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Identification and Expression of cGnRH-II Gene in Three Strains *Osphronemus gouramy* (Soang, Jepun and Bluesafir)

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ABSTRACT. Gouramy (*Osphronemus gouramy*) has very high economic value and is easy to cultivate. Currently there are about six strains that have been successfully cultivated based on their reproductive ability to produce eggs, namely goose (soang, goose gouramy), jepun (japan, japonica), blue sapphire, paris, bastar (broiler) and porcelain. One of the reasons for these differences in ability is internal factors which can be seen through the identification and expression of the cGnRH gene that each of these gouramy strains have. The cGnRH gene functions in signaling the pituitary gland to secrete the hormone GtH. This study aims to identify sequences and gene expression values resulting from three strains of gouramyat different age levels. The research method used was the exploration of three gouramy strains (soang, jepun, blue sapphire) at different age levels (4 months, 8 months, 12 months), and three gouramy strains were taken for each age level. This research was conducted through several stages, namely organ preparation, isolation, sequence identification and measurement of cGnRH gene expression. Sequence data was analyzed using phylogenetic trees and gene expression was analyzed using One Way ANOVA test. The sequence results showed that the soang strain had a sequence that was more similar to the jepun strain than the blue sapphire strain, and the resulting gene expression showed that the three gouramy strains with three different age levels did not give different results.

Keywords : cGnRH Gene, Gene Expression, Gouramy Reproduction, Gouramy Strains, Strain Identification

INTRODUCTION

Gouramy is one of the native Indonesian freshwater fish species that has a fairly high diversity. Currently, gouramy has many strains that have been widely cultivated. According to Lucas et al. (2015), there are six types of gouramy strains based on their egg production capacity, growth rate and maximum size or weight of mature gouramy. Each of these strains is goose (soang, *geese gouramy*), jepun (Japan, *Japonica*), blusafir, paris, bastar and porcelain.

The morphologically different gouramy strains include the soang, jepun and blue sapphire strains. The soang strain on its back is blackish brown while the color on the belly and chest is silvery white with rather large scales, the jepun strain is bluish and turns black when it starts to mature with scales smaller than the soang strain, while the blue sapphire strain has a black color reddish. The body length of the soang strain reaches 65 cm with a weight of 8 kg, the body size of the jepun strain only reaches a total weight of 3.5 kg with a length of only 45 cm and the body weight of the blusafir strain only reaches 2 kg/head with a length of about 35 cm (Andrian and Pratiwi, 2021).

Various strains of gouramy have different reproductive abilities. Reproduction in gouramy, as in other fish, is also heavily influenced by environmental factors (Burhanuddin, 2013). These environmental factors including pheromones are received by the central nervous system and continued to the hypothalamus. Neuroendocrine cells in the hypothalamus synthesize and secrete gonadotropin releasing hormone (GnRH) which activates the pituitary to synthesize and secrete gonadotropins. Gonadotropins are required for gametogenesis activity and the formation of gonadal hormones (Wijayanti et al., 2009; Prayogo et al., 2020).

The GnRH hormones that are commonly found in fish are cGnRH (GnRH II) and sGnRH (GnRH III) (Prayogo et al., 2012; Prayogo et al 2016). Functionally, cGnRH and sGnRh have the same function, which is to signal the pituitary gland to secrete the hormone GtH. But both work independently and have different strengths (Klausen, 2002).

Differences in reproductive ability among gouramy strains are also possible due to differences in reproductive genes possessed by gouramy. One of the reasons for the difference in reproductive ability is

due to differences in the sequence and expression of the GnRH gene which each of the gouramy strains have. According to Sari et al. (2014), several strains of gouramy that have different reproductive abilities include jepun, soang and blue sapphire strains. The three strains have different regional distributions, such as the jepun strain which is widely distributed in the Banyumas area, while the blue sapphire and soang strains are widely distributed in the West Java region. This study aims to identify the cGnRH-II gene sequence and detect cGnRH-II gene expression in gouramy soang, jepun and blue sapphire strains with different age levels.

