cycles. Furthermore, PCR results were analyzed using electrophoresis at 100 volts for 45 minutes. The electrophoresis results were then visualized using a UV transilluminator.

Cloning and sequencing of PCR products

cDNA 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 ng) (Takara) using ligation with T4 ligase. Furthermore, the plasmid was transfected into *Escherichia coli*, and the bacteria were spread on LB medium plates (Mohamed et al. 2007). The recombinant-positive colonies were screened using ampicillin, and the plasmid DNAs were purified from positive colonies with a mini-scale plasmid preparation. DNA sequences were determined using the Big Dye version 3.1 sequencing method with specific primers. The primers used for sequencing of Kiss-1 were FK1 and RK1, while those for Kiss-2 included FK2 and RK2, as shown in Table 1. The sequence data were then automatically collected on the ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems).

Basic Local Alignment Search Tools (BLAST) analysis

The results obtained from the previous process were in the form of DNA sequences, and they were analyzed using BLAST and multiple alignments (http://www.ebi.ac.uk/Tools/msa/clustalo/). The BLAST analysis used a web-based program, NCBI (http://ncbi.nlm.nih.gov/).

Phylogenetic tree

After aligning the sample sequences with comparison sequences, they were copied and processed using the FASTA format with the MEGA program or entered into the chat box on the application (http://www.phylogeny.fr/).

Expression of Kiss-1 and Kiss-2

The extracted RNA was reverse transcribed using the Primescript First-strand synthesis system (Takara) Kit. A total of 0.5 µL Random 6-mers (50 µM) was added with 0.5 µL dNTP-mixture (10 mM), 1.5 µg template RNA, and RNAse free dH_2O until the volume became 5 µL. The solution was heated for 5 minutes at 65°C and immediately cooled on ice. Subsequently, the solution was mixed with a 5 µL template RNA mixture, 2 µL of 5x Prime Script buffer, 0.25 µL of RNAse inhibitor (40 U/µL), 0.5 µL of Prime script R-tase (200 U/µL), and RNAse-free aquabides to bring the total volume to 10 µL. Next, the solutions were mixed and incubated at 30°C for 10 minutes and at 42°C for 30-60 minutes, followed by heating at 95°C.

RT-PCR

One Step qPCR setup started by synthesizing cDNA at 42°C for 5 minutes, followed by deactivation of RT (Reverse Transcriptase) at 95°C for 2-5 minutes, denaturation at 95°C for 3 seconds, annealing at 60°C for $\geq$20 seconds, and extension for 5 minutes, with a total of 40 cycles. Furthermore, specific real-time primers were used to amplify the kisspeptin gene in hard-lipped barb, and the housekeeping gene was amplified with beta-actin primers, namely FA and RA.

Table 1. Design of primers used in Real-Time PCR

Name/primer code	Complementary DNA sequence (primer)	Tm (°C)	PCR product
F- Kiss-1 (FK1)	ACA TCA CTC CRG GTK CTC A	56.1	
R -Kiss-1 (RK1)	CTG CTC TCT CTT TCC ATA ACG	51.7	189 bp
F -Kiss-2 (FK2)	RTG GTG GAG CGR AGG CAR TT	52.5	
R - Kiss-2 (RK2)	CAG GAA AAG CAT CAT TGG CAG	53.1	156 bp
F -Beta Actin (FA)	GAG CTA TGA GCT CCC TGA CGG	58.3	
R - Beta Actin (RA)	AAA CGC TCA TTG CCA ATG GT	55.6	

Real-Time PCR amplification results were then used to compare the number of DNA molecules amplified by kisspeptin encoding and β-Actin genes. The comparative values obtained were then compared with each sample based on the reference until R-value was reached (Forlenza et al. 2012). For the equation:

$$\Delta\Delta CT = (CT\ kiss - CT\ actin)\ sample - (CT\ kiss - CT\ actin)\ calibrator$$

$$R_{kiss} = 2^{-\Delta\Delta CT}$$

Where:

ΔΔCt : Cycles threshold
Ct kiss sample : Ct value kisspeptin sample -i
Ct *β-actin* sample : Ct value *β-actin sample*-i
Ct kiss calibrator : Ct value kisspeptin sample with lowest *β-actin*
Ct *β-actin* calibrator : Ct value *β-actin* lowest
R_{kiss} : gene kisspeptin value

Data analysis

All data gene expression (R) was analyzed ANOVA using the SPSS statistical package (version 10.0; SPSS Inc., Chicago, IL, USA). A one-way analysis of variance followed by Tukey's post hoc test was used to test for significant differences in the data ($p<0.05$). The values are expressed as the means ± Standard Error (SE).

