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Isolation and Sequences Analysis of Tight Junction Protein Claudin Encoding Genes in Intestinal Barrier of Common Carp (*Cyprinus carpio*)

Hamdan Syakuri[1] and Dieter Steinhagen[2]

[1] Department of Fisheries and Marine Science, Faculty of Science and Technology, Jenderal Soedirman University, Purwokerto, Indonesia
[2] Fish Diseases Research Unit, Center of Infectious Diseases, University of Veterinary Medicine Hanover, Bünteweg 17, D-30559 Hanover, Germany
Correspondence to: hsyakuri@gmail.com

Abstract

Hamdan Syakuri and Dieter Steinhagen. 2014. Isolation and Sequences Analysis of Tight Junction Protein Claudin Encoding Genes in Intestinal Barrier of Common Carp *(Cyprinus carpio)*. *Aquacultura Indonesiana, 15 (2) : 74-85.* Claudins multigene family encode proteins which forms main structures of tight junction among epithelial cells. In intestinal epithelium claudin proteins are proposed to have roles in ion balances regulation and forming barrier against pathogenic invasion. This work was done in order to identify claudin encoding genes in common carp (*Cyprinus carpio*), one of the most important cultured fish in freshwater aquaculture. Sample of RNA was isolated from the fish and transcribed into cDNA. Carp claudin genes were amplified, cloned, and sequenced. Carp claudin 1 and 2 were amplified with primers designed based on relevant known claudin genes in fish. On the basis of carp ESTs, other six carp claudin genes were amplified by using appropriate primers. Phylogenetic and sequence analysis confirmed that the eight identified genes are claudin genes and could be designated as carp claudin 1, 2, 3^b, 3^c, 7, 11, 23, and 30, respectively. This finding could be used to develop further study of carp claudin especially roles of claudin during pathogen infection and strategy to modulate its expression in order to protect diseases in carp aquaculture.

Keywords: Claudin; *Cyprinus carpio;* Intestinal barrier

Introduction

Common carp (*Cyprinus carpio*) is considered as one of the most important commodities of aquaculture, especially in freshwater fish culture in Asian and some European countries (FAO 2011). Common carp is not classified as a high-priced food; however, it is becoming an essential protein source in human diets. In the last two decades the annual production of common carp increased exponentially and reached more than 3 millions tones in 2010 (FAO 2011). Currently, it represents 14% of the global freshwater aquaculture production and is mainly cultivated in Asian countries especially in China which accounts for 70% of the world total production (FAO 2011). The development of common carp industry is challenged by many factors including protection against pathogenic invaders. With respect to this challenge, intestinal barrier functions have moved into the focus of research.

As the first barrier against external invaders from the lumen of the gut, the intestinal epithelium performs a broad range of innate defense mechanisms. The most outer layer, the mucus acts as physical and chemical barrier that protects the epithelium from microbial adherence and colonization, and this layer also might contain antimicrobial substances (Turner 2009). Another physical barrier is formed by the epithelial cells and their apical intercellular junctional structures, the tight junctions (TJs) which prevent microbial internalization (Lal-Nag and Morin, 2009). A major component of TJs is claudin proteins which are not only responsible for allowing ion and water transport but also for building the intestinal physical barrier against foreign antigens (Morita *et al.,* 1999; Lal-Nag and Morin 2009). According to many reports, claudin proteins are regulated during diseases or inflammation processes, and in some cases they become targets of pathogens (Berkes *et al.,* 2003; Prasad *et al.,* 2005; Groschwitz and Hogan 2009; Guttman and Finlay 2009; Schulzke *et al.,* 2009).

Claudin is a multiple genes family. A total of 24 claudin genes were reported from mammals and in particular humans and chimpanzees lack claudin-13 (Lal-Nag and Morin 2009). Fish claudins were at the first time identified in zebrafish, *Danio rerio* (Kollmar *et al.,* 2001) and then found in many fish species such as Japanese pufferfish, *Takifugu rubripes* (Loh *et al.* 2004); spotted green pufferfish,

Tetraodon nigroviridis (Bagherie-Lachidan *et al.*, 2008); Atlantic salmon, *Salmo salar* (Tipsmark *et al.*, 2008[a]); southern flounder, *Paralichthys lethostigma* (Tipsmark *et al.*, 2008[b]); rainbow trout, *Onchorhynchus mykiss* (Chasiotis and Kelly, 2011; Sandbichler *et al.*, 2011); and goldfish, *Carassius auratus* (Chasiotis and Kelly, 2011). However knowledge on fish claudin is still limited. The present work was done in order to molecularly identify claudin protein encoding genes in an important species in freshwater aquaculture, common carp (*Cyprinus carpio* L.).

