

Chondrichthyes Chitinase: Molecular Cloning, Distribution, and Phylogenetic Analysis

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Abstract

We have previously reported the presence of three types of chitinase (acidic fish chitinase-1: AFCCase-1, acidic fish chitinase-2: AFCCase-2, fish chitinase-3: FCCase-3) in Actinopterygii. In the present research, we report the identification of the novel chitinase genes *HjChi* (ORF: 1380 bp) and *DkChi* (ORF: 1440 bp) from the stomach of Chondrichthyes, Japanese bullhead shark (*Heterodontus japonicas*) and Kwangtung skate (*Dipturus kwangtungensis*), respectively. Organ-specific expression analysis identified the stomach-specific expression of *HjChi*, whereas *DkChi* was expressed widely in all organs. Chitinase activity was measured using *p*NP-(GlcNAc)_n (*n* = 2, 3) as a substrate and β -*N*-acetylhexosaminidase (Hex) activity was measured using *p*NP-GlcNAc. Relatively high values of chitinase activity were observed in the stomach, spleen, and gonads of the Japanese bullhead shark, *H. japonicas*, compared with that observed in the stomach of the Kwangtung skate *D. kwangtungensis*. However, Hex activity was detected throughout the body of both species. The optimal pH of chitinase in both the Japanese bullhead shark, *H. japonicas*, and the Kwangtung skate, *D. kwangtungensis*, were 3.5 - 5.5 and 3.5 - 4.0, respectively, and 4.0 for Hex in both species. Phylogenetic analysis revealed that Chondrichthyes chitinase forms a unique group (Chondrichthyes chitinase). These results suggested that the possibility of the formation of chitinase groups for each class in the phylogenetic analysis based on the observation of class-specific chitinase.

Keywords

Chitinase, Distribution, Chondrichthyes, cDNA Cloning, Phylogenetic Analysis

1. Introduction

Chitinase (EC 3.2.1.14) is an endo-chitinolytic enzyme that randomly hydrolyzes the β -1,4 glycosidic linkages of chitin to produce *N*-acetyl chitoooligosaccharides ((GlcNAc)_n) [1]. β -*N*-acetylhexosaminidase (Hex, EC 3.2.1.52) is an exo-type chitin-decomposing enzyme that sequentially hydrolyzes terminal residues, resulting in the formation of *N*-acetyl-D-glucosamine (GlcNAc) [2]. After cellulose, chitin is the second most abundant biopolymer and is found in the exoskeleton of arthropods, the cell walls of fungi, and the epithelium of nematodes [3]. (GlcNAc)_n, a degradation product of chitin, displays various physiological properties based on the length of its carbohydrate chain; similarly, GlcNAc also exhibits useful physiological activity [4]. The aforementioned points are a clear indication of the potential for the application of chitin in a wide range of fields and the research on chitinase enzymes was invaluable to their efficient use. A wide range of functions is suggested by the distribution of chitinase throughout a wide range of organisms. For example, the enzyme has been found to play a physiological role in digestion in mammals and fish [5] [6], plant and mammalian host defense [7] [8], and arthropod morphogenesis [9] [10].

It is reported that chitinase from marine animals displays characteristics that are unlike chitinase from other organisms. In particular, we made very interesting findings regarding chitinase in the stomach of fish that feed on organisms containing a chitin exoskeleton. For example, two or more kinds of chitinase isozymes have been discovered in the stomach of Actinopterygii, one of the most successful vertebrates on earth. The activity of these chitinase isozymes is dependent on an acidic pH and the crystalline α -chitin decomposition activity of these enzymes has been demonstrated to be superior to that of other organisms [6]. Two varieties of the chitinase genes were obtained from the stomach of the fish and subjected to phylogenetic analysis, based on the deduced amino acid sequence 2 fish-specific groups, acidic fish chitinase-1 (AFCase-1) and acidic fish chitinase-2 (AFCase-2) were identified [6]. In addition, chitinase activity has been observed in organs, excluding the stomach, in the class of fish classified as Actinopterygii [11] [12], and a new group fish chitinase-3 (FCase-3), has been identified based on an isoform found in the stomach and kidneys [12]. In contrast, not even one chitinase gene of the AFCase-1, AFCase-2, and FCase-3 groups has been detected in the stomach of either *Prionace glauca*, classified as a Chondrichthyes, or the stomach of *Latimeria chalmnae*, classified as Sarcopterygii [12]. These findings suggested the possibility that there may be a new chitinase group different from groups of AFCase-1, AFCase-2, and FCase-3, as there were large differences in the amino acid sequences of fish chitinases based on systematic positioning. Therefore, in this paper, two species of fish within the same phylogenetic class, but from different subclasses, that share a similar feeding habitat, feeding on molluscs and crustaceans, the Japanese bullhead shark *Heterodontus japonicus* and Kwangtung skate *Dipturus kwangtungensis* were selected for analysis. Compared with Actinopterygii, information on Chondrichthyes is

still limited; however, there are some reports on the occurrence of chitinase within the bodies of several varieties of sharks and stingrays [13], as well as reports of cDNA cloning of chitinase genes in the stomach of *P. glauca* [14]. In addition, there is no literature comparing chitinolytic activity and chitinase mRNA in the body of chondrichthyes. Moreover, it is thought that the examination of chitinase from both animals will assist in the clarification of the specialization of fish chitinase, as there are theories that indicate contrasting evolutionary paths for Chondrichthyes and Actinopterygii.

This is the first paper that seeks to clarify the characteristics of Chondrichthyes chitinase compared with the larger group of fish chitinase. This was performed through primary structural analysis, the investigation of chitinase activity, the expression status of mRNA to assess the physiological role in the body, and the use of genetic databases, including phylogenetic analysis.

2. Materials and Methods

2.1. Preparation of Samples

The Japanese bullhead shark *H. japonicus* and Kwangtung skate *D. kwangtungensis* were purchased from the fishing port in Miura City, Kanagawa Prefecture. Each organ was collected, washed with chilled distilled water, and stored at -80°C until use. Each organ was homogenized with five volumes of 20 mM sodium phosphate buffer (pH 7.3) and centrifuged at $9000 \times g$ for 20 min at 4°C . The supernatant was filtered through filter paper to remove floating fat and used as the crude extract solution. The crude extract solution was stored at -80°C until use.