RESULTS AND DISCUSSION

Identification of Kiss-1 and Kiss-2

Kiss-1 and Kiss-2 genes were identified and amplified successfully from the genomic DNA. The electrophoresis results on the agarose gel showed that DNA Kiss-1 and Kiss-2 were amplified with lengths of 200 bp and 180 bp, respectively, as shown in Figure 1.

Successful sequencing of the Kiss-1 sequence in the hard-lipped barb revealed a nucleotide sequence with a product length of 213 base pairs (bp), coding for 89 amino acids. At the same time, Kiss-2 consisted of 230 nucleotides that encoded 76 amino acids. That indicated the sample sequencing had been carried out successfully. The nucleotide base sequences of Kiss-1 and Kiss-2 genes were then tested for sequence homology using the BLA facility. This was in line with NCBI (2015) that BLAST could be used to find areas of similarity between sequences. Kiss-1 and Kiss-2 sequences in hard-lipped barb were similar to those obtained from other fish.

Based on the BLAST analysis, the nucleotide sequence of Kiss-1 in hard-lipped barb had the highest similarity of 93% with *Cyprinus carpio* Linnaeus, 1758, followed by *Labeo rohita* Hamilton, 1822 and *Catla catla* Hamilton, 1822 (92%), *Tor putitora* Hamilton, 1822 and *Clarias magur* Hamilton, 1822 (91%), and *D. rerio* (87%). Furthermore, the higher the value of identities, the higher the level of similarity.

Multiple alignment results showed that Kiss-2 in hard-lipped barb had the same sequence as other fish. This indicated the presence of kinship between the hard-lipped barb and other fish species, such as *C. carpio, C. catla, T. putitora, L. rohita, Sinocyclocheilus tingi* Fang, 1936, *Megalobrama amblycephala* Yih, 1955, *C. auratus*, and *D. rerio*. Based on these results, the test sample had the closest kinship with *C. carpio.*

According to Coulter (2013), catfish contained 13 to 14 protein-building acids, with tryptophan, asparagine, and glutamic acid being dominant. Furthermore, only four differences in the amino acids exist in hard-lipped barb and carp (*C. carpio).* These differences occurred at the 31st (Glycine), 37th (Histidine), 43rd (Lysine), and 92nd (Glycine) positions. Based on these results, the Kiss-1 gene was assumed to have the same function in both species.

The kiss-1 amino acid structure of the hard-lipped barb had a high similarity with several other fish species, indicating the presence of similar functions. In addition, kiss-1 was known to play a role as a regulator in initiating reproductive maturity through its involvement in GnRH secretion, gonadal development, and sex steroid production in mammals and other vertebrates (Bhattacharya and Babwah 2015).

The alignment results of Kiss-2 showed kisspeptin-10 (kp10), which was identified as a natural ligand for receptor activation (Mechaly et al. 2013). A comparison of the amino acid sequence of the peptide kp10 from various fish showed a similar structure. Furthermore, Kiss-2 gene was reported to play different functions in each fish sample. In hard-lipped barb, Kiss-2 played a role in gonadal maturation at an early stage, while in *C. auratus* (goldfish), it functioned in LH and FSH secretion. The gene also induced LH and growth hormone secretion in *C. carpio,* while it played a role in stimulating GnRH 1 in tilapia (Mechaly et al. 2011). Kiss-2 amino acid sequence of hard-lipped barb and other fish species showed they had the same ancestor, as shown in Figure 5B. The analysis revealed an amino acid sequence similarity of 70-80%.