The benefits of this research are expected to provide information about the important role of the cGnRH-II gene in gouramy reproduction. Knowing the reproductive ability of several gouramy strains (Osphronemus gouramy) through the GnRH-II gene with different age levels can be used as a first step in supporting aquaculture and conservation efforts, so that it can be used as material for consideration in designing reproductive intensification methods in gouramy farming businesses.

EXPERIMENTAL SECTION

Organ preparation

Three strains (soang, jepun, blue sapphire) of gouramy at each age level (4 months, 8 months, 12 months) were taken as many as 3 fish. The fish's brain and pituitary were taken with tweezers, then the sample was weighed and put into a *tub*e. The tube containing the sample was stored in the freezer -80 ºC until the organ sample was isolated.

DNA isolation and PCR

Approximately 35 mg of brain from each fish was taken and put into a 1.5 mL tube and then pulverized with a micropestle. After it was smooth, 200 μL GT buffer was added, then it was homogenized and pulverized again. After that, 20 μL Proteinase K was added to the sample then vortexed and incubated for 30 minutes at 60ºC (during incubation the sample was vortexed every 5 minutes).

Before the PCR stage is carried out, the composition mix must be prepared first. The composition mix is made in advance with the following composition. First, add 20 μL of My Taq, 4 μL of cGnRH-II (F and R) primer each, 8 μL of water and 4 μL of template into a 1.5 mL tube. The prepared composition mix was put into the PCR machine with initial denaturation settings of 95ºC for 1 minute, denaturation of 95ºC for 15 seconds, anneling of 47 ºC for 15 seconds, extension of 72ºC for 10 seconds, and final extension of 72ºC for 5 minutes. The primers used in this PCR were cGnRH-F (5'-TCCAGGAGGAAAGAGGGGTCTGGA-3') and cGnRH-R (5'- TGCGTCCATTTCCTCTGTCAGTGT-3') which were designed using Pirimer3web version 4.1.0 software (https://primer3.ut.ee/).

Making Buffers and Electrophoresis

The composition for making the media is 1.2 g of agarose, 8 mL of 10x TBE and 72 mL of distilled water. All ingredients are mixed and heated to a boil. After that, remove and let it get warm enough, then add 3 μL of syber safe. After that, the agar is poured into an electrophorensis device to be printed and waited for it to harden enough. After that, the sample was put into the mold and electrophorensised for a few minutes. The electrophorensis samples were then visualized using a UV transilluminator

Sequencing Analysis

The results of the sequencing were viewed using the BioEdit Sequence Alignment Editor application. Sequencing analysis was performed by examining the cDNA sequence for the cGnRH-II gene using a BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/) performed with the default setting on the nucleotide sequence of the GenBank database. After that, Multiple Sequences Alignment analysis was performed.

Phylogenetic Tree Analysis

Phylogenetic analysis was carried out to look at the cGnRH-II gene for each of these strains and compared with several other fish species. The relationship between gouramy strain and other fish species was carried out using CLUSTAL W software with the percentage assessment method.

Isolation of RNA and DNAse Treatment

Brain and pituitary samples were taken with tweezers and weighed ± 25 mg for each fish sample. Isolation of RNA samples used a product from Geneaid, namely the Total RNA Mini-Kit. DNAse Treatment using DNA-free™ Thermo Scientific-Kit. All DNAse components were put into a tube and then incubated at 37 °C for 30 minutes. After that, 1 mL of 50 mM EDTA was added and incubated at 65 °C for 10 minutes.