2.2. Cloning of *H. japonicus* chitinase cDNA (*HjChi*) and *D. Kwangtungensis* chitinase cDNA (*DkChi*)

The sequences of all primers are presented in **Table 1**. Total RNA was extracted from the stomach of *H. japonicus* and *D. kwangtungensis* using RNA extraction reagent (ISOGEN, Nippon Gene, Tokyo, Japan), in accordance with the manufacturer's instructions. The total RNA concentration and purity were measured using ultraviolet and visible spectrophotometry. cDNA was synthesized from 1.0 μg total RNA using a primer for reverse transcription (oligo dT) and cDNA synthesis kit (PrimeScript™ II First Strand cDNA Synthesis Kit, Takara Bio, Shiga, Japan), in accordance with the manufacturer's instructions. The internal sequences of *HjChi* and *DkChi* were amplified using the synthesized cDNA, DNA polymerase (Go Taq® Green Master Mix, Promega, Madison, USA), and degenerate primers were designed based on the conserved region of amino acid sequences of GH family 18 chitinases from several vertebrates, in accordance with the manufacturer's instructions. The forward and reverse primers were designed from the chitinase gene sequences obtained by the internal sequence amplification, and the upstream (5') and downstream (3') regions were amplified using the rapid amplification of cDNA ends (RACE) method. The 5' and 3' RACE

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Length	Usage
Oligo dT	CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTTTTT	40 mer	cDNA synthesis
HjChi-a	TGYTAYTTYACNAAAYTGG	18 mer	Conserved region PCR
HjChi-b	GAYATHGAYTGGGARTAYCC	20 mer	
HjChi-c	TTCCARTARTTCATNGCRTARTC	23 mer	
DkChi-a	GYNGGNGGNTGGAAYTTYGGNAC	23 mer	
DkChi-b	GAYTGGGARTAYCCNGG	17 mer	
DkChi-c	AYTTCATNGCRAARTCNAC	19 mer	
DkChi-d	TARTANGCNARRAANCCNGC	20 mer	
3'RACE	CTGTGAATGCTGCGACTACGAT	22 mer	3' RACE PCR
HjChi-1	CTGCGTCGTTATGGATTTGATGGT	24 mer	
DkChi-1	GACAAGCATCTCTACACTG	19 mer	
DkChi-2	TTCCCTCAGCTCGGCAAGGTAGTT	23 mer	
HjChi-2	AGMSSMRASATGGCAAAGCTT	21 mer	5' RACE PCR
HjChi-3	AGTCCAGGATTTGTCCAAGCTG	22 mer	
AAP	GGCCACGCGTCTGACTAGTACGGGIIGGGIIGGGIIG	36 mer	
AUAP	GGCCACGCGTCTGACTAGTACC	21 mer	
DkChi-3	TTGCTCATCCCACCAGCAAC	20 mer	
DkChi-4	ATCAGCTCCTGAGCCAG	17 mer	
DkChi-5	CGAGATCCAGGATATTCCC	19 mer	
DkChi-6	AAAGCCGTACCCTCTCAGG	19 mer	
DkChi-7	AACTACCTTGCCGAGCTGAGG	21 mer	
HjChi-4	AGAGGCGAGATGGCAAAGCTTCT	23 mer	Full length amplification
DkChi-8	CTCCTGCCCAGAACAGTAAC	20 mer	
DkChi-9	CCCAAGACTGTGGTCAAC	18 mer	
β -actin-a	GAAAGACAGTTACGTTGGTG	20 mer	Organ expression
β -actin-b	AGAATCTAGCACAATGCCAG	20 mer	
HjChi-5	TCCCAAATTCCCAAGACACT	20 mer	
HjChi-6	TCCAACCCATTTCATTCCAC	20 mer	
DkChi-10	CTGCCTAATGACAAGGATTAC	21 mer	
DkChi-11	GCCGACCCATATTCCATTCT	20 mer	

analyses were performed using custom kits (5' and 3' RACE System for Rapid Amplification of cDNA Ends, Invitrogen, Carlsbad, USA) in accordance with the manufacturer's instructions. The full-length chitinase genes were amplified using DNA polymerase (PrimeSTAR® Max DNA Polymerase, Takara Bio), in accordance with the manufacturer's instructions. The PCR products were electrophoresed

on a 2% agarose gel and the resolved DNA was extracted using Quantum Prep® Freeze 'N Squeeze spin columns (Bio Rad, Hercules, USA) and ligated into the pGEM-T Easy Vector (Promega). The base sequences were determined using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Waltham, USA) and domain prediction was performed using Inter Pro (EMBL-EBI: The European Bioinformatics Institute, European Molecular Biology Laboratory, Hinxton, England).

2.3. Organ-Specific Gene Expression

Total RNA was extracted from the organs of *H. japonicus* and *D. kwangtungensis*. cDNA was synthesized from 1.0 µg of total RNA obtained from each organ and an oligo dT primer; 1.0 µg of the synthesized cDNA was amplified using PCR and primers for *HjChi*, *DkChi*, and fish β -actin amplification primers. The PCR parameters were 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 20 s.

2.4. Chitinolytic Enzyme Activity Assay

Chitinolytic Activity was measured using *p*-Nitrophenyl-*N*-Acetyl- β -D-Glucosaminide (*p*NP-GlcNAc), *p*-Nitrophenyl Di-*N*-Acetyl- β -Chitobioside (*p*NP-(GlcNAc)₂), and *p*-Nitrophenyl Tri-*N*-Acetyl- β -Chitobioside (*p*NP-(GlcNAc)₃) (Yaizu Suisan Kagaku Industry Co., Ltd., Shizuoka, Japan) as substrates in accordance with the method described by Ohtakara [15], with slight modifications. Briefly, 2.5 µL of enzyme solution and 2.5 µL of substrate (4 mM solution) were added to 10.0 µL of 0.2 M phosphate-0.1 M citrate buffer (pH 6.0), and the solution was incubated at 37°C for 20 min. After incubation, 65 µL of 0.2 M sodium carbonate solution was added to the solution, and the absorbance of released *p*-nitrophenol was measured at 420 nm. A unit of chitinolytic enzyme activity (U) was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per minute.

2.5. Determination of Optimal pH

When 4 mM *p*NP-(GlcNAc)_n (n = 1 - 3) was used as a substrate, the optimal pH was determined by assaying enzyme activity. Specifically, the solution was incubated for 20 min at 37°C in 0.2 M phosphate-0.1 M citrate buffer (pH 2.5 - 8.0) using the same technique used for the measurement of chitinolytic activity.

