Thesis for the Degree of Master of Science

Characterization of Histone Gene Expression in Sevenband Grouper, *Hyporthodus septemfasciatus* against Nervous Necrosis Virus Infection

by

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School of Marine and Fisheries Life Science

(Major in Microbiology)

The Graduate School

Pukyong National University

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(신경괴사증바이러스에 감염된 능성어 Histone 유전자 발현 특성)

Advisor: Prof. Gun-Do Kim

by

Dong-Ryun Lee

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Abstract

Sevenband grouper (*Hyporthodus septemfasciatus*) is a high-quality fish that is one of the most popular seafood species for consumption. A group of deaths occurred in fish farms as a result of an infection by the Nervous Necrosis Virus (NNV), that causes viral nervous necrosis (VNN) disease. Studies of other fish species revealed that histone proteins are involved in innate immune responses during pathogen invasion as well as DNA packing. In this study, we characterized the histone ORF genes (H1, H2A, H2A.V, H3.3-1, H3.3-2) of sevenband groupers and analyzed histone gene expression in NNV-infected sevenband groupers. The histone gene amino acid sequences of sevenband grouper were similar among fishes and even with mammals and were an evolutionarily conservative. High gene expression of histone genes was observed in the tissues of sevenband grouper infected by NNV. Quantitative real time PCR analysis of cDNA showed that H1 had a high gene expression level in brain and eye on day 2 after infection, and H2A.V had a high gene expression level in brain and blood after NNV-infected. On day 2 after infection, H3.3-1 showed high gene expression in the liver and blood, and H3.3-2 showed the highest gene expression in the brain and blood. An increase in the amount of extracellular histone protein in the blood has been identified as a biomarker for vascular function in human, an increase in gene expression level of histone proteins in the blood of sevenband grouper implied that it is reasonable to assume that the histone gene is involved in the innate immune response in fish. More research is required to understand Histone's immune response at the protein level or in aquatic animals.

1. Introduction

One of the viral diseases of concern in aquaculture is viral nervous necrosis (VNN). The virus that causes this disease, nervous necrosis virus (NNV), is a member of the *Nodaviridae* family, which is further subdivided into *alphanodavirus* and *betanodavirus*. *Alphanodavirus* mainly infects insects and *betanodavirus* infects fish. NNV, which causes mass mortality in fish farms, belongs to the family of *betanodoviruses*. When fish were infected with NNV, vacuolization occurs in the gray matter of the brain, the spinal cord, and the granular layer of the retina, which gradually darkens and causes abnormal swimming behavior. NNV spreads not only horizontally but also vertically. it is extremely harmful to fish or fry in the hatching stage, with a 100 percent death rate, resulting in catastrophic ecosystem damage. [1,2].

NNV has an icosahedral structure without envelopment and has a diameter of 20-30 nm. It has two strands of positive sense RNA, RNA1 (about 3.1 kb) encodes RNA-dependent RNA polymerase required for viral genome replication, and RNA2 (1.4 kb) encodes capsid protein [3,4]. RNA3 is made from the 3'-terminus of RNA1 and encodes a non-structural protein, B2 protein [5]. The virus-like particle contains 180 capsid protein subunits, and each capsid protein (CP) has three major domains: (i) the N-terminal arm, which is an inter-subunit extension at the inner surface; (ii) the shell domain (S-domain), which is a jelly-roll structure; and (iii) the protrusion domain (P-domain), which is formed by three-fold trimeric protrusions. [6].

Based on the phylogenetic analysis of the T4 variable region, which consists of 427 bases of RNA2 sequence from 25 isolates, the fish *nodaviruses* are classified into four genotypes: tiger puffer NNV (TPNNV), striped jack NNV (SJNNV), barfin flounder NNV (BFNNV), and red-spotted grouper NNV (RGNNV). RGNNV is the most widely distributed virus affecting the highest number of tropical and temperate fishes where the water temperature ranges from 25 to 30 ◦C which is optimal for the virus replication [7].

Groupers are important warm temperate water fishes that live in tropical, subtropical, and temperate water zones and have a high economic value in aquaculture [8]. They are members of the *Epinephelidae* family, which includes 159 marine species divided into 15 genera. The sevenband grouper, *Hyporthodus septemfasciatus*, is a highly prized marine finfish with significant aquaculture potential in Southeast Asia. They live in water depths ranging from 5 to 30 meters and are found primarily in Korea, Japan, and China[9]. Because of their scarcity of resources and high economic value, sevenband groupers have been considered candidate species for aquaculture. Sevenband

grouper aquaculture took off in Korea 20 years ago, with 292 tons produced in 2018, 145 tons in 2019, and 79 tons in 2020 [10]. The use of wild seeds and bloodstocks in grouper aquaculture in Korea has resulted in outbreaks of viral nervous necrosis (VNN) in cages and offshore floating net-pens. The decrease in grouper production is due to an increase in larval mortality in hatcheries and cage cultured juveniles and adults. The VNN outbreak was first detected in cage-cultured seven band grouper on Korea's south coast in the summer of 1990[11]. NNV has since been isolated from a variety of cultured fish species, including rock bream (*Oplegnathus fasciatus*), olive flounder (*Paralichthys olivaceus*), grey mullet (*Mugil cephalus*), and red drum (*Sciaenops ocellatus*)[12] .

Neutrophils are immune phagocytes that play an important role in immune defense. Given that this neutrophil arsenal can also damage host tissues, its deployment is tightly regulated through three major strategies: phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs). NETs are a large extracellular web-shaped structure made up of cytoplasmic and granular proteins that is built on top of chromatin condensates. Although the majority of the DNA in NETs relates from the nucleus, these structures also have mitochondrial DNA. NETs are thought to trap, neutralize, and kill bacteria, fungi, viruses, and parasites, as well as prevent bacteria and fungi from spreading. There are two ways for neutrophil extracellular traps (NETs) to form. The first is an apoptotic pathway known as NETosis, which begins with nuclear envelope breakdown and fission and progresses to cell polarization loss, chromatin decondensation, and plasma membrane rupture. The second type of NETosis is insoluble NETosis, which can occur independently of cell death and involves the secreted release of nuclear chromatin followed by the degradation of granular proteins. These components combine to form outer lobes, leaving an active ocular nuclear membrane that continues to digest microbes[13]. NETs contain all core histones, known as H2A, H2B, H3, and H4, and account for 70% of all NET-associated proteins. [1]

Histone proteins are involved in DNA packing as well as regulation of gene expression and innate immune response to pathogens [14]. Histones are proteins found in eukaryotes and some archaea, and are classified into five types: H1/H5, H2A, H2B, H3, and H4. H1/H5 is a linker histone, which binds to the linker DNA that connects the nucleosome structures and plays a role in aggregating the nucleosome structures [15]. H2A, H2B, H3, and H4 are core histones, and these four proteins form an octamer and a nucleosome structure with DNA wrapped around the octamer. Each core histone has a "histone-fold" domain in the central portion, an N-terminal tail, and a C-terminal tail[16]. Post-translational modifications that occur frequently in tails include methylation, acetylation, propionylation, butylation, crontonylation, 2-hydroxyisobutylation, malonylation, succinylation, formylation, ubiquitination, citrullination, phosphorylation, O-GlcNAcylation, and ADP ribosylation [17].

There have not been many studies on the innate immunity of histone proteins in fish, and only a few studies have been reported. Olive flounder's histone H1-like protein showed strong antimicrobial activity against Gram positive and Gram-negative bacteria [18]. The H1 and H2B of european sea bass and gilthead seabream were found to relate to the immune response of NNV [19]. In *Carassius aurutus*, H2A was shown to play an important role in defense against invasion by *Aeromons hydrophila* [20]. Other studies have shown that histone mimics may not only potentiate the ability of the virus to interfere with the host, but also increase virus dependence on host transcription [21]. Furthermore, in the case of lytic infection by herpes simplex virus type 1, a study confirmed that histone strain H3.3 regulates gene expression [22]. However, little study has been done on the role of histone in NNV-infected sevenband grouper.

In this study, we studied the characteristics of the transcriptome of five histones (H1, H2A, H2A.V, H3.3-1, H3.3-2) of the sevenband grouper and analyzed the histone transcript expression level in each tissue of the sevenband grouper infected with NNV.

2. Materials & Methods

2.1. Fish

Juvenile sevenband grouper. *Hyporthodus septemfasciatus* were purchased from aquafarm where VNN had never occurred. Fishes were acclimated to the laboratory recirculating seawater system at 25℃ for two weeks and were fed twice daily before experimental manipulation. To confirm the absence of NNV, representative samples were screened by real-time PCR before use in experiments.

2.2. Virus preparation

In this study, SGYeosu08, an NNV isolate, was used. This isolate, belonging to the RGNNV(Red Grouper Nervous Necrosis Virus) genotype of the genus *Betanodavirus*, was obtained from a seven-band grouper affected by NNV in Yeosu in 2008. NNV was propagated in SSN-1 cell line, which had been established from whole fry tissue of striped snakehead *Ophicephalus striatus* [23]. To propagate NNV, SSN-1 cells were grown in 75-㎠ cell culture flasks until 90-95% confluence and NNV was inoculated onto SSN-1 cell monolayer. Once an extensive complete cytopathic effect(CPE) was observed, cell lysate was collected and centrifugated at 3,300 x g, for 15 min at 4℃. After centrifugation, the supernatant was aliquoted and stored at -80℃ as virus sample.

2.3. Histone gene identification

Sevenband grouper transcriptome database analyzed in the previous study was used [24]. The cDNA sequences, which indicated homology to known histone genes (H1, H2A.V H2A, H3.3-1, H3.3-2), were identified using BLAST (Basic Local Alignment Search Tool) pro from the National Center for Biotechnology Information (NCBI).

2.4. Polymerase chain reaction (PCR) amplification, cloning, and sequencing of the histone sequence in sevenband grouper

Primers were designed based on sevenband grouper transcriptome database (Table 1). The PCR amplified products were cloned and re-sequenced to evaluate the open reading frame (ORF). Total RNA was extracted from the brain of *H. septemfasciatus* using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. cDNA was synthesized using SuPrimeScript RT-premix (GeNet Bio, Daejeon, Korea). Then, cDNA was amplified using Table 1 primer set. The PCR reaction was performed in a 20 $\mu\ell$ volume containing AccuPower™ PCR Premix (Bioneer, Daejeon, Korea), 1 $\mu\ell$ of each specific primer pair, 2 $\mu\ell$ cDNA and 16 $\mu\ell$ ddH_20. The PCR conditions were 95℃ for 10 min, followed by 30 cycles of 95℃ 30 sec, 60℃ 30 sec, 72℃ 1 min. PCR product was eluted from the agarose gel with AccuPrep™ PCR/Gel Purification Kit (Bioneer, Daejeon, Korea) and ligated with pGEM®-T Easy Vector of TA cloning kit (Promega). Ligation mixture was incubated for overnight at 4℃ and transformed into BioFACT™ competent Cell(DH5α) (BioFACT, Daejeon, Korea) by heat shocked transformation. The transformants were placed on MacConkey agar plate containing ampicillin (50 mg/ml) and incubated at 37℃ for 18 hours. The single colony was inoculated to LB broth containing ampicillin (50 mg/ml) and plasmid DNA was extracted with AccuPrep™ Plasmid Mini Extraction Kit (Bioneer, Daejeon, Korea). Plasmid DNA was sequenced by Bioneer(Daejeon, Korea)

Table 1. Primers used for histone gene cloning.

