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Sequence and expression analysis of glucokinase mRNA from herbivorous Giant gourami (*Osphronemus goramy*)

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Abstract. *Sari DN, Nasrullah H, Ekasari J, Suprayudi MA, Alimuddin A. 2021. Sequence and expression analysis of glucokinase mRNA from herbivorous Giant gourami (*Osphronemus goramy*). Biodiversitas 22: 741-750.* Glucokinase (GCK) is one of the enzymes that play important roles in carbohydrate metabolism and high glucose homeostatic in fish. The information about the GCK mRNA sequence and its expression is limited in Giant gourami, one of the most important herbivorous aquaculture species in Indonesia. The present study aimed to characterize the GCK mRNA and analyze its mRNA expression and plasma glucose levels after high glucose injection in Giant gourami. We also compared its sequence variability among carnivorous and herbivorous fish. The GCK mRNA was identified using polymerase chain reaction (PCR) method from the fish liver. Its mRNA level was analyzed by real-time PCR (qPCR). Giant gourami GCK mRNA sequence was 2104 nucleotide long, encoding 478 amino acids, and shared high similarity with other fish. GCK was mainly expressed in the liver. The mRNA level of GCK was highly up-regulated after 6 hours of high glucose injection, in-line with the plasma glucose in the blood. There are no major differences observed in the GCK amino acid sequences among Giant gourami and other fish. The knowledge gained from this study could be used as a reference for further exploration of metabolic regulation in Giant gourami.

Keywords: Giant gourami, glucokinase, gene expression, herbivorous, *Osphronemus goramy*

INTRODUCTION

Giant gourami (*Osphronemus goramy* Lacapede 1801) is a freshwater fish species that are native to Southeast Asia's river and lake and has become one of the most important aquaculture species in Indonesia (Arifin et al. 2019). In Indonesia, Giant gourami is mainly cultured and harvested as a customary food, highly valued due to their thick flesh and pleasant flavor (Setijaningsih et al. 2007). Aside from table, the Giant gourami has been reported to be used as weed control, since it tends to have herbivory and algal-based diet in their nature (Ismail et al. 2018). Several studies reported that farmed Giant gourami is able to utilize fibrous plant-based feed like giant taro leaves *Alocasia macrorrhiza* and feed with high carbohydrate levels (Mokoginta et al. 2004; Slembrouck et al. 2019). Due to slow blood glucose clearance, most farmed fish are considered to be less tolerant of carbohydrate-rich meals. (Moon 2001; Polakof et al. 2012; Li et al. 2018). Culturing fish that can utilize high carbohydrates, like Giant gourami is very important for the sustainability of the aquaculture practices since the unit cost of carbohydrates as energy sources in fish feed is substantially lower than that of protein. (Prisingkorn et al. 2017; Wang et al. 2017; Coutinho et al. 2018).

Carbohydrate metabolism in fish is related to several factors, such as enzyme activity, glucose uptake, and the control of glycemia (Enes et al. 2012; Li et al. 2018). Glucokinase (GCK) is one of the hexokinase isoenzymes that play important roles in carbohydrate metabolism. It is mostly found in the liver and pancreatic cells of vertebrates including fish (Soengas et al. 2006). As a glucose sensor, it facilitates phosphorylation of glucose to glucose-6-phosphate, triggering shifts in metabolism in response to rising or falling levels of glucose (Panserat et al. 2014; Li et al. 2016a). The alteration of GCK mRNA and enzyme has a positive correlation with the alteration of blood glucose and has been evaluated in several fish species (Panserat et al. 2000; Li et al. 2016a; Zhou et al. 2018). The hepatic GCK mRNA was increased significantly after being fed with high dietary carbohydrate in tilapia *Oreochromis niloticus*, rainbow trout *Oncorhynchus mykiss*, and found to be higher expressed in herbivorous fish blunt snout bream *Megalobrama amblycephala* than carnivorous fish (Soengas et al. 2006; Li et al. 2016a; Chen et al. 2018). Thus, GCK is significantly related to high glucose tolerance and metabolism in fish (Nie et al. 2015; Li et al. 2016b).

Although the transcript and enzyme activities of GCK have been widely studied in fish (Soengas et al. 2006; Irwin and Tan 2014; Panserat et al. 2014), the information about the GCK mRNA sequence and its expression related to the high glucose load in Giant gourami is limited. Moreover, the comparison of GCK sequences between herbivorous and carnivorous fish is still scarce. The sequence variation within the GCK sequences might be lead to the different hyperglycemias activity as reported in the avian species (Velho et al. 2004). It might also be a

result in the variations of GCK activities and partly explains the metabolic differences in carbohydrate utilization among different species (Panserat et al. 2000). Giant gourami could become a potential model to understand the necessary key features and mechanism of GCK in herbivorous fish for utilizing high carbohydrates. The present study aimed to characterize the full-length mRNA sequence of GCK and to analyze the GCK mRNA expression and plasma glucose levels after high glucose injection. We also compared the sequence variability of Giant gourami with carnivorous and herbivorous fish to analyze the sequence variation with glucose utilization.

MATERIALS AND METHODS

Fish and experimental condition

Giant gourami (35.51±0.19 g) were obtained from local farm in Bogor, Indonesia. Prior to the experiment, fish were acclimatized in three tanks (60 × 40 × 40 cm3) for two weeks. The fish were fed by a commercial feed (protein 31.86% and nitrogen-free extract NFE 46.91%) twice a day and giant taro leaves *Alocasia macrorrhiza* (once a day in the evening). After acclimation, twenty-five fish were randomly selected from stock and housed in five tanks (60 × 40 × 40 cm^3) with five fish per-tank for glucose tolerant test (GTT). The fish were reared for two weeks and fed with commercial feed (protein 31.86% and NFE 46.91%) at-satiation three times a day under natural photoperiod conditions. The water temperature was maintained at 28-30 °C and water quality was maintained by changing water every two days. All animal experimental procedures were carried out in compliance with the national standard for fish experiment and welfare No. SNI 01-6489-2000 of the Republic of Indonesia.

