

# Molecular Characteristic of Giant Grouper (*Epinephelus Lanceolatus*) Vitellogenin

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## Abstract

The Vitellogenin (Vtg) gene sequence acts as an indicator to the fish reproduction, which can adapt to the environment factor or can influence the gonad development. The Vtg nucleotide sequence from Giant grouper was characterized using bioinformatics software. A homology search of the deduced amino acid sequence of the obtained Vtg DNAs (compare with 13 species) was carried out using the National Centre for Biotechnology Information website. Clustal W analysis was constructed a phylogenetic tree by using Molecular Evolutionary Genetic Analysis MEGA version 5.2.2. In order to verify the Vtg gene sequences obtained and elucidate structure-function relationship in Vtg, by using DELTA BLAST of 3-D structure of Giant grouper with others fishes.

Result of phylogenetic analysis using Maximum Likelihood (ML) and Neighbour Joining (NJ) showed tree analysis generated two separated tree topology. This similarity (0.015 distance matrix viewer) was closely related in terms of their Vtg gene sequence although from different environmental and ecological conditions. In general, showed that *Epinephelus lanceolatus* Vtg is evolutionary more related to *Poecilia latipinna*. *Epinephelus lanceolatus* shows four main domains (Vitellogenin-N, DUF1943, DUF1944 and VMD), similarly found in *Dicentrarchus labrax* but was different compare to *Clarias macrocephalus*, *Catla catla*, and *Danio rerio*. This indicates characteristic of Vtg domain for freshwater species is control by present of VMD in Vtg. The molecular approach can be done on Giant Grouper to understand the molecular respond towards fish growth and determine the individual of Giant grouper that has potential to increasing the Vtg production for increasing eggs quality.

**Keywords:** Nucleotide; Molecular; Environmental; Ecological; *Poecilia latipinna*

## Introduction

The development of molecular tools has recently opened new direction and facilitated the discovery of the genes involved in these processes and their evolutionary functional significance. Fish oocyte development attracted specific interest in the last century. Morphological investigations were followed by biochemical, physiological, and endocrinological analyses that extended our knowledge of dynamic events that take place during oocyte development and egg formation.

Molecular characterization of vitellogenin (Vtg) gene is important because it indirectly leads to the understanding of the role-play in the molecular basis of gonad development in terms of their structure and function [1]. Basically, each gene has its own molecular characteristic that is specific to their action. This includes the Vtg genes, which has certain features that are fundamental and responsible for its actions. The Vtg gene sequence acts as an indicator to the fish reproduction, which can adapt to the environment factor or can influence the gonad development [2]. Study on the molecular levels could permit the understanding of gonad development, gene regulation, structural-function relationships, evolution and adaptation to environment.

In teleost fish, as in other oviparous, Vtg is specifically incorporated in the oocyte by receptor-mediated endocytosis through receptors belonging to the low density lipoprotein receptor (LDLR) family, which have been named very low density lipoprotein receptors (VLDLRs), Vtg receptors (VtGRs), due to the presence of eight ligand-binding repeats [2]. The other members of the gene family bind various ligands and are involved in lipid metabolism in both vertebrates and invertebrates. Therefore, this function of Vtg component needs to clarify for better understanding in physiology process during oocyte development.

The objective of the present study was to characterize the Giant grouper Vtg gene, and to compare Vtg gene expression between other species as basic information to develop Vtg as biomarker indicator in sex identification of Giant grouper.

## Material and Methods

### Molecular phylogenetic analysis of giant grouper Vtg

The Vtg nucleotide sequence from Giant grouper was characterized using bioinformatics software. A homology search of the deduced amino acid sequence of the obtained Vtg DNAs was carried out using the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). The Vtg Giant grouper sequence (Figure 1) from previous study [3], was compared with 13 species such as lamprey (*Ichthyomyzon unicuspis*, GenBank; AAA49327.1), sailfin molly (*Poecilia latipinna*, GenBank; ACV65040.1), rohu (*Catla catla*; GenBank; ABP04034.2), tuna (*Thunnus thynnus*; GenBank;), catfish (*Clarias macrocephalus*; GenBank; ABW96364.1), carp (*Cyprinus carpio*; GenBank; AGZ80880.1), european seabass (*Dicentrarchus labrax*; GenBank; AFA26670.1), mummichog (*Fundulus heteroclitus*; GenBank; AAB17152.1), mangrove rivulus (*Kryptolebias marmoratus*;