Primer	Sequence(5'-3')
H1 Forward	ATGGCAGAAGTAGCTCCAGCTC
H1 Reverse	TCACTTCTTCTTGGGTGCTGCT
H2A.V Forward	ATGGCTGGTGGCAAGGCAG
H2A.V Reverse	CTATGCAGTCTTCTGCTGGCCC
H2A Forward	ATGTCTGGCAGAGGGAAAACCG
H2A Reverse	TTACTTGCTCTTGGCCGGTTTCT
H3.3-1 Forward	ATGGCCCGTACCAAGCAGA
H3.3-1 Reverse	CTAAGCGCGCTCTCCACG
H3.3-2 Forward	ATGGGGTGTAGAGGGACGGA
H3.3-2 Reverse	TCATTGGTTTTTTGTCTTCACCAAATGT

2.5. Sequence analysis

Conserved domains and signal peptides of the histones (H1, H2A.V, H2A, H3.3-1) polypeptide were analyzed using the PROSITE database searching tool. The molecular weight and isoelectric point of histones (H1, H2A.V, H2A, H3.3-1, H3.3-2) were predicted using the ProtParam tool on the ExPASy proteomics server(http://web.expasy.org/protparam/). The similarity of histones with other histones was analyzed using the BLASTN search program (http://www.ncbi.nlm.nih.gov/blast). Multiple-sequence alignment of the reported histones gene sequence was constructed by ClustalW and a phylogenetic tree was constructed using the MEGA 11 software based on maximum likelihood method with 1,000 bootstrap replicates. Acetylation sites, DNA binding sites, homodimerization interface, H2A-H2B dimerization interface and ubiquitination site were predicted using InterProScan sequence search (http://www.ebi.ac.uk/interpro/search/sequence-search).

2.6. Virus infection and sampling

Juvenile sevenband grouper (average weight 12.3 ± 0.7 g) were intramuscularly injected with NNV (SGYeosu08 strain, RGNNV genotype) at a dose of 10^4 $TCID_{50}$/100 ㎕/fish, whereas negative control fishes were injected with L15 medium at 100 ㎕/fish. After the challenge, animals (n = 3 from each tank) from infected and control groups were collected at five time points (24h, 48h, 72h, 96h, 120h) post challenge. Tissues (spleen, heart, gill, brain, eye, liver, kidney, and blood) were collected aseptically from the sampled fishes.

2.7. In vivo quantitative real-time PCR analysis of histone expression

Total RNA was extracted from healthy sevenband grouper spleen, heart, gill, brain, eye, liver, kidney and blood with *TransZol* Up (Transgenbiotech, Beijing, China). cDNA was synthesized using M-MLV Reverse Transcriptase (Bioneer, Daejeon, Korea). Quantitative real-time PCR was performed to determine expression levels of histones in different tissues on *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). The PCR reaction was performed in a 20㎕ volume containing 10㎕ AccuPower 2X Greenstar qPCR Mastermix, 1㎕ of each specific primer pair, 2㎕ cDNA, 6㎕ ddH_20. The PCR conditions

were 95℃ for 5 min, followed by 40 cycles of 95℃ 30 sec, 60℃ 30 sec, 72℃ 30 sec. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified. The expression level of histones was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with EF1α as an internal reference. EF1α was amplified as an internal control using primers EF1α_F and EF1α_R [25], while histones amplify used primers. The primers used, specific for the histone (H1, H2A.V, H2A, H3.3-1, H3.3-2) were designed using the Primer3Plus tool (Primer3Plus - Pick Primers) and are shown in Table 2.

Table 2. Primers used for in vivo qPCR analysis of histone expression.

Primer	Sequence(5'-3')
EF1α qPCR F	CGAGAAGTTCGAGAAGGAAGC
EF1α qPCR R	GATGAGCTGCTTCACACCAAG
H1 qPCR F	TTCTTGGGTGCTGCTTTCTT
H1 qPCR R	CGAGAAAGTTGCCAAAAAGG
H2A.V qPCR F	AGCGGTGAGGTACTCAAGGA
H2A.V qPCR R	GGTCGTATCCACAGGCACTT
H2A qPCR F	AGCCAGGGACAACAAGAAGA
H2A qPCR R	AGCTTGTTGAGCTCCTCGTC
H3.3-1 qPCR F	TGGAGGAAAAGCTCCTCGTA
H3.3-1 qPCR R	CGACGGATTTCTCTCAAAGC
H3.3-2 qPCR F	CTTTGCAGATCAGAGGCACA
H3.3-2 qPCR R	CTGTTTGCCACACAAACCAC

3. Results

3.1. Cloning and sequencing of *Hyporthodus septemfasciatus* histone genes

Five histone gene sequences were retrieved using PCR, cloning and sequencing from the NNV-infected sevenband grouper brain transcriptome data obtained in the previous study. The InterProScan tool was used to examine the properties of each gene.

A band of about 627 bp was obtained by PCR using primers that were designed based on histone H1 gene. Based on the ORF, the encoded protein histone H1 was predicted to contain 208 amino acid residues, with a molecular weight of 21.2 kDa. The histone H1/H5 domain is represented by a 24-99 amino acid sequences. A red line denotes the start of the sequence. (Figure 1)

The sequence length of H2A.V ORF gene is 387 bp, encoding a protein of 128 amino acid residues, with a molecular weight of 13.5 kDa. A red line indicates the 7-94 amino acid sequences of the histone H2A/H2B/H3 binding domain. Sky blue lines denote the 95-127 amino acid sequences that make up the histone H2A_C binding region. The ubiquitination site is represented by the 122th amino acid sequence, lysine. The acetylation site is represented by the 8th and 12th amino acid sequences, lysine. The ubiquitination and acetylation sites are highlighted in yellow and green, respectively. A bright green line denotes the homodimerization interface, while a black line denotes the DNA binding site. A purple line indicates the H2A-H2B dimerization interface. (Figure 2)

The sequence length of H2A ORF gene is 387 bp, encoding a protein of 128 amino acid residues, with a molecular weight of 13.7 kDa. A red line indicates the 5-89 amino acid sequences of the histone H2A/H2B/H3 binding domain. Sky blue lines denote the 92-126 amino acid sequences that make up the histone H2A_C binding region. The ubiquitination site is represented by the 120th amino acid sequence, lysine. The acetylation site is represented by the 6th and 10th amino acid sequences, lysine. The ubiquitination and acetylation sites are highlighted in yellow and green, respectively. A bright green line denotes the homodimerization interface, while a black line denotes the DNA binding site. A purple line indicates the H2A-H2B dimerization interface. (Figure 3)

The sequence length of H3.3-1 ORF gene is 411 bp, encoding a protein of 136 amino acid residues, with a molecular weight of 15.3 kDa. The histone H2A/H2B/H3 binding domain is represented by a 1-132 amino acid sequence. A red line denotes the start of the sequence. H3 and its variation, CENP-A (Centromere protein A), are included in the H3.3-1 amino acid sequence. (Figure 4)

The sequence length of H3.3-2 ORF gene is 360 bp, encoding a protein of 119 amino acid residues, with a molecular weight of 13.4 kDa. The InterProScan program did not give analysis findings for H3.3-2 amino acid sequences. (Figure 5)

Figure 1. The nucleotide and amino acid sequences of *H. septemfasciatus* histone H1

H1 gene consists of a 627-bp ORF encoding 208 amino acids. Histone H1/H5 binding domain is represented by the red line.

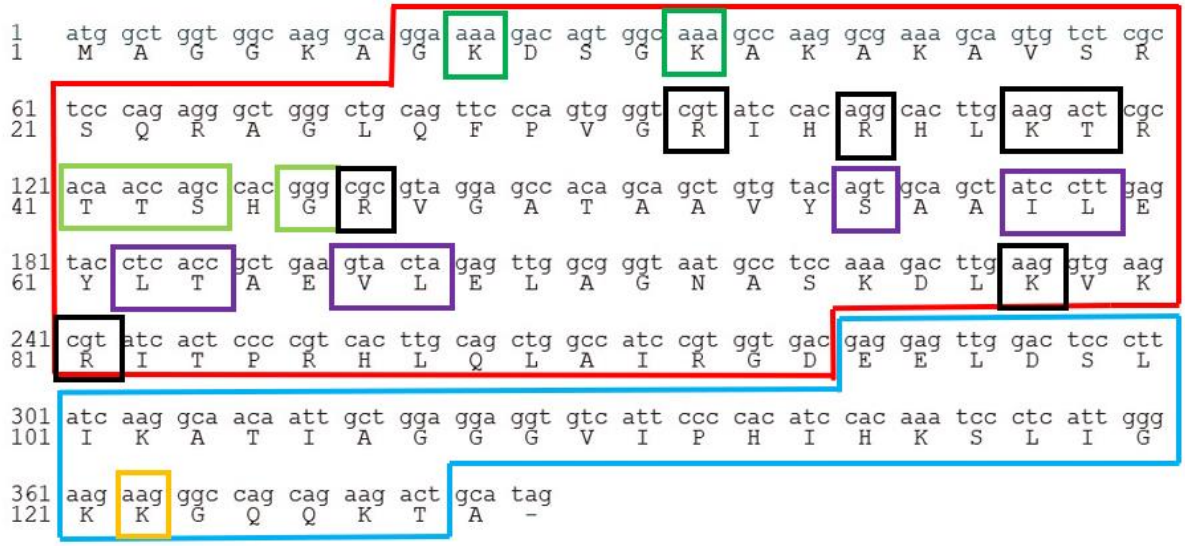

Figure 2. The nucleotide and amino acid sequences of *H. septemfasciatus* histone H2A.V

H2A.V gene consists of a 387-bp ORF encoding 128 amino acids.