2.6. Phylogenetic Analysis of *HjChi* and *DkChi*

Phylogenetic analysis based on the deduced amino acid sequences of *HjChi* and *DkChi* was performed using the chitinase genes obtained from many organisms. The analysis was performed using ClustalW2 program (EMBL-EBI: The European Bioinformatics Institute, European Molecular Biology Laboratory, Hinxton, England) and the tree view program. A bacterial chitinase (GenBank: X03657) was used as an outgroup.

3. Results and Discussion

3.1. Cloning of *H. japonicas* chitinase cDNA (HjChi) and *D. kwangtungensis* chitinase cDNA (DkChi)

Using degenerate primers designed based on the deduced amino acid sequences of several vertebrate chitinases, the internal sequences of the cDNA of the stomach of *H. japonicas* and *D. kwangtungensis* were amplified by RT-PCR and fragments of 450 bp and 400 bp were obtained, respectively. From the nucleotide sequence of the obtained fragments, upstream and downstream amplification primers were designed. Using the 5'RACE method, the upstream region of the gene fragments, 300 bp and 500 bp, were amplified and the nucleotide sequence of the start codon was included in the gene fragments. In addition, gene fragments of 1300 bp and 1000 bp were obtained by the 3'RACE method, and the analysis of the nucleotide sequence identified a stop codon and polyadenylation signals (AATAAA), each found in the 3'UTR. Based on the untranslated regions of these nucleotide sequences, the full-length primer was designed to sandwich an open reading frame (ORF) and using an enzyme with 3'→5' exonuclease activity, the full-length gene of chitinase was amplified. Based on these methods, we obtained full length chitinase genes from the stomach of both *H. japonicas* and *D. kwangtungensis*. The full-length gene of the stomach chitinase of the *H. japonicas* (*HjChi*), consisted of 1592 bp and included a 1380 bp ORF encoding 480 amino acids (**Figure 1**). For *D. kwangtungensis*, a 1440 bp ORF encoding 478 amino acids was included in the 1601 bp full length stomach chitinase gene (**Figure 2**). The nucleotide sequences of the full length chitinase genes were registered in DBJ and the accession numbers were (*HjChi*: LC215400, *DkChi*: LC215399) issued. The nucleotide sequences of *HjChi* and *DkChi* were analyzed using NCBI BLAST search. The paper claims that *HjChi* and *DkChi* demonstrated approximately 70% homology with other Chondrichthyes chitinase (*Rhinocodon typus*: XP_020390951, *P. glauca*: AB872008, *Scyliorhinus torazame*: BAU51439) and approximately 60% with Actinopterygii (*Hexagrammos otakii*: AB626093, *Scophthalmus maximus*: KY421058, *Paralichthys olivaceus*: AB121732). The computed pI/Mw tool was used to estimate the isoelectric point and molecular weight of the deduced amino acid sequence (*HjChi* and *DkChi*) of *HjChi* and *DkChi*, which were 8.52 and 51.6 kDa and 6.59 and 50.6 kDa, respectively. Using the same calculation method, the isoelectric point and molecular weight of chitinase of *R. typus* were 8.33 and 51.0 kDa (DDBJ: XM_020535362) and *P. glauca* were 8.42 and 52.0 kDa (DDBJ: AB872008) [14], respectively. For *P. olivaceus*, Actinopterygii, the isoelectric points and molecular weights of the chitinase varieties were 6.31, 52.6 kDa (*fChi1*); 5.39, 53.2 kDa (*fChi2*); and 6.52, 53.1 kDa (*fChi3*), respectively. These results identified similar molecular weights of the chitinases of Chondrichthyes and Actinopterygii and the differing chitinase of the shark species, which tended to have a higher isoelectric point.

A comparison of the deduced amino acid sequences of several varieties of

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AGAGGCGAG
ATGGCAAAGCTTCTACTTGGAAACAGTTGCGGTCTTGTGGCATGTCTGGAACCTGGGCTCT 60
M A K L L L G T V A V L L A C L E L G S
GCCTACAGACTGGTTTGTACTTTACAAACTGGGCTCAGTACAGACCAGGAGAGGGGAAG 120
A Y R L V C Y F T N W A Q Y R P G E G K
TACATGCCCGAAGTGTGGACCTTGTGTGCACGCATCTGATCTATGCTTTTGTGGG 180
Y M P E N V D P C L C T H L I Y A F A G
ATGAAGGACAATCAAATTACCACTATGAATGGAACGACGCAACTCTACCACTATC 240
M K D N Q I T T Y E W N D A T L Y H S F
AATGGCCTAAAAACAATAATGCAAACTTAAGACACTCCTGGCCATCGGAGGCTGGAAAC 300
N G L K N K N A N L K T L L A I G G W N
TTTGGAACTCAGAAGTTCAGCGCTATGGTTTCCACAGCAGCAACTCGCCAAACTTTTCATA 360
F G T Q K F S A M V S T A A T R Q T F I
AAATCTGTGATTAGCTTCTCGTCTGTTATGGATTTGATGGTTTTGATCTAGATTGGGAA 420
K S V I S F L R R Y G F D G F D L D W E
TATCCTGGGTCAAGAGGAAGTCCCCACAAGACAACATCTCTTCTACTGTGGTGGTGCAG 480
Y P G S R G S P P Q D K H L P T V L V Q
GAACTGATGGCAGCTTTGAAGCTGAGGGGAAAAGCTCTGGGAGCACAAGACTGCTGCTC 540
E L M A A F E A E G K S S G R P R L L L
TCGGCTGCAGTTGTGGTGGGAAGATACCATTGATTCTGGCTATGAGATTCTCAGCTT 600
S A A V A G G K S T I D S G Y E I P Q L
GGACAACTCTGGACTTTTTCAACGTGATGACGTATGATTTCTTTGGTTCCTGGAGTGTCT 660
G Q I L D F F N V M T Y D F F G S W S A
GTCACCTGGAGAAAATAGCCCTCTTTATCCTCTCCCAAATTCCEAAGCACTACCAACCTA 720
V T G E N S P L Y P L P N S Q G D T T N L
TACTTTAATGTGGATTTTGCATGAAATACTGGAAAGATCAGGGAGCTCTGCAGAGAAA 780
Y F N V D F A M K Y W K D Q G A P A E K
TTGAATGTTGGATTCCCTACATATGGCCATACCTTCAGACTGGCTCATCCCAACTGGG 840
L N V G F P T Y G H T F R L A S S N T G
ATTGGAGCCCCAGCAGCAGGTCCTGGAGCAGCTGGGAAAGTACACCAGGAGGCTGGATTC 900
I G A P A A G P G A A G K Y T R Q A G F
CTGGCCTATTATGAGATCTGCACATTTTGAACCGTGCACCGTGAATGGAATGCTCCA 960
L A Y Y E I C T F L K R A T V K W N A P
CAAATGGTTCATATGCCTCAGTGGAAATGAATGGGTTGGATATGCAATGGAATAAG 1020
Q M V P Y A Y S G N E W V G Y D N V K S
TTGGCGACAAGACTGAGTGGCTGAAAAAGAACAACTATGGAGGAGCTATGGTGGGAT 1080
F G D K T E W L K K N N Y G G A M V W D
CTGTCTGGATGATTTCTCTGGCACTTCTGTGACCAAGGACCTTATCTCTCATTAA 1140
L A L D D F S G T F C D Q G P Y P L I N
AAACTGCACACTGGGCTTGGAAATTTCTGCAGCTTGTGTTCCATCCAAAACAACACTGCCA 1200
K L H T G L G I S A A C V P S K T T L P
CCAACTGTTCTGGTGGTGGCAGCAGTGGCTGTGGGTTCTGCACAGCAAAAGCTAAATGG 1260
P T V P G G G S S G C G F C T D K A N G
TACTACCTTATCCAAAAGACAGATAAATTTATCATTGTGTGATGGAGTGCACCTAC 1320
Y Y P Y P K D K S K F Y H C V D G V T Y
CAGATGGACTGTTCTCTGGATTAGTCTTTGATCCTTCTGTGACTGTTGAGCTGGCCG 1380
Q M D C S P G L V F D P S C D C S W P
TAAATGAGAACGCAGATGTTTGGCATTACTCTTCATTGGTTATCATGAAGGTGCACCTGCT
CTTCAGTTTAACTACTGAAATGGCAGCACTTGGTGGATAAAAACATTAATGCACCACTG
TAATTTGTCTCAACTGAAAGATAAACTGTAGTTTAAACAGCAAATAAATCATATTGATTC
ATACAGCAAAAAAAAAAAAAAAAAA