The results showed that *D. rerio* had the farthest genetic distance from Kiss-1, with a genetic coefficient value of

0.163, while *C. carpio* and *T. putitora* were the closest, with 0.044. Meanwhile, Kiss-2 showed the closest and farthest genetic distance to *C. carpio* (0.83) and *D. rerio* (5.454), respectively. Based on these results, the genetic distance between the species indicated the presence of close kinship. A small coefficient value indicated a close kinship relationship, while a large value showed a distant one. Furthermore, the genetic distance affected the role of kisspeptin in these species, where the closer the genetic distance, the higher the similarity in the roles of kisspeptin, and vice versa. The phylogenetic tree describes the evolutionary lineage of a species and its relationship with other organisms with a common ancestor (Radhakrishnan et al. 2013). The phylogenetic tree created in this study revealed the genetic relationship between species in one population and between populations. Phylogeny was also useful for organizing knowledge of biological diversity for structural classification and providing insight into events that occurred during evolution (Taylor 2014).

Table 2. BLAST analysis of the Kiss-1 gene *Osteochilus vittatus* in NCBI

Species	Access number	Similarity (%)
Cyprinus carpio	LN590717.1	93.45%
Labeo rohita	KF737179.1	92.24%
Tor putitora	KM504155.1	91.62%
Catla catla	KM212970.1	92.04%
Clarias magur	MF373409.1	91.45%
Danio rerio	GQ228091.1	87.62%

Table 3. BLAST analysis of Kiss-2 gene *Osteochilus vittatus* in NCBI

Species	Access number	Similarity (%)
Cyprinus carpio	JQ715608.1	95.65
Catla catla	KM212969.1	93.24
Tor putitora	KX024449.1	93.39
Labeo rohita	KF695115.1	92.07
Sinocyclocheilus tingi	KU500629.1	89.33
Megalobrama amblycephala	KC136218.1	88.60
Carassius auratus	GQ141877.1	85.78
Danio rerio	NM001142585.1	85.46

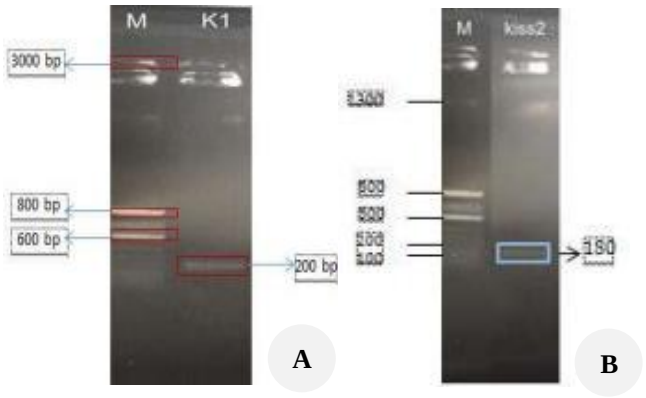

Figure 1. Electrophoretic results of the PCR products of Kiss-1 and Kiss-2 *genes Osteochilus vittatus.* A. Kiss-1 gene, B. Kiss-2 gene, M: marker

ACA	TCA	CTC	CGG	GTT	CTC	AGG	GGA	ACT	GAT	ACT	CAT	CCC	ACA
T	S	L	R	V	L	R	G	T	D	T	H	P	T
GCT	GGA	TCT	CCT	TCT	GGC	AAG	CTC	TCA	GTG	CAC	TTC	TCC	ATG
A	D	G	P	S	G	K	L	S	V	H	F	S	M
GAT	GCA	GGT	CCT	CAG	CGA	AAC	ACA	TGG	TGG	TGG	TCT	CCA	
D	A	G	P	Q	R	N	T	W	W	W	S	P	
GGA	AAG	CCT	TAC	AGG	AAA	AGG	AGG	CAG	AAT	GTT	GCT	TAT	
E	K	P	Y	R	K	R	R	Q	N	V	A	Y	
TAC	AAT	CTC	AAT	TCC	TCC	GGC	CTC	CGT	TAT	GGA	AAG	AGA	
Y	N	L	N	S	F	G	L	R	Y	G	K	K	
GAG	CAG												
E	Q												

