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Molecular Cloning and Phylogenetic Analysis of Vitellogenin Gene in *Hemibagrus nemurus*

Ang Pay Ling[*1], S.A. Harmin[1], M.Y. Ina-Salwany[2], O. Roshani[1], Z. Zulperi[2], S.N. Baharum[3] and M.Z. Mohd Syahril[4]

[1]Faculty of Science and Biotechnology, Universiti Selangor, Bestari Jaya Campus, Jalan Timur Tambahan, 45600 Batang Berjuntai, Selangor, Malaysia
***Email: jocelyn.love@hotmail.com**
[2]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia
[3]Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 Bangi Selanggor, Malaysia
[4]Faculty of Applied Science, Universiti Teknologi MARA, Kuala Pilah Campus, Pekan Parit Tinggi, 72000 Kuala Pilah, Negeri Sembilan, Malaysia

ABSTRACT

Vitellogenin is an egg yolk precursor glycophospolipoprotein, which it can be used as a biomarker indicator in sexual maturation. In this study, total of the nucleotide size for this partial VTG sequence is 574bp. The partial VTG nucleotide homology of *Hemibagrus nemurus* is high; showing 88-91% identity between *Clarias macrocephalus* and *Ariopsis felis*. The VTG sequences analysis has shown the highest homologous portion of the VTG gene between local *H. nemurus* with *Ariopsis felis* and *Bagre marinus*. VTG from different species are conserved domains, and phylogenetically related. Studies as such will certainly provide new insights to better understand the biological role of VTG in river catfish.

Keywords: *Hemibagrus nemurus,* Vitellogenin, cDNA, PCR, Phylogenetic

INTRODUCTION

Hemibagrus nemurus is a freshwater fish, and locally known as Malaysian river catfish. It is widely distributed throughout Southeast Asia-Thailand, Laos, Vietnam, Cambodia, Indonesia and Malaysia. In order to evaluate the reproductive cycle in fish, the vitellogenin should be characterized. Vitellogenin (VTG) is a kind of lipophosphoglycoprotein, which it can be used as a biomarker for evaluating the reproductive condition in the female fish. According to Panprommin, Poompuang, and Srisapoome (2008), complete VTG synthesis is vital to the reproductive success of egg-breeders, however insufficient amounts of yolk proteins that was deposited into the oocytes may cause larval development to be incomplete, resulting in high mortality as eggs or sac-fry. Thus, this research is important to clone and identify the VTG gene which can be helpful in future management of broodstock for spawning purposes. Phylogenetic analysis can be used to compare the results of multiple sequences from previous studies of other fishes. Molecular technique was applied throughout the isolation, cloning and phylogenetic analysis of VTG gene.

MATERIALS AND METHODS

Fish and RNA Isolation. Adult females of the river catfish, *H. nemurus* were obtained from commercial supplier and acclimitized for one month in the fiber tank located in Aquaculture Laboratory of Faculty of Science and Biotechnology in UNISEL. The females were then dissected to collect the liver. After collected, the liver was properly labeled and kept in -80^{0}C until use for RNA isolation. The total RNA was extracted from 100 mg of liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

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cDNA synthesis and Polymerase Chain Reaction (PCR) amplification. One microgram of total RNA was used as template for cDNA synthesis by using Quantitect Reverse Transcription Kit (QIAGEN, GmbH, Hilden) following the instructions of the manufacturer. The fragment was amplified in a 25 µL reaction volume containing 10 x PCR reaction buffers, 50 mM $MgCl_2$, 10 mM dNTPs mix, 20 pmol forward (F1) and reverse (R1) primer and 5 U Taq DNA Polymerase (Vivantis, Oceanside, CA). F1-MTGGRYAAYGCTGGWCAYCCTKCWA, and R1- AKMRGCTCCTGCCAKGTAAGYACGK were the primer used in Kim et al. (2010) study. The sequence size was 574 bp. The PCR conditions used for initial denaturation were 95^{0}C for 10 minutes, 30 cycles of denaturation at 95^{0}C for 1 minute, annealing at 47^{0}C for 1 minute and extension at 72^{0}C for 1 minute 30 seconds, followed by final extension at 72^{0}C for 10 minutes (Mastercycler Gradient, Eppendorf, Germany).The obtained PCR products were separated on 1.2% agarose gel, and purified with GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania, EU).

cDNA cloning and sequencing. The purified PCR amplified product was then cloned into TOPO vector using TOPO TA Cloning kits (Invitrogen) following the manufacturer's protocol. The cloned product was purified using the EZ-10 Spin Column DNA plasmid kits (Bio Basic, Canada). Purified products were sent for sequencing (Firstbase, Malaysia).

