Agriculture and Natural Resources 51 (2017) 117–130

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original Article

Molecular characterization and expression analysis of *Cyclin B* and *Cell division cycle 2* in gonads of diploid and triploid bighead catfish, *Clarias macrocephalus* Günther, 1864

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ARTICLE INFO

Article history:
Received 2 March 2016
Accepted 29 May 2016
Available online 24 May 2017

Keywords:
Cdc2
Clarias macrocephalus
Cyclin B
Diploids
Expression
Triploids

ABSTRACT

This study investigated the differential expression of genes associated with reproduction in sterile triploid and normal diploid bighead catfish (*Clarias macrocephalus* Günther, 1864). The triploid fish were produced using cold shock and were reared in the same conditions as the diploid counterpart. The histomicrographs showed completely retarded triploid gonads across the samples aged 2–12 mth, whereas the gonads of the diploids were in developing stages during 2–4 mth, reached the early maturing stage at 6 mth, matured at 8 mth and showed signs of atresia at 10–12 mth. In parallel, the full-length cDNAs of *cyclin B1* (*CmCcnb1*; 1539 bp in length with an open reading frame (ORF) of 1194 bp corresponding to 397 amino acids) and *cell division cycle 2* (*CmCdc2*; 1355 bp, an ORF of 909 bp, 302 amino acids) of bighead catfish (*C. macrocephalus* Günther, 1864) were isolated. Phylogenetic analysis revealed that the newly characterized *CmCcnb1* should be regarded as a member of *cyclin B1* rather than *cyclin B2*. The expression level of *CmCcnb1* mRNA was limited in different stages of the ovaries and testes of triploids. In diploid ovaries, its expression was significantly higher than that in triploid ovaries in fish aged 2 mth (513.43 ± 82.22 fold) and in fish aged 8 mth (2430.87 ± 900.06 fold). The *CmCcnb1* level in the testes of diploids was significantly greater than that in triploids in fish aged 2 mth (928.85 ± 208.72 fold). Similarly, expression of *CmCdc2* mRNA was also reduced in triploids. Its expression was significantly lower than that in diploid females aged 2 mth (7.66 ± 3.42 fold), 4 mth (59.42 ± 10.50 fold) and 8 mth (42.74 ± 8.36 fold). In males, significantly greater expression of *CmCdc2* was observed at age 6 mth (58.61 ± 34.64 fold) and 8 mth (72.70 ± 4.36 fold) diploids compared to triploids. The results illustrated that *CmCcnb1* and *CmCdc2* are functionally involved in oogenesis and spermatogenesis and reduced expression levels of these transcripts affected the reproductive development of triploid *C. macrocephalus*.

Introduction

Investigation into the reproductive physiology of fish has been receiving much attention (Lubzens et al., 2010; Nagahama, 1997; Nagahama and Yamashita, 2008; Schulz et al., 2010) because of its wide range of applications, for example in the development of techniques for breeding and biological containment. Currently, the advancement of molecular genetics technologies and the availability of gonadal retarded triploid fish allow for comparative gene expression studies of diploids and triploids, and hence provide valuable detailed information on these issues (Dheilly et al., 2014; Xu et al., 2015).

Triploidy has been induced in bighead catfish (*Clarias macrocephalus* Günther, 1864) resulting in reproductively sterile triploids (Na-Nakorn and Lakhaanantakun, 1993; Fast et al., 1995). Triploid *C. macrocephalus* were used in this study for the comparative gene

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http://dx.doi.org/10.1016/j.anres.2016.05.004

expression study of genes related with the reproduction of fish including *cyclin B* (*CmCcnb1*) and *Cdc2* (*CmCdc2*).