Red line: histone H2A/H2B/H3 binding domain,

Sky blue line: histone H2A_C binding domain

Yellow line: ubiquitination site

Green line: acetylation site

Bright green line: homodimerization interface

Black line: DNA binding site

Purple line: H2A-H2B dimerization interface

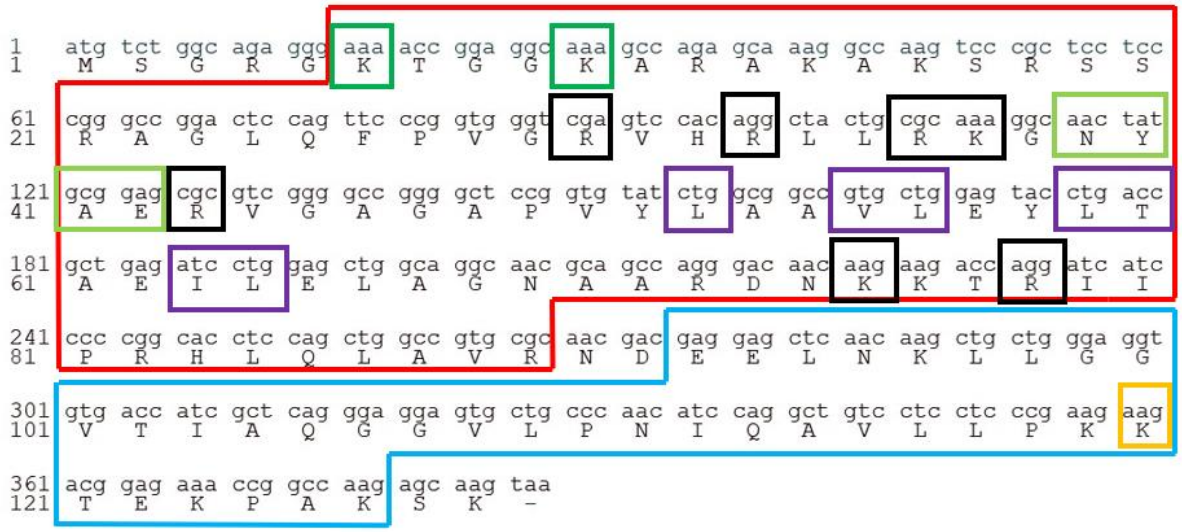

Figure 3. The nucleotide and amino acid sequences of *H. septemfasciatus* histone H2A.

H2A gene consists of a 387-bp ORF encoding 128 amino acids

Red line: histone H2A/H2B/H3 binding domain,

Sky blue line: histone H2A_C binding domain

Yellow line: ubiquitination site

Green line: acetylation site

Bright green line: homodimerization interface

Black line: DNA binding site

Purple line: H2A-H2B dimerization interface

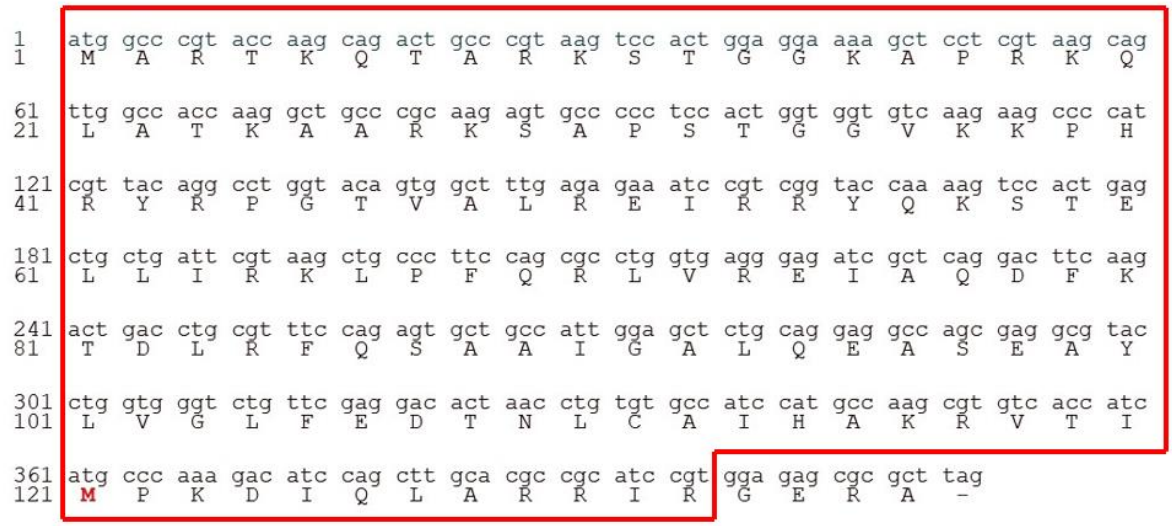

Figure 4. The nucleotide and amino acid sequences of *H. septemfasciatus* histone H3.3-1

H3.3-1 gene consists of a 411-bp ORF encoding 136 amino acids. Histone H2A/H2B/H3 binding domain is represented by the red line. CENP-A is present throughout the sequence.

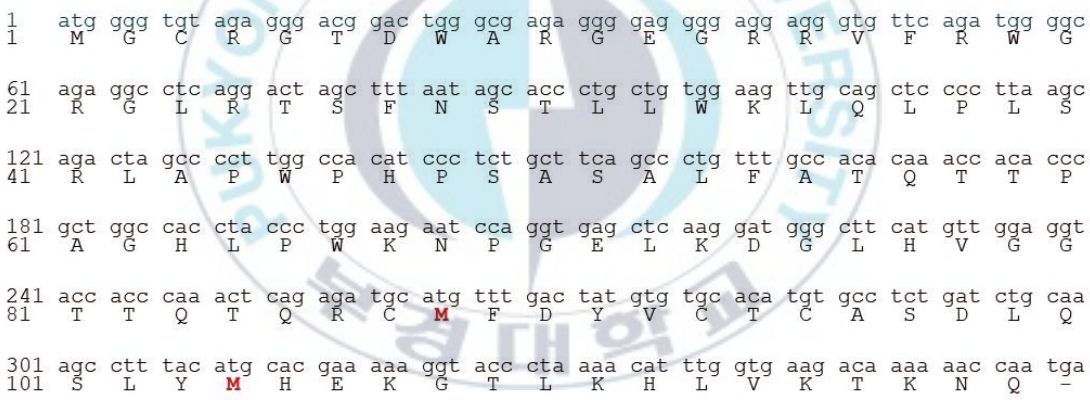

Figure 5. The nucleotide and amino acid sequences of *H. septemfasciatus* histone H3.3-2

H3.3-2 gene consists of a 360-bp ORF encoding 119 amino acids.

3.2. H1, H2A.V, H2A protein sequence alignment and phylogenetic analysis

The histone H1, H2A.V, H2A amino acid sequence was aligned by comparing it to that of other species, and phylogenetic analysis was used to analyze the similarities across species(Figure 6 ~ 11). The *H.septemfasciatus* histone amino acid sequence was found to be comparable to that of other species and to have a common domain. The phylogenetic tree confirmed the difference in lineage between fish and other species. Phylogenetic tree of complete amino acid sequence of histones. Phylogenetic tree was constructed by maximum likelihood tree method using MEGA11. The number at each node indicates the percentage of bootstrapping after 1,000 replicates.

Figure 6. Multiple alignments of H1 with other known H1 proteins.

Gray shading indicates an amino acid sequence that matches more than 60% of the time, while black shading indicates a sequence that matches 100% of the time. A red line indicates the H1/H5 DNA binding domain.

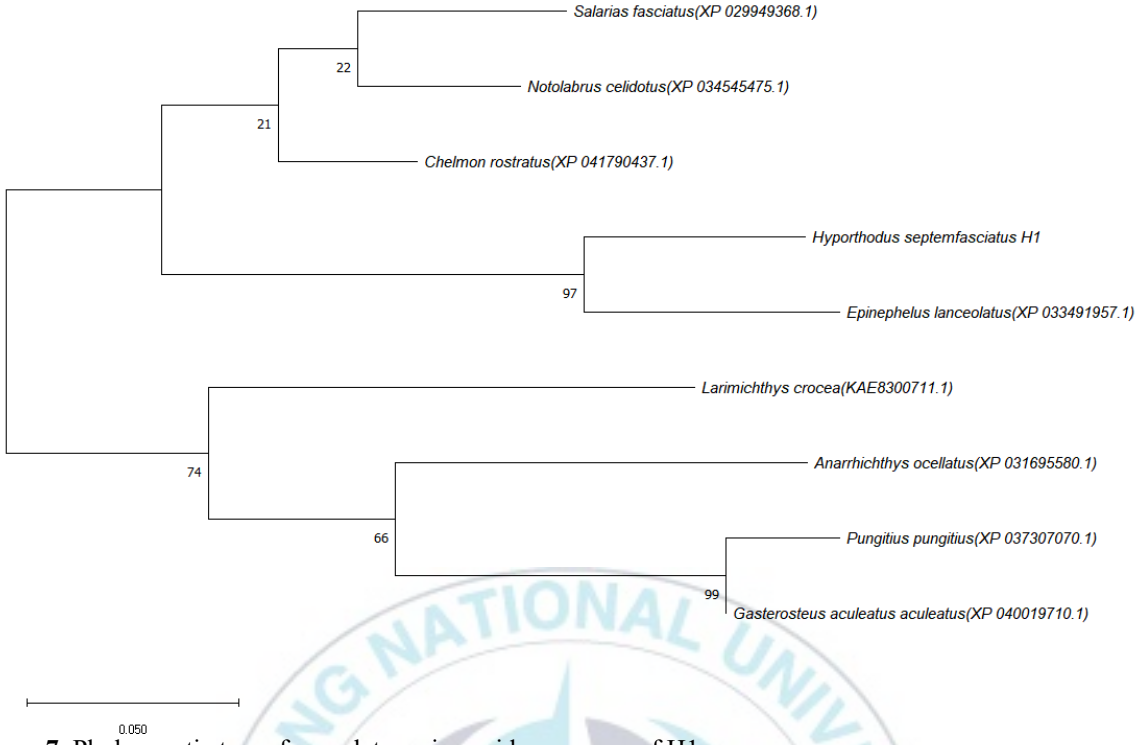

Figure 7. Phylogenetic tree of complete amino acid sequences of H1.