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MKAVV LALTL AFVAG QNFAP EFAAG KTYVY KYEAL ILGGL PEEGL ARAGL 90
KI STK LLLSA ADQNT YMLKL VEPEL SEYSG I WPKD PAVPA TKLTA ALAPQ 100
LAI PI KFEYT NGVVG KVFAP EEVST LVLNI YRGI L NILQL NIKKT HKVYD 150
LQEVG TQGVG KTLYS ISEDA RIENI LLTKT RDLSN CQERL NKDIG LAYTE 200
KCDKC QEETK NLRGT TTLSY VLKPV ADAVM ILKAY VNELI QFSPF SEANG 250
AAQMR TKQSL EFLEI EKEPI PSVKA EYRHR GSLKY EFSDE LLQTP LQLIK 300
I SDAP AQVAE VLKHL ATYNI EDVHE NAPLK FLELV QLLRI ARYED LEMYV 350
NQYKK MSPHR HWFLD TIPAT GTFAG LRFIX EKFM A EEI TI AEA AQ AFIT A 400
VHMVT ADPEV I KLF E SLVDS DKVVE NP LLR EVVFL GYGT M NKYCNKTV D 450
CPVEL IKPI Q QRLSD AI AKN E EENI I LYIK VLGNA GHPSS FKS LT KIMPI 500
HGTA A VSLPM TIHVE AIMAL RNIAK KESRM VQELA LQLYM DKALH PELRM 550
LSCIV LFETS PSMGL VTTVA NSVKT EENLQ VASFT YSHMK SL SRS PATI H 600
PDVAA ACSAA MKI LG TKLDR LSLRY SKAVH VDLYN SSLAV GAAAT AFYIN 650
DAATF MPKSF VAKTK GFI AG STA EV LEIGA NIEGL QELI LKNPAL SESTD 700
RITKM KRVIK ALSEWRSLPT SKPLA SVYVK FFGQE IGFAN IDKPM IDKAV 750
KFGKE LFIQE YGREA LKALL LSGIN FHYAK FVLA A EMRRI LPTVA GIPME 800
LSLVS AAVAA ASVEI KPNTS PRLSA OFDVK TLEET DVELK AEIRP MVAND 850
TYAVM GLNTD IFQAA LVARA KLHSV VPAKI AARLN I KEGD FKLEA LPVDV 900
PENIT SMNVT TFAYA RNIEE PLVER I TPLL PTKVL VPIPI RRHTS KL DPT 950
RNSML DSSEL LPME E EDVEP I PEYK FRRFA KKYCA KHIGV GLKAC KFKAS 1000
QNGAS IQDIV LYKLA GSHNF SFSVT PIEGE VVERL EMEVK VGAKA AEKLV 1050
KRI NL SEDEE TEEGG PVLVK LNKI L SRRRN SSSSS SSSSS SSSSS CSRSS 1100
RSSSS SSSSS SSSSR KIDLA ARTNS SSSSS SRRNS SSSSS SSSSS SSSSS 1150
SSSSS SSSSR SSSSS SSSSS SSSSS SSSSR RVNST RSSSS SRTS SASLL 1200
ASFFS DSSSS SSSSD RRSKE VMEKF QRLHK KMVAS GSSAS SVNEA IYKEK 1250
KYLGE EAVVA VILRA VKADK RMVGY QLGFY LDKPN ARVQI IVANI SSSDS 1300
NWRI C ADAVV LSKHK VTTKI SWGEQ CRKYS TNVGT ETGI V SSSPA ARLRV 1350
SWERL PSTLK RYGMK VNKYV PVKIL SDLIH TKREN STRNI SVI AV ATSEK 1400
TIDI I TKT PM SSSVY NVTMH LPMCI PIDEI KGLSP EFDE V IDKIH FMVSK 1450
AAAAE CSFVE DTLTY FNNRS YKJNK M PSSCY QVAAQ DCTDE LKF MV LLR KD 1500
SSEQH HINVK ISEID IDMF P KDDNV TVKVN EMEI P PPA CL TATQQ LPLKI 1550
KTKRR GLAVY APSHG LQEVY FDRKT WRIKV ADWMK GKTCG LCGKA DGEIR 1600
QEYHT PNGRV AKNSI SFAHS WILPA ESCRD ASECR LKLES VQLEK QLTIH 1650
GEDST CFSVE PVP RC LPGCL PVKTT PVTVG FSCLA SDPQT SVYDR SVDLR 1700
QTTQA HLACS CNTKC S 1750

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Figure 1: Amino acid sequence (1,704) in single letter code of Giant grouper vitellogenin peptide showing sequence coverage after trypsin digest.