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Figure 1. Deduced amino acid sequences and bases for *HjChi* from the stomach of *H. japonicus*. DDBJ accession No. LC215400.

previously reported chitinase from *P. olivaceus* (DDBJ: AB121732, AB121733, AB121734) [16], *Homo sapiens* (DDBJ: AF290004, U29615) [8] [17], and *Mus musculus* (DDBJ: EF094027, AY458654) [18] is shown in **Figure 3**. Based on the domain prediction, beginning at the N-terminal side, all sequences were composed of a signal peptide, a catalytic domain, a linker region, and a chitin-binding domain. The domain structure predicted from the deduced amino acid sequence was a common structure in vertebrate chitinases [19] [20]. The (DXXDXDXE) array, a conserved sequence within the active site of the GH family18 chitinases [21], was identified in the active site of the catalytic domain and was thought to possess chitin-degrading activity. The linker region of *HjChi* and *DkChi* was rich in glycine, and a distance between the catalytic domain and the chitin-binding domain was observed. The flexible, glycine-rich amino acid linker region allows for the flexibility in determining the appropriate distances between domains, which enabled the full demonstration of domain function [22]. Therefore, based on enzymatic degradation, it was inferred that *HjChi* and *DkChi* possessed a rational structure. In contrast, a different scenario was predicted for the linker regions of *AMCase* [8], *fChi1*, and *fChi2* [16], in which serine-containing repetitive sequences are present in addition to glycine.

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CGAGATCCAGGATATTCCCCTCCTGCCAGAACAGTAACAAG
ATGGCAAAGCTACTTCTCGGAACAGCGGTGGTCTTACTGGCCTGCAACTGGGCTCT 60
M A K L L L G T A V V L L A C L Q L G S
GCCTACAGGCTGGTGTACTTCACTTCACTGCGCAGTACAGACCAGGAGGGTAAA 120
A Y R L V C Y F T N W A Q Y R P G B G K
TACATGCCCCAGAGCGTGGACCTTGCCTTGCACACATCATCGCCTTCGCTGGA 180
Y M P Q S V D P C L C T H I I Y A F A G
ATGAGGAGCAATCAGATCGCCACCTATGAATGGAACGACCCAAACTCTACAAGGAGATC 240
M R S N Q I A T Y E W N D P K L Y K B I
AACAGCCTGAAGAACAAGAATTCAAACCTCAAGACTCTGCTGTCCATTGGAGGCTGGAAC 300
N S L K N K N S N L K T L L S I G G W N
TTTGGCACCAGAAATTCACCGCGATGGTTGCCTCATCAGGAACCTCGGAAATCTTTCATC 360
F G T Q K F T A M V A S S G T R Q I F I
AAGTCAGTCATCGCCTTCTGAGAGGGTACGGCTTTGACGGTCTGGACATCGATTGGGAA 420
K S V I A F L R R G Y G F D G L D I D W E
TATCCTGGATCTCGAGGAAGTCCCTCCGTTGACAAGCATCTCTACACTGTCTGGCTCAG 480
Y F G S R G S P P V D K H L Y G T V L A Q
GAGCTGATGGCTGCGTTTCGAGGCGGAGGGGAAAAGCACAGGACAGCCAGACTGCTGCTC 540
B L M A A F B A B G K S T G Q P R L L L
TCAGCTGCCGTTGGTGGGATGAGCAACATTGATACTGGCTACGAGGTTCTCAGCTC 600
S A A V A G G M S N I D T G Y E V P Q L
GGCAAAGTAACTTCTTCAACGTGATGACCTACGATTTCTATGGTCCCTGGAGCCAC 660
G K V V D F F N V M T Y D F Y G P W S H
GTTACTGGAGAGAACAGCCCTTATGCGCTGCTAATGACAAGGATTACACCAATC 720
V T G B N S P L Y A L P N D K D Y T N I
TACTTCAATGTGGAATACGCCATGAACACTACTGGAAGAGCAAGGGGGCTCCAGTGGACAAG 780
Y F N V E Y A M N Y W K S K G A P V D K
CTGAATGTGGGCTTCCCCACCTATGGACACACCTTACAGACTGACCTCGTCCAGCAGCGCC 840
L N V G P P T Y G H T F R R L T S S T A
GTGGGTGCCCCAGCAGGAGGCGCTGGACCAGCCGGCAAAATACACCCGCAAGCCGGCTTC 900
V G A P A G G A G P A G K Y T R Q A G F
CTGGCCTACTATGAGATCTGCGGGTTCCTGAACGGTGCACCCGCAATGGAAACGCCCTC 960
L A Y Y E I C G F L N G A T A E W N A P
CAGGACACCCGTACGCTTCAAGAATGGAATATGGTTCGGCTATGACAACATCAAGAGC 1020
Q D T P Y A F K N G I W V G Y D N I K S
TTTGGAGACAAGGTTGAGTGGCTGAAAGAACAACCTTTGGAGGAGCTATGGTCTGGGAT 1080
F G D K V E W L K K N N F G G A M V W D
CTGGCTCTGGATGACTTCTGCTGGTGCCTTCTGTACCAGGGACCTACCTCTCATCAAC 1140
L A L D D F S G A F C H Q G P Y P L I N
ACTCTGCACTTGGCCTTGGCATCTCTGGAGCTTGGCTCCCATCCAAGACAACATTGAAG 1200
T L H S G L G I S G A C V P S K T T L K
CCAGCGGTCCACCTACTGATGCTCCTAGTGGTGGTGGAGGTGGGGTGGTGGAGGC 1260
P A V P P T D A P S G G G G G G G G G
AGCAGTGGTGGAGGCGAGTGGCAGTGGAGGCGAGTGGCTTCTGTAAGGGCAAGCGAAGCGGT 1320
S S G G G S G S G G S G F C K G K A N G
ACCTTCCCGATCCCAAGGACAAGAACAAGTCTACAGTGGCCCAATGGTTCGGACCTAC 1380
T F P D P K D K N K F Y Q C A N G R T Y
CTGAAGAGCTGTGCCACTGGATTAGTCTTCGATTATCCTGTAATGCTTGCACACTGGCT 1440
L K S C A T G L V F D S S C K C N W P
TAA
*
GCTGTTAAAAAATAAAAAAAGTCATAATTTAAACATAAAAAAAAAAAAAAAAAAAAA

