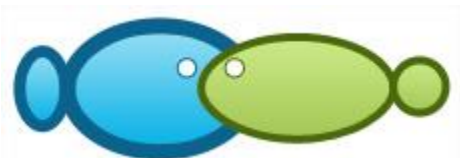

Characterization of genes encoding follicle-stimulating hormone β-subunit (fsh-β) and luteinizing hormone β-subunit (lh-β) from Indonesian leaffish *Pristolepis grootii*

[1,2]Muslim Muslim, [3]Agus O. Sudrajat, [3]Muhammad Zairin Jr., [3]Muhammad A. Suprayudi, [4]Arief Boediono, [3]Iis Diatin, [3]Alimuddin Alimuddin

[1] Doctoral Program, Aquaculture Science Study Program, Postgraduate School, IPB, Bogor, Indonesia; [2] Aquaculture Study Program, Faculty of Agriculture, Sriwijaya University, Palembang, Indonesia; [3] Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB, Bogor, Indonesia; [4] Department of Anatomy, Physiology, and Pharmacology, Faculty of Veterinary Medicine, IPB, Bogor, Indonesia. Corresponding author: A. Alimuddin, alimuddin@apps.ipb.ac.id

Abstract. The Indonesian leaffish *Pristolepis grootii* (Bleeker, 1852) is an indigenous fish species. The purpose of this study was to analyze the characteristics of the β-subunit of follicle-stimulating hormone (FSH-β) and the β-subunit of luteinizing hormone (LH-β) of *P. grotii*. Complementary DNA (cDNA) was synthesized from mRNA that was extracted from *P. grootii* pituitary glands. Twelve fish were reared in two aquaria and injected with luteinizing hormone-releasing hormone analog (LHRHa) for the analysis of FSH-β and LH-β gene expression. The results showed that *P. grootii* FSH-β was 227 bp in length, consists of 93 amino acid residues, while LH-β was 300 bp in length and consists of 100 amino acid residues. *P. grootii* FSH revealed the highest similarity (91%) with climbing perch *Anabas testudineus*, while LH-β with *Betta splendens* (90%). Two putative N-glycosylation sites in FSH-β and one site in LH-β were conserved in *P. grootii*. The expression of FSH-β and LH-β in fish injected with LHRHa was higher than that of LHRHa not injected fish. The ovary of *P. grootii* had various sizes of oocytes. The injection of LHRHa affected the development of oocytes; there was a trend for higher doses LHRHa to develop oocytes faster. Thus, it is most likely that the isolated genes are FSH-β and LH-β of *P. grootii*.
Key Words: DNA cloning, gonadotropin, gene expression, pituitary hormone.

Introduction. The reproductive system in fish is regulated by the brain-pituitary-gonadal axis. This axis consists of neuroendocrine circuits in the brain that regulate the synthesis and release of the two gonadotropins in the pituitary gland, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These gonadotropins regulate the development and function of the gonads for reproductive activities (Chi et al 2015; Zulperi et al 2015). FSH plays a primary role in gametogenesis and gonadal development, while LH plays a role in gonadal maturation and spermiation and/or ovulation (Sambroni et al 2013; Zhang et al 2015; Nguyen et al 2019).

Gonadotropins (FSH and LH) are members of the glycoprotein hormones family and each member is a heterodimer consisting of α-subunit and β-subunit (Pierce & Parsons 1981). The α-subunit is the common part of hormones, and the β-subunit is the specific part. The β-subunit has a unique character that is connected to the carboxyl group with a terminal peptide (Klausen et al 2001). Both subunits bind non-covalently into gonadotroph, to configure dimeric hormones that are biologically active (Zhou et al 2010). Both hormones are synthesized in the pituitary gland (Molés et al 2011; Horie et al 2014). The action of the pituitary gland is controlled in a large portion by several neurohormones including neuropeptides, neurotransmitters that are synthesized by specific neuronal populations and extend the pituitary (Zohar et al 2010).

Structurally, fish FSH and LH are different, but homologous to other vertebrates (Levavi-Sivan et al 2010; Yaron & Levavi-Sivan 2011). The major characteristic of the FSH and LH subunits was the presence of a conserved cysteine series. The α and -β subunits contain a common cystine knot core, indicating that LH and FSH belong to a larger superfamily of multimeric proteins characterized by the presence of a cystine knot motif (Hearn & Gomme 2000; Levavi-Sivan et al 2010). Gonadotropin hormones are heterodimers that range from 30-50 kDa (Levavi-Sivan et al 2010). FSH and LH are important hormones in regulating reproduction in vertebrates, including fish (Yaron & Levavi-Sivan 2011).