Measurement of cGnRH-II Gene Expression

RNA samples from each strain were then evaluated for the expression of the cGnRH-II encoding gene using KAPA ™ SYBR® *FAST One-Step qRT-PCR Kit,* using the primer : cGnRH-F (5'-TCCAGGAGGAAAGAGGGGTCTGGA-3') and cGnRH-R (5'- TGCGTCCATTTCCTCTGTCAGTGT-3') and primers BA-F (5'- TGACGGAAGGTCATCACC -3') and BA-R (5'- CTCATCGTACTCCTGCTTGC -3'). The primers used in this study used specific real time primers to amplify the cGnRH-II gene in gouramy. Meanwhile, for the house keeping gene, beta actin primers are used which are general in nature, according to research (Novitasari et. al., 2021). The One Step qPCR setting starts from synthesizing cDNA at 42 °C for 5 minutes, then inactivating RT (*Reverse Transcript*) at 95 °C for 2-5 minutes. Then denaturation process at 95 °C for 3 seconds and annealing at 60 °C for ≥ 20 seconds for 40 cycles. The amplification results using *Real Time* PCR were

then used to compare the number of DNA molecules amplified by the cGnRH-II encoding gene with those of the β-Actin gene amplified. The comparison value obtained is then calculated using the formula:

ΔΔCT = (CT cGnRH – CT actin) sample – (CT cGnRH – CT actin) Calibrator, RcGnRH = $2^{-\Delta\Delta CT}$

Description :

ΔΔCt : Threshold cycle
of Ct cGnRH sample : value of Ct cGnRH sample i
Ct *β-actin* sample : value of Ct *β-actin* sample i
Ct cGnRH calibrator : value of Ct cGnRH sample with *actin* Ct *β-actin*
calibrator : the lowest Ct *β-actin* a result of RcGnRH
amplification : cGnRH gene expression level Forlenza et al. (2012)

RESULTS AND DISCUSSION

Identification of the cGnRH-II Gene in Gouramy

The process of identifying the cGnRH-II gene in three gouramy strains was carried out by DNA extraction, then amplified by the PCR method which is a method for duplicating a DNA fragment (Setianingtyas et al., 2015) . In this PCR process, it will amplify the DNA fragment with the target of the cGnRH-II gene sequence. After that, electrophoresis was carried out with the results shown in **Figure 1**.

Based on the results of DNA amplification using one pair of primers (cGnRH- II R and cGnRH–II F) of the cGnRH–II gene obtained band values of about 400 bp for the soang strain, jepun strain and blue sapphire strain. The thickness of the band formed indicates the quality of the genetic character of the sample being analyzed. In well 1 (soang strain), well 2 (jepun strain) and well 3 (blue sapphire strain) the DNA bands formed were quite thick and clumped together. According to Klug and Hazel (1998), the formation of thick bands indicates the number of amplified DNA fragments.

The sequencing results of the three gouramy strains that have been obtained are then aligned using multiple alignments to show *conserved* areas and areas that vary based on the alignment of several sequences. The *conserved* itself has high alignment between sequences (Iqbal et al*.,* 2016). The results for *multiple alignment analysis* performed on the three gouramy strains are shown in **Figure 2**.

Figure 2 shows the alignment results between the three gouramy strains and other fish species from BLAST results, namely *Anabas testudineus* (XM_026348872.1), *Monopterus albus* (XM_020592994.1), *Stegastes partitus* (XM_008280536.1), *Oreochromis niloticus* (XM_003442697.5), *Pundamilia nyererei* (XM_005731234) and *Maylandia zebra* (XM_004545260.1). The structure for the cGnRH-II gene after alignment shows the similarities and differences in the nucleotide of the three strains. Overall, the three gouramy strains show quite a lot of *conserved* which are owned by the three gouramy strains and several other fish species. The conserved itself is shown in red, while the other colors are *diverging*. This is in accordance with the opinion of Dharmayanti (2011), where when the nucleotide or protein sequences of two different organisms are similar, then they are thought to originate from common ancestor or ancestor sequence. Sequence alignment will show sequence positions that do not change or conserved, and sequences that develop or *diverge* to be different from the common ancestor.