GenBank; AAQ16635.1), striped bass (*Morone saxatilis*; GenBank; ADZ57172.1), rainbow trout (*Oncorhynchus mykiss*; GenBank; CAA63421.1), zebra fish (*Danio rerio*; GenBank; NP 001157843.1), and Japanese eel (*Anguilla japonica*; GenBank; AAV48826.1).

The deduced amino acid sequences were aligned using the ClustalW [4] program hosted by the DNA Data Bank of Japan (<http://clustalw.ddbj.nig.ac.jp/top-j.html>) and subjected to ClustalW analysis to construct a phylogenetic tree using the bootstrapped neighbor-joining method [5]. The sequence obtained was exported to FASTA format in notepad and then, was edited using Bioedit software to remove the unwanted and vector sequences to identify the location of the insert sequence. Multiple alignments from 14 fish peptide sequences of Vtg were conducted using eBiox 5.2.2 program and it were used in the phylogenetic analysis. The phylogenetic analysis was carried out using Molecular Evolutionary Genetic Analysis MEGA version 5.2.2 [6] with Maximum Likelihood and Neighbor Joining algorithms in order to estimate the phylogeny.

### Domain

In order to verify the Vtg gene sequences obtained and elucidate structure-function relationship in Vtg, the conserved and essential domains and residues in Vtg and other members of the gene family such as Lipovitellin I (Lv-I) and II (Lv-II), phosvitin (Pv), polyserine track (PT), von Willebrand-factor type-D domain (VWD) were determined by using DELTA BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The molecular characterization of primary structure Giant grouper Vtg gene such as protein domain, families and functional sites were determined by comparing the sequence to other fish.

### Three dimensional (3D) structure prediction

Furthermore, in this study the prediction of 3-D structure of Giant grouper with others fishes from different orders were also viewed using protein homology/analogy recognition engine v 2.0 Phyre2 server <http://www.sbg.bio.ic.ac.uk/> [7].

## Results

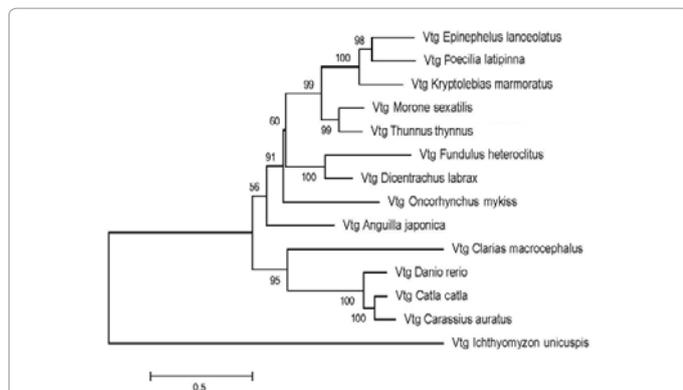
### Molecular phylogenetic analysis

Result of phylogenetic analysis using maximum likelihood (ML) and neighbor joining (NJ) methods showed tree analysis generated two separated tree topology (Figure 2). In general, showed that *Epinephelus lanceolatus* Vtg is evolutionary more related to *Poecilia latipinna* and *Kryptolebias marmoratus*. It is noted that the distribution of Vtg phylogenetic was significantly different, between freshwater species (*Carassius auratus*, *Catla catla*, *Danio rerio* and *Clarias macrocheilus*) as one group of Vtg compare to seawater and euryhaline species (*Epinephelus lanceolatus*, *Poecilia latipinna*, *Kryptobias marmorata*, *Morone saxatilis*, *Thunnus thynnus*, *Fundulus heterocittus*, *Dicentrarchus labrax* and *Anguilla japonica*) for another group. The constructed a phylogenetic tree that places closely related sequences under the same interior node and whose branch lengths closely reproduce the observed distances between sequences.