Basic LocaL Alignment Search Tool (BLAST). The sequenced results from Firstbase were analyzed using BLASTn, a program from the website: https://blast.ncbi.nlm.nih.gov/Blast.cgi, developed by National Center for Biotechnology Information (NCBI), USA.

Sequence analysis and phylogenetic analysis. To compare sequence similarities of *H. nemurus* VTG with other fishes, the nucleotide data were collected from Genbank: (https://blast.ncbi.nlm.nih.gov). Pairwise sequence identities between *H. nemurus* and other species were assessed using MEGA6 (Molecular Evolutionary Genetic Analysis) software with CLUSTAL W set as the default parameter. The phylogenetic tree was constructed by using the Maximum-likelihood (ML) method from MEGA6 software.

RESULTS AND DISCUSSION

Partial amplification of cDNA encoding *H. nemurus* VTG gene was done using published primer F1-MTGGRYAAYGCTGGWCAYCCTKCWA, and R1- AKMRGCTCCTGCCAKGTAAGYACGK in Kim et al. (2010), and they were successful in amplifying the conserved region of the gene. Figure 1 shows the fragment obtained in 1.2% of agarose gel electrophoresis, which was PCR-amplified vitellogenin gene from *H. nemurus* liver. VTG is usually synthesized in extraovarian tissues such as liver in non mammalian vertebrates, in fat body of insects, in hepatopancreas for crustacean, and intestine for sea urchin (Wu et al., 2015). The result for nucleotide sequence from primers (F1, R1) is as below. The nucleotide size for this partial VTG is 574 bp.

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1     ATGGGTAATGCTGGTCATCCTGCTAGCCTTAAAACCATCATGAAACTCCTGCCTGGATTT
61    GGAAATGCTGCTGCTGCCGTTCCCCTGAGGGTCCAGATTGATGCCATCCTGGCTCTTAGG
121   AACATTGCCAAGAAGGAACCAAAGATGGTTCAGCCAGTGGCACTGCAACTTTTCATGGAC
181   AAGGCTTTCCATCCTGAACTGCGCATGGTTGCTTGTATTGTGCTCTTTGAGACCAAACCA
241   TCAGTAGCCCTCATGGCCCCACTTGGTGGTGCTTTGGAGAAGGAGACCAACATGCATGTT
301   GTCAGTTTTGTTTATTCTCATATCAAGTCTCTGACCAGAAGCATGGCCCCTGATTATATG
361   CATGTGGCTGCTGCAGCAAATGTTGCCATCAGGATGTTGAGCCCCAAACTGGACAGACTG
421   AGTCACCAATTTAGCAGAGCCATCCATTACGATCTCTACATCTCTCCTTTCATGGTTGGT
481   GCTGCTGGTAGCGCTTACTTGATCAATGATGCTGCCACCACCTTGCCCAGAGCTGTTGTG
541   ACTAGAGCACGTGCTTACCTGGCAGGAGCTGCTA
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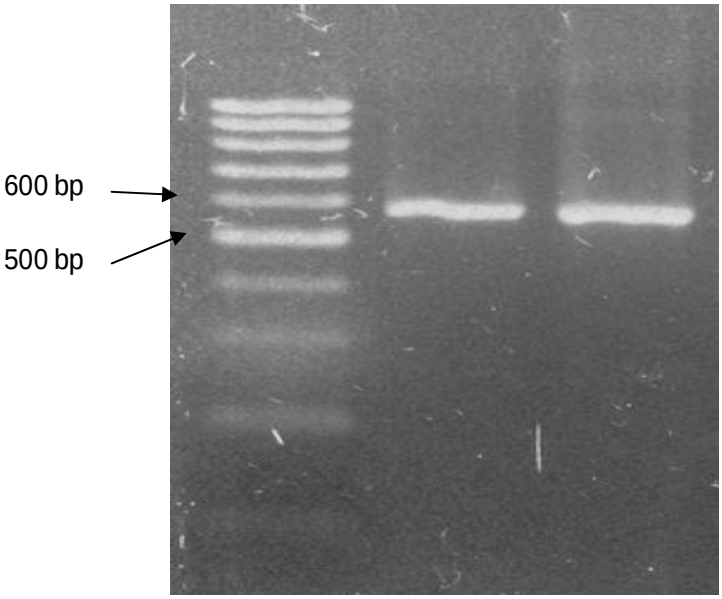

Figure 1. The fragment (574 bp) obtained from PCR amplification of *H. nemurus* vitellogenin using primer F1 and R1

The PCR amplified product that goes through sequencing process was analyzed using BLASTn. Figure 2(a) shows the blast hits for the PCR product sequence while in figure 2(b), BLAST result shows the sequences producing significant alignments. The *H. nemurus* partial sequence was 88% similar to *Clarias macrocephalus*, and 91% identical to *Ariopsis felis*.