The Indonesian leaffish *Pristolepis grootii* is one of the many native fish species in Indonesia (Muslim et al 2021). This species is present in the islands of Sumatra and Kalimantan (Indonesia) and Malaysia. The habitat of the fish is freshwater, namely rivers, lakes, and swamps. The fisherman families use this species as a side dish for their daily household consumption. In addition, urban communities consider this species an ornamental fish. The supply of this fish still depends on capture from the wild, because the aquaculture of this species does not yet exist. There have not been many studies on this species. A study on the subunit gonadotropin genes (FSH-β and LH-β) of *P. grootii* has not been carried out to our knowledge. The purpose of this study was to analyze the nucleotide characters of partial genes of FSH-β and LH-β of *P. grootii*. Information about the characteristics of the FSH-β and LH-β of *P. grootii* will be very important for the development of this species using hatchery technology in the future.

Material and Method

Pituitary collection. This study was conducted from May to August 2020. Twelve female *P. grootii* (weight 20±0.5 g) were obtained from the Kelekar River, Ogan Ilir Regency, South Sumatra, Indonesia and transported to the Aquatic Organism Reproduction Laboratory, IPB University, Bogor, Indonesia. The fish were separated in two aquaria and then injected intramuscularly with luteinizing hormone-releasing hormone analog (LHRHa) at doses of 10 μg kg^{-1} and 50 μg kg^{-1}. Six fish were not injected as a control. Fourteen days after injection, the fish were dissected, and the pituitary removed. The pituitary was collected from each fish and immediately stored in liquid nitrogen until further use.

Total RNA extraction. The pituitary samples were put in a microtube (1.5 mL), then dissolved in 200 μL GENEzolTM Reagent (Geneaid, Taiwan), iced, and then crushed. The extraction procedure followed the manufacturer instructions. Measurement of the purity of total RNA content was determined using a UV-VIS spectrophotometer with a wavelength of 260 nm and 280 nm. Purity was determined based on the comparison of the absorption value at a wavelength of 260 nm to 280 nm, while the DNA concentration was determined based on the absorption value at a wavelength of 260 nm.

Synthesis of cDNA using RT-PCR technique. Synthesis of cDNA was performed using the ReverTraAce®qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan) with the Reverse transcription-polymerase Chain Reaction (RT-PCR) technique. The RNA concentration was equalized (50 ng μL^{-1}) and dissolved in nuclease-free water (NFW) and then homogenized with a slow vortex in a microtube. The synthesis procedure followed the manufacturer instructions. The result of cDNA synthesis was then diluted with NFW 10 times and then stored at -20°C until used in the PCR.

cDNA amplification. The isolation of FSH-β and LH-β genes was carried out by using cDNA as a DNA template. Specific primers were made using a program available on the NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and the β-actin gene of *Pristolepis fasciata* was used as an internal control for RNA loading. The primers used in this study are presented in Table 1. The amplification of FSH-β and LH-β genes was carried out using a thermal cycler machine (peqSTAR, UK) with the MyTaqTMHS Mix Red (Bioline) kit. The procedure followed the manufacturer instructions. For the amplification

of target DNA fragments, 1.5 μL of PCR results was electrophoresed on 1% agarose gel at a voltage of 120 volts for 30 min and documented using the Gel Documentation System. Determination of the standard of the DNA fragments used the VC 100 bp Plus DNA Ladder marker.

Table 1

Primer oligonucleotide sequences used in this study

Genes	*Primary sequence*
FSH-β	F: 5′-TGGTTGTCATGGCAGCAGTG-3′ R: 5′-GCAGTTTCTGGCCACAGGGTA-3′
LH-β	F: 5′-TTTCTGGGAGCCTCATCTTCCATTT-3′ R: 5′-CTCAAAGCCACAGGGTAGGTGAC-3′
β-actin	F: 5′-CCTCCTCCCTGGAGAAAAGC-3′ R:5′-TACCGCAGGACTCCATACCA-3′

Cloning and sequencing. The positive results of the target gene amplification were then purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purification results were then ligated to the cloning vector pTA2 (TOYOBO, Japan) according to the manual kit instructions. The formed plasmid (pTGI) was transformed into competent cells of *Escherichia coli* DH5α (Geneaid, Taiwan). The results of the transformation were then spread on 2x YT medium containing ampicillin (10 μg μL^{-1}) and incubated at 37°C for 16 hours. The growing colonies were isolated by the cracking method and then verified by PCR and electrophoresis methods. The presence of a DNA band with a size larger than the size of pTG1 indicates that the cloning and transformation processes were successful and the PCR amplification results showed a DNA band the same size as the ligated DNA fragment. The positive colony results were further verified by sequencing methods using the services of the 1st Base Laboratory, Singapore.