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Figure 2. Deduced amino acid sequences and bases for *DkChi* from the stomach of *D. kwangtungensis*. DDBJ accession No. LC215399.

3.2. Organ-Specific Gene Expression

Organ-specific expression analysis of *HjChi* and *DkChi* chitinase genes from the stomach, intestines, liver, kidney, spleen, and gonads of *H. japonicas* and *D. kwangtungensis* was performed (Figure 4). *HjChi* was expressed only in the stomach of *H. japonicas*. This was similar to the observed expression conditions of digestion-related genes, classified as AFCase-1 and AFCase-2 in the stomach of Actinopterygii [16]. However, their expression in *D. kwangtungensis* displayed a similar profile to that of *P. glauca* [14] and *Latimeria chalumnae* [23], with expression in all organs, including metabolic and detoxification organs such as the liver and kidney, hematopoietic organs such as the spleen, and hormone-secreting organs (endocrine organs) such as the gonads. The expression conditions of *DkChi* were similar to that of FCCase-3 obtained from the kidney and expressed in various parts of the body of *Sebastes marmoratus*. These results suggested that, similar to fish chitinase classified as AFCase-1 and AFCase-2, *HjChi* is mainly responsible for the digestion of food *H. japonicas*. In *D. kwangtungensis*, *DkChi* was responsible not only for digestion, but is also involved in the metabolism of chitin oligosaccharides in the liver and kidney, and the possible role

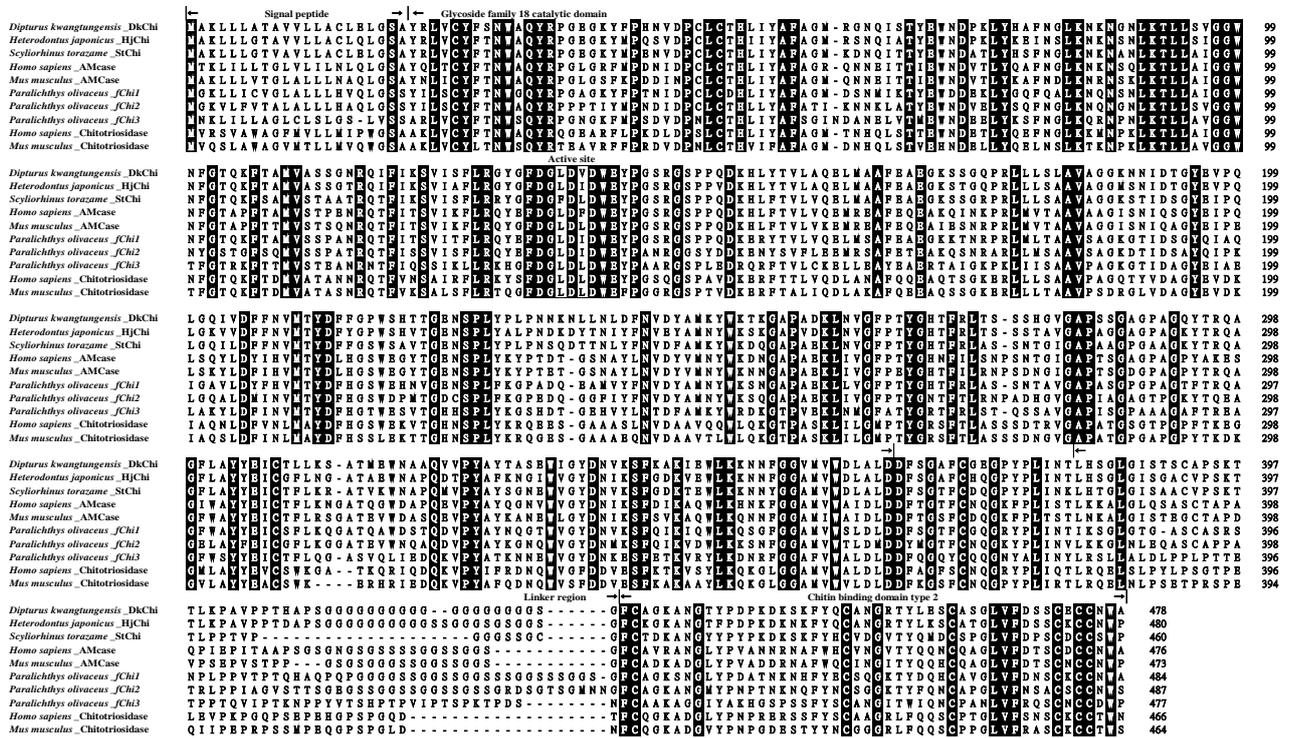


Figure 3. Alignment of amino acid sequences of chitinase. *D. kwangtungensis* (DkChi: stomach LC215399), *H. japonicus* (HjChi: stomach LC215400), *S. torazame* (StChi: stomach LC018996), *P. olivaceus* (fChi-1: stomach AB121732, fChi-2: stomach AB121733, fChi-3: pancreas AB121734), *H. sapiens* (AMCase: stomach and lung AF290004, Chitotriosidase: U29615), *M. musculus* (AMCase: stomach EF094027, Chitotriosidase: Tongue AY458654).