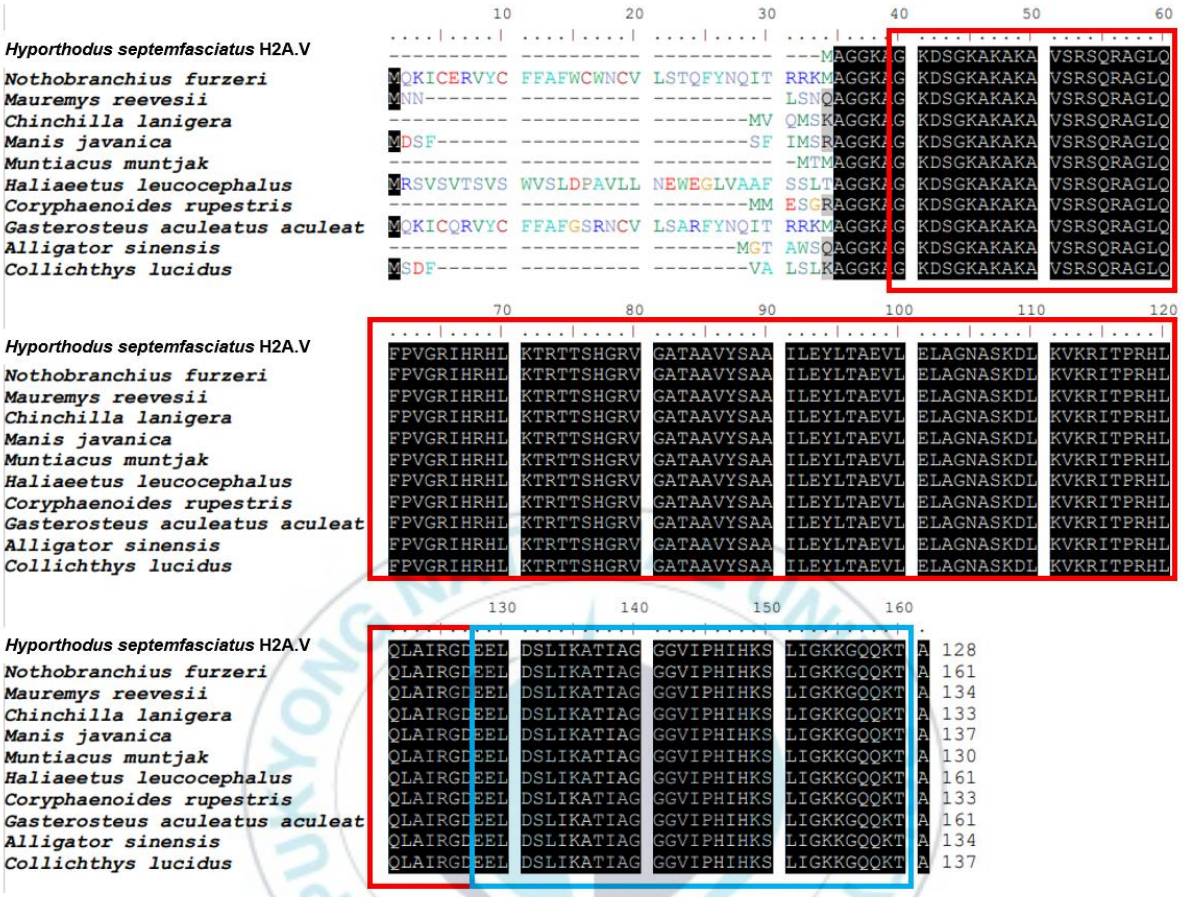

Figure 8. Multiple alignments of H2A.V with other known H2A.V proteins.

A red line indicates the H2A/H2B/H3 DNA binding domain. H2A_C binding domain is represented by the sky-blue line.

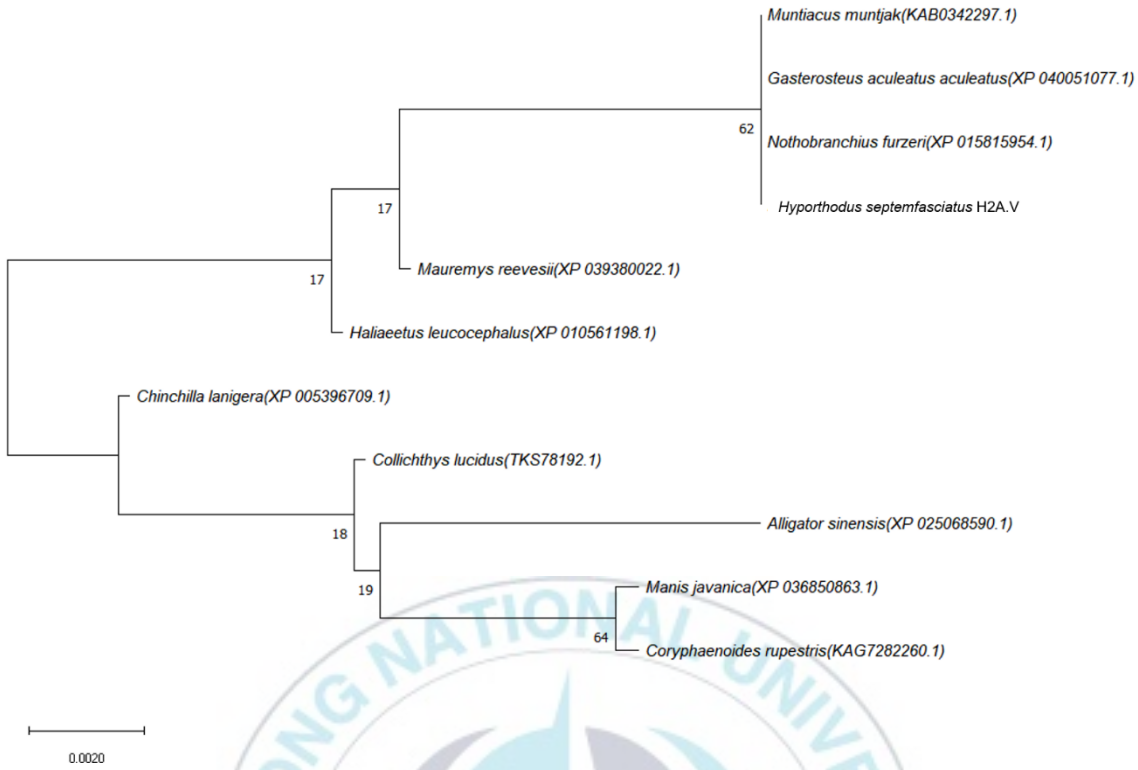

Figure 9. Phylogenetic tree of complete amino acid sequences of H2A.V.

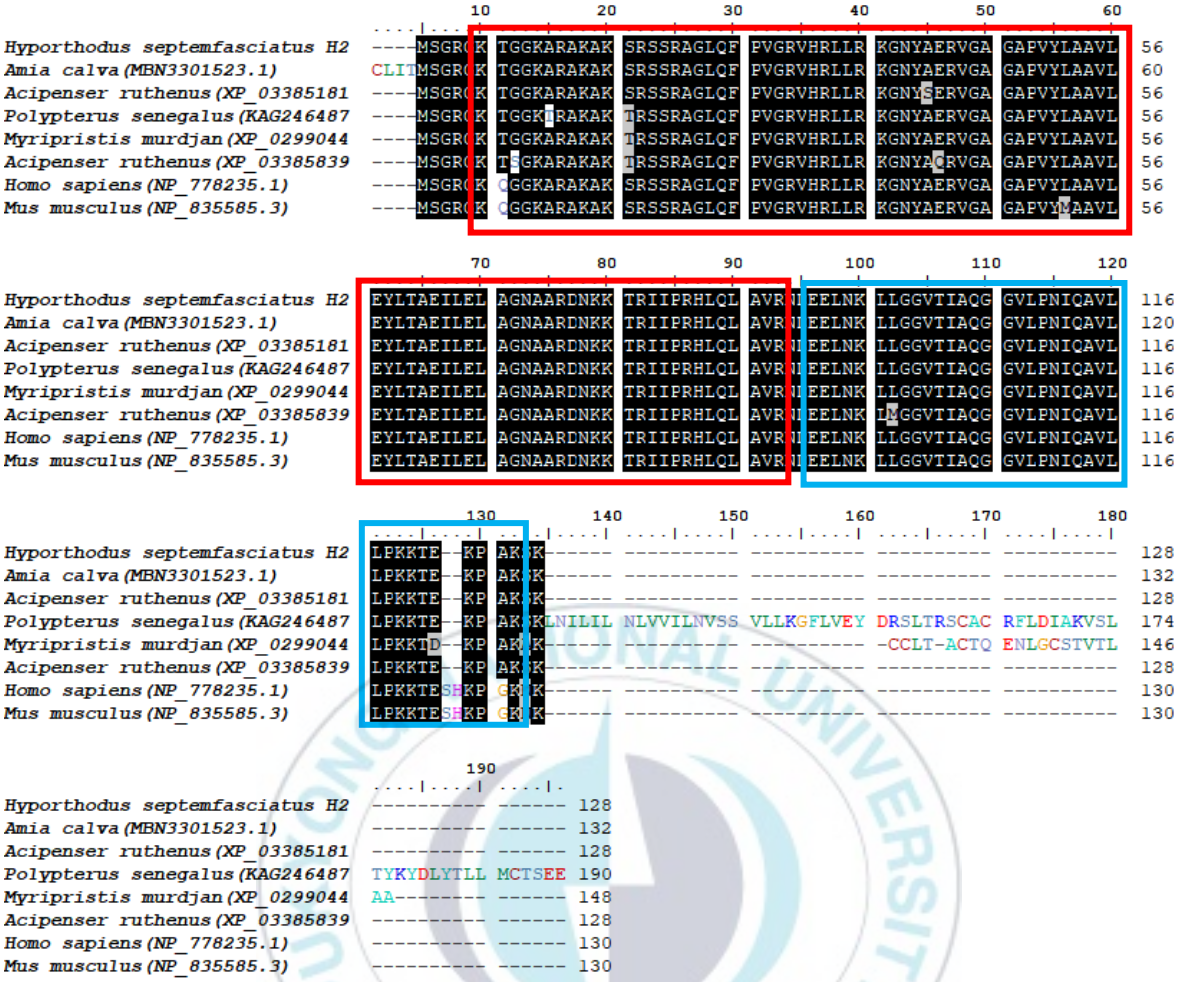

Figure 10. Multiple alignments of H2A with other known H2A proteins.

A red line indicates the H2A/H2B/H3 DNA binding domain. H2A_C binding domain is represented by the sky-blue line.

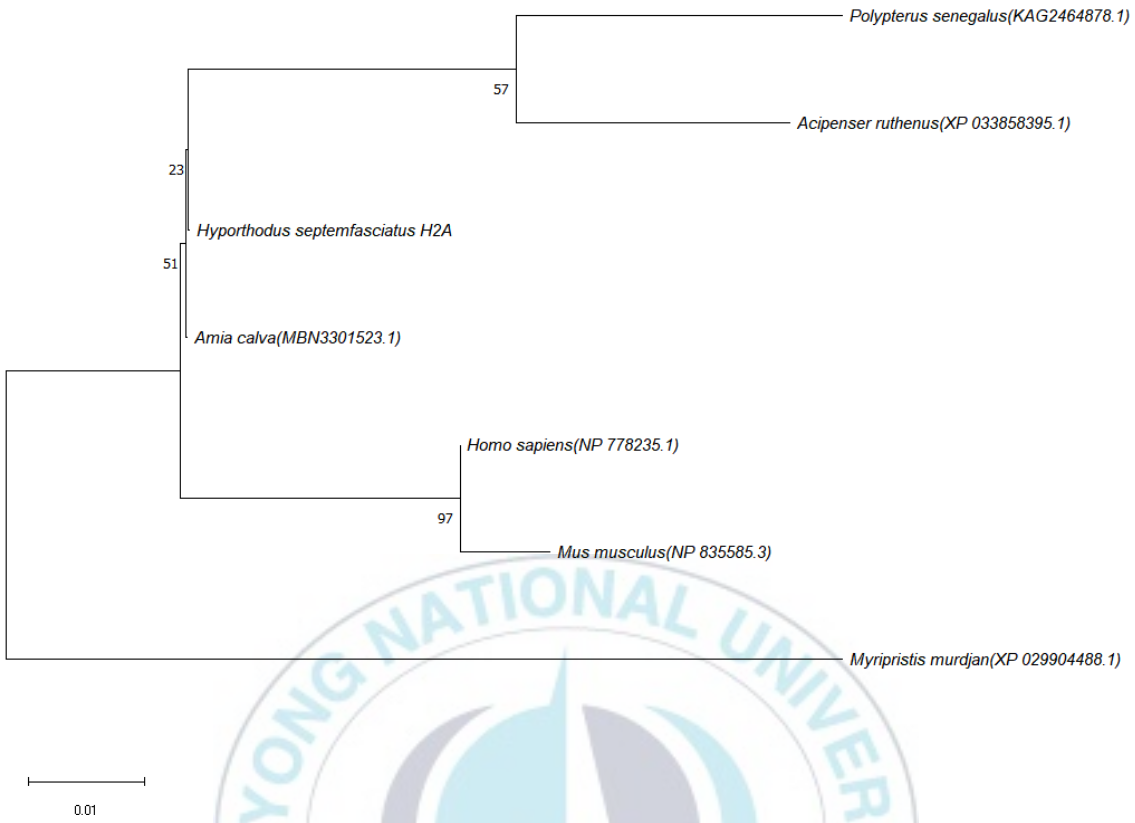

Figure 11. Phylogenetic tree of complete amino acid sequences of H2A.