Materials and Methods

Sampling of Intestinal Tissues and RNA Extraction

After fish was killed, intestinal pieces were collected and placed directly in 1.5 mL-tubes filled with 1 mL of RNA isolation reagents TriZol (Life Technologies) and then stored at -80^{o}C until further use. RNA was extracted according to protocol from the manufacturer. Tissue samples were ground using a Tissue lyser (Qiagen). The resulting suspension was transferred to a new 1.5-mL tube and incubated for 5 minutes at room temperature. After separation with 0.2 mL chloroform addition, RNA was precipitated from upper aqueous phase with 0.5 mL of isoprophanol. The RNA pellet was washed twice with 1 mL of 75% ethanol and then was dissolved with RNase-free water and heated at 55^{o}C for 10 minutes. Finally, quantity and purity of the samples were determined in a Nanodrop spectrophotometer and then the samples were kept at -80^{o}C until use. Samples with a ratio of absorbance $A_{260/280}$ around 2.00 and $A_{260/230}$ = 2.00-2.20 were accepted as pure RNA samples.

Digestion of Genomic DNA and Construction of cDNA

Prior to the construction of cDNA a possible contamination with genomic DNA was removed by using RNase-free DNase I (Fermentas) according to manufacturer's protocol. Briefly, a mix of RNA (1 µg), 1 µL of 10x buffer with $MgCl_2$, 2 units of DNase, and 10 units of ribolock (RNase inhibitor) in DEPC-treated water (up to 10 µL of final volume) was incubated for 30 minutes at 37^{o}C. Subsequently, the DNase was inactivated by adding 1 µL of 25 mm EDTA and incubation at 65^{o}C for 10 minutes.

The cDNA was constructed using Maxima Reverse Transcriptase (Fermentas) as described in the manufacturer's protocol. RNA samples were mixed with a mix of 0.25 µL oligo dT (100 pmol) and 0.25 µL random hexamer (100 pmol), 1 µL dNTPs (10 mm each), 4 µL of 5x RT buffer, 0.5 µL ribolock (20 u), 1 µL Maxima Reverse Transcriptase (200 u) and nuclease-free water (until a total volume of 20 µL). The mixture was incubated for 10 minutes at 25^{o}C followed by 30 minutes at 50^{o}C. At the end, the reaction was terminated by heating at 85^{o}C for 5 minutes. Then the cDNA samples were preserved at -20^{o}C until further analysis. Amplification of β-actin by using primers which cover intron on few non reverse transcribed samples confirmed no genomic DNA contamination.

Primers Design

Primers for carp claudin 1 and 2 were designed based on gene specific sequences of other fish species and primers for carp claudin 3[b], 3[c], 7, 11, 23, and 30[c] were designed on the basis of relevant expressed sequences tags (ESTs) of carp available in gene bank (see Table 1). The primers were designed using the software Primer3 (Rozen and Skaletsky, 1999) and the melting temperature of the primers was calculated using the software of Integrated DNA technologies OligoAnalyzer 3.1 available at eu.idtdna.com. All primers used in the present study are listed in **Error! Reference source not found.**

Table 1. Primers used for gene amplification

Gene	Name	Sequence (`5→`3)	Product size(bp)
Claudin-1	Cyca_cl1F	tgccaggagaggttcagg	147
	Cyca_cl1R	cagccacagccactttattc	
Claudin-2	Cyca_cl2F	gctctggagttgatgggtttc	189
	Cyca_cl2R	gtaggtctcgcattggaagg	
Claudin-3[b]	Cyca_cl3BF	atatgggagggaatctggatg	288
	Cyca_cl3BR	gtgagcagaccagcatacag	
Claudin-3[c]	Cyca_cl3CF	tcacggcacaagtcatctgg	342
	Cyca_cl3CR	cggtggacagtaaccgggttg	
Claudin-7	Cyca_cl7F	ccccaatggaagatgtctgc	547
	Cyca_cl7R	aaacgtactccttgctgctg	
Claudin-11	Cyca_cl11F	cttatcatcgccactgccac	416
	Cyca_cl11R	tacagggagaagccaaaggac	
Claudin-23	Cyca_cl23F	agggaatctgggacatctgc	290
	Cyca_cl23R	gctggtgaggatatgagtgtacc	
Claudin-30[c]	Cyca_cl30F	atcggcagcaacatcgtcac	350
	Cyca_cl30R	aacagagggttgtagaagtcc	
β-actin	BF	ggtatgggacagaaggacagc	300
	BR	ggcatacagggacagcac	

Claudin Genes Amplification

For PCR, the standard reaction mix consisted of 1X PCR buffer (Invitrogen), 0.2 mm of each dNTPs (Fermentas), 1.5 mm of $MgCl_2$ (Invitrogen), 0.2 µm of each primer, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen), 2 µL of template (cDNA), and in total volume of 20 µL. The PCR assays were performed in a Mastercycler gradient (Eppendorf). The cycle's program was: initial denaturation, 95^{o}C, 10 min.; denaturation, 95^{o}C, 30 s.; annealing, 55-59^{o}C, 45 s.; extension, 72^{o}C, 30 s.; 40 cycles; and an additional extension, 72^{o}C, 5 min. Amplification products were confirmed by electrophoresis on ethidium bromide or GelRed pre stained 1.5% agarose gels and visualized with UV light. The rest of the amplification products were kept at -20^{o}C until further use.