Sequences analysis. The nucleotide sequence of FSH-β and LH-β results were analyzed using BLASTn (Basic Local Alignment Search Tool), a computer software available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, developed by the National Center for Biotechnology Information (NCBI), USA. To compare sequence similarities between FSH-β and LH-β of *P. grootii* with other vertebrate and fish orders, the amino acid sequence data of other vertebrates and fish were collected from the GenBank (http://www.ncbi.nlm.nih.gov). Pairwise sequence identities between *P. grootii* and other species were assessed using the Clustal Omega program (version 10; ebi.ac.uk; Hinxton, Cambridgeshire, UK), and their sequence identities were calculated. Protein structure modeling was conducted using the Swiss-Model program (swissmodel.expasy.org; Basel). Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0; Megasofware.net; Pennsylvania, US) applying the Neighbor-Joining method. The robustness of the inferred nodes was evaluated by using bootstrapping procedure with 1000 replicates.

Gene expression analysis. The analysis of FSH-β and LH-β gene expression was carried out quantitatively using the real-time PCR (RT-qPCR) method with a RotorGene 6000 machine (Corbett, USA). The amplification process was carried out using the KAPA SYBR® FAST qPCR kit (KAPA, USA). The PCR process was run at 95ºC pre-denaturation for 2 min; 40 cycles for denaturing at 95ºC for 15 seconds, annealing at 60ºC for 15 seconds, extension at 72ºC for 10 seconds. FS-β and LH-β gene expression was normalized by β-actin gene expression.

Gonadal histology. The ovaries were fixed in Bouin's solution (25% formaldehyde solution, 70% picric acid, 5% acetic acid), embedded in paraffin after dehydration and infiltration, cut at 5 mm, and stained with hematoxylin and eosin for histological examination with binocular microscope (Olympus, Tokyo) at 40x10 magnification. The

oocytes were observed under a microscope, then an image was taken. The images were analyzed using ImageJ software, which measured the diameter of all oocytes present in the image. For each fish, a hundred oocytes were measured.

Statistical analysis. Data of the mRNA level are presented as mean ± SEM. The significance of the differences between group means of mRNA levels was determined by a one-way analysis of variance. To compare between three doses of LHRHa (0, 10, 50 μg kg^{-1}), Student's t-test data analysis was performed using the Statistical Package for the Social Sciences software (version 17.0; SPSS Inc.; Chicago, IL, USA). The comparison differences were considered to be statistically significant at $p<0.05$.

Results and Discussion

Genes sequence and predicted protein structure. The partial coding sequences of gonadotropin subunits (FSH-β and LH-β) were determined from the pituitary gland of *P. grootii*. Partial amplification of cDNA encoding *P. grootii* FSH-β was 279 bp in length. There were 93 amino acids, two N-linked glycosylation sites, two proline residues, and 8 cysteine residues. A putative N-linked glycosylation site was identified at amino acid 25-27 (NIS: asparagine, isoleucine, serine), and a second N-linked glycosylation site at amino acid 77-79 (YTT: tyrosine, threonine, threonine). Partial amplification of cDNA encoding *P. grootii* LHβ was 300 bp in length. There were 100 amino, one N-linked glycosylation site, and 7 cysteine residues. N-linked glycosylation site was identified at amino acid residues of 44-46 (NCT: asparagine, cysteine, threonine). Both genes have been registered in the gene bank (NCBI) with gene accession numbers: MN688328 and MN688329 for FSH-β and LH-β, respectively. Amino acid residues of FSH-β and LH-β of *P. grootii* have high identities with other fish species as well as with the amphibian group *Lithobates pipiens* (Figure 1). The identities of the FSH-β and LH-β sequences of *P. grootii*, compared to the amphibian sequence *L. pipiens*, were 36% and 41%, respectively. The identity percentage of FSH-β and LH-β of *P. grootii* with several other fish species is presented in Table 2.