3.3. H3.3-1, H3.3-2 gene sequence alignment and phylogenetic analysis

There was no change between the H3.3-1 amino acid sequence and a previously known sequence when compared to other species. The amino acid sequences of H3.3-2 could not be found in NCBI. As a result, the two genes were used to examine sequences and lineage between species by Gene sequence. H3.3-1 confirmed that similar sequences existed between species (Figure 12), and that similar species were categorized as a result of the systematic analysis (Figure 13). Due to the substantial variance in amino acid sequences between heterogeneous species, H3.3-2 confirmed similar sequences between heterogeneous species (Figure 14), but systematic analysis confirmed that only the *H.septemfasciatus* was categorized individually (Figure 15). Phylogenetic tree of complete gene sequence of histones. Phylogenetic tree was constructed by maximum likelihood tree method using MEGA11. The number at each node indicates the percentage of bootstrapping after 1,000 replicates.

Figure 12. Multiple alignments of H3.3-1 with other known H3.3-1 genes.

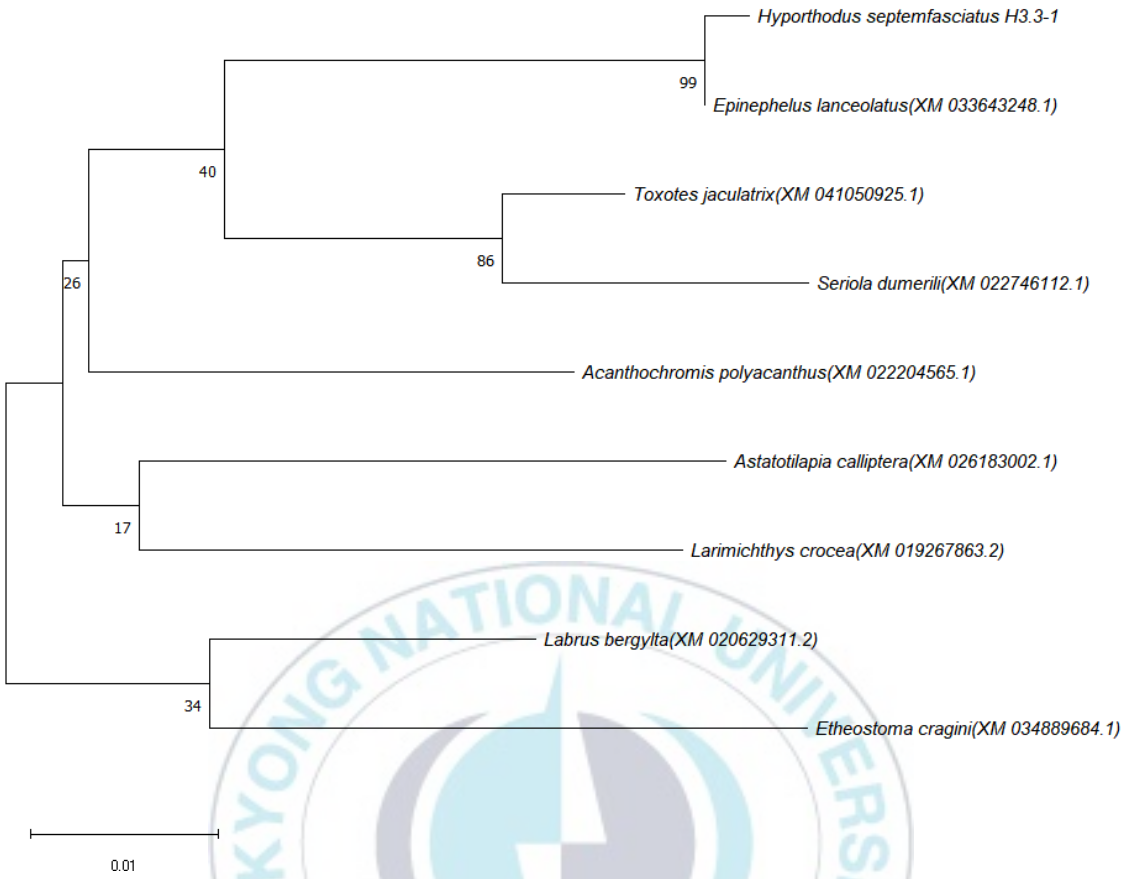

Figure 13. Phylogenetic tree of gene sequences of H3.3-1.

Figure 14. Multiple alignments of H3.3-2 with other known H3.3-2 genes.

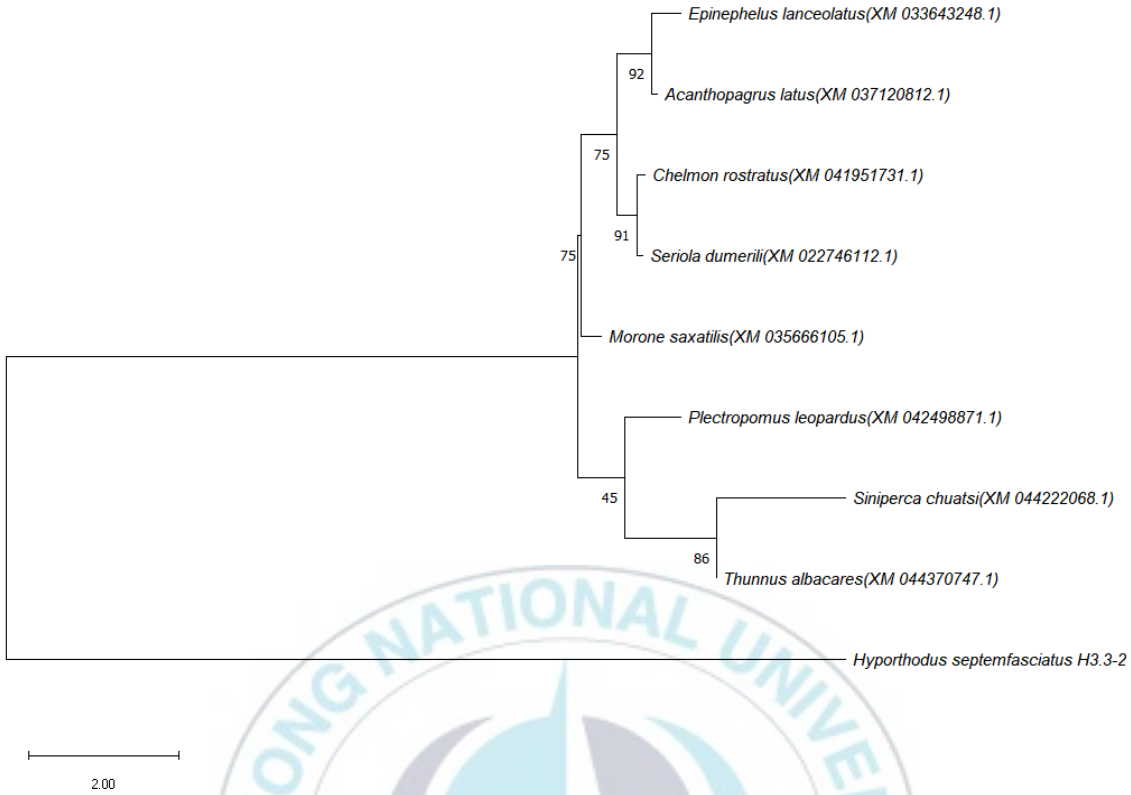

Figure 15. Phylogenetic tree of gene sequences of H3.3-2.

3.4. Expression of histones under natural conditions

Tissue specific expression analysis showed that each of the 5 histones was expressed in all eight tissues (spleen, heart, gill, brain, eye, liver, kidney and blood) examined, although the level of expression differed.

The tissue with the highest expression of H1, H2A genes was spleen followed by gill (Figure 16, 18). However, H2A.V, H3.3-1, H3.3-2(Figure 17, 19, 20) showed highest gene expression levels at heart. Expression levels calculated relative to blood expression. Values are shown as mean ± SEM (N = 3). N, the number of times the experiment was performed.

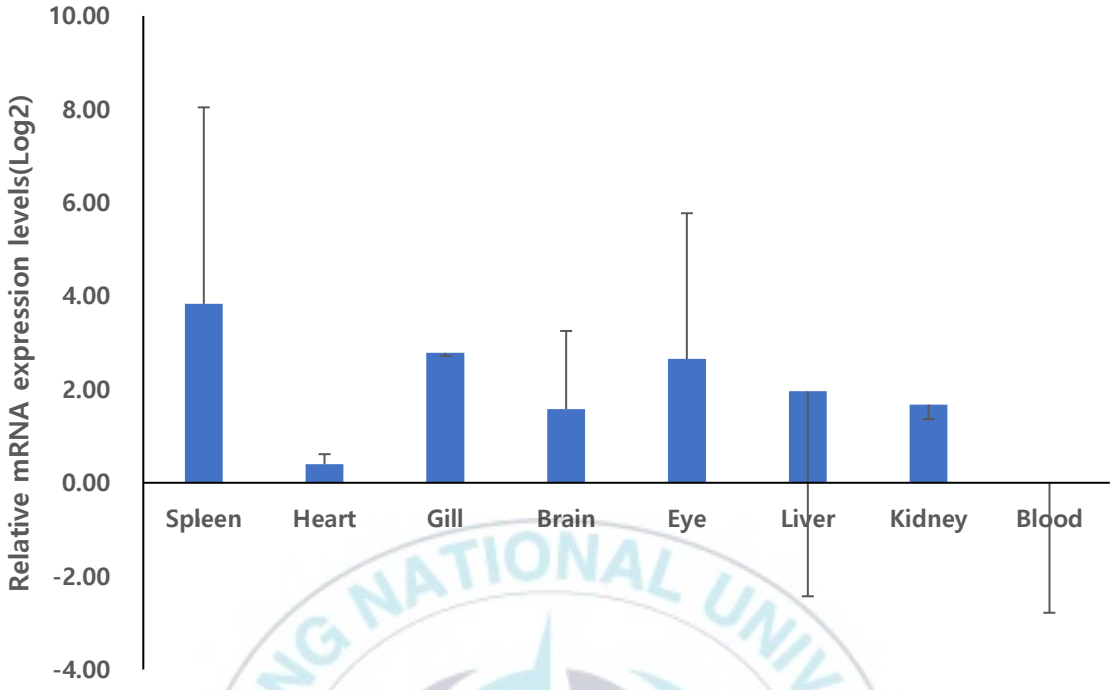

Figure 16. Histone H1 expression in *H.septemfasciatus* tissues under normal physiological condition.