A

V	V	E	R	R	Q	F	D	E	P
GTG	GTG	GAG	CGG	AGG	CAG	TIT	GAC	GAA	CCC
S	A	A	D	D	A	S	L	C	F
AGC	GCT	TCG	GAC	GAC	GCA	AGC	CTC	TGC	TTT
F	F	Q	D	K	D	E	S	R	H
TTC	TTT	CAA	GAC	AAA	GAC	GAA	TCG	CGT	CAT
I	S	C	K	H	R	Stop	T	R	S
ATT	TCC	TGC	AAA	CAT	CGA	TAA	ACA	CGA	AGT
K	F	N	Y	N	P	F	G	L	R
AAA	TTC	AAC	TAC	AAC	CCG	TTT	GGG	CTG	CGC
F	G	K	R	N	E	A	T	T	D
TTT	GGG	AAG	CGA	AAT	GAA	GCG	ACT	ACT	GAC
T	D	R	P	K	H	K	H	L	L
ACC	GAC	AGA	CCC	AAA	CAC	AAG	GAC	CTG	CTG
P	Met	Met	L	F	L				
CCA	ATG	ATG	CTT	TTC	CTG				

B

Figure 2. Nucleotide and amino acid sequences that code for Kiss. A. Kiss-1 gene, B. Kiss-2 gene

The phylogenetic tree of Kiss-1 showed that kinship was divided into two aspects, namely *D. rerio* on one aspect and *O. vittatus* with several other species on the other. The Kiss-1 gene in hard-lipped barb showed the closest kinship to *C. carpio* with a similarity percentage of 62% (0.62). It also had 37% (0.37) similarity with several species on the same branch, including *L. rohita*, *T. putitora*, *C. catla*, and *C. magur*. Meanwhile, the result showed it had the most distant kinship with *D. rerio* in a different cluster. Based on the phylogenetic tree, *O. vittatus* was not in the same branch as *D. rerio* due to a gap in the alignment results. Kiss-2 showed closeness with *O. vittatus* and other fish, indicating a similar function of the Kiss-2 gene. The *O. vittatus*, as a sub-group, indicated the genetic variation between individuals in each group.

Table 4. Genetic distance of hard-lipped barb (*Osteochillus hasseltii*) and other species

Species	1	2	3	4	5	6	
Kiss-1 gene							
Osteochilus hasseltii							
Cyprinus carpio	0.083						
Labeo rohita	0.078	0.082					
Tor Putitora	0.084	0.090	0.084				
Catla catla	5.433	5.209	5.439	5.091			
Clarias magur	5.784	5.796	5.999	5.424	2.448		
Danio rerio	0.161	0.174	0.175	0.161	5.054	5.454	
Kiss-2 gene							
Osteochilus hasseltii							
Cyprinus carpio	0.044						
Catla catla	0.057	0.037					
Labeo rohita	0.064	0.044	0.006				
Sinocyclocheilus tingi	0.097	0.077	0.070	0.077			
Megalobrama amblycephala	0.119	0.083	0.084	0.091	0.119		
Tor putitora	0.044	0.012	0.025	0.031	0.076	0.076	
Danio rerio	0.163	0.125	0.126	0.134	0.142	0.141	0.118
Carassius auratus	0.098	0.077	0.071	0.077	0.064	0.113	0.077	0.149

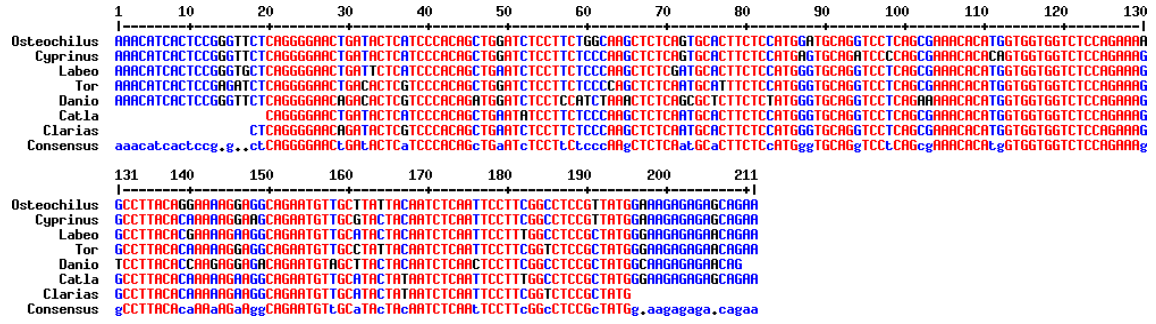

Figure 3. Alignment of gene sequences of Kiss-1 between *Osteochilus vittatus.* Red: conserved area, Blue: non-conserved area