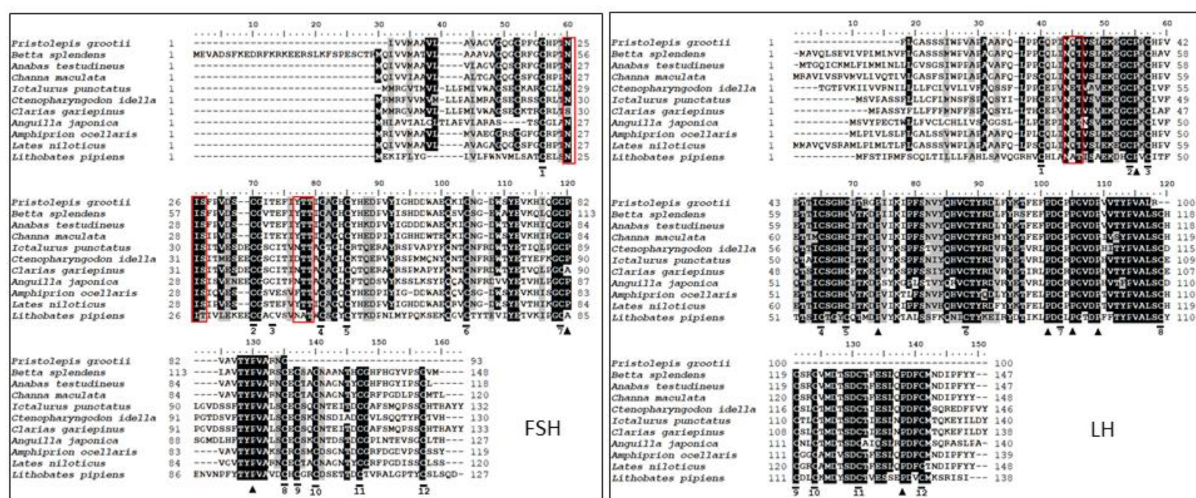

Figure 1. Alignment of the amino acid residues of the FSH-β and LH-β genes of *P. grootii* with other species. Identical residues were given a black background and sequences with a 75% similarity level were given a gray background. Gaps between sequences are separated (-). Bottom line: cysteine residue, red box: N-glycosylation site, triangle: proline residue.

Table 2
Percent identity of FSH-β and LH-β peptides of *P. grootii* compared to some other fish species (LH-β above the diagonal)

No	*Fish species*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1	*Pristolepis grootii*		90	83	88	83	86	54	58	54	58
2	*Betta splendens*	82		83	87	84	85	55	58	56	59
3	*Channa maculata*	86	72		88	82	89	57	58	57	60
4	*Anabas testudineus*	91	80	81		88	89	57	62	57	60
5	*Amphiprion ocellaris*	73	69	70	71		91	53	59	54	58
6	*Lates niloticus*	87	76	82	81	77		57	62	57	61
7	*Anguilla japonica*	39	41	41	42	41	44		76	70	72
8	*Ctenopharyngodon idella*	37	42	38	39	37	42	56		76	81
9	*Clarias gariepinus*	39	39	39	39	39	39	59	74		88
10	*Ictalurus punctatus*	35	39	37	37	41	41	57	71	89	

The phylogenetic tree was constructed from the aligned sequences of the amino acid residues for FSH and LH using the neighbor-joining method. The four fish orders and one class of different vertebrates were divided into two groups. For FSH-β and LH-β, the first group contained Perciformes, Siluriformes, Cypriniformes, Anguilliformes, while the second group consisted of tetrapods (amphibian). The FSH-β of *P. grootii* revealed the highest similarity (bootstrap value 80%) with *A. testudineus*, compared with other species, while LH-β with *B. splendens*) (86%). Phylogenetic trees for FSH-β and LH-β of *P. grootii* are shown in Figure 2.