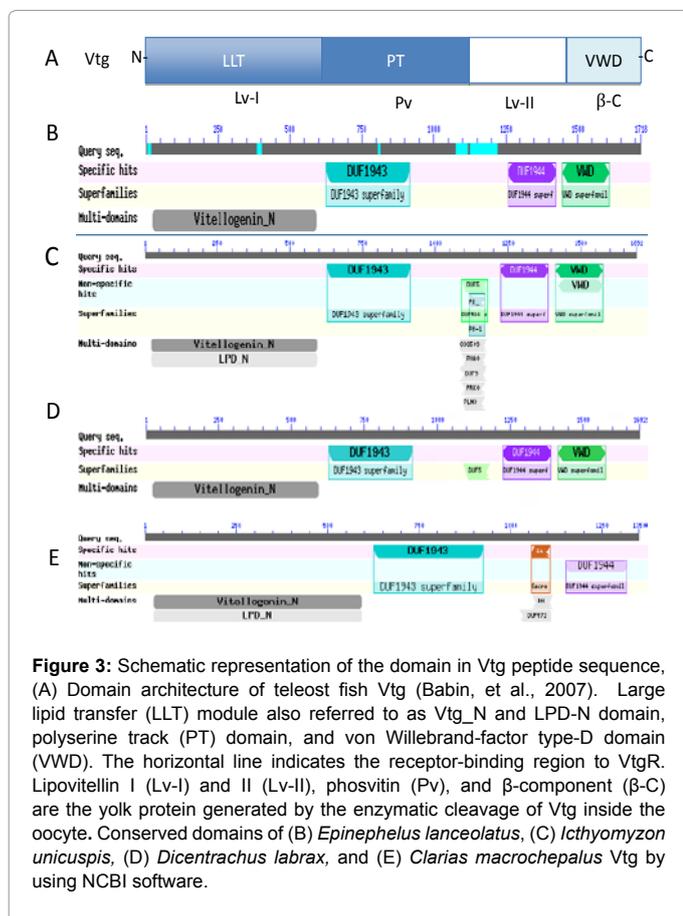
The results from evolutionary distance estimations are displayed in the distance matrix explorer (Table 1). Results describe the accuracy of pair wise alignment by Clustal under the specific simulation conditions and alignment parameters. Estimation of evolutionary distances between Vtg sequences is important for constructing phylogenetic trees (Figure 1), dating species divergences and understanding the mechanism of evolution of protein. Vtg sequence of giant grouper (*Epinephelus lanceolatus*) was closed (0.015) with *Poecilia latipinna* (Genbank: ACV65040.1) and very far from *Ichthyomyzon unicuspis* (Genbank: AAA49327.1 (1.041). Estimating the number of nucleotide or amino acid substitutions needed to compute evolutionary distances is one of the most important subjects in molecular evolutionary genetics and comparative genomics. Estimation of evolutionary distance of Giant grouper Vtg with alignment of 13 other fish homologous sequence, revealed that Giant grouper Vtg was belongs to the marine fishes species rather than freshwater species group.

### Domain architecture of Vtg

Study by Babin et al. [8], has proposed the domain architecture



**Figure 2:** Polygenetic distributions of 14 species of Vtg sequences. Numbers besides nodes indicate the percent of bootstrap values for each branch of the tree in the 1,000 bootstrap trials.



**Figure 3:** Schematic representation of the domain in Vtg peptide sequence. (A) Domain architecture of teleost fish Vtg (Babin, et al., 2007). Large lipid transfer (LLT) module also referred to as Vtg<sub>N</sub> and LPD-N domain, polyserine track (PT) domain, and von Willebrand-factor type-D domain (VWD). The horizontal line indicates the receptor-binding region to VtgR. Lipovitellin I (Lv-I) and II (Lv-II), phosphitin (Pv), and  $\beta$ -component ( $\beta$ -C) are the yolk protein generated by the enzymatic cleavage of Vtg inside the oocyte. Conserved domains of (B) *Epinephelus lanceolatus*, (C) *Ichthyomyzon unicuspis*, (D) *Dicentrarchus labrax*, and (E) *Clarias macrocephalus* Vtg by using NCBI software.