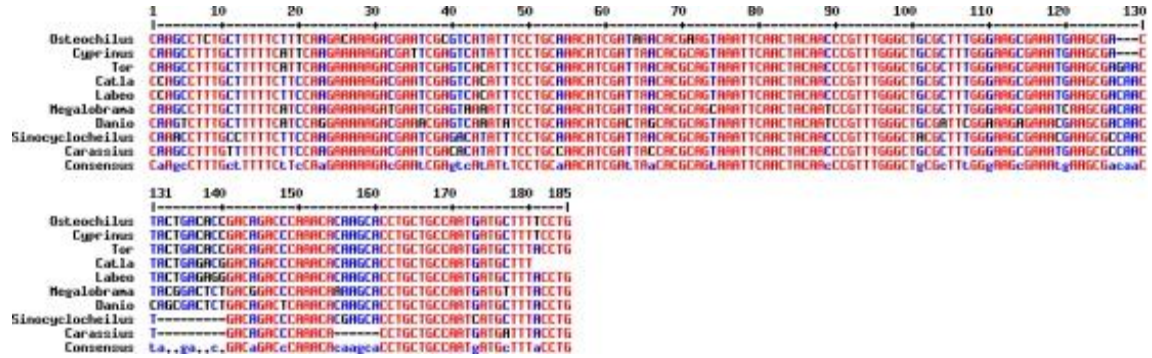

Figure 4. Alignment of Kiss-2 gene sequences between *Osteochilus* vittatus. Red: conserved area, Blue: non-conserved area