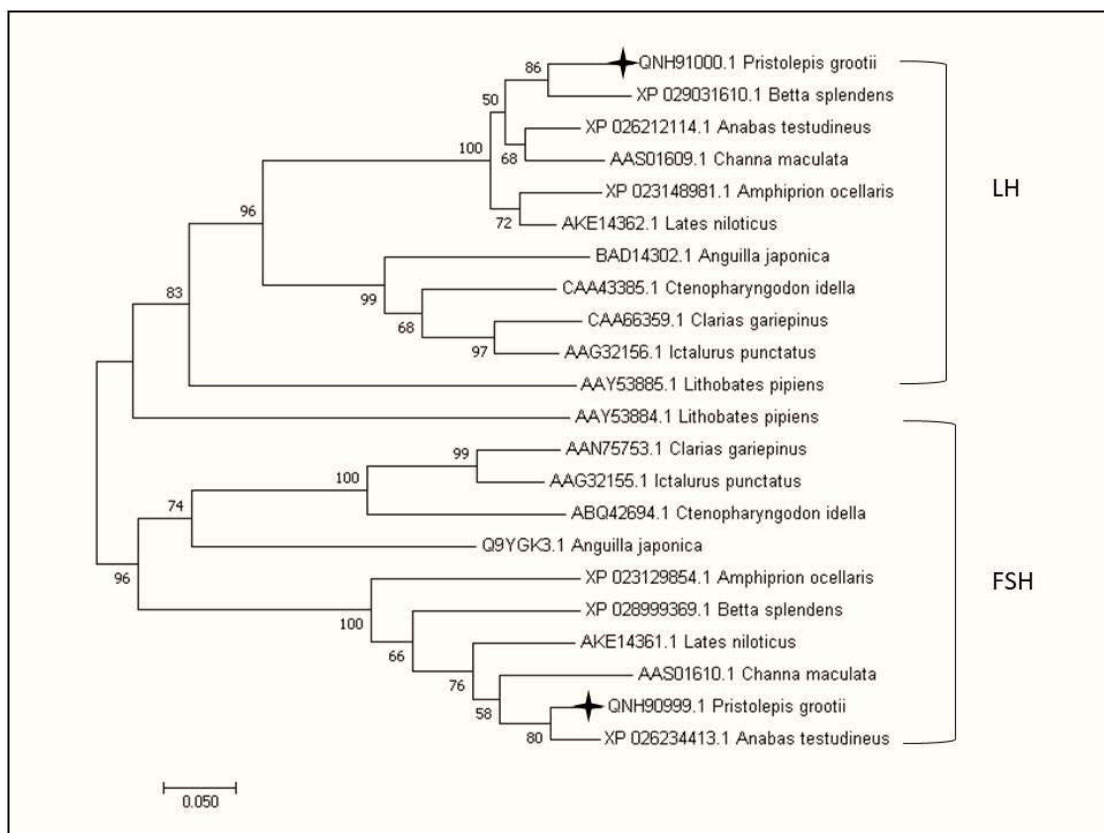

Figure 2. Phylogenetic trees for the FSH-β and LH-β. Bootstrap analysis was performed using the neighbor-joining (NJ) method and the number of bootstrap replications was 1000.

The amino acid residues of the FSH-β and LH-β were then used to construct a secondary protein structure. The protein structure of the FSH-β and LH-β of *P. grootii* was different from that of other fish. Differences of protein structure with other fish species are presented in Figure 3 (especially on the parts marked with arrows).

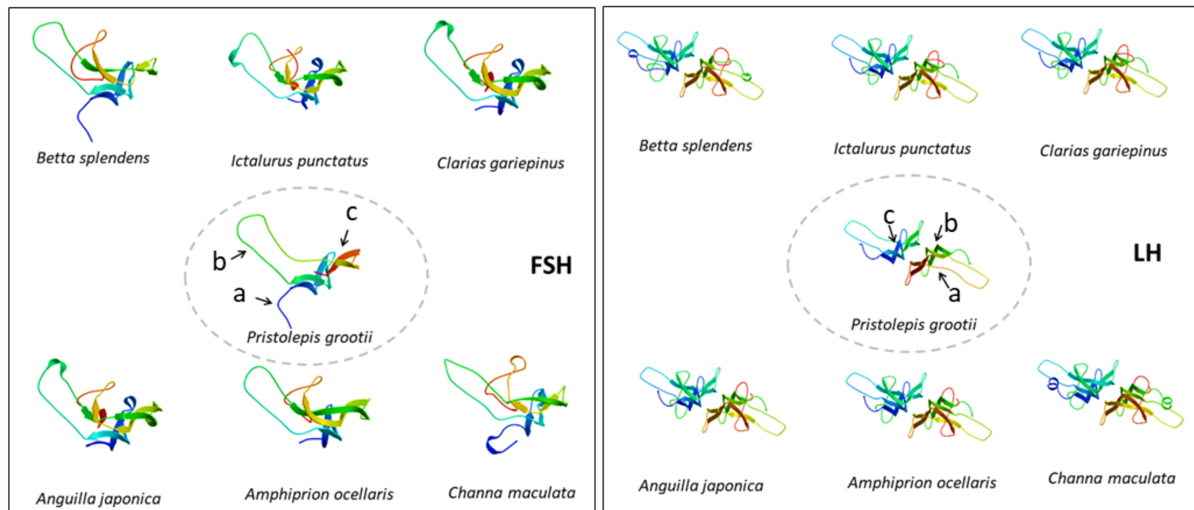

Figure 3. The structure of the FSH-β and LH-β protein in *P. grootii* and several fish from other genera. The FSH structure is like a "rocking chair": (a) "rocker" is blue, (b) "headrest" is green, (c) "armrest" is red. The LH structure is like a "pair of sandals": (a) "sole" is yellow, (b-c) "strap" is green and blue.