Cloning of The Genes

Amplification products of each gene were cloned into the pGEM-T easy vector (Promega) according to the manufacture's protocol. Briefly, 2 µL of PCR product was mixed with 1 µL of ligase (3 U/µL), 0.5 µL vector (25 ng), and 5 µL of 5x ligation buffer in a total volume of 10 µL. After incubation at 4°C over night, 2 µL of the ligation product was mixed with 25 µL of *Escherichia coli* JM109 High-Efficiency Competent Cells (Promega) and placed on ice for 20 minutes. The mix then was heat-shocked at 42^{o}C for 45 seconds and placed back on ice for 2 minutes. After adding of 970 µL of LBB and incubated at 37^{o}C for 2 hours, 50 µL of the concentrated bacterial suspension was poured on LB/ampicillin/IPTG/X-gal plates. White colonies were collected, cultured in LBB for 16-18 hours and then the recombinant plasmid was isolated.

The recombinant plasmid was isolated using the GeneJet plasmid miniprep kit (Fermentas) according to the protocol from the manufacturer. Briefly, pelleted cells were resuspended in 250 µL of resuspension solution and lysed using 250 µL of lysis solution. After the process was terminated using 350 µL of neutralization solution, the samples were centrifugated at more than 12000 x g for 5 minutes and the supernatant was transferred to a separation column. After two washing steps, the plasmid samples were eluted and stored at -20^{o}C until further use.

The presence of a DNA insert in the recombinant plasmid was confirmed by an endonuclease digestion assay. Four µL of every plasmid were mixed with 0.5 µL of *Eco*R1 and 1 µL of buffer in a total volume of 10 µL. After incubation at 37^{o}C for 2 hours, the DNA plasmid and the DNA insert were separated on a 1.5% agarose gel pre stained with GelRed (Biotium) in 100 mV for 30 minutes and visualized under UV light.

Sequencing and Sequence Analysis

Recombinant plasmids were directly sequenced (LGC Genomicy GmbH, Germany). Nucleotide sequences of carp claudin encoding genes were confirmed as part of targeted genes using a BLASTn analysis (Altschul *et al.*, 1990)

and translated in silico into an amino acid sequence with a DNA translation tool available online at www.expasy.org (Gasteiger *et al.,* 2003). Further confirmation of carp claudins was performed based on local and global models Pfam HMMs as motif sources by MyHits motif scan analysis (Pagni *et al.,* 2004; Pagni *et al.,* 2007) available at www.myhits.isb-sib.ch. Transmembrane domains of claudins were predicted by SOSUI (Hirokawa *et al.,* 1998; Mitaku and Hirokawa 1999; Mitaku *et al.,* 2002) available at bp.nuap.nagoya-u.ac.jp. Protein motifs of carp claudins were compared with those of relevant animals by means of multiple sequence alignment analysis using ClustalW (Larkin *et al.,* 2007) in default settings (www.ebi.ac.uk). The Phylogenetic relationship of carp claudin genes with appropriate genes of other fish were analyzed by following steps and methods: alignment, MUSCLE; curation, Gblock; phylogeny, phyML; and tree rendering, treeDyn at www.phylogeny.fr (Guindon and Gascuel 2003; Edgar 2004; Anisimova and Gascuel 2006; Dereeper *et al.,* 2008; Dereeper *et al.,* 2010).

Results

From common carp intestinal tissues, partial fragments of eight carp claudin encoding genes were successfully amplified, cloned, sequenced, and confirmed as parts of claudin encoding genes by using BLASTn analysis (www.ncbi.nlm.nih.gov) and/or by motif scan analysis (www.myhits.isb-sib.ch). Two claudin genes, claudin-1 and -2 were amplified from cDNA of carp by using primers designed on the basis of the same genes of other animals. Sequences of the other six claudin genes of carp: claudin-3^{b}, -3^{c}, -7, -11, -23, and -30 were identified by using primers designed on the basis of claudin-like ESTs from carp, which were obtained by performing BLASTn analysis of all zebrafish and pufferfish claudins on the EST database available in the GenBank.

A phylogenetic tree, constructed on the basis of the nucleotide sequences of claudins and shown in Figure 1, describes the relationship of the eight carp claudin genes from this study with other claudin genes identified in teleost species. According to the consensus phylogenetic tree of claudin proteins (Loh *et al.,* 2004), six carp claudin genes were assigned to the first gene cluster and two carp claudin genes were assigned to the second gene cluster. On the basis of the phylogenetic analysis and according to the claudin nomenclature proposed for Japanese pufferfish claudins (Loh *et al.,* 2004), names for carp claudins were considered. In cases when only one isoform was identified, the carp claudin gene was named according to the main name of the pufferfish claudin and not to the name for an isoform. Six carp claudin genes sequences were deposited in the GenBank (accession number JQ767156- JQ767160). Only fragments of less than 200 bp were obtained from claudin-1 and claudin-2 and therefore the sequences of these two genes could not be deposited in the GenBank because of an internal policy of this data base.