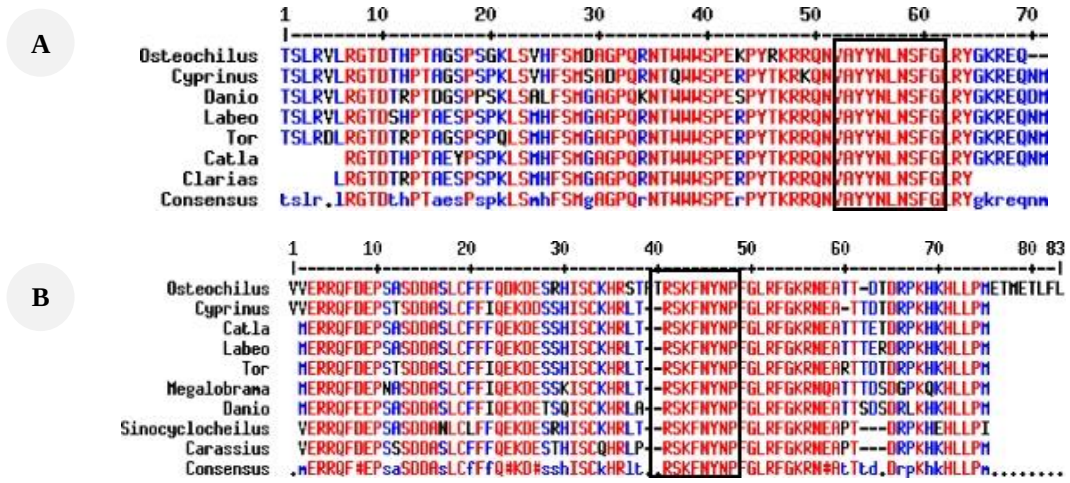

Figure 5. Amino acids alignment of Kiss-1. A. Kiss -1 gene, B. Kiss-2 gene

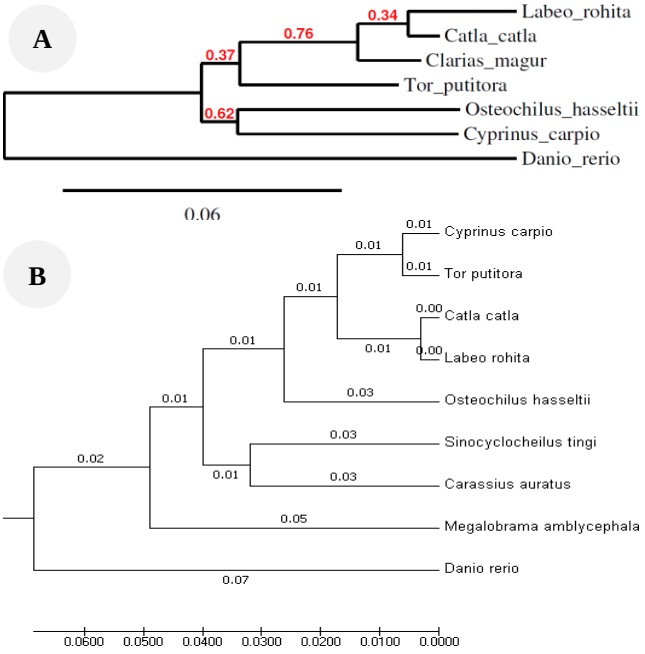

Figure 6. Phylogenic tree *Osteochilus vittatus* on nucleotides from several fish species. A. Kiss-1 gene, B. Kiss-2 gene

Expression of Kiss-1 and Kiss-2 Genes

Based on quantitative real-time PCR analysis in female hard-lipped barb, two major types of kisspeptin genes were expressed, namely Kiss-1 and Kiss-2. These genes were observed in the hypothalamus and gonads during the juvenile, adult, and gonadal phases.

In the hypothalamus, the highest Kiss-1 gene expression value was observed during the juvenile phase (8.7543), while the lowest was found during the adult phase (3.3974). The gene was expressed in the hypothalamus from the early fish's development. Furthermore, this was consistent with Biran's et al. (2008) study on zebrafish (*D. rerio*) that the level of Kiss-1 mRNA gradually increased during the first 2-8 weeks of life until it reached a peak with mature vitellogenic follicles at 12 weeks. Similar results were also obtained in a report on the hard-lipped barb, where the decreased expression of the gene in the hypothalamus during the adult phase was caused by its higher concentration in the gonads in preparation for the reproductive period or gonadal maturation. Biran et al. (2008) stated that the mRNA levels of Kiss-1 and Kiss-2 in the hypothalamus peaked 30 days after fertilization and remained high in the spawning period. Ogawa and Parhar (2018) also revealed the high expression value of Kiss-1, which indicated that the gene had a specific role in the hypothalamus. It was further conveyed by Gahete et al. (2016) that kisspeptin regulated reproductive processes, including spawning and ovulation, specifically by activating the surface kiss-1 receptor (aka GPR54). This receptor was reported to be present in the neurons of the hypothalamic gonadotropin-releasing hormone (GnRH).

Furthermore, the highest expression value of the Kiss-1 gene in the gonads was obtained during the juvenile phase (3.2007), followed by the adult (2.8376) and maturation (1.1779) phases. A previous study showed that kisspeptin-10 had a major role in the maturation of the gonads of *Channa striatus* Bloch, 1793 (Francis et al. 2011).

The ANOVA test results revealed that organs significantly influenced the expression level of the Kiss-1 gene ($P<0.05$). However, the developmental phase alone or combined with organs had no influence. These findings were consistent with Zmora et al. (2015) that Kiss-1 was expressed in the brain, pituitary, and gonads. Ogawa and Parhar (2018) also revealed that regardless of the type of kisspeptin, its presence in fish could stimulate various reproductive functions depending on the reproduction stage, sex (gender), fish species, and treatment methods.