Plasma glucose levels

Two weeks after adaptation with commercial feed, the fish were fasted for 48 hours and then slightly anesthetized with 0.5 mL L^{-1} tranquilizer (Super Stabilizer, Taiwan) for five minutes. The fish were injected intraperitoneally with 0.9% saline solution containing 300 mg glucose mL^{-1} (1 g of glucose kg^{-1} body weight) for glucose tolerance test (GTT). The control group was injected with an equal volume of 0.9% saline solution. After injection, the fish were transferred immediately into four tanks (36 L each) at a density of three fish per tank. Blood was taken at 0, 6, 18 hours post-injection using syringes rinsed with 3.8% Na-citrate. Blood was taken from fish in the different tanks at each sampling time to minimize the stress. Blood was centrifuged at 3000 rpm for 10 min at 4 °C. Blood plasma was separated into a new tube and then stored at -20 °C for further analysis. Plasma glucose analysis was analyzed according to Wedemeyer and Yasutake (1977) using the spectrophotometry method at 635 nm. The fish liver was also collected at 0, 6, and 18 hours post-injection for mRNA expression analysis (n=3). Various fish tissues including liver, intestine, pancreas, heart, spleen, white muscle, and brain were collected before injection from the stock for further mRNA expression distribution analysis (n=3 for each tissue).

Total RNA extraction and GCK amplification

Total RNA was extracted from seven tissues (liver, intestine, pancreas, heart, spleen, muscle, and brain) of Giant gourami using GENEzol™ Reagent Kit (Geneaid, Taiwan) according to the manufacturer's manual. Its purity and quantity were later determined by absorbance measures at 260 and 280 nm, and its integrity was tested by electrophoresis in 1.0% agarose gels. For sequence identification, hepatic RNA of Giant gourami (50 ng μL^{-1}) was used in the first-strand cDNA synthesis by ReverTraAce®qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan). The cDNA amplification was conducted using the polymerase chain reaction (PCR) method from the diluted first-strand cDNA product. The amplification was performed using the degenerate primers of GCK that were designed based on the partial GCK sequence of several fish species. The PCR reaction was set at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, at 51 °C for 30 s, and 72 °C for 50 s. The final elongation step of 5 min was done at 72 °C. The PCR products were purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan), cloned into the pTA2 vector (Toyobo, Japan), and then transformed into *Escherichia coli* DH5α competent cells following the method from our previous study (Nasrullah et al. 2019). Plasmid from positive clones was isolated and then delivered to 1st BASE Laboratory (Selangor, Malaysia) for sequencing procedure.

RACE-PCR

The full-length sequence of Giant gourami GCK was identified using the rapid amplification of cDNA ends (RACE) method (Fukada et al. 2012). The primers for RACE PCR were designed according to the obtained partial sequence from the initial amplification. The 5' RACE PCR was performed by RACE System for Rapid Amplification of cDNA Ends Kit (Invitrogen, USA) according to the manufacturer's manual. Briefly, reverse transcription of 0.5 µg total RNA was performed with a gene-specific primer 5'GSP-1 to obtain the first-strand cDNA. The cDNA was purified by RNase treatment, and Oligo (dC) tail at the 5' end was added using terminal deoxynucleotidyl transferase (Invitrogen, USA). The resulting product was amplified with a universal abridged anchor primer (AAP) and primer 5'GSP-2 to obtain the 5' untranslated region (UTR) sequence of Giant gourami GCK. To obtain a 3'UTR sequence, a nested PCR was performed using the Oligo (dT) and AP2 primers. Firstly, cDNA was amplified using a specific forward primer 3GSP-1 and Oligo (dT) as the reverse primer and followed by nested PCR with 3GSP-1 and AP2 primers from the 1:10 diluted PCR product. The purified RACE PCR products were cloned into the pTA2 vector (Toyobo, Japan), transformed into the competent cell *Escherichia coli* DH5α and then sequenced, respectively. A set of qPCR primers was then designed based on the obtained full-length sequence of Giant gourami GCK. All primers used in this study are presented in Table 1.

Table 1. List of primers used in this study

Primer name	Sense/Anti-sense	Sequence (5'-3')	Application
FwOgGCK	Sense	GGTTGGTAATTTCCTGGCACTG	Partial identification
RvOgGCK	Anti-sense	CACAACTTTGTGGAACCTGTC	
5'GSP-1	Anti-sense	ATTGAGTACATTTGGTT	5'RACE
AAP	Sense	GGCCACGCGTCGACTAGTAC $(G)_{14}$	5'RACE anchor
5'GSP-2	Anti-sense	CGCCCAGATCCAGTGCCAGGAAAT	Nested 5'RACE
Oligo (dT)	Anti-sense	GTAATACGAATAACTATAGGGCACGCGTGGT CGACGGCCCGGGCTGG $(T)_{17}$	3'RACE anchor
AP2	Anti-sense	CTATAGGGCACGCGTGG	Nested 3'RACE
3GSP-1	Sense	ATTGCGACATTGTGCGTCTGGTCT	3'RACE
qGCKOg-F	Sense	GGAGTATGACAGAGTGGTGGACGAG	qPCR analysis
qGCKOg-R	Anti-sense	CAGAACAAGCCTGACCAGCTCACC	
qACTB-F	Sense	ACCGGAGTCCATCACAATACCAGT	Internal control, qPCR normalization
qACTB-R	Anti-sense	GAGCTGCGTGTTGCCCCTGAG	