The cGnRH–II gene sequence data from the three strains were then compared. The results of this sequence comparison are then visualized in the form of a phylogenetic tree which can show the kinship relationship between the observed strains. The phylogenetic tree makes branches in the form of roots and branches. The root of the tree is a point that acts as an ancestor, while the branch is a point that describes species that are related to each other. The closer the branches, the closer the relationship between species (Baldauf, 2003). Branch relationships in this part of the tree reflect the degree to which the different sequences are related. Two similar sequences will be located as *neighboring outside* of the branches and connected in a*common branch* (Seprianto, 2017).

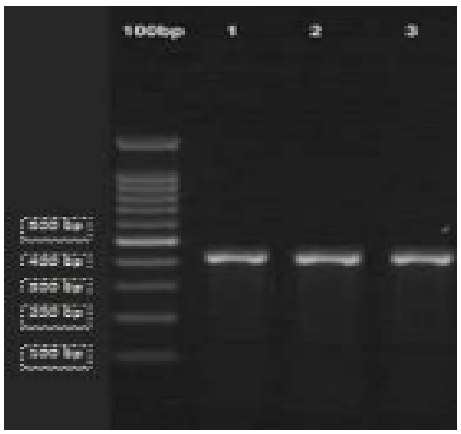

Figure 1. Electrophoretic results of the PCR product of the cGnRH-II gene in three strains of gouramy (*Osphronemus gouramy*) (**Note** : 1 = Soang Strain, 2 = Jepun Strain, 3 = Blue sapphire Strain)

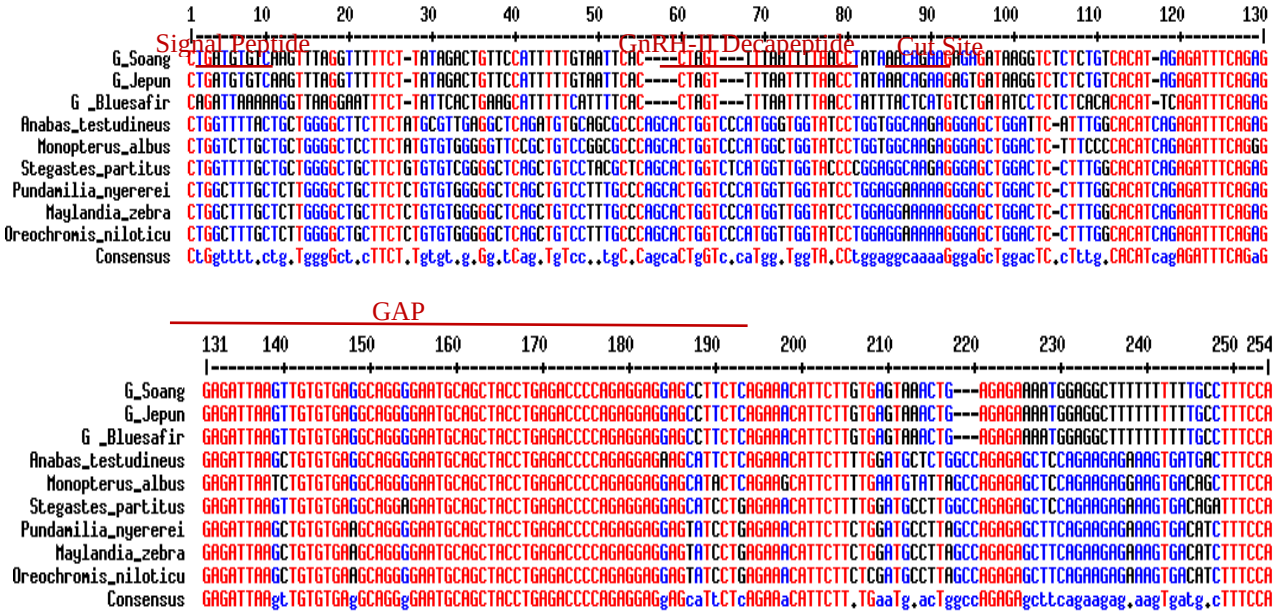

Figure 2. Results of multiple alignment of cGnRH-II gene sequences in three strains of gouramy (*Osphronemus gouramy*)