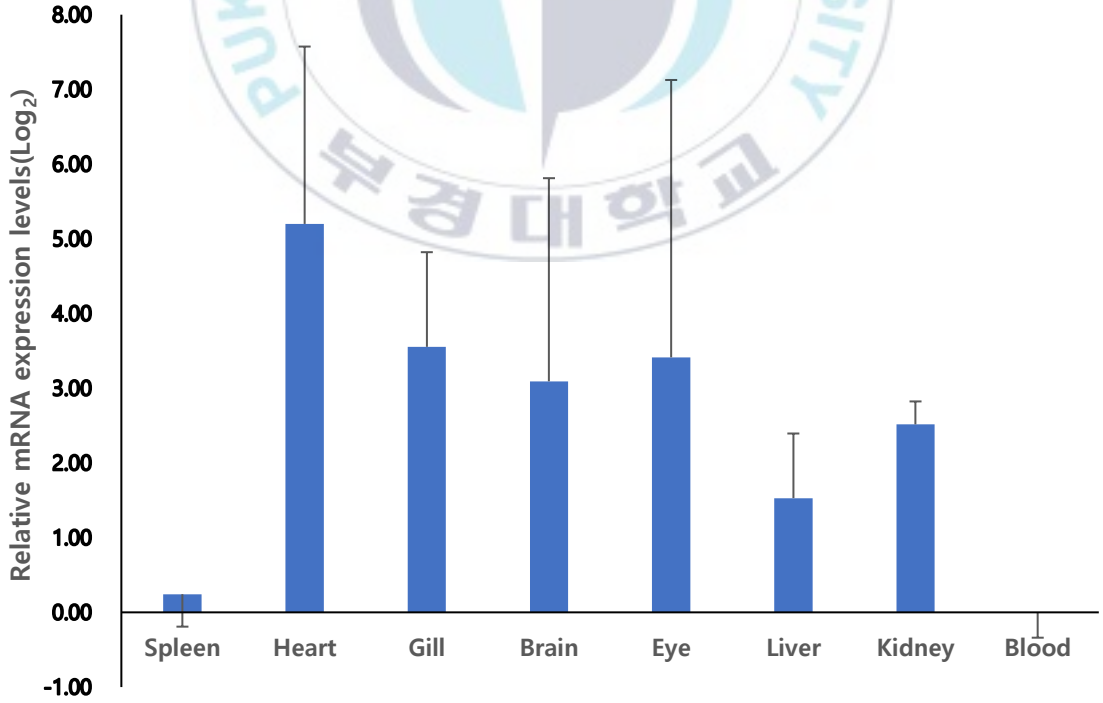

Figure 17. Histone H2A.V expression in *H.septemfasciatus* tissues under normal physiological condition.

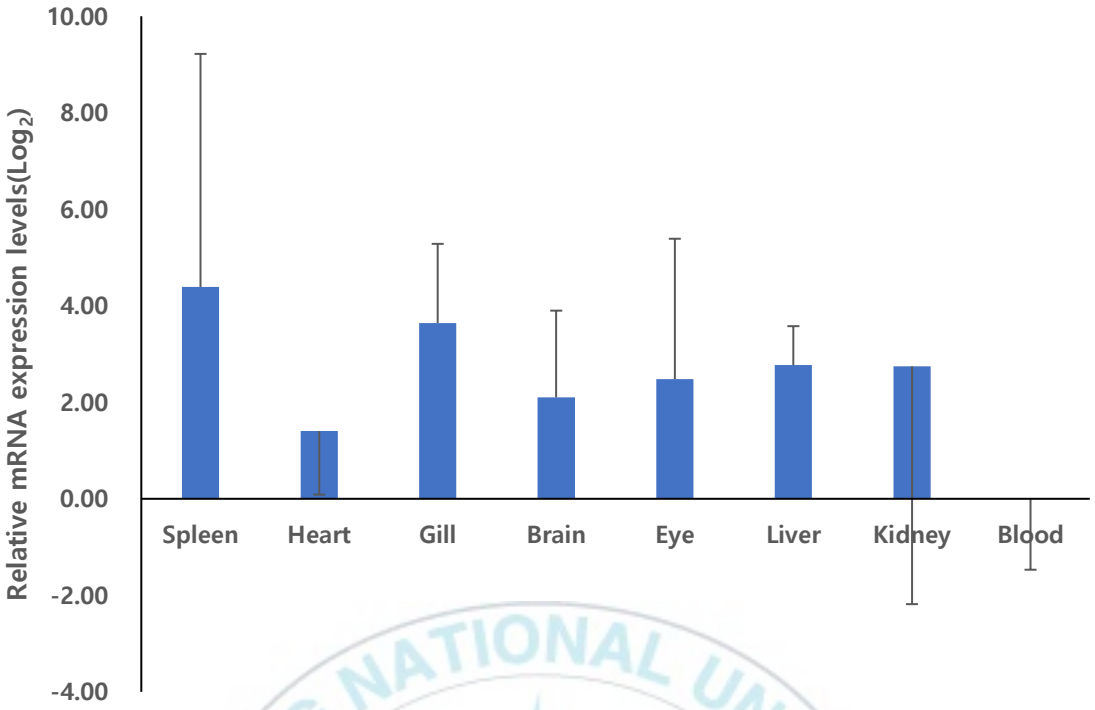

Figure 18. Histone H2A expression in *H.septemfasciatus* tissues under normal physiological condition.

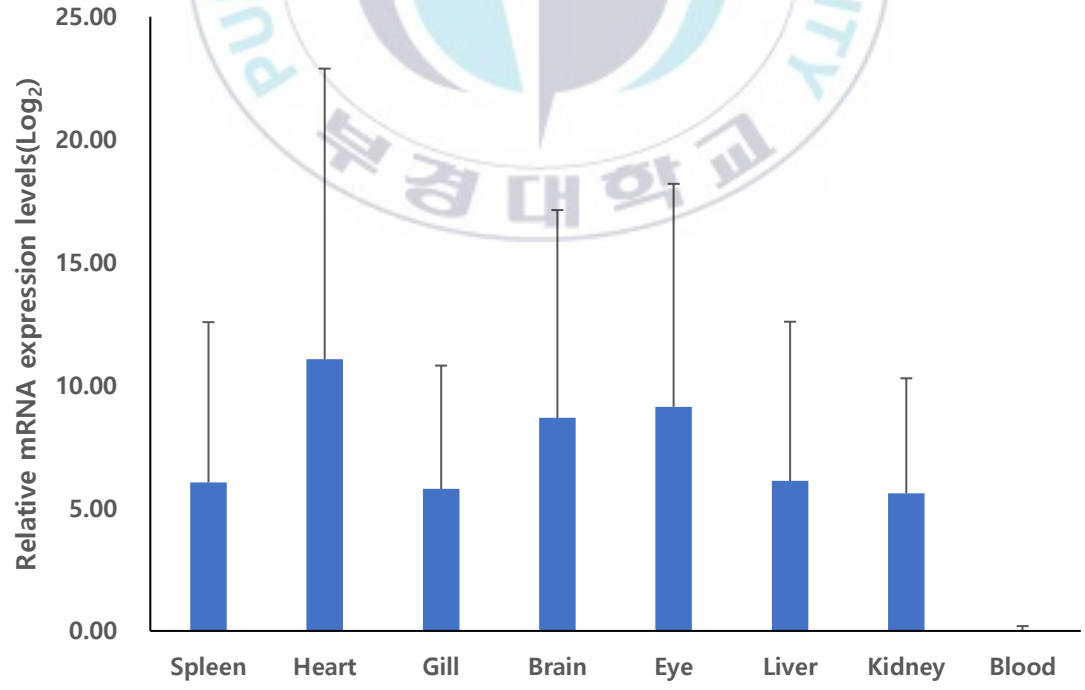

Figure 19. Histone H3.3-1 expression in *H.septemfasciatus* tissues under normal physiological condition.

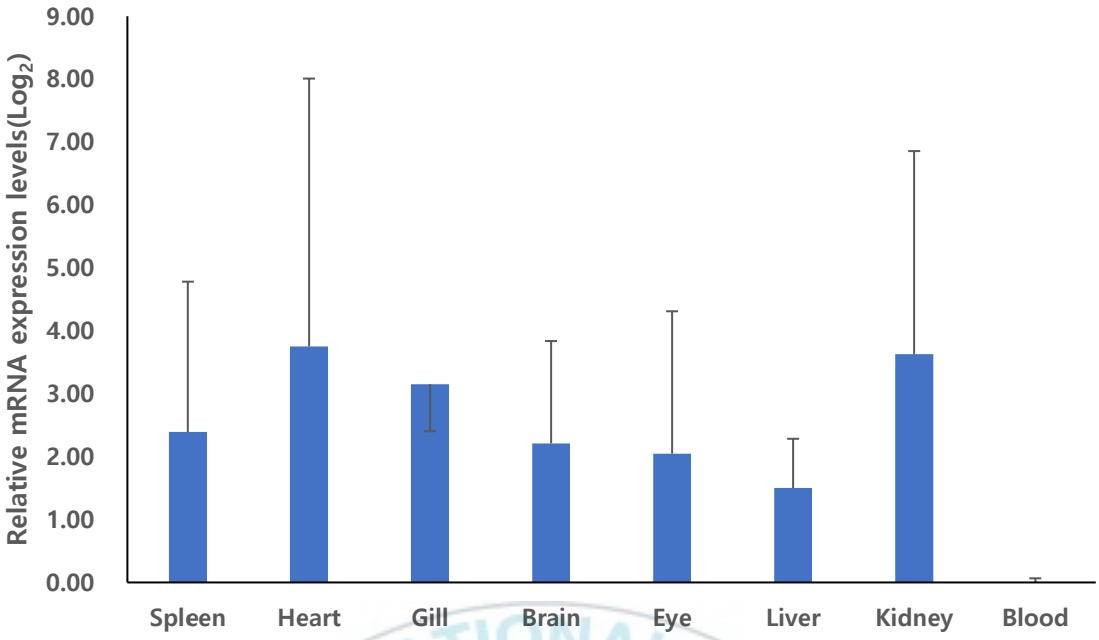

Figure 20. Histone H3.3-2 expression in *H.septemfasciatus* tissues under normal physiological condition.