Gene expression and gonad histology. The FSH-β and LH-β genes of *P. grootii* injected with LHRHa had a higher expression than in the control fish that were not injected with LHRHa (Figure 4). Furthermore, there was a trend for the expression of FSH-β and LH-β genes to increase with the increasing dose of LHRHa injected.

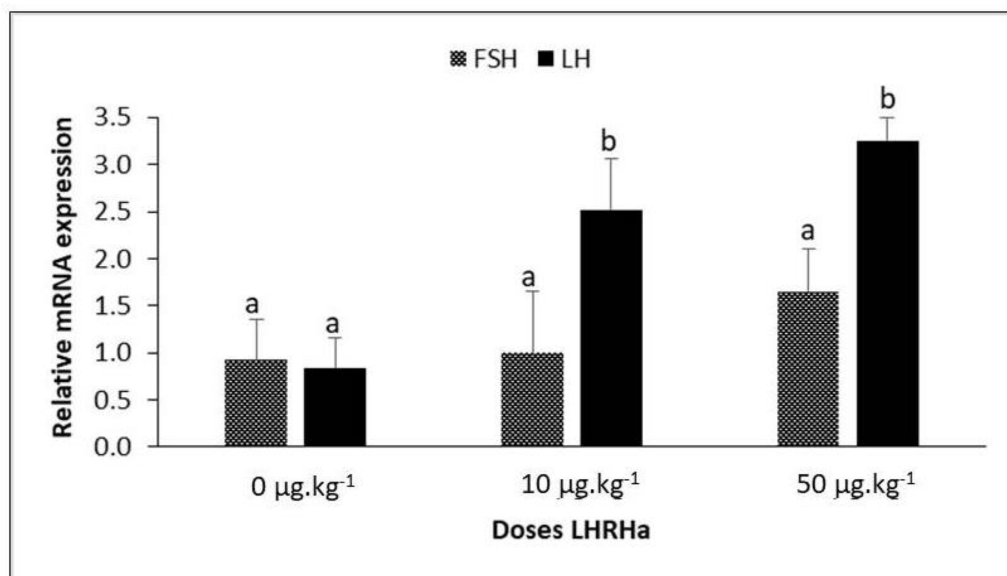

Figure 4. Expression of the FSH-β and LH-β genes of *P. grootii* which were not injected (control) and injected with different doses of luteinizing hormone-releasing hormone analog (LHRHa). Different letters on top of the two histograms indicate that the difference is statistically significant ($p<0.05$).

The ovary of *P. grootii* had various sizes of oocytes (Figure 5). The diameter of the oocytes in the fish that were not injected was 20-460 µm, while those of the fish that were injected ranged between 30-700 µm. For fish that were injected, the average oocyte diameter was larger than that of those not injected. The diameter of the oocytes from fish injected with a dose of 50 µg kg^{-1} was higher than that of fish injected with 10 µg kg^{-1}, namely 30-700 µm and 30-650, respectively.