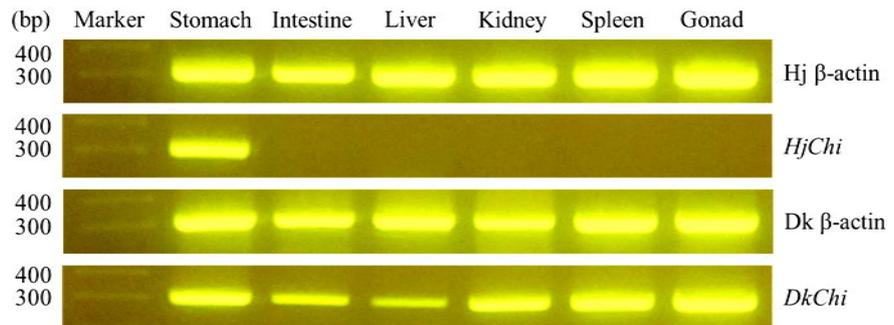


Figure 4. Expression of *DkChi*, *HjChi*, and β -actin mRNA in organs of *H. japonicus* and *D. kwangtungensis* by RT-PCR; β -actin was used as a control.

of biological defense is also suggested in the spleen and gonads for one variety of chitinase.

3.3. Distribution of Chitinolytic Enzyme Activity

The distribution of chitinolytic enzyme activity in *H. japonicus* and *D. kwangtungensis* was investigated using pNP -(GlcNAc)_n ($n = 2, 3$) as a substrate for the measurement of chitinase activity (Figure 5(a), Figure 5(c)). The highest activity for pNP -(GlcNAc)₃ was observed in the gonads, followed by the stomach and spleen in *H. japonicus*. However, with the substrate pNP -(GlcNAc)₂, the activity

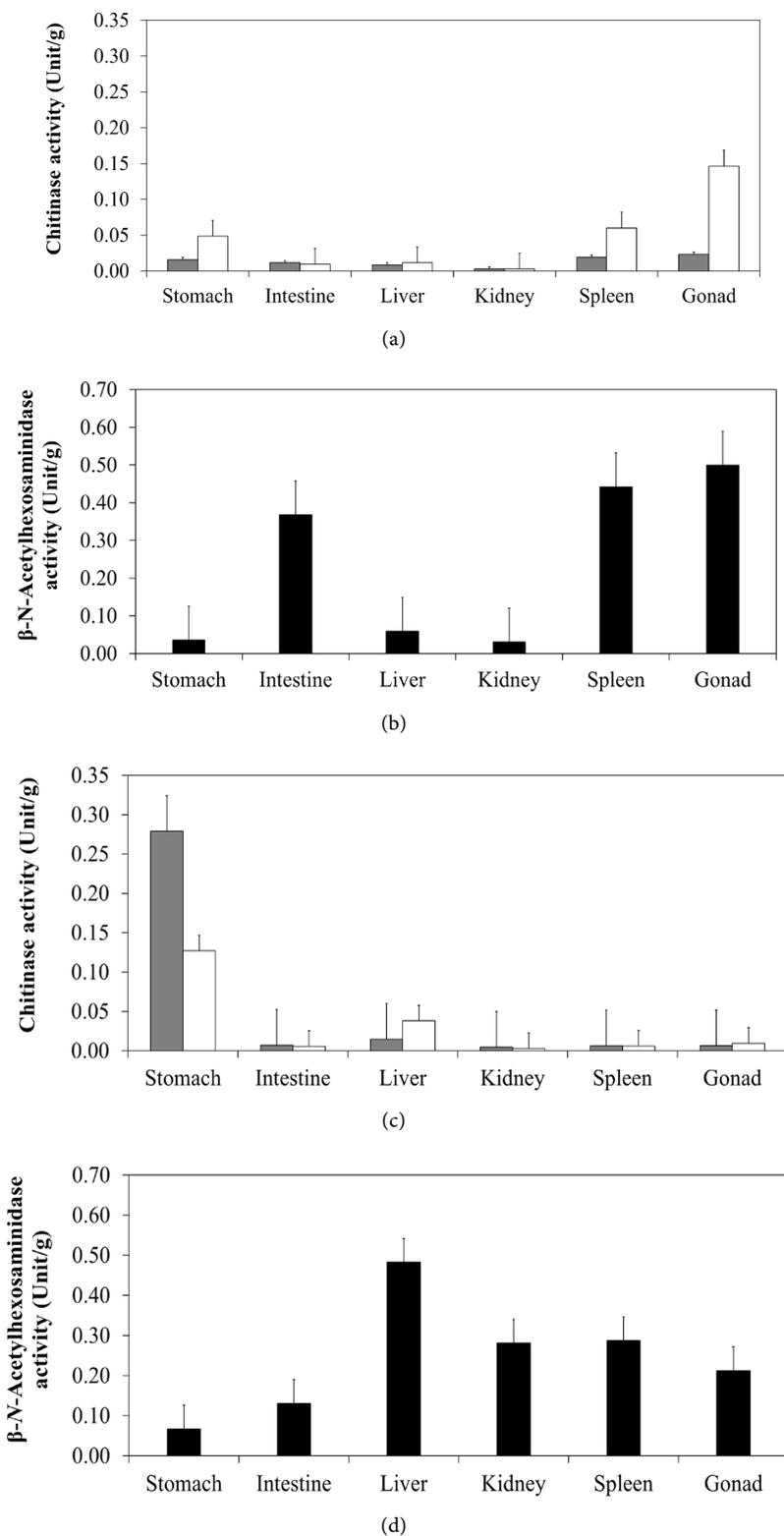


Figure 5. The distribution of the chitinolytic enzyme activities in the organs of *H. japonicus* and *D. kwangtungensis*. (a) Chitinase activity of *H. japonicus*; (b) β -N-Acetylhexosaminidase activity of *H. japonicus*; (c) Chitinase activity of *D. kwangtungensis*; (d) β -N-Acetylhexosaminidase activity of *D. kwangtungensis*, ■: pNP -(GlcNAc), □: pNP -(GlcNAc)₂. Bars represent the standard deviation (n = 3).