Typical molecular properties of the multiple genes family of tight junction protein claudins include two cytoplasmic tails, four transmembrane (TM) domains, one intracellular loop (IL), two extracellular loops (ELs), and two highly conserved cysteine residues in the first extracellular part (Lal-Nag and Morin 2009). In order to confirm identified genes in this work, partial protein sequences of carp claudin genes were in silico transcribed, their molecular motifs were predicted, and the level of identity among other fish claudins was analysed. A fragment of carp claudin-1 consists of an incomplete domain of TM-2, IL, and few amino acid residues of TM-3 (Figure 2). Parts of TM-1 and the extracellular loop 1 (EL-1) of carp claudin-2 were identified (Figure 3). The identified fragment of carp claudin-3b showed parts of EL-1, TM-2, IL, and TM-3 (Figure 4). Features of EL-1, TM-2, IL, TM-3, and EL-2 were determined from a partial protein sequence of claudin-3c (Figure 5). All properties of claudin-7 except of the amino-tail, TM-1, and few residues of EL-1 were identified in carp (Figure 6). A fragment that is starting from EL-1 until carboxy-tail of carp claudin-11 was reported (Figure 7). Fragments consisting of EL-1, TM-2, IL, and TM-3 were identified as parts of carp claudin-23 (Figure 8), and claudin-30 (Figure 9). In comparison to the same genes of other fish species, claudin genes of carp showed different level of identity. For examples; carp claudin-30 was 93% identical to claudin-b of goldfish (Figure 9) and carp claudin-11 was 92% similar with claudin-11^{a} of zebrafish (Figure 7). In contrast, carp claudin-1 was only 63% and 60% identical with the genes of zebrafish and Japanese pufferfish, respectively (Figure 2) and the sequence of carp claudin-3^{b} had a low identity to claudin-3^{b} of the Japanese pufferfish or claudin-3 of Atlantic salmon with 62% and 67%, respectively (Figure 4).