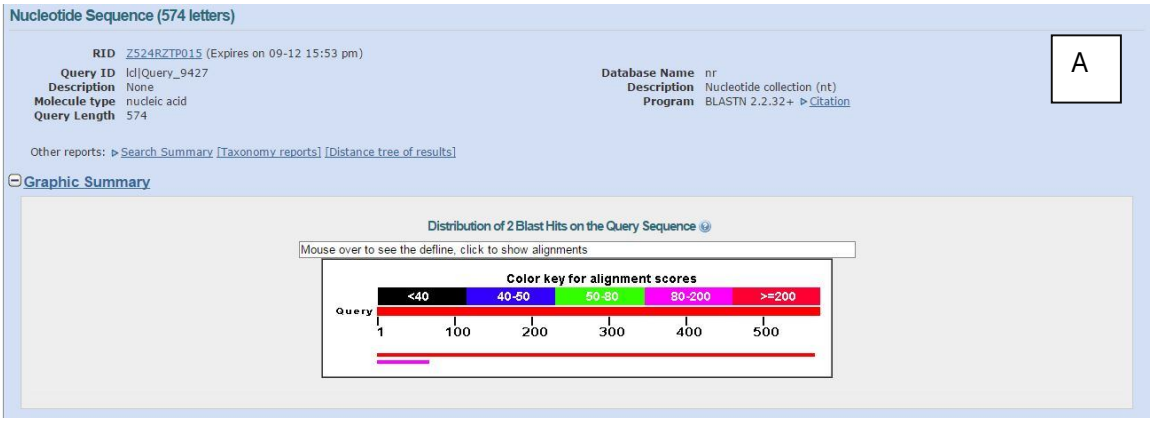

Figure 2. (a) Distribution of two blast hits on the query sequence; (b) Sequences producing significant alignment.

For each successful amplification of the cDNA, the purified PCR products was cloned into TOPO TA Cloning kit. Figure 3 shows the white colonies and blue colonies which, grew on the LB plate that

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was supplemented with ampicillin and X-gal. The positive clones were PCR screened, and grown in culture medias containing ampicillin before proceeded to plasmid extraction.

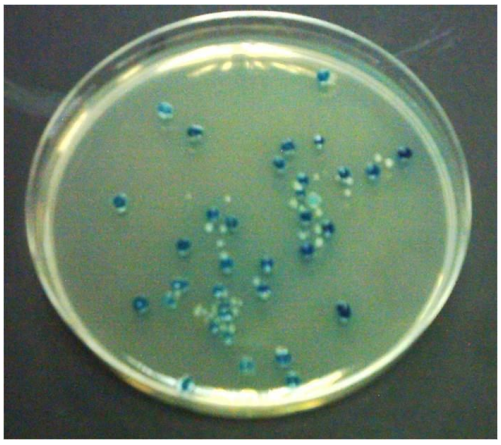

Figure 3. White colonies and blue colonies grew on LB plate.

The evolutionary analyses were conducted in MEGA6 software (Tamura et al., 2013). Evolutionary history was inferred using the Maximum-likelihood method. Figure 4 shows the partial VTG gene from *H. nemurus* was clade together with *Bagre marinus, Ariopsis felis*, *Cathorops melanopus, Clarias batrachus,* and *Clarias macrocephalus.* These fishes were from the same order of siluriformes, the catfishes category. However, *H. nemurus* partial sequence shows that it is more similar to *Bagre marinus* and *Ariopsis felis*.

The phylogenetic analysis in the studies of Kim et al. (2010) for the *Acheilognathus yamatsutae* (slender bitterling), where nucleotide of the VTG was aligned to those of other known teleosts, shared the closest nucleotide similarity (87.5%) with *Pimephales promelas* (fathead minnow), which belongs to the same family of Cyprinidae. In the studies of Panprommin et al. (2008) for VTG of *Clarias macrocephalus* (Gunther walking catfish), the phylogenetic tree showed clustering with those of *Cyprinus carpio* (common carp), *Pimephales promelas* (fathead minnow), and *Danio rerio* (zebra fish), which should be expected given their membership in Superorder Ostariophysi. The phylogenetic tree in Zheng et al. (2012) for *Chlamys nobilis* (noble scallop) was constructed based on the VTG sequences from 20 species. *Chlamys nobilis* was firstly clustered with its sister species *Chlamys farreri* (scallop) and another scallop *Mizuhopecten yessoensis*, which, *Chlamys farreri* and *Mizuhopecten yessoensis* had closer relationship. According to Jia et al. (2013), that studies the VTG gene for *Scylla paramamosain* (crab), 24 VTG sequences including 4 vertebrate and 20 invertebrate (17 decapods, 3 insects) were used for phylogenetic tree analysis. The result revealed that, *Scylla paramamosain* VTG is closely related to the *Portunus trituberculatus* (crab) followed by the VTG of *Charybdis feriatus* (crab) and *Callinectes sapidus* (crab). Interestingly, the VTG from decapods crustaceans are more closely related to vertebrate, rather than with insects.