Note: AP2 primer sequence is underlined

Sequence and phylogenetic analysis

The core fragment, 3' end, and 5' end sequences were assembled using MEGA 7.0 software (www.megasoftware.net/). Open Reading Frame (ORF) prediction and mRNA translation were performed in BioEdit Sequence Alignment Editor Version 7.2.5 (www.mbio.ncsu.edu/BioEdit/BioEdit.html) using standard genetic codes. The result was compared against all available fish GCK nucleotide databases in the GenBank using BLAST (blast.ncbi.nlm.nih.gov/). The similarity of predicted amino acid sequence was analyzed using MUSCLE (www.ebi.ac.uk/Tools/ msa/muscle/). The amino acid sequence similarity between herbivorous and carnivorous fish was generated using WebLogo 3 tool (https://weblogo.berkeley.edu/logo.cgi). The molecular weight (MW) and isoelectric point (PI) of GCK protein were both predicted using the pI/Mw software at https://web.expasy.org/cgi-bin/compute_pi/pi_tool and the predicted protein model of GCK was generated using SWISS-MODEL (https://swissmodel.expasy.org). A phylogenetic tree was constructed using the MEGA 7.0 software from the deduced amino acid of GCK using the Neighbor-Joining method with 1000× bootstraps. The separation between herbivorous and carnivorous fish was referring to several previous studies listed in Table S1.

Gene expression analysis

The GCK mRNA expression was determined by the real-time PCR method (qPCR). The qPCR assays were carried out with a 20 µL reaction volume containing 10 µL SensiFAST SYBR®NO-ROX kit (Bioline, UK), 0.8 µL of each primer (10 µM), 4 µL cDNA template (5 ng μL^{-1}), and 4.4 µL nucleases free water. The reaction was performed in the RotorGene 6000 machine (Corbett, USA). The PCR protocols were initiated at 95 °C for 5 min; followed by 40 cycles at 95 °C for 5 s, at 60 °C for 15 s and 72 °C for 15 s; and then followed by melting curve analysis. The expression levels were normalized with the β-actin gene (ACTB) and then analyzed using the optimized comparative Ct ($2^{-\Delta\Delta ct}$) value method by Livak and Schmittgen (2001).

Data and statistical analysis

All the obtained data were tabulated and analyzed using MS. Excel 2016 (Microsoft, USA) and SPSS v.21 (SPSS Inc, USA). The GCK expression level was calibrated to the values at 0 h (GTT test) or calibrated to the value in the heart (tissue distribution analysis) after normalized with the β-actin gene. Data were analyzed using the one-way analysis of variance (ANOVA) with Duncan's multiple range tests ($P<0.05$), while the non-homogeneous data were analyzed using the nonparametric test with Mann-Whitney test ($P<0.05$). The differences between injection groups at the same sampling time were analyzed using the independent sample t-test ($P<0.05$).

RESULTS AND DISCUSSION

GCK mRNA sequence

The full-length GCK cDNA sequence from Giant gourami liver was 2104 nucleotide long, encoding 478 amino acids. The cDNA sequence consists of 1437 bp of ORF, 96 bp of 5'UTR, and 571 bp of 3'UTR including one AATAAA motifs, which represent putative polyadenylation signals. TAA stop codon was present 572 nucleotides upstream of the 3'end (Figure S1). The GCK sequence of Giant gourami has been submitted to GenBank under accession number MW194320. The Giant gourami's amino acid sequence contains several conserved functional sites, including two ATP-binding domains (Asp90-Lys102 and Glu114; Giant gourami's GCK numbering), one regulatory protein binding site (His153-Leu156), one conserved hexokinase signature sequence (Leu158-Phe183), two potential conserved N-linked glycosylation sites (Asn178-Thr180 and Asn216-Thr218), several glucose binding sites (Pro163-Pro165, Asn178-Thr181, Asn216-Thr218, Ile237-Asn243, Asn266-Gly270, Gln299, and Glu302), one cell attachment sequence (Arg204-

Asp206) and one glycosaminoglycan attachment site (Ser457-Gly460) (Figure 1). The deduced amino acid sequence of GCK is highly similar between Giant gourami and other fish species. The protein of Giant gourami GCK has a predicted molecular weight of 53.9 kDa and an isoelectric point of 4.81 that shares a similar structure with other fish species. The predicted protein structure of Giant gourami has high similarity with other herbivorous and carnivorous fish like *M. amblycephala* (84.7%) and *O. mykiss* (86.8%) (Figure 1).

Nucleotide and amino acid sequence of Giant gourami's GCK shows high similarity with several fish species (Table 2). The highest similarity was found with *Anabas testudineus*, 90.4 %, and 92.1%, respectively. Generally, the similarity was ranged from 69.6-90.4% for nucleotide and 84.1-92.1% for amino acid residues.

Phylogenetic analysis among fish species based on GCK amino acid sequences is presented in Figure 2. Tree topology shows that Giant gourami has the closest distant relationship with GCK of *Betta splendens*, which belongs to the same family. Then, Giant gourami's GCK formed a clade with other fish from Anabantidae, Gobiidae, Apagonidae, Cichlidae, and Sciaenidae while separated with the fish from family Carangidae, Lateolabracidae, Paralichthyidae, and Serranidae. Herbivorous and carnivorous fish seems not significantly separated.

Plasma glucose and GCK gene expression

The Giant gourami's GCK mRNA expression was distributed in all examined tissues (Figure 3.A). The GCK was mainly expressed in the liver, intestine, and pancreas, and least expressed in the brain, spleen, and heart. The mRNA level of GCK was also analyzed after the glucose tolerant test by injecting a high dose of glucose. The result shows that GCK mRNA was highly up-regulated ($P<0.05$) after six hours of high glucose injection, which is in line with the alteration of the plasma glucose in the blood (Figure 3.B). There is no significant alteration at control injection ($P>0.05$). The mRNA expression and plasma glucose levels were rapidly decreased to their basal level within 18 hours post-injection.

Functional sites comparison

Several active and signature sites of GCK of Giant gourami were compared with other herbivorous and carnivorous fish, presented in Figure 4. The compared regions were the ATP-binding domain, glucose binding site, hexokinase signature sequence, and glycosaminoglycan attachment site.