was remarkably low in all organs. Similarly, in *D. kwangtungensis*, the highest activity for the substrate $pNP-(GlcNAc)_n$ ($n = 2, 3$) was observed in the stomach and minimal activity against $pNP-(GlcNAc)_2$ was also detected in the liver. In *H. japonicas*, the activity of $pNP-(GlcNAc)_3$ was higher than $pNP-(GlcNAc)_2$ in almost all organs, and the chitinase displayed a similar degradation pattern to the chitinase encoded by the gene AFCase-2 [6]. In addition, chitinase activity observed in the stomach of the *D. kwangtungensis* was similar to that at the degradation site of the chitinase coded by the gene classified as an AFCase-1 [6]. Chitinase activity is present in the lymphomyeloid tissue of the *Chimaera monstrosa*, in the esophagus and lymphomyeloidepigonol organs of *Etmopterus spinax* and *Raja pulchra* [13], and in the stomach, liver, and spleen of *Mustelus manazo* [24]. These findings indicated that chitinase was found in most rays and sharks, where it participates in physiological roles. Organ expression analysis identified *HjChi* only in the stomach and chitinase activity in *H. japonicas* was thought to be a result of the chitinase encoded by *HjChi*. Further, chitinase activity in the spleen and gonads is thought to be a result of chitinase encoded by a gene other than *HjChi*. In contrast, *DkChi* was expressed in all organs of *D. kwangtungensis*, but chitinase activity was observed only in the stomach, and based on the feeding status of the three samples used in the current study, the possibility of adjustment for expression confined to the stomach exists. Conversely, Hex activity, which was measured using $pNP-GlcNAc$ as the substrate, was detected in all organs, similar to previous reports [11] [12], with high values in the intestine, spleen, and gonads, and low values in the stomach, liver, kidney of *H. japonicas*, whereas in *D. kwangtungensis*, higher activity was found in the liver, kidney, spleen, and gonads and lower activity in the stomach and intestine (Figure 5(b), Figure 5(d)).

3.4. Determination of Optimal pH

The effect of pH on chitinolytic activity was determined in the stomach of the *H. japonicus* and *D. kwangtungensis* using $pNP-(GlcNAc)_n$ ($n = 2, 3$) as substrate (Figure 6(a), Figure 6(c)). Optimal values of pH 5.5 and 5.0 for $pNP-(GlcNAc)_2$ and $pNP-(GlcNAc)_3$, respectively, were observed in *H. japonicas*; for the same substrates in *D. kwangtungensis*, pH 4.0 and 3.5 were the optimal values, respectively. For both substrates, the relative activity in the stomach of *H. japonicas* and the *D. kwangtungensis* decreased to 50% or less of the maximal activity from pH 6.0 - 6.5 to basic pH values. Further, in the investigation of the effect of pH on Hex activity, $pNP-GlcNAc$ was used as the substrate, and the optimal pH for Hex activity was pH 4.0 in *H. japonicas* and *D. kwangtungensis* (Figure 6(b), Figure 6(d)). The aforementioned results suggested that the optimal pH of stomach chitinase of *H. japonicas* and *D. kwangtungensis* was similar to that reported for the stomach of Actinopterygii [6], and that it may be involved in feeding and digestion, based on the acidic environment because of the stomach acid.

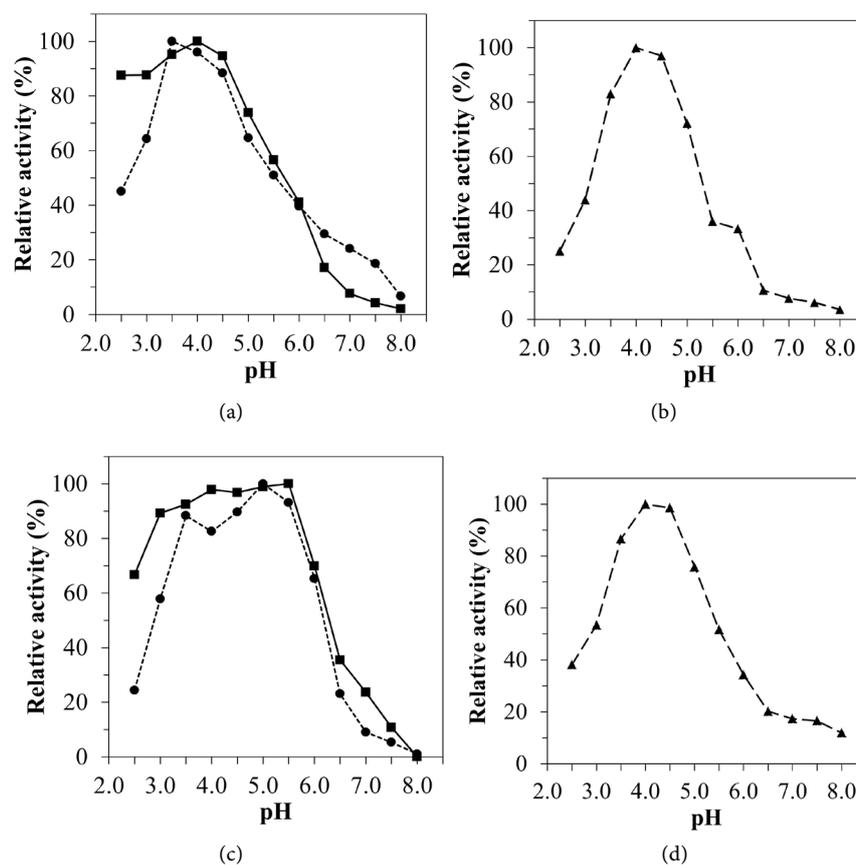


Figure 6. Effect of pH on the chitinolytic enzyme activities in the stomach of *H. japonicus* and *D. kwangtungensis*. (a) and (b) *H. japonicus*; (c) and (d) *D. kwangtungensis*, ▲: pNP-(GlcNAc), ■: pNP-(GlcNAc)₂, ●: pNP-(GlcNAc)₃.