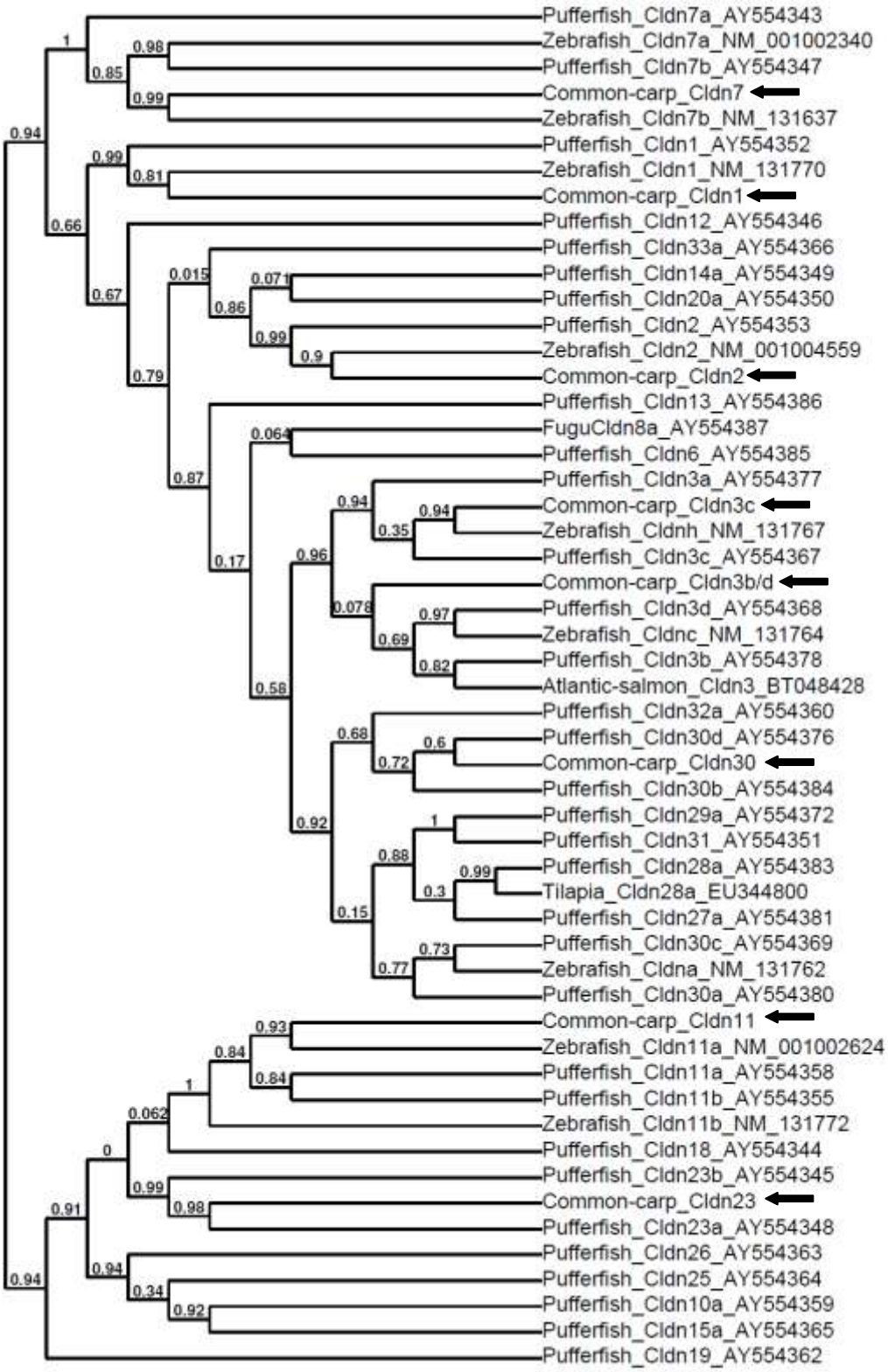


Figure. 1. Unrooted cladogram of fish claudin genes.
The phylogenetic analysis included carp claudin-1, -2, -3^{b} (JQ767156), -3^{c} (JQ767157), -7 (JQ767155), -11 (JQ767158), -23 (JQ767159), and -30 (JQ767160) encoding genes, and its relevant genes from pufferfish (*Takifugu rubripes*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and tilapia (*Oreochromis mosambicus*). The carp claudin gene sequences were obtained from this study and the other sequences were retrieved from GenBank. The accession numbers are mentioned in the leaves names. The carp claudin genes are indicated by the arrows.

```
                                TM-1                                EL-1
ZebrafishCldn1      MAHAGLQMLGYCLGFLGLLGLIASTAMAEWKMSSYAGDNIITAQAQYEGLWQSCVSQSTG 60
PufferfishCldn1     MANAGIQLLGFILAFLGLIGTIASTIMVEWKASSYAGDNIITAQAMYEGLWKSCVSQSTG 60
CarpCldn1           ------------------------------------------------------------

                                    TM-2                        IL
ZebrafishCldn1      QLQCKKYDSLLKLPGEIQGARGLMLTGIFLCGLSTLVSFVGMKCTTCLSEAPQVKSKVAL 120
PufferfishCldn1     QIQCKVYDSLLQLPGIVQGTRGLMLASVLLSVICLLVEMVGMRCTTFMAEEPEQKDKVAL 120
CarpCldn1           ------------------------------GVGLLVAAVGMKCTTCLSDEKEQKNKVAV 29
                                                   :  **  ***:*** :::  : *.***:
                          TM-3                      EL-2              TM-4
ZebrafishCldn1      AGGVLFITGGLFALIATSWYGEKIRQKFFDPFTPTNARYEFGKALYVGWGSSALSIIGGS 180
PufferfishCldn1     AGGVIFIIAGLLALVGTSWYGHRIAREFYDPFTPTNSRYEFGSALYVGWGAACLTLIGGG 180
CarpCldn1           AGGV-------------------------------------------------------- 33
                    ****

ZebrafishCldn1      LLCCICGSEASEK-PSYPPARAAGRPGTDRV 210
PufferfishCldn1     FLCCSCPKKGSQKSPRYPPTRSSGPQGKDYV 211
CarpCldn1           ------------------------------
```

A

B

SeqA: Name (length)	SeqB: Name (length)	Score
CarpCldn1 (33 aa)	ZebrafishCldn1 (210 aa)	63.0
CarpCldn1 (33 aa)	PufferfishCldn1 (211 aa)	60.0
ZebrafishCldn1 (210 aa)	PufferfishCldn1 (211 aa)	65.0

Figure 2. Multiple alignment of deduced protein sequences of fish claudin-1 genes (A) and their pairwise scores (B). The analysis included claudin-1 genes of common carp (*Cyprinus carpio*; present work), zebrafish (*Danio rerio*; accession number: NM_131770), and pufferfish (*Takifugu rubripes*; accession number: AY554352). Domains of four transmembranes (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

```
                                TM-1                                EL-1
CarpCldn2           -----------FCGLFGMLGTFVATLLPYWEMSAHVGSNIVTAVESMKGLWIECVYQSTG 49
ZebrafishCldn2      MAILALELMGFFFGLIGMLGTLVATLLPYWATSAHVGPNIVTAVVSMKGLWMECVYQSTG 60
PufferfishCldn2     MASAALELMGFFLGLFGLLGTMVSTVLPYWQISAHIGSNIVTAVGNMRGLWMECVYQSTG 60
                             * **:*:***:*:*:****  ***:*.****** .*:***:********

                                    TM-2                        IL
CarpCldn2           ------------------------------------------------------------
ZebrafishCldn2      AFQCETYNTLLGLTVDLQAARAMMVMSSIFSVMACAVSTVGMQCTVCMDGS-SVKTKVAG 119
PufferfishCldn2     AFQCETYNSMLALPSDMQASRALMVISLVLSILAIALAVLGMQCTVCLEGLGAVKGRVAG 120

                          TM-3                      EL-2              TM-4
CarpCldn2           ------------------------------------------------------------
ZebrafishCldn2      VGGSMFLLAGLLSLIPVAWKTHEVVQTFYMPNMPASLKFEIGDCLYVGLASSLLSMLGGG 179
PufferfishCldn2     VGGGLFLTAGFLSLIPVAWTTHEVVQTFYRPDLPSSLKFELGRCLYVGLASALMSMLGGG 180

CarpCldn2           ------------------------------------------------------------
ZebrafishCldn2      LLSASCCDDIDGNRGSRRHYPYPERNALRGPSHSMTYQPAALHSTTNPNITNKTQTLNSH 239
PufferfishCldn2     MLCVSCSEENDGRRRNGRGYPYPLGANVR--TTSQTYRNPTLQVGGIGAANRGRAVVRTI 238

CarpCldn2           ------------------------------
ZebrafishCldn2      TSSGTHSIQAQDSRKMTRQNTAAGYDVTGYV 270
PufferfishCldn2     SDSSHPSLHGNQGGKKP---AAAGYDITGYV 266
```