The comparison shows that the sequences of the GCK functional domain were quite similar among Giant gourami, herbivorous, and carnivorous fish. There are no major differences in the GCK sequences among Giant gourami and other fish, except a site in the glucose binding site region. Giant gourami has Proline163 at the early glucose binding site, while other fish have Serine at the same site. In Giant gourami and herbivorous fish, the amino acid at site 102 in the ATP-binding domain was Lysine; while in carnivorous fish, it was Lysine or Isoleucine. At the hexokinase signature site, herbivorous fish tend to have Asp171 and Lys172 while Giant gourami and carnivorous fish are Asp171 and Ile172 or Leu172, respectively.

Table 2. Percent identity of nucleotide and amino acid sequence of Giant gourami against other species.

	Species	Amino acid																		
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1	*Osphronemus goramy*		**85.1**	**84.7**	**85.3**	**84.3**	**86.8**	**86.8**	**91.4**	**88.0**	**89.3**	**92.1**	**84.1**	**90.4**	**91.2**	**91.9**	**91.6**	**91.4**	**88.9**	**91.2**
2	*Ctenopharyngodon idella*	**71.5**		98.5	91.0	82.4	88.3	87.8	85.7	86.2	84.9	85.7	89.7	86.1	85.9	88.8	88.2	87.0	85.3	87.0
3	*Megalobrama amblycephala*	**71.8**	96.9		89.9	82.1	87.9	87.4	85.1	86.2	85.1	85.3	89.5	85.5	85.7	89.0	87.6	86.6	85.3	86.6
4	*Chanos chanos*	**69.6**	70.0	70.3		83.2	88.9	88.5	85.5	86.6	84.9	86.1	88.9	86.6	86.3	87.7	88.7	86.8	86.8	87.2
5	*Boleophthalmus pectinirostris*	**78.2**	74.2	74.4	74.3		84.1	83.9	84.9	85.0	86.2	86.8	83.1	87.5	84.9	88.8	87.0	85.6	84.3	86.4
6	*Oncorhynchus mykiss*	**74.1**	68.4	68.7	68.7	76.5		98.5	86.4	87.1	87.7	87.5	86.2	89.2	89.0	89.9	90.2	89.2	87.7	89.4
7	*Salvelinus alpinus*	**74.5**	68.6	69.1	69.1	76.6	97.0		86.2	86.9	87.0	87.7	85.6	89.3	89.3	90.1	90.0	89.8	87.5	89.5
8	*Betta splendens*	**82.7**	69.2	69.1	69.1	78.4	72.5	73.9		87.7	89.1	92.1	84.1	89.8	90.2	91.4	90.8	90.8	87.9	89.8
9	*Cynoglossus semilaevis*	**79.0**	69.7	69.6	69.5	77.8	71.0	71.5	74.1		89.9	89.0	85.0	89.4	90.3	92.7	91.8	90.7	96.4	91.1
10	*Neolamprologus brichardi*	**82.1**	68.8	69.1	68.1	79.4	71.7	72.5	77.0	77.8		89.3	84.3	90.2	91.4	92.7	92.3	92.1	89.3	91.4
11	*Anabas testudineus*	**90.4**	71.0	71.0	69.9	78.6	73.9	74.2	80.9	78.2	82.2		84.9	91.0	91.2	92.3	92.7	91.6	88.5	90.6
12	*Ictalurus punctatus*	**70.0**	70.5	70.6	68.8	73.5	67.4	69.3	69.0	69.0	68.4	70.2		84.9	84.9	87.2	86.2	84.9	84.3	85.2
13	*Sphaeramia orbicularis*	**84.5**	76.5	77.1	79.1	80.8	81.1	81.0	83.9	82.3	83.7	85.2	76.1		91.8	93.6	93.7	93.1	90.2	93.9
14	*Paralichthys olivaceus*	**84.9**	69.7	70.2	70.1	78.8	74.2	74.0	80.6	81.2	80.6	84.0	69.1	85.1		93.8	94.1	94.8	91.0	93.9
15	*Larimichthys crocea*	**85.8**	77.2	77.0	75.1	79.8	78.4	80.8	78.1	83.9	85.7	86.7	74.3	83.4	85.0		94.9	94.9	93.0	94.5
16	*Epinephelus lanceolatus*	**86.5**	71.2	71.7	71.4	79.7	74.9	75.6	80.2	82.0	84.1	85.7	70.3	86.9	86.1	89.8		95.6	92.9	95.0
17	*Lateolabrax japonicus*	**87.7**	72.1	72.5	71.5	80.0	76.1	76.5	81.0	82.3	85.2	87.2	69.4	87.0	87.0	89.0	89.8		91.8	95.2
18	*Trachinotus ovatus*	**85.2**	70.0	70.4	69.3	78.9	72.9	73.3	77.7	86.0	81.6	83.8	68.9	83.9	86.1	87.6	86.8	88.6		92.9
19	*Seriola dumerili*	**86.6**	70.5	70.4	70.0	80.4	74.1	74.4	80.3	82.1	83.6	86.1	70.2	85.9	88.1	88.8	88.8	91.1	90.8	