In most animals, oocytes are arrested at prophase I and meiosis is resumed near or at the end of oogenesis (Kishimoto, 2003; Lubzens et al., 2010). The meiotic resumption is triggered by the maturation-promoting factor (MPF) which is a complex of cell division cycle 2 (Cdc2 also called cyclin-dependent kinase 1, Cdk1) and cyclin B (Ccnb) (Okano-Uchida et al., 1998; Kishimoto, 2003; Voronina and Wessel, 2003; Basu et al., 2004; Nagahama and Yamashita, 2008; Qiu et al., 2008). Activation of MPF cascade is induced by the maturation-inducing hormone (MIH) which is 17α,20β-dihydroxyprogesterone (17α,20β-P) or 17α,20β,21-trihydroxyprogesterone (20β-S) in most female fish (Nagahama, 1997; Nagahama and Yamashita, 2008). In males, the functions of *Cdc2* and *Cyclin B* genes/proteins are not well defined. However, they were suggested to play roles in mitotic and meiotic cell divisions, based on the fluctuated expression patterns of *Cdc2* and *Cyclin B* during spermatogenesis (Han et al., 2012; Mita et al., 2000; Qiu et al., 2008; Zhang et al., 2008). The current study provided the first evidence of the relationship between these genes and triploid sterility, which have not been studied in fish and other animals. This study isolated the full-length cDNAs and examined the expression level of *cyclin B* (*CmCcnb1*) and *Cdc2* (*CmCdc2*) during the ovarian and testicular development of diploid and triploid *C. macrocephalus*. Comparative results at the transcriptional level of *CmCcnb1* and *CmCdc2* provide insightful information on the functional involvement of these genes on the reproductive development of this economically important species.

Materials and methods

Experimental animals and design

Five individuals each of gravid females and males, aged 8 mth of *C. macrocephalus* were acclimated under laboratory conditions prior to an intramuscular injection with 30 μg/kg or 10 μg/kg buserellin acetate (LHRHa; Suprefact) plus 5 mg/kg dopamine antagonist (motilium), in females and males, respectively. At 12–16 h after injection, eggs from each female were stripped and pooled. They were fertilized with pooled sperm solution obtained from sacrificed males. Soon after water activation, fertilized eggs were separated into two portions and subjected to either cold shock (7 °C at 4.5 min after water activation for 14 min duration; Na-Nakorn et al., 2004) or incubation in water at ambient temperature (27 °C). Hatching was conducted in 1.5 $m^2 \times 2$ m^2 concrete tanks. The diploid or triploid status of the treated catfish was determined using chromosome counting (Netto et al., 2007). Fish were sampled every 2 mth from 2 mth to 12 mth ($N = 8$ & 7, 10 & 8, 10 & 6, 10 & 6, 8 & 5 and 8 & 6 for diploid and triploid females and $N = 8$ & 6, 8 & 5, 10 & 5, 8 & 6, 8 & 5 and 8 & 6 for diploid and triploid males, respectively). Verification of triploidy using chromosome counts was performed across all samples used in further studies. The developmental stages of the gonads of representative fish from each age were examined using conventional histology (Humason, 1979).

For gene expression analysis, different ages (2 mth, 4 mth, 6 mth, 8 mth, 10 mth and 12 mth; $N = 3$–5 for each group) of diploid and triploid *C. macrocephalus* were collected. The ovaries or testes of each fish were dissected out, weighed and immediately placed in liquid N_2 before being transferred to −80 °C until used. The gonadosomatic index (GSI) of the ovaries or testes of each fish (gonad weight/body weight × 100) was calculated. The developmental stages of ovaries were histologically classified as immature, maturing, mature and spent, which corresponded to ovaries predominated with previtellogenic oocytes, vitellogenic oocytes, postvitellogenic oocytes and atretic oocytes, respectively. Testes were also identified into four stages—immature (no spermatozoa found in seminiferous tubules), maturing (spermatozoa were observed in a small amount), mature (testes were filled with spermatozoa) and regression (similar to maturing but with some empty seminiferous tubules) phases.

Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from the gonads of *C. macrocephalus* using TRI Reagent following the protocol recommended by the manufacturer (Molecular Research Center; Cincinnati, OH, USA). The concentration of extracted total RNA was measured using spectrophotometry (Sambrook and Russell, 2001). Total RNA (1.5 μg) was reverse-transcribed using an Improm-II™ Reverse Transcription System (Promega; Madison, WI, USA).