The results of the phylogenetic tree analysis carried out on three gouramy strains are shown in **Figure 3**. Based on **Figure 3**, the kinship values of the three gouramy strains with other fish species differ. The results of the topological reconstruction of the phylogenetic tree show that the soang strain has the closest kinship with the jepun strain because it is on the same branch with a bootstrap 0.92, which is on a different branch from the blue sapphire strain with a *bootstrap* 1. In addition, gouramy strains are also on a different branch from other fish species with a *bootstrap* 1. *Pundamilia nyererei* and *Maylandia zebra* are on the same branch with *bootstrap* 0.98 and 0.72 for *Stegastes partitus* and 0.2 for *Anabas testudineus* and *Monopterus albus*. The value of *distance scale* in the phylogenetic tree above is 0.1, where *distance scale* is a scale that represents the number of differences between sequences (Vierstraete, 1999).

This phylogenetic tree can be used to see the kinship and genetic distance of a species or strain. Genetic distance is a measure of genetic differences between populations due to mutation, selection, random crossing and gene drift that will lead to evolution. In addition, the phylogenetic tree also describes the changes that occur in the marker genes for each species. The longer a branch means the more changes that occur in marker genes during the evolutionary process, as a result the species that are on that branch can be said to be more advanced (Baldauf, 2003).

Gene Expression of cGnRH-II in Gouramy

Gene expression is the process of transcription of genetic material (DNA) in cells into RNA and then translated into specific polypeptides (Madingan *et al.*, 2009). Michelle (2011) stated that gene expression is the process of the flow of genetic information in determining the nature of an individual through the mechanisms of transcription and translation. Transcription is the process of copying the genetic code in DNA into an RNA molecule, while translation is the process of translating the nucleotide sequence in an mRNA molecule into a sequence of amino acids that make up a polyeptide or protein.

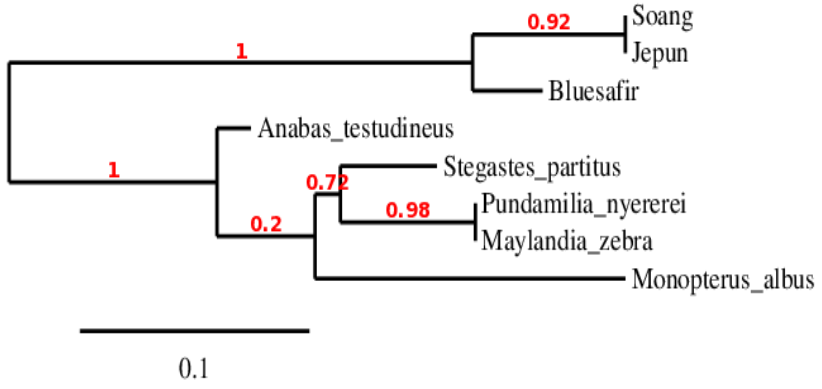

Figure 3. Phylogenic tree of the cGnRH–II gene in three strains of gouramy (*Osphronemus gouramy*)

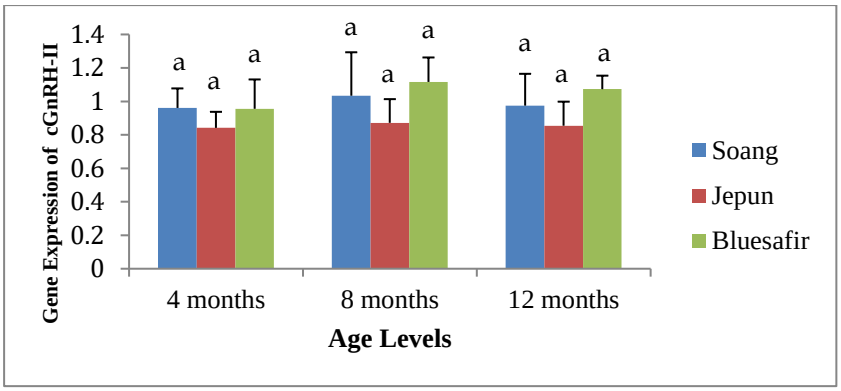

Figure 4. Gene expression level cGnRH-II (±SD) gouramy soang, jepun and blue sapphire strain at different age levels