A

B

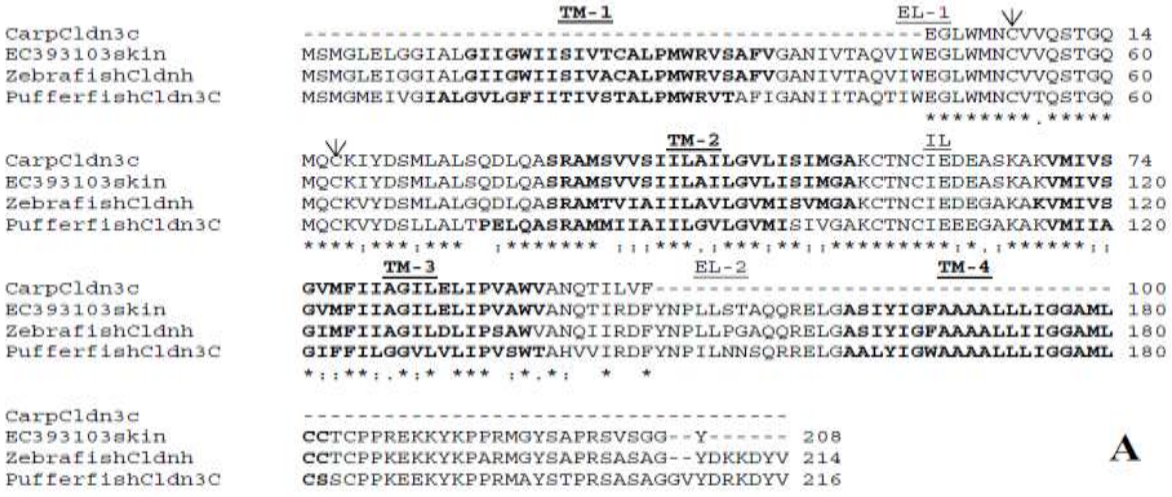
SeqA: Name (length)	SeqB: Name	Score
CarpCldn2 (49 aa)	ZebrafishCldn2 (270 aa)	83.0
CarpCldn2 (49 aa)	PufferfishCldn2 (266 aa)	75.0
ZebrafishCldn2 (270 aa)	PufferfishCldn2 (266 aa)	56.0

Figure 3. Multiple alignment of deduced protein sequences of fish claudin-2 genes (A) and their pairwise scores (B). The analysis included claudin-2 genes of common carp (*Cyprinus carpio*; present work), zebrafish (*Danio rerio*; accession number: NM_001004559), and pufferfish (*Takifugu rubripes*; accession number: AY554353). Domains of four transmembranes (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

B

SeqA: Name (length)	SeqB: Name (length)	Score
CarpCldn3b (82 aa)	CF662816mix (205 aa)	89.0
CarpCldn3b (82 aa)	PufferfishCldn3b (217 aa)	62.0
CarpCldn3b (82 aa)	AtlanticsalmonCldn3 (219 aa)	67.0
CF662816mix (205 aa)	PufferfishCldn3b (217 aa)	59.0
CF662816mix (205 aa)	AtlanticsalmonCldn3 (219 aa)	63.0
PufferfishCldn3b (217 aa)	AtlanticsalmonCldn3 (219 aa)	73.0