Degenerate primer design and PCR

Protein sequences of *Ccnb* of *Danio rerio* (accession no. AAP47013), *Oncorhynchus mykiss* (AAN40513), *Carassius auratus* (AAF82780), *Cyprinus carpio* (ABX89586) and *Carassius gibelio* (AAF82779), and *Cdc2* of *Ictalurus punctatus* (NP_001187396), *Oryzias latipes* (BAB13720), *D. rerio* (AAP47014), *Anabas testudineus* (AAS59851), *Larimichthys crocea* (ACO35040) and *Salmo salar* (NP_001134623) were retrieved from GenBank (http://ncbi.nlm.nih.gov). Multiple alignments were performed using Clustal W (Thompson et al., 1994). Degenerate primers for amplification of the partial ORFs of *CmCdc2* (CmCdc2-Degen-F/R) and *CmCcnb1* (CmCcnb-Degen-F/R; Table 1) were designed and used for PCR amplification. The PCR profile was predenaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 90 s and final extension at 72 °C for 7 min. The resulting products were size-fractionated through agarose gels. The amplification fragment was eluted from the gel, cloned into pGEM-T Easy vector and sequenced in both directions. Nucleotide sequence of the cloned fragment was searched against data previously deposited in GenBank using Blast*N* and Blast*X* (Altschul et al., 1990).

Isolation of CmCcnb1 and CmCdc2 cDNAs using rapid amplification of cDNA ends-polymerase chain reaction

Total RNA was extracted from the ovaries of mature females and mRNA was further purified using rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR) and an illustra™QuickPrep Micro mRNA purification kit (GE Healthcare; Chicago, IL, USA). The 5′- and 3′-RACE-PCR cDNA template was synthesized from purified mRNA using a SMART RACE cDNA amplification kit following the protocol recommended by the manufacturer (Clontech; Mountain View, CA, USA). Gene-specific primers (GSP) were designed for RACE-PCR analysis of *CmCcnb1* (primers CmCcnb-RACE-I and CmCcnb-RACE-II) and *CmCdc2* (primers CmCdc2- RACE-I and CmCdc2-RACE-II) (Table 1). The 5′ and 3′ RACE-PCR of each gene was carried out. The resulting products were gel-eluted, cloned and sequenced in both directions. Nucleotide sequences of the original sequence and 5′ and 3′ RACE-PCR were assembled and searched against previously deposited sequences using Blast*N* and Blast*X* (Altschul et al., 1990). The p*I* value and molecular weight of the deduced protein were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The protein domain in the deduced proteins was predicted using SMART (http://smart.embl-heidelberg.de).

Table 1
Nucleotide sequences and positions of primers used for characterization and gene expression analysis of *Clarias macrocephalus Ccnb1* and *Cdc2*.

Primer name	Sequence	Position[a]	Amplicon size
Degenerate PCR			
CmCdc2-Degen-F	5′-AAGATTCGTCTGGAGAGTGA-3′	148–168	614 bp
CmCdc2-Degen-R	5′-TTRTAGTCAGGYAGNSWCTC-3′	742–761	
CmCcnb-Degen-F	5′-GTWGGTGTRACRGCCATGTTC-3′	690–710	498 bp
CmCcnb-Degen-R	5′-AATTCTCATCTGYTTYTGACT-3′	1166–1187	
RACE-PCR			
CmCdc2- RACE-I.	5′-ATGTACTGGCCTGAGGGAATGGAGTC-3′	322–347	347 bp
CmCdc2-RACE-II	5′-CTTCTCGTTACTCCACTCCAGTAGATGTTTG-3′	580–611	775 bp
CmCcnb-RACE-I	5′-AGGCGATGAACATGGCTGTTACACCAAC-3′	690–717	740 bp
CmCcnb-RACE-II	5′-AGCCATGTTCATCGCCTCCAAATAC-3′	700–725	941 bp
Quantitative real-time PCR			
CmCdc2-qPCR-F	5′-CGTTCCTAGCACTGCTGTCC-3′	177–196	208 bp
CmCdc2-qPCR-R	5′-GATCTGATACAGATAACTCTTG-3′	363–384	
CmCcnb-qPCR-F	5′-ATATGGAGATGAACATCCTGAG-3′	802–823	245 bp
CmCcnb-qPCR-R	5′-CAGTGTGGGGGTCCATTCAC-3′	1027–1047	
18S rRNA-qPCR-F	5′-AAACGGCTACCACATCCAAG-3′	37–56	247 bp
18S rRNA-qPCR-R	5′-TCCCGAGATCCAACTACGAG-3′	263–283	

[a] According to *CmCcnb1* and *CmCdc2* transcripts isolated in this study.