Nucleotide

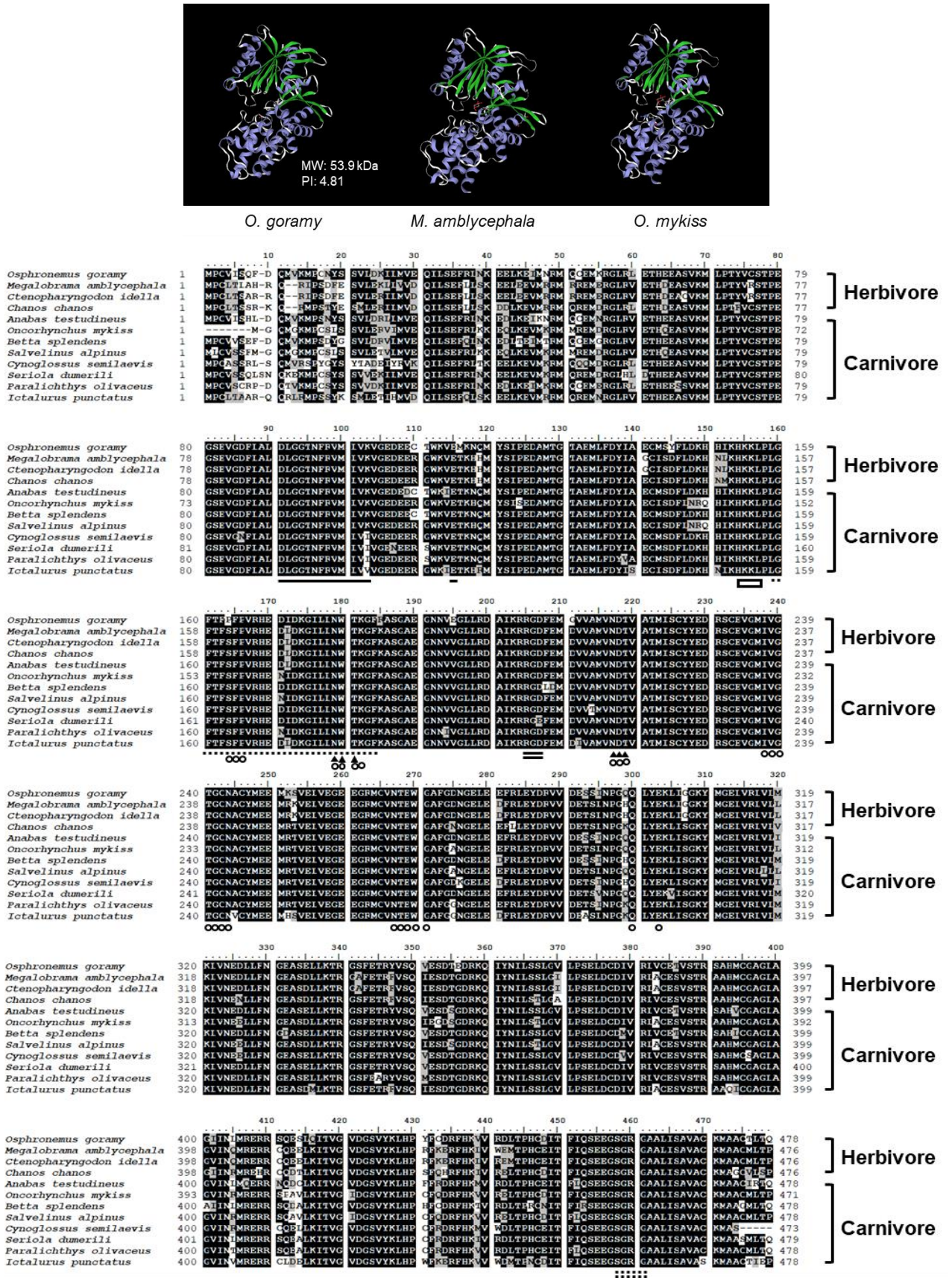

Figure 1. Predicted protein structure and multiple alignments of the GCK amino acid sequence from Giant gourami and other fish. Identical amino acids are marked in black and homologous amino acids in grey (50% threshold). ATP-binding domain (underlined), regulatory protein binding sites (boxed), hexokinase signature (dot underlined), N-linked glycosylation (triangle), glucose-binding sites (circle), cell attachment sequence (double-underlined), and glycosaminoglycan attachment site (double dot underlined) are also presented

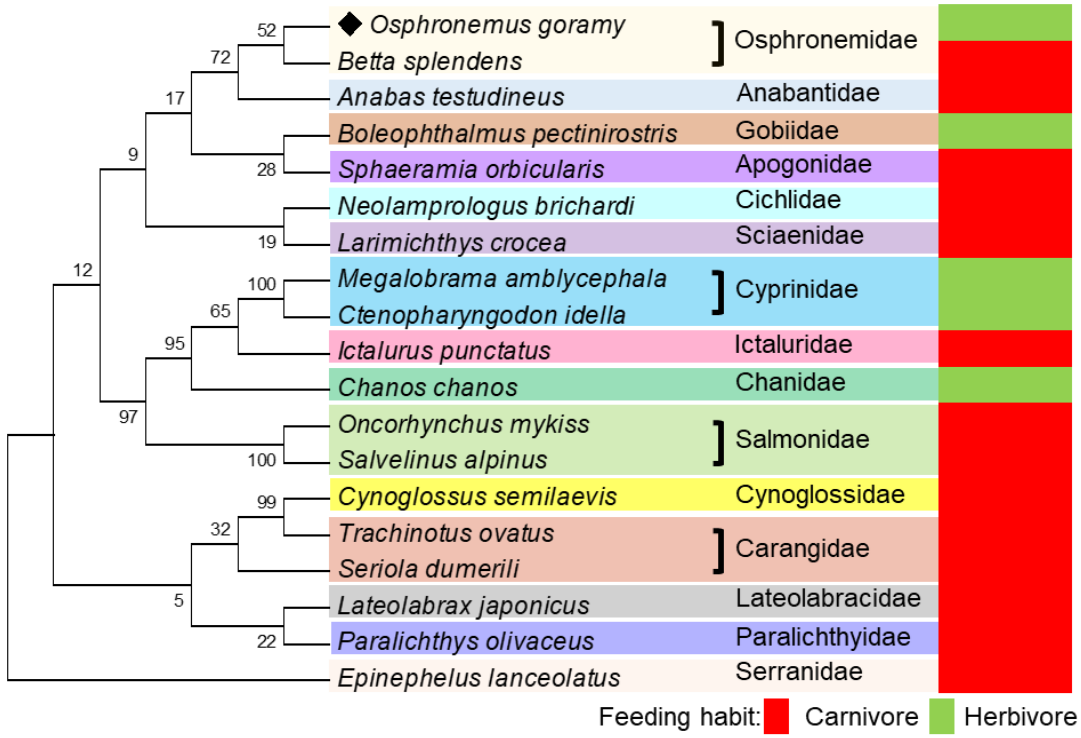

Figure 2. Phylogenetic tree based on GCK amino acid sequences using the Neighbor-joining method (bootstrap 1000×). Fish were labeled by their feeding habit referred to several studies listed in Table S1.