3.5. Expression of histones upon NNV infection in *H. septemfasciatus*

After incubated NNV-infected *H. septemfasciatus* from day 1 to day 5, eight tissues (spleen, heart, gill, brain, eye, liver, kidney and blood) were obtained. cDNA synthesis was completed after extracting RNA from the collected tissue, and relative quantitative analysis was performed in comparison to normal *H. septemfasciatus* tissue. On day 2 of infection, the H1 gene showed the highest level of expression in the brain, with a declining trend. The level of expression increased again on Day 5. Eye confirmed a high overall expression following infection. Gill expression level has been proven to have reduced. On the second day of infection, H2A.V confirmed high expression in Brain and Blood, and Blood showed a steady drop, while Brain confirmed a certain degree of gene expression on the third day of infection. H2A exhibited a lower expression in the spleen, gill, liver, and kidney than H2A.V. On the second day of infection, H3.3-1 confirmed the maximum level of expression in liver and blood, and liver showed a considerable decline in the amount of expression on the third day of infection, although blood confirmed a certain level of decrease in the amount of expression. After the first day of infection, the remaining tissues demonstrated a drop in expression and down expression. On the second day of infection, H3.3-2 confirmed strong expression in the brain and blood, and on the third day of infection, H3.3-2 proved a considerable level of decline in expression. Values are shown as mean ± SEM (N = 3). N, the number of times the experiment was performed.

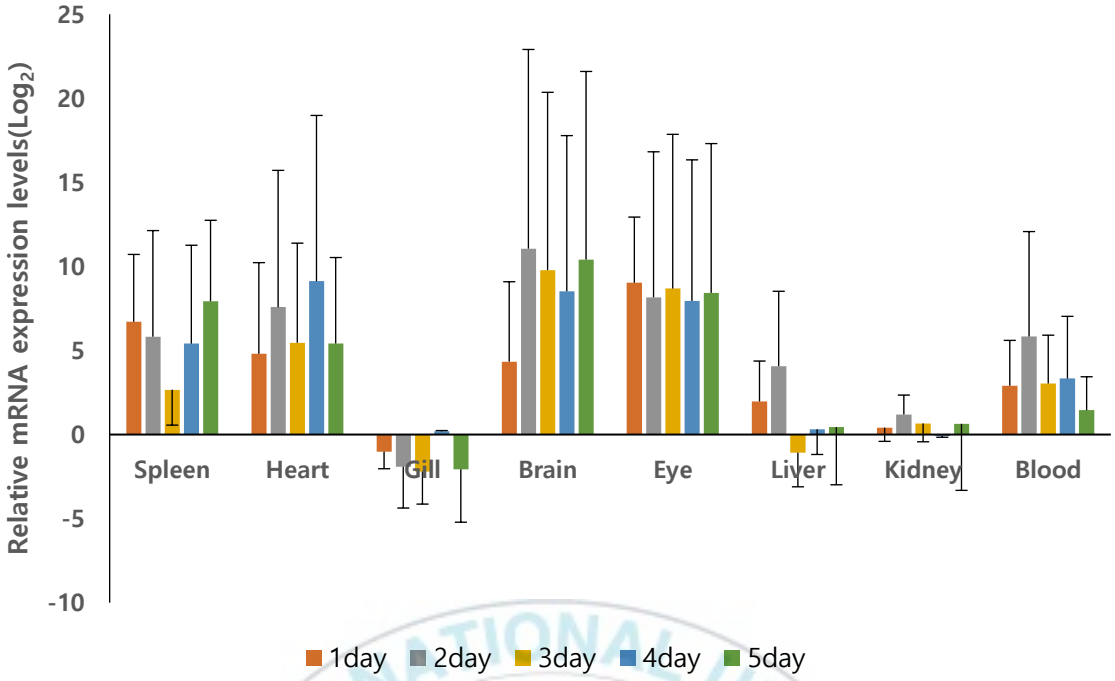

Figure 21. Histone H1 gene expression in *H. septemfasciatus* infected with NNV was compared by tissue.

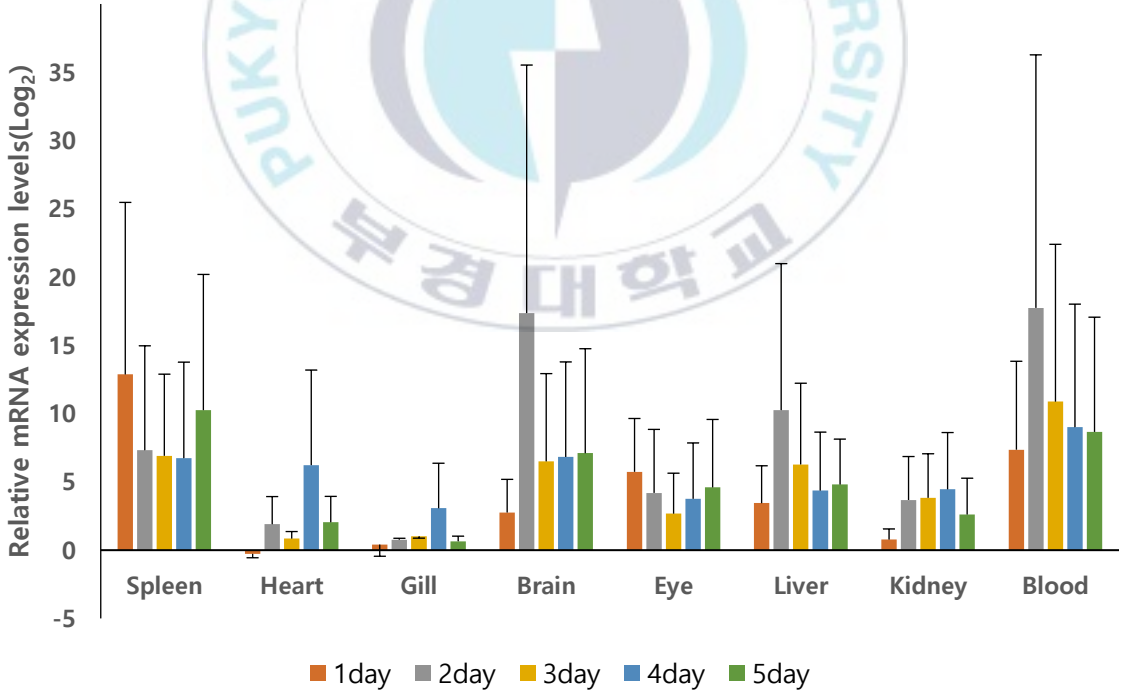

Figure 22. Histone H2A.V gene expression in *H. septemfasciatus* infected with NNV was compared by tissue.

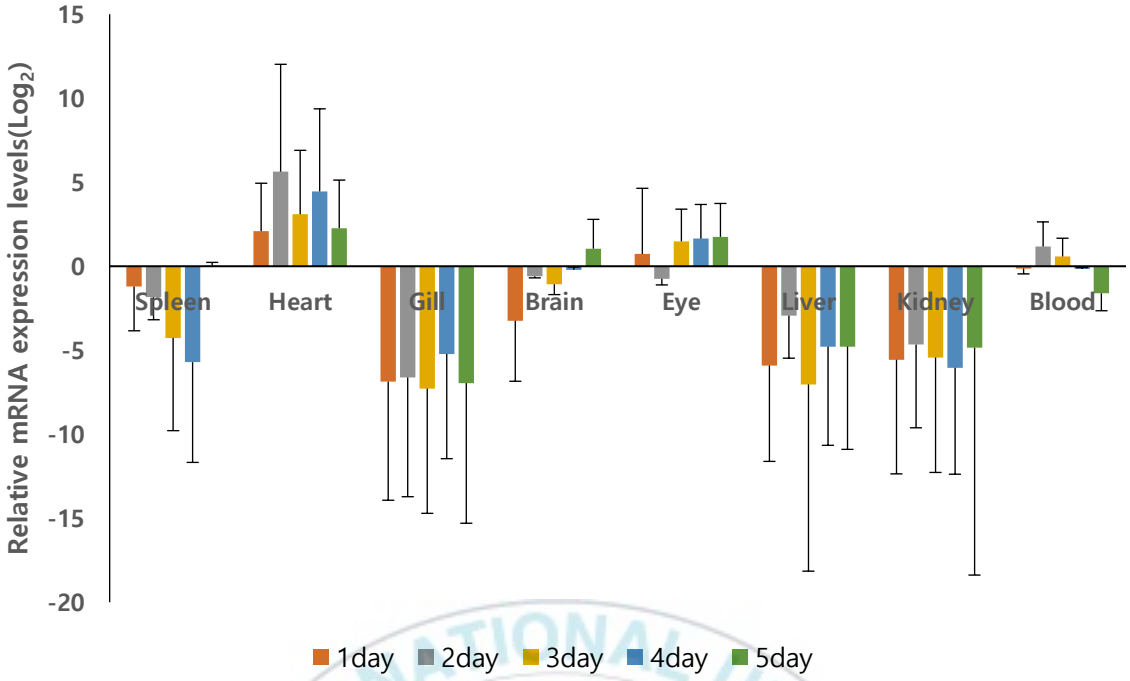

Figure 23. Histone H2A gene expression in *H. septemfasciatus* infected with NNV was compared by tissue.

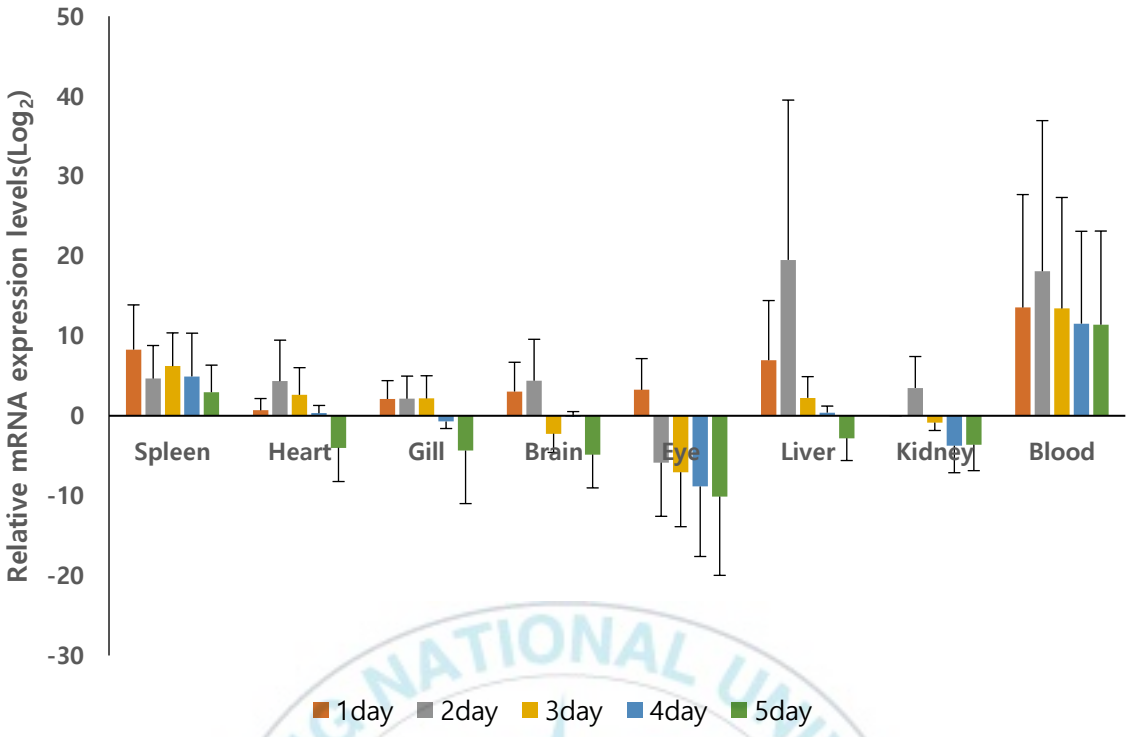

Figure 24. Histone H3.3-1 gene expression in *H. septemfasciatus* infected with NNV was compared by tissue.