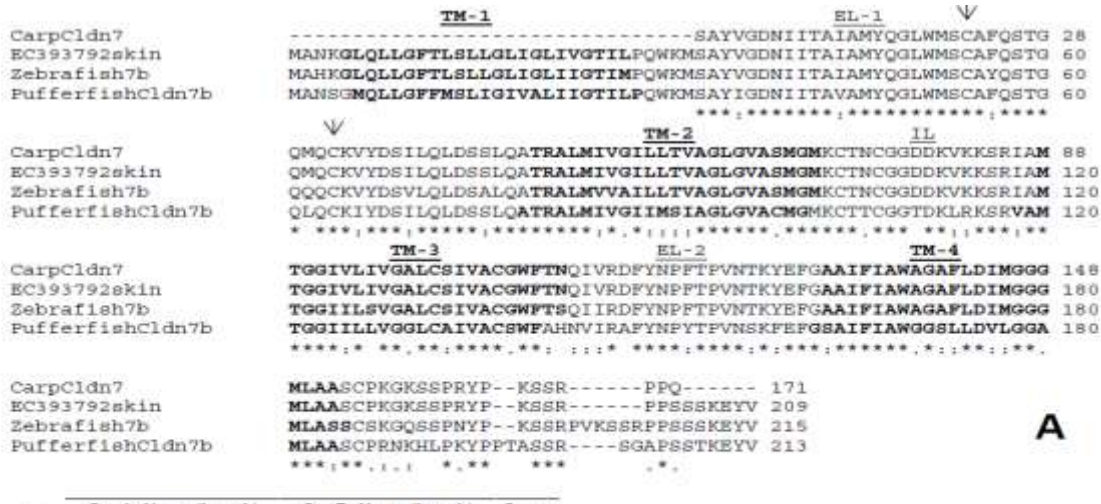
Figure 4. Multiple alignment of deduced protein sequences of fish claudin-3[b] genes (A) and their pairwise scores (B). The analysis included carp EST (CF662816; a mix library of skeletal white muscle, cardiac muscle, brain, gill, kidney and intestinal mucosa tissues) and claudin-3[b] genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767156), Atlantic salmon (*Salmo salar*; accession number: BT048428) and pufferfish (*Takifugu rubripes*; accession number: AY554378). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.



B

SeqA: Name (length)	SeqB: Name (length)	Score
CarpCldn3c (100 aa)	EC393103skin (208 aa)	98.0
CarpCldn3c (100 aa)	PufferfishCldn3C (216 aa)	68.0
CarpCldn3c (100 aa)	ZebrafishCldnh (214 aa)	84.0
EC393103skin (208 aa)	PufferfishCldn3C (216 aa)	71.0
EC393103skin (208 aa)	ZebrafishCldnh (214 aa)	88.0
PufferfishCldn3C (216 aa)	ZebrafishCldnh (214 aa)	75.0

Figure 5. Multiple alignment of deduced protein sequences of fish claudin-3[c] genes (A) and their pairwise scores (B). The analysis included carp EST (EC393103, skin library) and claudin-3[c] genes of carp (*Cyprinus carpio*; present work; accession number: JQ767157), zebrafish (*Danio rerio*; accession number: NM_131767), and pufferfish (*Takifugu rubripes*; accession number: AY554367). Domains of Four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.



B

SeqA: Name (length)	SeqB: Name (length)	Score
CarpCldn7 (171 aa)	EC393792skin (209 aa)	99.0
CarpCldn7 (171 aa)	PufferfishCldn7b (213 aa)	72.0
CarpCldn7 (171 aa)	Zebrafish7b (215 aa)	90.0
EC393792skin (209 aa)	PufferfishCldn7b (213 aa)	74.0
EC393792skin (209 aa)	Zebrafish7b (215 aa)	91.0
PufferfishCldn7b (213 aa)	Zebrafish7b (215 aa)	69.0

Figure 6. Multiple alignment of deduced protein sequences of fish claudin-7 genes (A) and their pairwise scores (B).
The analysis included carp EST (EC393792, skin library) and claudin-7 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767155), zebrafish (*Danio rerio*; accession number: NM_131637), and pufferfish (*Takifugu rubripes*; accession number: AY554347). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

B

SeqA: Name (length)	SeqB: Name (length)	Score
CarpCldn11 (126 aa)	DW722473testis (163 aa)	96.0
CarpCldn11 (126 aa)	PufferfishCldn11a (216 aa)	65.0
CarpCldn11 (126 aa)	ZebrafishCldn11a (215 aa)	92.0
DW722473testis (163 aa)	PufferfishCldn11a (216 aa)	65.0
DW722473testis (163 aa)	ZebrafishCldn11a (215 aa)	89.0
PufferfishCldn11a (216 aa)	ZebrafishCldn11a (215 aa)	66.0

Figure 7. Multiple alignment of deduced protein sequences of fish claudin-11 genes (A) and their pairwise scores (B).
The analysis included carp EST (DW722473, testis library) and claudin-11 genes of common carp (*Cyprinus carpio*; present work; accession number JQ767158), zebrafish (*Danio rerio*; accession number: NM_001002624), and pufferfish (*Takifugu rubripes*; accession number: AY554358). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

Figure 8. Multiple alignment of deduced protein sequences of fish claudin-23 genes (A) and their pairwise scores (B). The analysis included carp EST (CA964745, a mix library of skeletal white muscle, cardiac muscle, brain, gill, kidney and intestinal mucosa tissues) and claudin-23 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767159), and pufferfish (*Takifugu rubripes*; accession number: AY554348). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