and conserved sequence of teleost fish Vtg (Figure 3A). Based on the analysis, *Epinephelus lanceolatus* shows four main domains (Vitellogenin-N, DUF1943, DUF1944 and VMD) (Figure 3B), similarly found in *Dicentrarchus labrax* but different compare to *Clarias macrocephalus*, *Catla catla*, and *Danio rerio* (figure not show) where VMD domains, was absent. This indicate characteristic of Vtg domain for freshwater species is control by present of VMD in Vtg. Analysis on domain of Vtg with PROSITE (<http://www.expasy.org/prosite>) clarified the amino acid sequence for Giant grouper was from sequence number 22 till number 660 (Figure 4).

The predicted secondary structures of Giant grouper Vtg are show in Figure 3. It was clearly seen that  $\alpha$ -helix was predominantly present in the Giant grouper Vtg sequence and helix can be grouped into four major groups, which are located in domain region. Analysis indicated that the  $\alpha$ -helix,  $\beta$ -sheet and the coil structure configurations have 39.96%, 25.54% and 34.48% respectively. As it can see in Figure 4, the 4-helics can recognize in the different color of domain region.

### Three dimensional (3-D) structure prediction

Analysis of the 3-D structure found that *E. lanceolatus* Vtg gene shows this protein has the typical 4 $\alpha$ -helices bundle protein that runs in anti-parallel (Figure 5A). Based on the color, its can categorized in 4 helix structure, which is Blue, Red, Light green and Green respectively. In the present study, the main structure of Vtg gene in Giant grouper from different species was similar at the 4-helic region (Figures 5B-5F). However, the difference can be seen in Helix-1 (blue) and Helix-4 (red) where the structure was totally different in Lamprey (*Ichthyomyzon unicuspis*) but similar in Catfish (*Clarias macrocephalus*), Japanese Eel (*Anguilla japonica*) and European Seabass (*Dicentrarchus labrax*). However, Zebra fish (*Danio rerio*) 3-D vtg structure was different with giant grouper in the position of reddish color Helix-4.

### Discussion

Vitellogenin (Vtg) is an egg yolk precursor expressed in the females of nearly all oviparous species including fish, amphibians, reptiles, birds, most invertebrates, and the platypus. Vtg is the precursor of the lipoprotein and phosphoproteins that make up most of the protein content of yolk. There is potential of Vtg as a biomarker for measuring exposure of oviparous animals to estrogen or estrogen mimics, by using several fish species for which both *in vivo* and *in vitro* assays have been developed [9-11], Vtg functions as a nutritional source for the developing embryo, rather than as an important functional protein.