Phylogenetic analysis

The deduced amino acid sequence of *CmCcnb1* (accession no. KR674084) was phylogenetically compared with *Ccnb1* of *C. gibelio* (AAF82779.1), *C. carpio* (ABX89586.1), *C. auratus* (AAB24163.1), *D. rerio* (AAI53627.1), *Esox lucius* (XP_010876173.1), *S. salar* (ACI68598.1), *Anguilla japonica* (BAD52076.1), *O. latipes* (BAA89697.1), *Xenopus laevis* (NP_001080196.1), *Homo sapiens* (EAW51306.1), and *cyclin B2* of *O. latipes* (BAA89700.1), *D. rerio* (AAH66507.1), *O. mykiss* (NP_001118131.1), *A. japonica* (BAD52077.1), *X. laevis* (AAA49697.1), *Xenopus tropicalis* (NP_001138848.1) and *H. sapiens* (EAW77563.1).

In addition, a phylogenetic tree of *CmCdc2* (accession no. KR674083) was compared with *Cdc2* of *I. punctatus* (NP_001187396.1), *C. auratus* (BAA04605.1), *D. rerio* (AAH79527.1), *E. lucius* (ACO13346.1), *S. salar* (ACI68251.1), *O. mykiss* (ABI54409.1), *Oreochromis niloticus* (XP_003455045.1), *Anabus testudineus* (AAS59851.2), *X. laevis* (NP_001080554.1) and *H. sapiens* (AAI07751.1) found in GenBank (http://ncbi.nlm.nih.gov). Multiple alignments were carried out with ClustalW (Thompson et al., 1994). A bootstrapped neighbor-joining tree (Saitou and Nei, 1987) was constructed using the MEGA 6.0 software (Tamura et al., 2013).

Expression levels of CmCcnb1 and CmCdc2 mRNAs

The intron/exon boundaries of *CmCcnb1* and *CmCdc2* cDNA segments were characterized. Primers for amplification of *CmCcnb1* (CmCcnb-qPCR-F/R; Table 1) and *CmCdc2* (Cm-Cdc2-qPCR-F and CmCdc2-ExIn-R: 5′-GGCAGTGACAGAACAGGATC-3′) were designed from nucleotide sequences of degenerate polymerase chain reaction (PCR) products of respective cDNAs as previously described. The amplification reaction was carried out against the ovarian first-strand cDNA and genomic DNA template. In addition, primers for amplification of the partial *18S rRNA* cDNA (18S rRNA-qPCR-F/R; Table 1) were designed from the conserved region of *Clarias batrachus* (KM018296) and *Clarias gariepinus* (GQ465837) *18S rRNAs*. PCR was performed and the resulting fragments of each gene were cloned and sequenced. To prevent the possible amplification of contaminated genomic DNA, the CmCcnb-qPCR-F located in the exon region and CmCcnb-qPCR-R which covered the other exon/intron boundaries where 8 bases at the 3′ terminus of the primer were located in a different exon, were used. In addition, a CmCdc2-qPCR-R primer which covered the exon/intron boundary where 4 bases at the 3′ terminus of this primer were located in a different exon (Table 1), was designed and used in combination with CmCdc2-qPCR-F. For *18S rRNA*, the original primers did not generate the amplification product when tested against the genomic DNA template. Accordingly, 18S rRNA-qPCR-F/R primers (Table 1) were further applied for quantitative real-time PCR analysis.