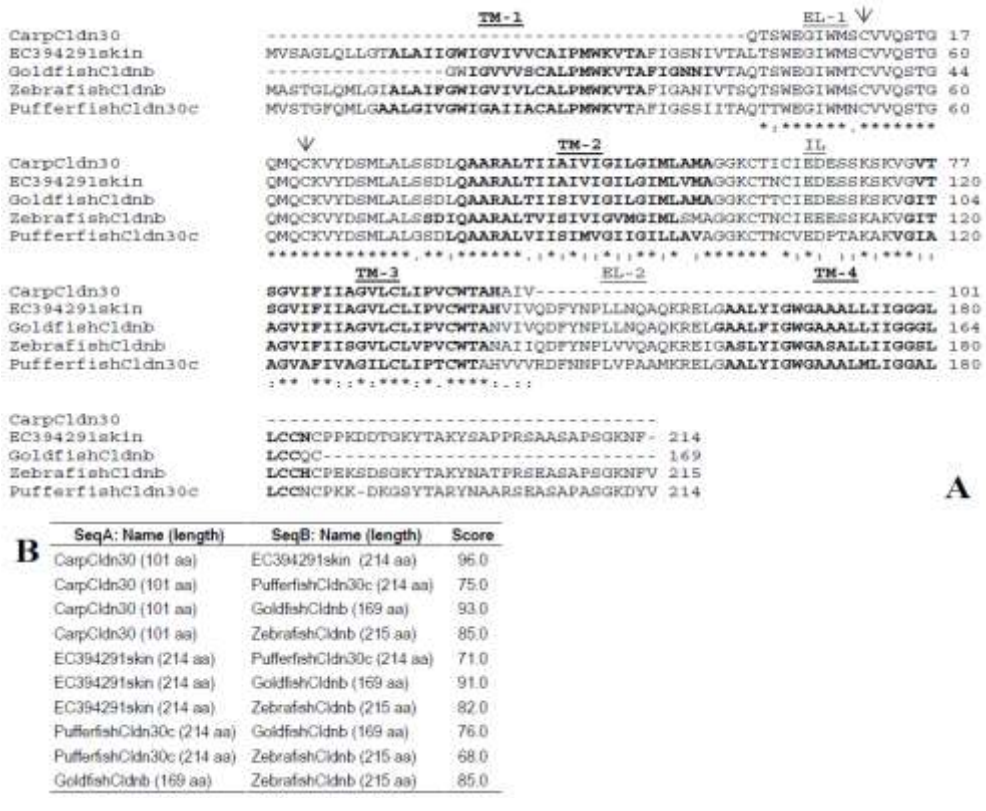


Figure 9. Multiple alignment of deduced protein sequences of fish claudin-30 genes (A) and their pairwise scores (B). The analysis included carp EST (EC394291, skin library) and claudin-30 genes of common carp (*Cyprinus carpio*; present work; accession number: JQ767160), zebrafish (*Danio rerio*; accession number: AF359426), Gold fish (Carassius auratus; accession number: HQ656008) and pufferfish (*Takifugu rubripes*; accession number: AY554369). Domains of four transmembrane (TM, bold letters), two extracellular loops (EL), and an intracellular loop (IL) are indicated. Arrows show two highly conserved cysteine residues.

Discussion

Correspond to the current state of typical profiles of members in claudin multiple genes family (Lal-Nag and Morin, 2009) fish claudins genes are characterized by four transmembrane helices and two extracellular loops (ELs). The two extracellular structures of claudins allow them to build intercellular junctional structures which mainly involve the second EL and mediate selectively barrier permeability functions of TJs that are mainly facilitated by the first EL (Van Itallie and Anderson, 2004, 2006). Roles of the first extracellular domain in size and charge paracellular selectivity have been at least described in claudin-2, -4 (Colegio *et al.,* 2003), -5 (Wen *et al.,* 2004), and -7 (Alexandre *et al.,* 2007). Furthermore two conserved cysteine residues that were predicted to form intramolecular disulfide bond for protein stabilization (Wen *et al.,* 2004; Angelow *et al.,* 2008; Lal-Nag and Morin 2009) are consistently present in EL-1 domains of fish claudins.

In piscine studies, the first identification of claudin genes was made in Zebrafish (Kollmar *et al.,* 2001). Thereafter, molecules from the claudin gene family were found in other fishes (Loh *et al.,* 2004; Bagherie-Lachidan *et al.,* 2008; Tipsmark *et al.,* 2008[a]; Tipsmark *et al.,* 2008[b]; Chasiotis and Kelly 2011; Sandbichler *et al.,* 2011). Interestingly, in the Japanese pufferfish the claudin gene family comprises a higher number of members (56) than in mammals and some of them represent fish specific claudin genes (Loh *et al.,* 2004). In the present study, eight claudin genes, claudin-1, -2, -3[b], -3[c], -7, -11, -23, and -30, were identified from common carp. In gene expression studies, transcript level of some claudin genes in common carp was modulated under Koi Herpesvirus (KHV) infection (Adamek *et al.,* 2013, Syakuri *et al.,* 2013) and after feeding beta-glucan and *Aeromonas hydrophila* intubation (Syakuri *et al.,* 2014). Therefore further studies are needed and modulation of the genes expression could be considered as a part of diseases protection strategies in common carp cultivation.

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