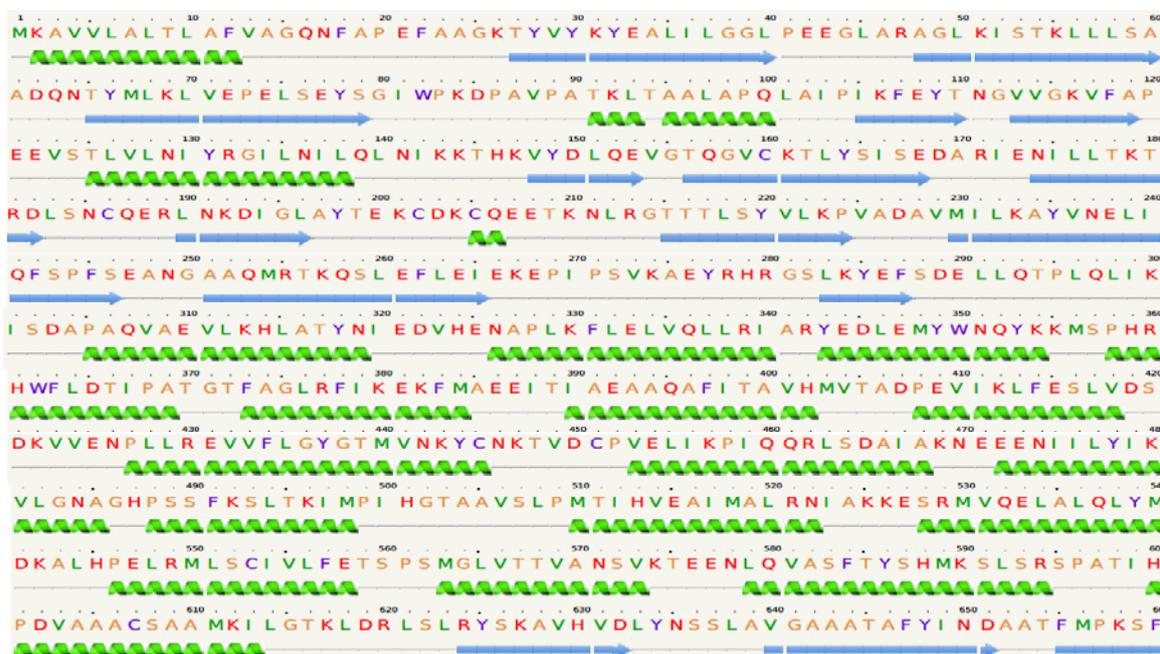
Three types of vitellogenin (Vtg) namely vitellogenin A (Vt gA), vitellogenin B (vtg B) and vitellogenin C (vtg C) have been identified in fishes. Paracanthopterygii and Achantopterygii generally express three types of Vtg at transcription level [12]. Advanced teleost fishes (Acanthomorpha) produced two complete types of Vtg (VtgAa and VtgAb) with five linear yolk protein domains organized from the amino-terminus as follows lipovitellin heavy chain (LvH), phosphitin (Pv), lipovitellin light chain (LvL),  $\beta$ -component ( $\beta$ '-c), and C-terminal peptide (C-t)) Reading [13].

Generally, assessment with Blast analysis showed that the Vtg amino acid protein sequences were similar with Vtg gene in the GeneBank database, it is very likely that 14 sequences obtained were Vtg gene fragments. Molecular characteristic of Vtg Giant grouper showed phylogenetic analysis by using maximum likelihood (ML) and neighbor joining (NJ) was generated two separated tree topology. The phylogenetic grouping showed the Giant grouper were closed to *Poecilia latipinna* and *Kryptolebias marmoratus* than other group fish at 98-100% similarity in terms of nucleotide and amino acids sequences respectively (Figure 1). It was shown that Giant grouper Vtg gene had the highest homology with *Poecilia latipinna*. These similarities (0.015 distance matric viewer) were closely related in terms of their Vtg gene sequence although from different environmental and ecological conditions.

Biological similarities are seen between lipoprotein and Vtg from the point of view of binding of hydrophobic molecules, cell specific uptake, and the possibility that these proteins may have a common ancestor [14-15]. In this investigation, the region of Giant grouper Vtg, which

(	1	2	3	4	5	6	7	8	9	10	11	12	13	14	)
(1)		(0.021)	(0.026)	(0.026)	(0.21)	(0.025)	(0.026)	(0.024)	(0.024)	(0.021)	(0.038)	(0.025)	(0.026)	(0.021)	
(2)	0.435		(0.026)	(0.021)	(0.022)	(0.026)	(0.027)	(0.025)	(0.026)	(0.023)	(0.038)	(0.021)	(0.022)	(0.012)	
(3)	0.614	0.604		(0.03)	(0.02)	(0.029)	(0.03)	(0.028)	(0.028)	(0.027)	(0.040)	(0.030)	(0.030)	(0.026)	
(4)	0.602	0.443	0.734		(0.026)	(0.029)	(0.03)	(0.029)	(0.029)	(0.028)	(0.039)	(0.017)	(0.015)	(0.021)	
(5)	0.426	0.474	0.417	0.614		(0.026)	(0.027)	(0.025)	(0.026)	(0.023)	(0.038)	(0.026)	(0.026)	(0.022)	
(6)	0.565	0.608	0.701	0.706	0.619		(0.023)	(0.014)	(0.014)	(0.025)	(0.040)	(0.029)	(0.029)	(0.026)	
(7)	0.614	0.652	0.76	0.755	0.647	0.514		(0.023)	(0.023)	(0.027)	(0.040)	(0.030)	(0.030)	(0.027)	
(8)	0.544	0.583	0.695	0.709	0.586	0.207	0.5		(0.011)	(0.024)	(0.040)	(0.029)	(0.029)	(0.026)	
(9)	0.556	0.608	0.692	0.723	0.616	0.225	0.516	0.146		(0.025)	(0.040)	(0.029)	(0.029)	(0.026)	
(10)	0.433	0.503	0.632	0.672	0.496	0.57	0.629	0.541	0.591		(0.038)	(0.027)	(0.028)	(0.023)	
(11)	1.043	1.041	1.083	1.064	1.036	1.085	1.099	1.104	1.102	1.018		(0.039)	(0.040)	(0.038)	
(12)	0.589	0.428	0.743	0.34	0.595	0.713	0.736	0.698	0.709	0.635	1.059		(0.019)	(0.020)	
(13)	0.607	0.464	0.733	0.237	0.625	0.711	0.753	0.723	0.729	0.676	1.083	0.382		(0.021)	
(14)	0.443	0.162	0.625	0.422	0.464	0.611	0.64	0.62	0.62	0.52	1.032	0.391	0.441		