Gene expression can be analyzed using *Real Time* PCR or qPCR. Polymerase Chain Reaction (PCR) is a method that can be used to reproduce the DNA of an organism (Pertiwi, et al. 2015). Measurement of the expression value of the cGnRH-II gene in gouramy in several different strains can be seen from the R cGnRH-II value obtained from the calculation of the delta CT formula. According to Rahardianti and Nur (2017), the control genes used in qPCR are *housekeeping* constitutively expressed.

The results of measuring the expression value of the cGnRH-II gene in soang, jepun and blue sapphire gouramy can be seen in **Figure 4.** The cGnRH-II gene expression values in the three strains above had an average range of 0.8418 – 1.1163. The soang strain showed cGnRH-II gene expression values ranging from 0.09609 to 1.0341. In the jepun strain, the cGnRH-II gene expression ranged from 0.8418 to 0.8720. In the blue sapphire strain, the cGnRH-II gene expression ranged from 0.9558 to 1.1163. In relative gene quantification, a change in relative expression level (fold change) > 1 indicates an increase in expression; conversely, if the change in the change in expression level is <1, then it is considered that there is a decrease in expression. The value of number 1 is the agreed quantity for determining the target gene (Rahardiati and Nur, 2017). In each gouramy strain, the value of the cGnRH-II gene was expressed at several different age levels. This shows that the cGnRH-II gene is still produced at different ages. According to Fornies *et al.* (2003), the cGnRH-II gene in fish began to be expressed 4 days after hatching. Then, the cGnRH-II gene was still expressed in the pituitary of gonad immature fish even though the levels were very low.

Based on **Figure 4**, it also shows that there were fluctuations in the expression value of the cGnRH-II gene of the three gouramy strains at the age level of 4 to 12 months. At the age of 4 months, 8 months and 12 months the blue sapphire strain showed higher cGnRH-II gene expression values compared to the soang and jepun strains. However, the cGnRH-II gene expression values of the three strains at that age level showed no different results (P>0.05).

The cGnRH-II gene expression values observed from three gouramy strains with different age levels indicated that the blue sapphire strain tended to have high gene expression values, while the strain with low gene expression values was jepun. This is in accordance with the opinion of Agromedia (2007), that the blue sapphire strain has a higher egg-producing ability than the soang and jepun strains. However, the difference in gene expression values did not show different results between the three strains.

Another factor that is thought to be causing the different reproductive abilities of the soang, jepun and blue sapphire strains is that the DNA sequences of each strain are also different. Every organism has a unique genetic makeup that reflects the characteristics of living things. This is because during the translation process, different DNA sequences will produce different protein codes (Sutanto *et al.*, 2013). According to Sahabuddin (2014), genetically no two individuals in one species are exactly the same. Even though two individuals are members of the same species, they can be different due to variations in various factors, including genetic factors, age, sex, food, habitat and others.

It is well known that the cGnRH-II gene is one of the key genes in the reproductive process. These genes play a role in gonad maturation and neuromodulator (signal delivery neurons). This gene is secreted by the preoptic part of the hypothalamus nerve, which will then trigger the synthesis and secretion of the gonadotropins, namely LH and FSH (Branco *et al.*, 2019).

CONCLUSIONS

The sequences of the three gouramy strains have the same (conserved) sequence and different (divergent) parts, where the soang strain is closer to the jepun strain, while the blue sapphire strain is on a different branch from the other two strains. Meanwhile, the gene expression values of three gouramy strains with different age levels showed varied results, where the highest cGnRH-II gene expression value was the blue sapphire strain at 8

months old and the lowest was the jepun strain at 4 months old. However, the three strains showed no different results.

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