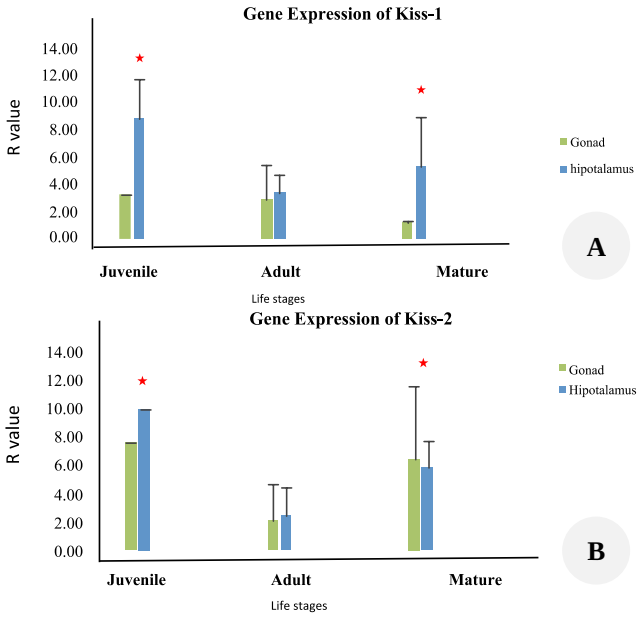

Figure 7. Expression value of Kiss gene in the gonads and hypothalamus of female hard-lipped barb (*Osteochilus vittatus*). A. Kiss-1 gene, B. Kiss-2 gene). ★: significantly different

The highest value of Kiss-2 gene expression was found in the hypothalamus (9.8492) and gonads (7.5904) during the juvenile phase compared to the adult (hypothalamus 2.4040 and gonads 2.0530) and mature gonads (hypothalamus 5.7673 and gonads 6.3888) phases. According to Park et al. (2016), Kiss-2, GPR54, and rGnRH I mRNA were simultaneously increased during the early life stages of tilapia. That indicates these genes work together to regulate essential reproductive events, such as sex differentiation and spawning onset. These findings were consistent with Mohamed et al. (2007), where increased GPR54 and GnRH mRNA simultaneously occurred at 26 day post hatch in a teleost, cobia, and *Rachycentron canadum*. A significant amount of rGnRH I and Kiss-2 neurons were also observed in the brain of *red seabream* female *Pagrus major* during the first spawning period (Shimizu et al. 2012), while increased GPR54 and rGnRHI gene expression was found in red tilapia at 11 week post hatch (Martinez-Chavez et al. 2008).

The results of the ANOVA test showed that the gonads and hypothalamus in the juvenile phase significantly affected the value of the Kiss-2 gene expression level ($P<0.05$). Furthermore, the stages of fish development gave a more significant impact on the expression of the gene ($P<0.05$). The value obtained was higher in the hypothalamus for both the juvenile and adult phases than in the gonad. That was because Kiss-2 gene was expressed only in the brain (Zmora et al. 2015), pituitary, and testes but not in the ovaries (Ohga et al. 2013). The Kiss-2 gene acted through two different pathways to directly modulate gonadotrophic function. Therefore, apart from its role as neuropeptide hypophysiotropic, it could be an autocrine/paracrine factor influencing gonadotrophic activity (Espigares et al. 2015).

The value of Kiss-2 gene expression in the hypothalamus was relatively high in each phase compared to Kiss-1. This was because Kiss-1 gene was expressed in the habenular. At the same time, Kiss-2 in the hypothalamus could regulate reproduction, including spawning, and stimulate and regulate gonadotropin gene expression in adult female fish (Ohga et al. 2018). Furthermore, Mechaly et al. (2013) stated that this kisspeptin gene expression indicated the potential involvement of the kisspeptin system (Kiss-1 and Kiss-2) during early fish development and gonadal sex differentiation.

In conclusion, based on the structure of Kiss-1 gene in hard-lipped barb, the nucleotide sequence had a product length of 213, coding for 89 amino acids. Meanwhile, Kiss-2 consisted of 230 nucleotides, coding for 76 amino acids, and had 70-80% of the same sequence as other fish. The expression values of Kiss-1 and Kiss-2 genes produced in the hypothalamus were higher than those in the gonads and lowest in the adult phase but highest in the Juvenile phase. Furthermore, the kisspeptin gene can increase reproduction in hard-lipped barbs.

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