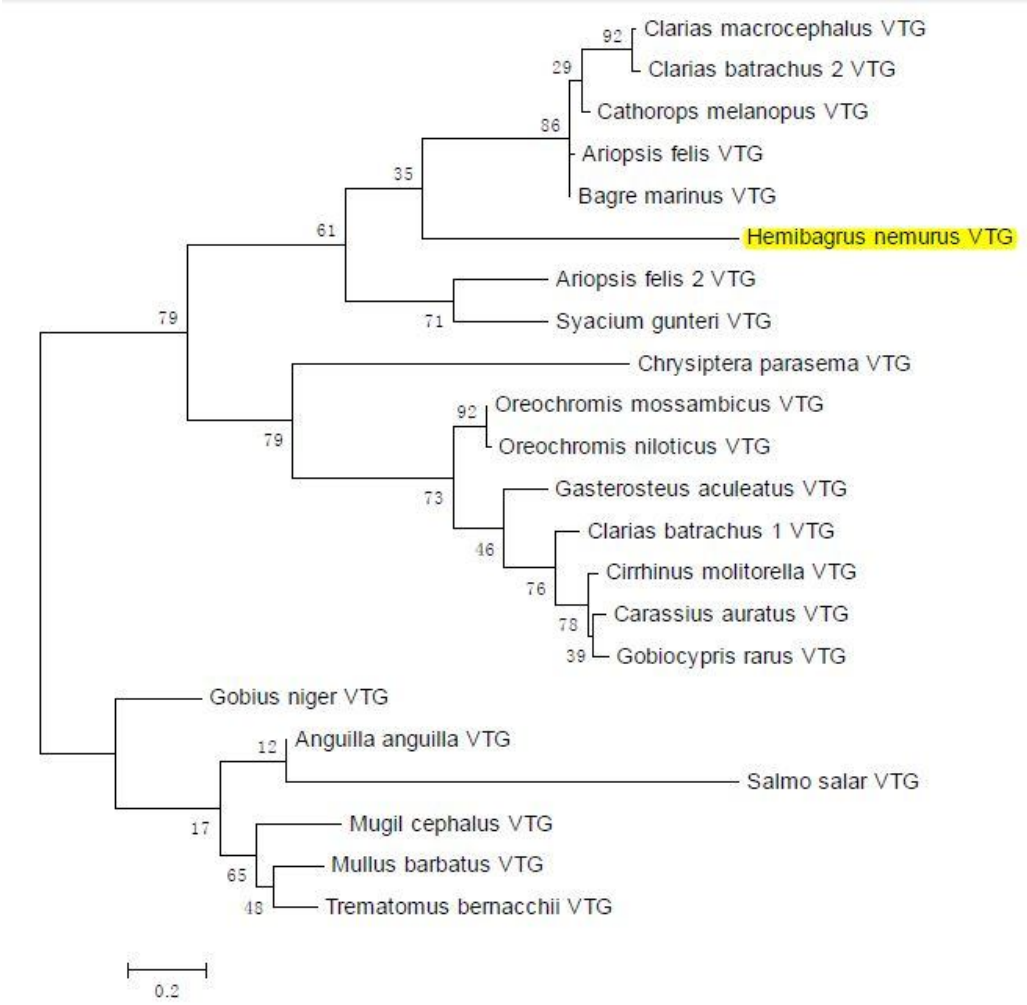

Figure 4. Phylogenetic tree based on VTG family members of *Hemibagrus nemurus* and other fishes constructed with Maximum-likelihood. The numbers on each brand suggest the bootstrap values (%) for 1000 replications, the bars represent the distance.

CONCLUSIONS

This study confirms that vitellogenin identification via sequence analysis can be used to partially determine the different forms of vitellogenin for different species. The estimation of evolutionary distance of *H. nemurus* VTG with 19 species fish homologous sequence revealed that river catfish VTG belongs to the Siluriformes order. Future investigation of the sequence can help to study the various forms of vitellogenin genes.

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