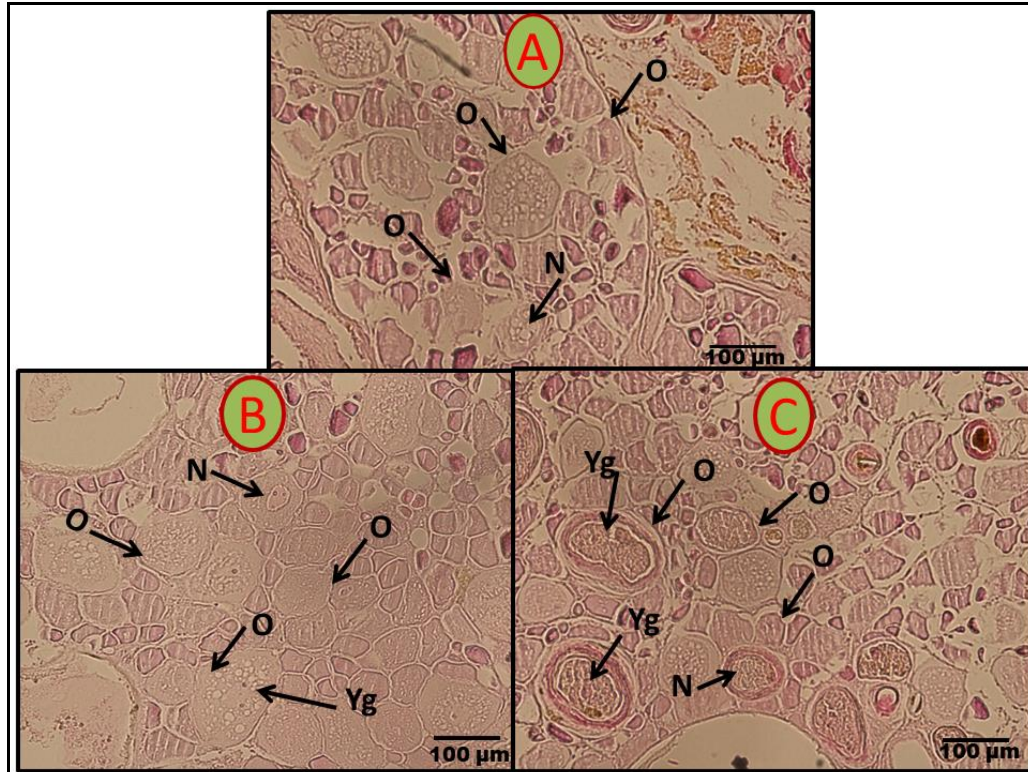

Figure 5. Photomicrographs of the ovaries of *P. grootii*: (A) fish not injected; (B) fish injected with luteinizing hormone-releasing hormone analog (LHRHa) at dose 10 µg kg^{-1} of fish; (C) LHRHa at dose 50 µg kg^{-1} of fish (O - oocyte, N - nucleus, Yg - yolk granule).

This study encompassed the molecular cloning and sequence analysis of the FSH-β and LH-β from *P. grootii*. Two partial cDNA genes, FSH-β and LH-β were cloned from *P. grootii*. The sequences of FSH-β of *P. grootii* have the highest identity with *A. testudineus* (91%), *L. niloticus* (87%), *C. maculata* (86%), and LH-β with *B. splendens* (90%), *A. testudineus* (88%), *L. niloticus* (86%). Both sequences of *P. grootii* identified cysteine residues. According to Sevilhano et al (2017), the main characteristic of the primary structure of the FSH-β and LH-β is the preserved cysteine residues. These residues are important for determining the structure of the cysteine knot, stabilizing heterodimers, and hormone activity. The N-glycosylation sites are also found in both beta subunits. The N-glycosylation sites play a role in the process of biosynthesis and hormone regulation (Chatterjee et al 2005; Shi et al 2015). Glycosylation is the most abundant post-translational modification of proteins and its impact may range from very refined to pivotal, since glycoproteins are involved in various biological roles (Sevilhano et al 2017).

Analysis of vertebrate glycoprotein hormones (i.e. FSH and LH) has revealed that the evolutionary rates of these hormones are not constant and may differ in each condition (Levavi-Sivan et al 2010). The phylogenetic analysis of the gonadotropin subunits of FSH-β and LH-β in selected references, based on the amino acid homology, was presented in Figure 2. The rooted phylogenetic tree revealed that FSH-β and LH-β of *P. grootii* formed clusters together with fishes belonging to the Cypriniformes (*Ctenopharyngodon idella*), Anguilliformes (*Anguilla japonica*), and Siluriformes (*Ictalurus punctatus, Clarias gariepinus*). Based on the phylogenetic trees of the composition of the

amino acid residues of the FSH-β gene, it can be seen that the closest kinship relationship was between *P. grootii* and *A. testudineus* (bootstrap value of 80%). The LH-β gene of *P. grootii* has the closest relationship with *B. splendens* (bootstrap value of 86%). The FSH-β and LH-β of *P. grootii* have separate kinship from the amphibian group *L. pipiens*. The fish species that have high homology of FSH-β and LH-β genes can be used as a reference in selecting donor fish for the application of the hypophysation. The hypophysation technique can use the pituitary gland donor from the same fish species (homoplastic donor) and/or from different fish species (heteroplastic donor) (Marte 1989).

FSH and LH are glycoprotein hormones (Pierce & Parsons 1981; Hearn & Gomme 2000). The amino acid sequence of the β-subunit of the glycoprotein hormone creates a secondary structure. The structure of the protein FSH-β and LH-β genes of *P. grootii* is different from that of *B. splendens*, *I. punctatus*, *C. gariepinus*, *C. maculata*, *A. ocelaris*, and *A. japonica*. The dominant physiological behavior of the glycoprotein hormones is connecting with the rule of a range of endocrinological pathways (Hearn & Gomme 2000).