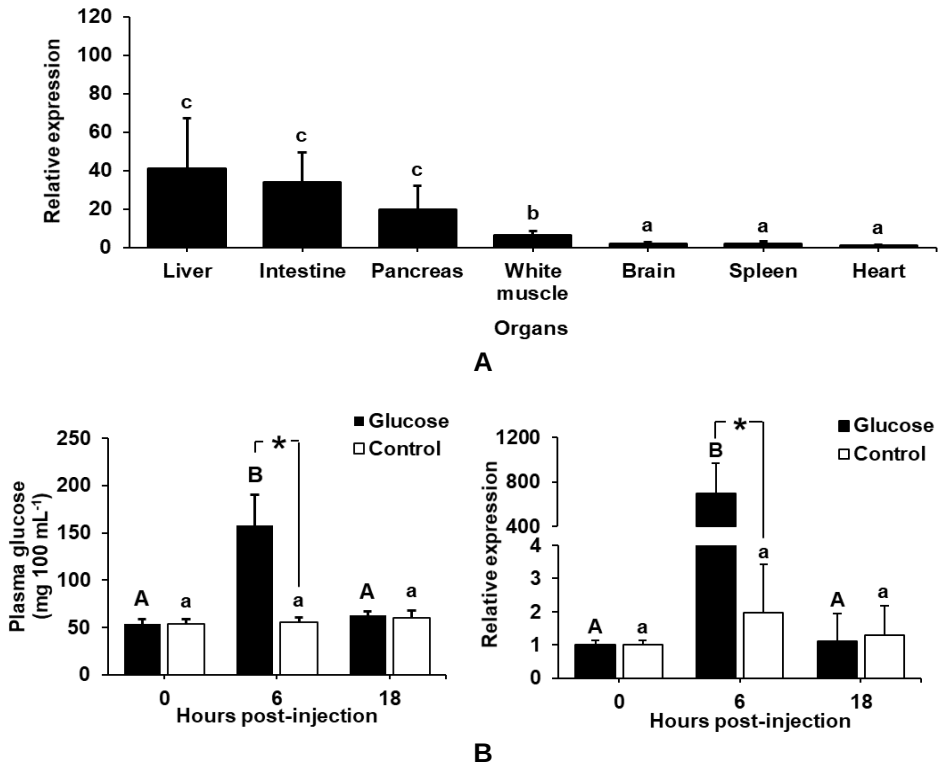

Figure 3. Tissue distribution (A) and plasma glucose and relative expressions of GCK mRNA of Giant gourami after 1 g kg^{-1} glucose injection (B). All values represent the mean ± SD (n=3). Significant differences (P<0.05) are indicated by different letters (uppercase for glucose injection and lowercase for control injection). Asterisks indicate significant differences between the group injection at each sampling time (P<0.05)

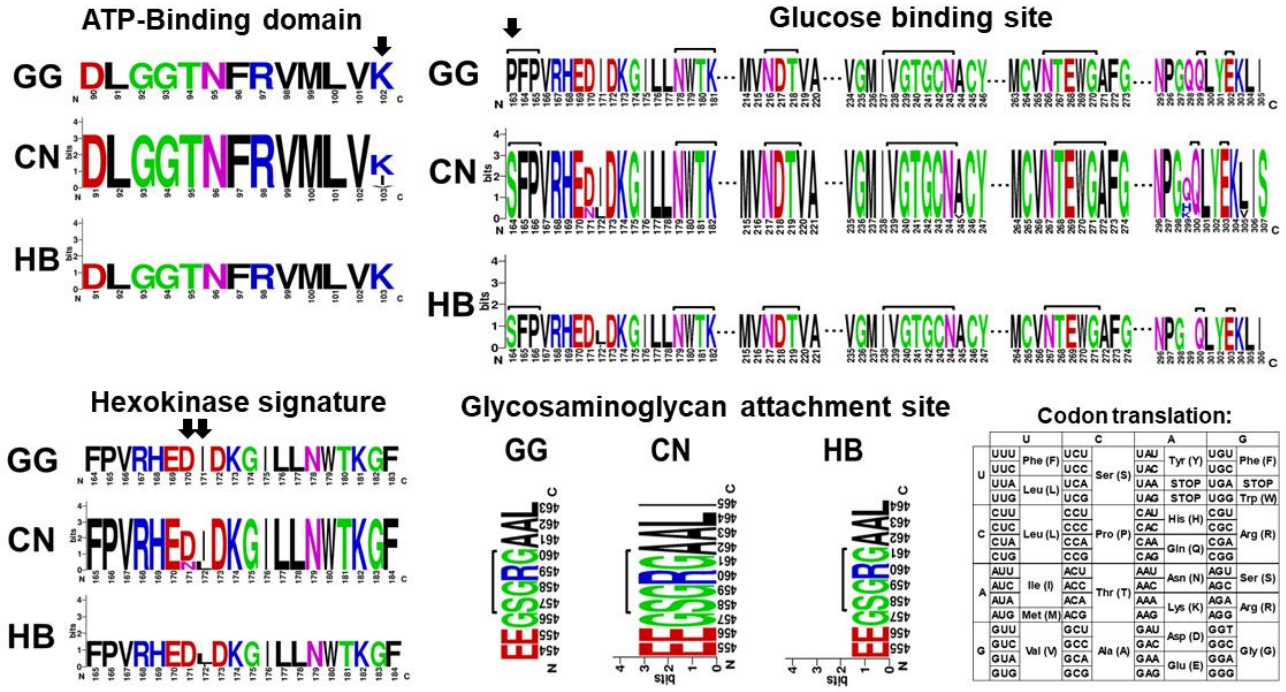

Figure 4. Comparison of amino acid sequence in the functional domain of Giant gourami GCK with other carnivorous and herbivorous fish species. GG= Giant gourami, CN: carnivorous fish, HB= herbivorous fish. Sequence variations are marked with an arrow