Standard curves representing 1×10^3–1×10^8 copies (in duplicate) of recombinant plasmids of the target genes (*CmCdc2* and *CmCcnb1*) and the reference gene (*18S rRNA*) were then constructed (Table 1; amplification efficiency >95%). The target genes and *18S rRNA* in the ovaries of each fish were separately amplified in a 15 μL reaction volume containing 7.5 μL of 2× LightCycler 480 SYBR Green I Master (Roche; Basel, Switzerland), 500 (*CmCdc2* and *CmCcnb1*) or 50 (*18S rRNA*) ng first strand cDNA template, 0.2 μM each primer. The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 53 °C for 15 s and 72 °C for 30 s. The specificity of PCR products was confirmed using melting curve analysis performed at 95 °C for 15 s, 65 °C for 1 min and 98 °C for a continuous fluorescent reading (ramp time = 0.11 °C/s). The cooling process was carried out at 40 °C for 30 s. Quantitative real-time PCR of each specimen was carried out in duplicate. The average quantification of *CmCdc2*, *CmCcnb1* and *18S rRNA* mRNA of each sample was evaluated from the respective standard curves. The relative expression level (copy number of *CmCdc2* or *CmCcnb1*/that of *18S rRNA*) between fish with different ages were statistically tested using one way ANOVA followed by Duncan's new multiple range test. Significant differences between groups of samples were considered when $p < 0.05$.

Results

Gonad development of diploid and triploid females and males of C. macrocephalus

Diploid and triploid *C. macrocephalus* females and males were successfully produced as revealed by chromosome counting (2n = 54 and 3n = 81, Fig. 1) with a success rate of 65%. The GSI values of diploid females were significantly greater than those of triploid females at age 6 mth onwards (Fig. 2). Within diploid samples, the average GSI values at age 2 mth and 4 mth were low (0.036 ± 0.010 and 0.029 ± 0.011%) which were consonant with histological results where ovarian samples were dominated by previtellogenic oocytes without more advanced stages of oocytes (Fig. 3A and B). Subsequently, the GSI value reached its highest