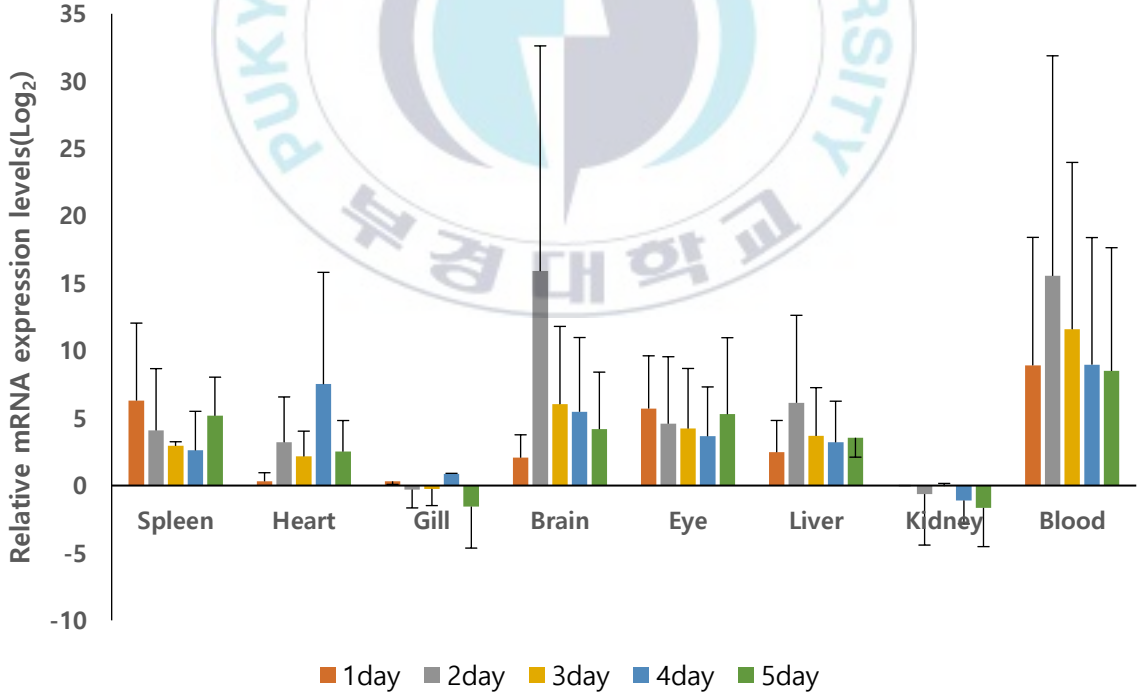

Figure 25. Histone H3.3-2 gene expression in *H. septemfasciatus* infected with NNV was compared by tissue

4. Discussion

Histone is a basic protein that has been conserved throughout evolution and is found in all eukaryotic cells. They play an important role in the organization and regulation of DNA as an important component of chromosomes [26]. It forms antimicrobial activity and neutrophil extracellular traps (NETs) in addition to the DNA conditioning role to play a congenital immune role that feeds on pathogens invading the body [27]. The histone genes found in *H. septemfasciatus* were identified in this study, and the characteristics of each histone gene were determined using sequence analysis. A systematic comparison with the histone gene of similar species was carried out. After infecting *H. septemfasciatus* with nervous necrosis virus, the expression of histone genes in each tissue was compared and analyzed over time to see if the histone gene was involved in the innate immune response.

The H1 amino acid sequence analysis confirmed the presence of a binding domain region in 24-99 amino acids, and the multiple alignment result with other species confirmed the presence of similar sequences in the binding domain region. The pedigree analysis revealed a difference of 21 percent to 97 percent from other fish species, confirming that it was an H1 sequence from a related species. The amino acid sequence domain patterns of H2A and H2A.V were found to be similar. Both genes had an H2A/H2B/H3 binding domain as well as modification sites. Multiple alignments with other species confirmed that H2A.V has the same amino acid sequence as fish and mammals, indicating that the interspecies sequence is very conservative. Because H2A has a very similar amino acid sequence in fish, the interspecies sequence was confirmed to be conservative. H3.3-1, like H2A.V, has the same amino acid sequence in different species such as fish, mammals, and annular animals. Because the difference between species could not be confirmed by amino acid sequence, it was determined that they had a very similar sequence after comparing multiple alignments with other species with gene sequence. The CENP-A (centromere protein A) sequence was retained as a singularity in the H3.3-1 amino acid sequence. CENP-A is a protein that is similar to histone H3 (histone H3) and is found in concentric nucleosomes. CENP-A protein contains a domain associated with histone H3, which replaces the histone H3 that forms the existing nucleosome at the center of mobilization and function. By substituting one or both histone H3 in the existing histone (H3-H4) 2 structure in the nucleosome at the concentric site, the CENP-A protein forms a modified nucleosome or nucleosome-like structure. It is encoded by the CENP-A gene in humans and can produce distinct isomorphic proteins via selective splicing [28]. Because the H3.3-2 amino acid sequences were not registered with NCBI, accurate analysis was impossible. Similar sequences were confirmed by comparing multiple alignments with other species with gene

sequences, but systematic analysis after changing amino acid sequences revealed that there were many differences from other fish species. This suggests that more research into the H3.3-2 gene is required.

Histone gene expression in normal *H. septemfasciatus* tissues was investigated. The study found that many H1 and H2A genes were expressed in the spleen, and that H2A.V, H3.3-1, and H3.3-2 genes were most expressed in the heart. When the results of *H. septemfasciatus* histone expression was compared to the results of european seabass, olive flounder and gilthead seabream histone expression, it was confirmed that there was a difference in expression amount for each tissue. This suggests that differences in the amount of histone gene expression by tissue may exist between fish species [19,29].

Histone gene expression in *H. septemfasciatus* infected with NNV was studied over time. The amount of expression was confirmed to be high in certain tissues on the 2nd day after NNV infection, and the amount of expression gradually decreased after the 2nd day. H1 was confirmed to have a high expression amount in the brain on the 2nd day after infection, and eye was confirmed to have a high expression amount continuously from infection. Blood has a lower expression level than brain, but it increased on the second day after infection and then gradually declined. Other research has found that the mammalian histone H1 protein inhibits the adhesion of mammalian cells to the *norwalk virus* [30]. On the 2nd day after infection, H2A.V confirmed a high expression volume in the brain and blood, which then gradually decreased. The amount of expression in liver was lower than in brain and blood, but it increased on the second day after infection and then gradually decreased. H2A, on the other hand, confirmed downregulation in the sleep, gill, liver, and kidney challenging NNV infection. Because H2A.V is an isoform of H2A, histone variants play a variety of additional roles in chromosome metabolism and may differ from canonical histones in terms of stability, DNA wrapping, and specialized domain [31]. On day 2 after infection, the highest amount of gene expression was confirmed in H3.3-1 liver and blood. Liver confirmed a significant decrease in gene expression beginning on day 3 after infection, and in blood, the expression amount decreased to a certain level beginning on day 3 after infection, but a similar expression amount was confirmed beginning on day 5 after infection. On the 2nd day after infection, high levels of gene expression were found in the brain and blood of H3.3-2. It was confirmed that the amount of expression decreased to a certain level on the third day after infection and was maintained on the 4th and 5th days. Histone is classified into two groups: lysine-rich (H1, H2A, H2B) and arginine-rich (H3, H4) [32]. Antiviral activity of arginine-rich histone H3 against influenza A virus has been confirmed in studies [33]. As a result, it can be deduced that the amount of gene expression in H3-series genes was high in brain, blood, and liver, and was strongly involved in the immune response.

NNV is a virus that causes necrosis of the brain and retina [34]. The increased expression of H1, H2A.V, H3.3-1, and H3.3-2 genes in the brain, eye suggests that histone is involved in innate immune response. However, the differences in tissues and amounts of expression increased for each gene was confirmed. A biomarker indicating vascular dysfunction, such as severe trauma or sepsis, is an increase in the amount of extracellular histone in the bloodstream [35]. Increased histone expression in the blood suggests that histones play a role in innate immune responses.

In conclusion, this study identified and analyzed each characteristic of the *H. septemfasciatus* histone gene infected with NNV. Furthermore, H1, H2A.V, H3.3-1, and H3.3-2 genes were found to be involved in the immune response of *H. septemfasciatus* infected with NNV by analyzing the histone gene expression of each tissue by infection time. More research is needed to identify histone's innate immune response mechanism and to explain the immune response at the protein level of the histone gene. Histone clearly indicates that it is a critical point in the study of innate immune responses as well as DNA packing.

5. 국문 초록

능성어는 고급 양식어종으로 식용으로 인기있는 해산어종 중의 하나이다. Viral nervous necrosis(VNN) 질병을 일으키는 Nervous necrosis virus(NNV) 감염으로 인해 양식장에 집단 폐사를 일으켜 많은 피해를 입히고 있다. 최근 histone protein이 DNA packing 뿐 아니라 선천적 면역 반응에 관여한다는 연구들이 보고되어, 능성어의 histone ORF genes(H1, H2A, H2A.V, H3.3-1, H3.3-2)들을 확인하고, NNV에 감염된 능성어의 histone genes의 조직 별 발현 양을 분석하였다. Histone gene들은 각 종 간 amino acid 서열이 유사하여, 진화적으로 보존적인 서열을 가지고 있음을 확인하였다. NNV에 감염된 능성어의 조직 별 발현 양 분석 결과 감염 후 2일차에 H1은 brain, eye에서 높은 유전자 발현양을 확인하였으며, H2A.V는 brain, blood에서 높은 유전자 발현양을 확인하였다. H3.3-1은 감염 후 2일차에 liver, blood에서 높은 유전자 발현양을 확인하였고, H3.3-2 유전자에서 brain, blood에서 유전자 발현양이 가장 높음을 확인하였다. 혈액 내 세포 외 histone protein 양의 증가는 혈관 기능 이상의 바이오마커로 알려져 있어, histone 유전자는 선천적인 면역반응에 관여함을 추측할 수 있다. Histone의 면역반응을 단백질 수준에서 설명하기 위해서는 추가적인 연구가 필요하다.

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