Discussion

The full-length sequence of GCK mRNA of Giant gourami was successfully amplified from the liver tissue. The 5' UTR, 3'UTR, and ORF regions were able to be characterized. To our knowledge, this is the first study to report the sequence of GCK mRNA in Giant gourami. Giant gourami's GCK functional sites such as ATP-binding domain, regulatory protein binding site, hexokinase signature sequence, N-linked glycosylation, the glucose binding sites, the cell attachment sequence, and the glycosaminoglycan attachment site. These sites shared high similarity with blunt snout bream *Megalobrama amblycephala* (Li et al. 2016a) and golden pompano *Trachinotus ovatus* (Zhou et al. 2018), and other fish species (Panserat et al. 2000; Nie et al. 2015). The GCK gene has been known to have an important role in fish carbohydrate utilization and is mainly expressed in the liver (Li et al. 2016a; Zhou et al. 2018). Giant gourami's GCK transcripts were mostly distributed in the liver, followed by the intestine, pancreas, and muscle (Fig. 3). This pattern was similar to other fish species (Blasco et al. 2001; Matschinsky 2002; Polakof et al. 2011). GCK plays role in the first and important step of glycolysis and as a glucose sensor in the regulation of glucose homeostasis (Polakof et al. 2012). Fish with high carbohydrate utilization ability is characterized by the rapid glucose homeostatic metabolism, indicated by the plasma glucose and GCK expression that rapidly returning to its basal level within 24 hours after carbohydrate administration (Panserat et al. 2001). These characteristics are often found in herbivorous fish (Li et al. 2016a). The molecular weight and isoelectric point of predicted GCK protein of Giant gourami were 53.9 kDa and 4.81, respectively. The molecular weight of predicted GCK protein in Giant gourami is similar to turbot *Scophthalmus maximus* (Nie et al. 2015), blunt snout bream *M. amblycephala* (Li et al. 2016a), and golden pompano *T. ovatus* (Zhou et al. 2018).

Following the high glucose injection, Giant gourami's plasma glucose was highly up-regulated after six hours post-injection ($P<0.05$) and returned to normal after 18 hours ($P>0.05$). In concert with plasma glucose alteration, the GCK mRNA level was also significantly increased after the high glucose injection ($P<0.05$), and decreased to basal expression after 18 hours post-injection ($P>0.05$). In other herbivorous fish, blunt snout bream *M. amblycephala*, the plasma glucose, and GCK transcripts were highly up-regulated after 1 hour and 2 hours of high glucose injection (1.67 g glucose kg^{-1}), respectively. The plasma glucose was rapidly decreased to the basal level after 6 hours post-injection and GCK mRNA was detected at its basal expression after 12 hours post-injection (Li et al. 2016a). In contrast, carnivorous fish tend to have a longer time to control high glucose levels. The plasma glucose of carnivorous rainbow trout *O. mykiss* and seabream *Sparus aurata* was returned to its basal levels after 30 and 24 hours of high-glucose administration, while their GCK expression was peaked at 24 hours and returned to normal after 39 hours (Peres et al. 1999; Panserat et al. 2001). Herbivorous fish were also reported to have higher GCK alteration at their peak expression after carbohydrate exposures compared to the carnivorous fish (Nie et al. 2015; Li et al. 2016a; Li et al. 2016b) as also shown in the Giant gourami. These results suggest that Giant gourami have a high capability to maintain the glucose homeostatic

rapidly, similar to other herbivorous fish. GCK expression may tightly be correlated with the carbohydrate metabolism in Giant gourami and have a significant role in the fish's ability to utilize the high carbohydrate levels.

We further assessed the differences in the GCK sequences between gourami, herbivorous and carnivorous fish since these sites were thought to become the factor that could affect the enzyme affinity and catalytic rate (Mahalingam et al. 1999; Panserat et al. 2000). Our results show that there were no major differences in the GCK sequences among Giant gourami and other fish (Fig. 4). To investigate it more, we aligned the gourami sequence with other seventy-three GCK sequences from other teleost species available at GenBank (not presented). We found that only Giant gourami has Proline on the glucose binding site, while the other fish have Serine. We hypothesized that this alternative sequence of GCK might be correlated with the carbohydrate complexity in the taro leaves that were given as supplementary feed during the rearing periods before the experiment. The giant taro leaf is known to contain high fiber, starch, and several complex carbohydrates (Temesgen and Retta 2015). The carbohydrate complexity was reported to be able to affect the activity of enzymes related to carbohydrate metabolism in fish (Enes et al. 2010) and thus might affect this GCK glucose binding site sequence modification in Giant gourami. This phenomenon was not further investigated in this study but is potentially studied in the future. Compared to herbivore fish, carnivore fish had several changes in the ATP-binding domain, glucose binding site, and hexokinase signature. In carnivorous fish, Lysine (LV<u>K</u>) was replaced with Isoleucine and Valine; Alanine (<u>A</u>CY) was replaced with Valine; and Aspartic Acid (HE<u>D</u>) was replaced with Asparagine, in ATP-binding domain, glucose binding site, and hexokinase signature, respectively. This change is assumed to be one of the causes of differences in carbohydrates metabolism capability between herbivorous and carnivorous fish (Panserat et al. 2000).

In conclusion, the present study successfully cloned and characterized the full sequence of the Giant gourami's GCK mRNA from the liver. Giant gourami has a relatively high ability to maintain glucose homeostasis. The GCK mRNA expression was up-regulated after glucose injection, demonstrating that Giant gourami has an effective hepatic glycolysis adaptation to high glucose levels. However, there are no major differences observed in the GCK amino acid sequences among herbivorous fish, carnivorous fish, and Giant gourami. The information gained from this study could be used as a reference for further exploration of metabolic regulation in Giant gourami.