**Table 1:** Distance matrix viewer showing distance and their standard errors for sequence pairs. (Each number in bracket refer to each species below), (1)Vtg Oncorhynchus mykiss; (2)Vtg Morone saxatilis; (3)Vtg Fundulus heteroclitus; (4) Vtg Epinephelus lanceolatus; (5) Vtg Dicentarchus labrax; (6)Vtg Danio rerio; (7) Vtg Clarias macrocephalus; (8) Vtg Catla catla ; (9) Vtg Carassius auratus; (10) Vtg Anguilla japonica; (11) Vtg Ichthyomyzon unicuspis; (12) Vtg Krytolebias marmoratus; (13) Vtg Poecilia latipina; (14) Vtg Thannus thynnus.

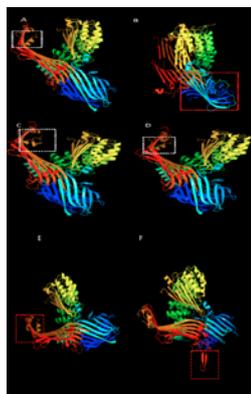


**Figure 4:** Secondary structure of *Epinephelus lanceolatus* Vtg as predicted by the Phyre2 software. The green, blue color and the faint lines symbols represent  $\alpha$ -helix,  $\beta$ -sheet and coil respectively.

showed homology, was limited to the N-terminal half of the molecule corresponding to the 660 domain profile. However, there is different in domain profile between seawater species and freshwater species. The catfish, (*Clarias macrocephalus*) was absent in von Willebrand-factor type-D domain (VWD), similarly finding [16] in Zebrafish. It should be noted that an additional DUF 1061 domain of unknown function was identified in the last region of Vtg peptide sequence.

The information from identification of Vtg (such as molecular

mass and sequencing) could be useful during preparation of Vtg antibody production. Antibodies production is generated by *in vivo* or *in vitro* approaches, their identification relies mainly on screening of hybridoma supernatants or bacterially expressed antibody fragments. The molecular approach can be done on Giant grouper to understand the molecular respond towards fish growth and determine the individual of Giant grouper that has potential to increasing the Vtg production for increase eggs quality.



**Figure 5:** The 3-D structure of Vtg in fishes from different species. Figure A, B, C, D, E, and F are the 3-D structure of *Epinephelus lanceolatus*, *Ichthyomyzon unicuspis*, *Dicentrarchus labrax*, *Clarias macrocephalus*, *Anguilla japonica* and *Danio rerio* respectively. The location of helix; blue: helix1; green: helix 2; light green: helix 3; red: helix 4.

Application of Vtg gene in aquaculture is promising in many aspects especially in molecular approach. This includes in the production of GMOs to improve the fish performance and gene regulation study to produce the high-quality eggs and determination of SNPs that can be used as genetic marker. Nutritional genomics is an area of science to studying how genes influence response of genetically to feed. Knowledge of these interactions and variations can be applied in the field of nutrigenetics to improved maturation diet for broodstock.

The results of this investigation will enable in further studies on the elucidation of the hormonal regulation of vitellogenesis including the physiological functioning with vitellogenin-stimulating ovarian hormone. This information can be used for improving the production of giant grouper for broodstock management.

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