Study on the expression of FSH-β and LH-β genes in fish has been widely carried out, including on *Trichogaster trichopterus* (Degani et al 2003), *C. idella* (Zhou et al 2010), *Carassius auratus* (Klausen et al 2001) and *Oryzias latipes* (Horie et al 2014). In this work, a study of the expression of FSH-β and LH-β genes from *P. grootii* was conducted. FSH-β and LH-β were expressed simultaneously in the pituitary of *P. grootii*. The expression patterns of FSH and LH differed between asynchronous and synchronous fish species. The expression pattern of FSH and LH of salmonids is different from that of cyprinids and anabantids. In the asynchronous fishes, gonadotropin secretion occurs simultaneously during the spawning period. According to Aizen et al (2012), FSH and LH are secreted differentially during the reproductive cycle. In salmonids, FSH levels are high during the early stages of gametogenesis and decrease during vitellogenesis and maturation, whereas LH is undetectable during gametogenesis, and increases rapidly before ovulation (Gomez et al 1999). Secretion of FSH and LH in *C. idella* is simultaneous, at the early of stages of gametogenesis, FSH levels are higher than LH, and in early ovulation, LH levels increase rapidly (Zhou et al 2010). In *T. trichopterus*, the mRNA levels of FSH and LH are very high during vitellogenesis and maturation (Degani 2020). The expression pattern of FSH and LH in *P. grootii* is similar to that of *T. trichopterus*. The spawning type of *P. grootii* is asynchronous. In the ovary of *P. grootii* there are oocytes of various sizes. Oocyte development consists of a proliferation phase, a primary growth phase, a secondary growth phase, and a maturation phase (Kagawa 2013; Gardner et al 2015). Therefore, in the gonads, there are various sizes of oocytes, so that FSH and LH play a role simultaneously. The FSH is involved in the initiation of gametogenesis and regulation of gonadal growth, whereas LH mainly regulates gonadal maturation and spermiation or ovulation (Mateos et al 2002; Gen et al 2003).

Based on the results of ovarian histology, several generations of oocytes at various stages of development are present in the ovary, both in the fish injected with LHRHa and in those not injected. This indicates that the *P. grootii* is an asynchronous spawner. According to Yaron et al (2003), an asynchronous fish contains several sizes of oocytes, indicating the various stages of oocyte development. Therefore, these species show off spawning cycles of sundry durations. In this species, the vitellogenic phase of one oocyte generation may be simultaneous with the final maturation of a more progressive generation forming the sequence of hormone appearance over complicated. Therefore, such a variation in reproductive strategies is associated with substantial provisional differences in the gonadotropic hormones (FSH and LH) appearance. Histological results showed that the gonad development phase of the sample fish was in the secondary growth stage or in the vitellogenic phase. Administration of LHRHa enhances oocytes development. The results Çek & Gökçe (2006), on female sea bream *Sparus aurata* were similar to this study. The higher the dose of hormone injected, the faster the oocyte develops. The ovary histology of *P. grooti* that was injected showed that many yolk granules and the nucleus had fused, while the opposite condition was observed in fish that were not injected. Not injected fish were in the previtelogic phase,

while the injected fish were in the vitellogenic phase. According to Feist et al (2015), the characteristics of the gonads in the vitellogenic phase include yolk granules becoming larger and the nucleus becoming irregular in configuration.

Conclusions. This study has successfully cloned and analyzed the gonadotropin subunit from *P. grootii*. The results of this study serve as the basis for further studies to explain the role of FSH and LH in the regulation of gametogenesis and steroidogenesis in *P. grootii*. In addition, information on the homology of sequences with other species can be used for the selection of donor fish on hypophysation of *P. grootii*. In the future, this data will allow the development of specific tests for measuring this hormone *in vivo*, as well as to design recombinant FSH and LH to improve fish hatchery technology.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Muslim Muslim, Aquaculture Study Program, Faculty of Agriculture, Sriwijaya University, 30663 Palembang, Indonesia; Doctoral Program, Aquaculture Science Study Program, Postgraduate School, IPB University, 166680 Bogor, Indonesia, e-mail: muslim_bda@unsri.ac.id
Agus Oman Sudrajat, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB, 16680 Bogor, Indonesia, e-mail:omanipb@gmail.com
Muhammad Zairin Junior, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB, 16680 Bogor, Indonesia, e-mail: zairinmz@live.com
Muhammad Agus Suprayudi, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, 16680 Bogor, Indonesia, e-mail: agus.suprayudi1965@gmail.com
Arief Boediono, Department of Anatomy, Physiology, and Pharmacology, Faculty of Veterinary Medicine, IPB University, 16680 Bogor, Indonesia, e-mail: aboedi17@gmail.com
Iis Diatin, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, 16680 Bogor, Indonesia, e-mail: iisdiatin@gmail.com
Alimuddin Alimuddin, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, 16690 Bogor, Indonesia e-mail: alimuddin@apps.ipb.ac.id
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