3.5. Phylogenetic Analysis of HjChi and DkChi

In order to determine the systematic position of the chitinases of Chondrichthyes, HjChi and DkChi, discovered in this study, and that of other fish chitinases, we performed phylogenetic analysis based on the homology of the deduced amino acid sequence of family 18 chitinases of other organisms (Figure 7). The accession number and the names of all organisms included in the phylogenetic tree are shown in Figure 7. The results indicated that, similar to previous reports [6] [12], the chitinases expressed in mammalian stomach were classified as members of the AMCCase [8] [25], chitinases produced by mammalian macrophages were classified as members of the chitotriosidases [17] [18], stomach chitinases of Actinopterygii were classified as members of the AFCCase-1 and AFCCase-2 [6] [16], chitinases from the kidney of Actinopterygii were classified as members of the FCase-3 [12]; however, HjChi and DkChi were not classified as members of any of the aforementioned groups. In addition, *L. chalumnae* (Sarcopterygii) [23], *Protopterus aethiopicus* (LC350289), and *Lethenteron japonicum* (Cyclostomata) (EU741679) did not belong to these groups. A new group was created to classify Chondrichthyes in which HjChi and DkChi were detected, including *R. typus* (XM_020535362), *P. glauca* (AB872008) [14],

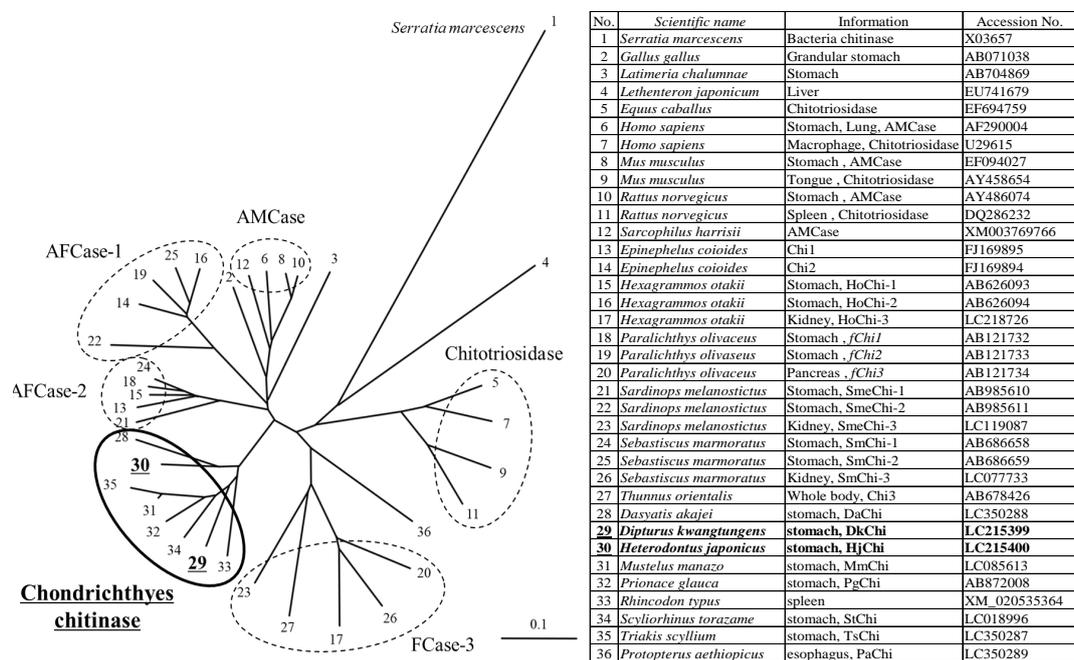


Figure 7. Phylogenetic tree for chitinase amino acid sequences developed using the neighbor joining method in the ClustalW program. The right table lists the scientific name, information, and accession No. of chitinase used in the phylogenetic tree analysis. The numbers in the phylogenetic tree and table correspond to each other. A chitinase from bacteria was used as an outgroup. The scale bar indicates the substitution rate per residue.

Dasyatis akajei (LC350288), *Triakis scyllium* (LC350287), *M. manazo* (LC085613), and *S. torazame*. The chitinases of Chondrichthyes group were identified to be similar to stomach chitinases and were more similar to the chitinases produced mainly within the stomach, such as AMCase, AFCase-1, and AFCase-2, more than the mammalian macrophage-produced chitotriosidase and the Actinopterygii produced FCCase-3, and was particularly similar to the AFCase-2. Further, as in this study, the chitinases of Chondrichthyes were placed in the same group, whether of shark or ray origin; thus, we named the four chitinases obtained from sharks and rays as Chondrichthyes chitinase. These results indicated that fish possess chitinases with different functions and structures because of adaptation and that this was dependent on the feeding habitat and environment. Consequently, each chitinase group was formed based on a network classification by phylogenetic analysis.

4. Conclusion

Novel chitinase genes were obtained from the stomach of *H. japonicas*, *HjChi* (ORF: 1380 bp) and the stomach of *D. kwangtungensis*, *DkChi* (ORF: 1440 bp). Although the amino acid sequence of the linker region was unique, the domain structure predicted from the deduced amino acid sequence was a common structure in vertebrate chitinases. Organ-specific analysis identified the expression of *HjChi* occurred mainly in the stomach of *H. japonicas*, which indicated a primary role in the digestion of food, whereas *DkChi* was expressed in all organs

of *D. kwangtungensis*, which was suggestive of a variety of physiological roles. Moreover, chitinase activity against *p*NP-(GlcNAc)₃ was comparatively high in the stomach, spleen, and gonads, whereas chitinase activity against *p*NP-(GlcNAc)₂ was substantially lower in all organs. In contrast, a very high value was obtained for chitinase activity against *p*NP-(GlcNAc)_{*n*} (*n* = 2, 3) in the stomach of *D. kwangtungensis*. Hex activity was widely observed throughout the body of both species of fish. In addition, the optimal pH of the stomach chitinase of both *H. japonicas* and *D. kwangtungensis* was similar to that reported for the stomach chitinase of Actinopterygii. Based on our phylogenetic analysis, HjChi and DkChi do not belong to previously reported groups; thus, a new group, Chondrichthyes chitinase, was established owing to the similarities with AM-Case, AFCase-1, and AFCase-2, which are mainly expressed in the stomach, unlike chitotriosidase and FCase-3. These results suggested the existence of class-specific chitinase in the fish kingdom. Hence, it was possible to classify chitinase groups, along with the application of phylogenetic analysis. Our studies are expected to lead to efficient chitin degradation and production of chitin oligosaccharides of specific chain length by obtaining knowledge of chitinase of various properties of fish kingdom. In addition, we are going to try expression of protein in future.

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