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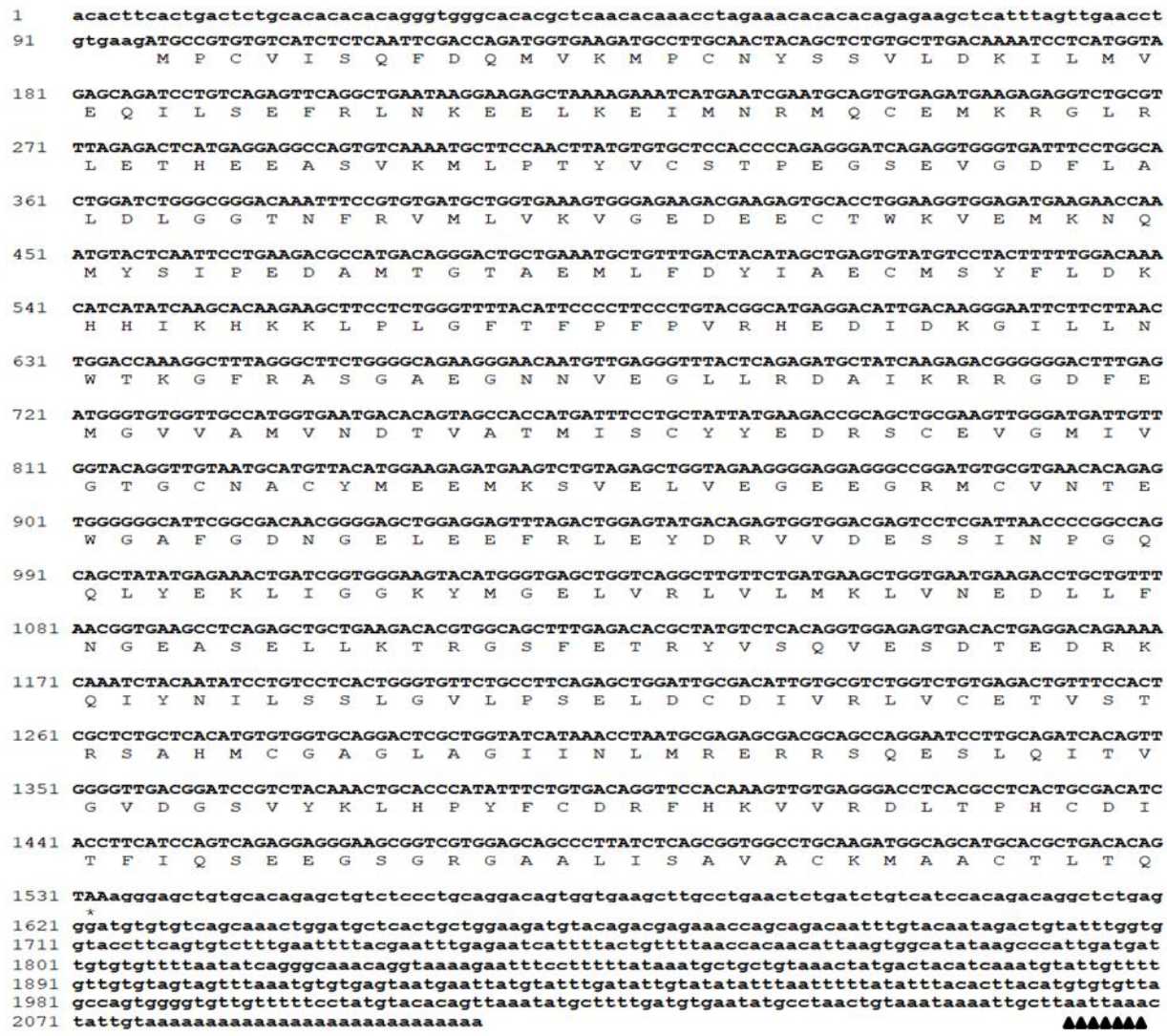

Figure S1. Nucleotide sequence of Giant gourami's GCK cDNA and the deduced amino acid sequence. Uppercase letters: translated region, lowercase letters: untranslated region, triangles: polyadenylation signal. The Giant gourami's GCK sequence was submitted to NCBI GenBank, accession no. MW194320

Table S1. List of fish feeding habit reference in several studies

No	Species	Feeding habit	Reference
1	*Osphronemus goramy*	Herbivorous	Azrita and Syandri 2015
2	*Ctenopharyngodon idella*	Herbivorous	Cai et al. 2018
3	*Megalobrama amblycephala*	Herbivorous	Li et al. 2016
4	*Chanos chanos*	Herbivorous	Makmur et al. 2020
5	*Boleophthalmus pectinirostris*	Herbivorous	Tran et al. 2020
6	*Oncorhynchus mykiss*	Carnivorous	Polakof et al. 2010
7	*Salvelinus alpinus*	Carnivorous	Langeland et al. 2013
8	*Betta splendens*	Carnivorous	Saekhow et al. 2018
9	*Cynoglossus semilaevis*	Carnivorous	Liu et al. 2013
10	*Neolamprologus brichardi*	Carnivorous	Kohda 1995
11	*Anabas testudineus*	Carnivorous	Morioka et al. 2008
12	*Ictalurus punctatus*	Carnivorous	Gao et al. 2017
13	*Sphaeramia orbicularis*	Carnivorous	Mees et al. 1999
14	*Paralichthys olivaceus*	Carnivorous	Liu et al. 2019
15	*Larimichthys crocea*	Carnivorous	Wang et al. 2017
16	*Epinephelus lanceolatus*	Carnivorous	Lu et al. 2018
17	*Lateolabrax japonicus*	Carnivorous	Zhang et al. 2018
18	*Trachinotus ovatus*	Carnivorous	Zhou et al. 2018
19	*Seriola dumerili*	Carnivorous	Hossain et al. 2018