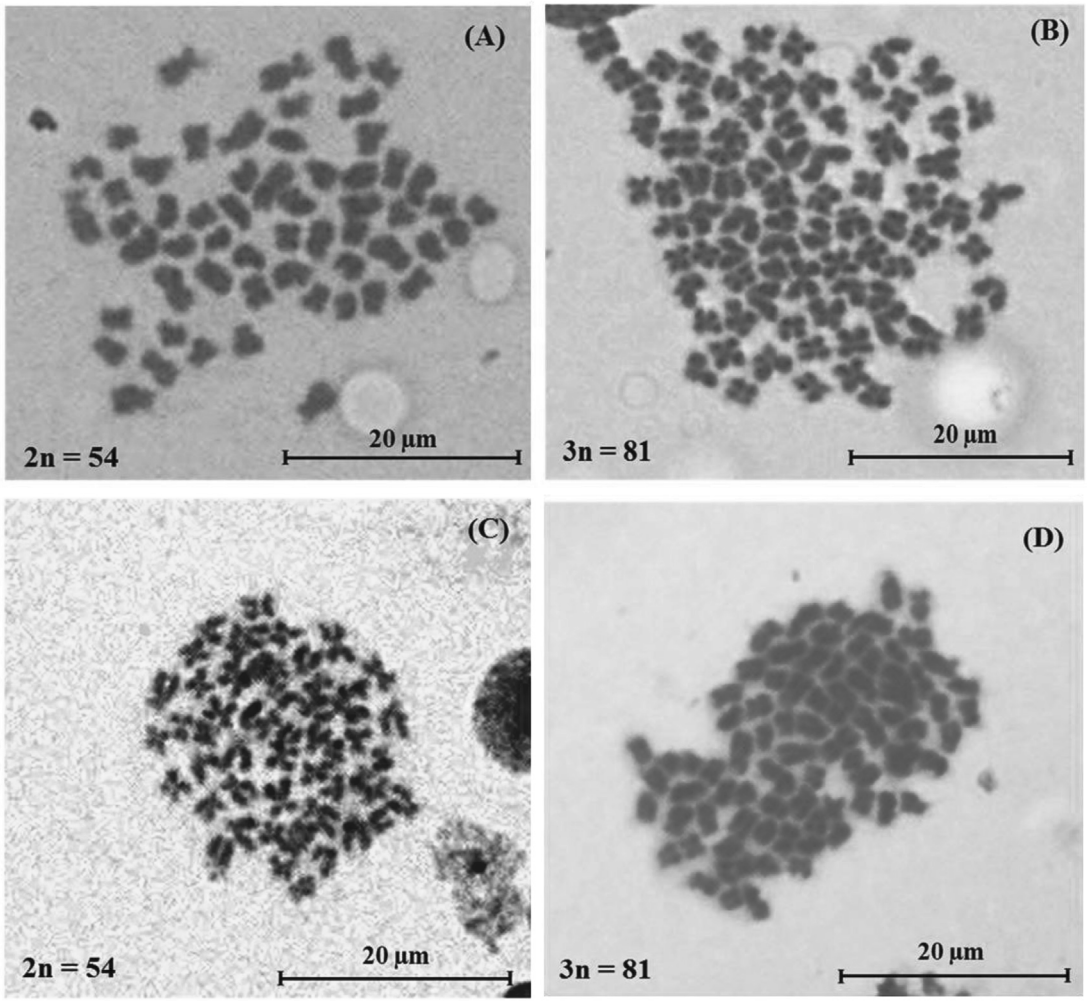

Fig. 1. Chromosome number of representative individuals of diploid and triploid females (A and B) and males (C and D) of *Clarias macrocephalus*.

value (12.06 ± 1.90%; $p < 0.05$) at age 6 mth. Then, it was significantly reduced at age 8 mth and retained similar values at ages 10 mth and 12 mth (6.711 ± 0.513 to 8.197 ± 1.122%; $p < 0.05$), but was still higher than those of catfish aged 2 mth and 4 mth ($p < 0.05$). The GSI values of triploid females at different ages were low and not significantly different (GSI = 0.021 ± 0.004 to 0.069 ± 0.016%) as shown in Fig. 2.

The histological study of diploids aged 2–12-mth showed normal development of oocytes and spermatozoa (Tan-Fermin et al., 1997a, 1997b) while triploid females and males showed retarded gonads (Figs. 3 and 4). Previtellogenic oocytes predominated in the ovaries of fish aged 2 mth (Fig. 3A) and a greater proportion of vitellogenic oocytes was found in *C. macrocephalus* aged 4 mth (Fig. 3B). Ovarian samples of catfish aged 6 mth onwards were occupied with oocytes in postvitellogenic stages with small amounts of previtellogenic oocytes (data not shown). A few atretic oocytes were observed in diploid samples aged 12 mth. Histologically, ovaries of female triploids were filled with connective tissues without developed oocytes at any stages (Fig. 3C,D and F–H)—only one of the 38 examined triploid samples showed a few previtellogenic oocytes (Fig. 3E).

In males, the GSI values in diploids were greater than those in triploids at age 4 mth onwards ($p < 0.05$, Fig. 2B). Within diploid males, the lowest GSI value was observed in samples aged 2 mth ($p < 0.05$) and the GSI of diploid males was not significantly different from age 4 mth onwards (0.129 ± 0.048 to 0.213 ± 0.016%; $p > 0.05$). The histology of testes revealed that spermatogonia predominated in diploids aged 2 mth and 4 mth (Fig. 4A) while diploids aged 6 mth showed enlarged seminiferous tubules partially filled with spermatogenic cells (Fig. 4B). The testes of diploids aged 8 mth were similar to those of diploids aged 6 mth with the exception of the amount of spermatozoa which had substantially increased (Fig. 4C). The amount of spermatozoa slightly declined in samples aged 10 mth (Fig. 4D) and was significantly decreased in samples aged 12 mth (data not shown). Some empty seminiferous tubules were observed in diploids aged 12 mth and thus marked the regression of testes. In triploids, the GSI value of catfish aged 6 mth (0.0700 ± 0.0078%) was significantly greater than that of males aged 2 mth and 10 mth (0.0115 ± 0.0042 and 0.0192 ± 0.0093%, respectively). Nevertheless, the testes of triploids were completely retarded as revealed by conventional histology where only connective tissues were observed without spermatogenic cells throughout the developmental periods (Fig. 4E–H).

Isolation and primary structure of full-length cDNAs of CmCcnb1 and CmCdc2

In this study, full-length cDNAs of *CmCcnb1* and *CmCdc2* were successfully isolated and reported for the first time in this species. The predicted translational start site based on the best-known

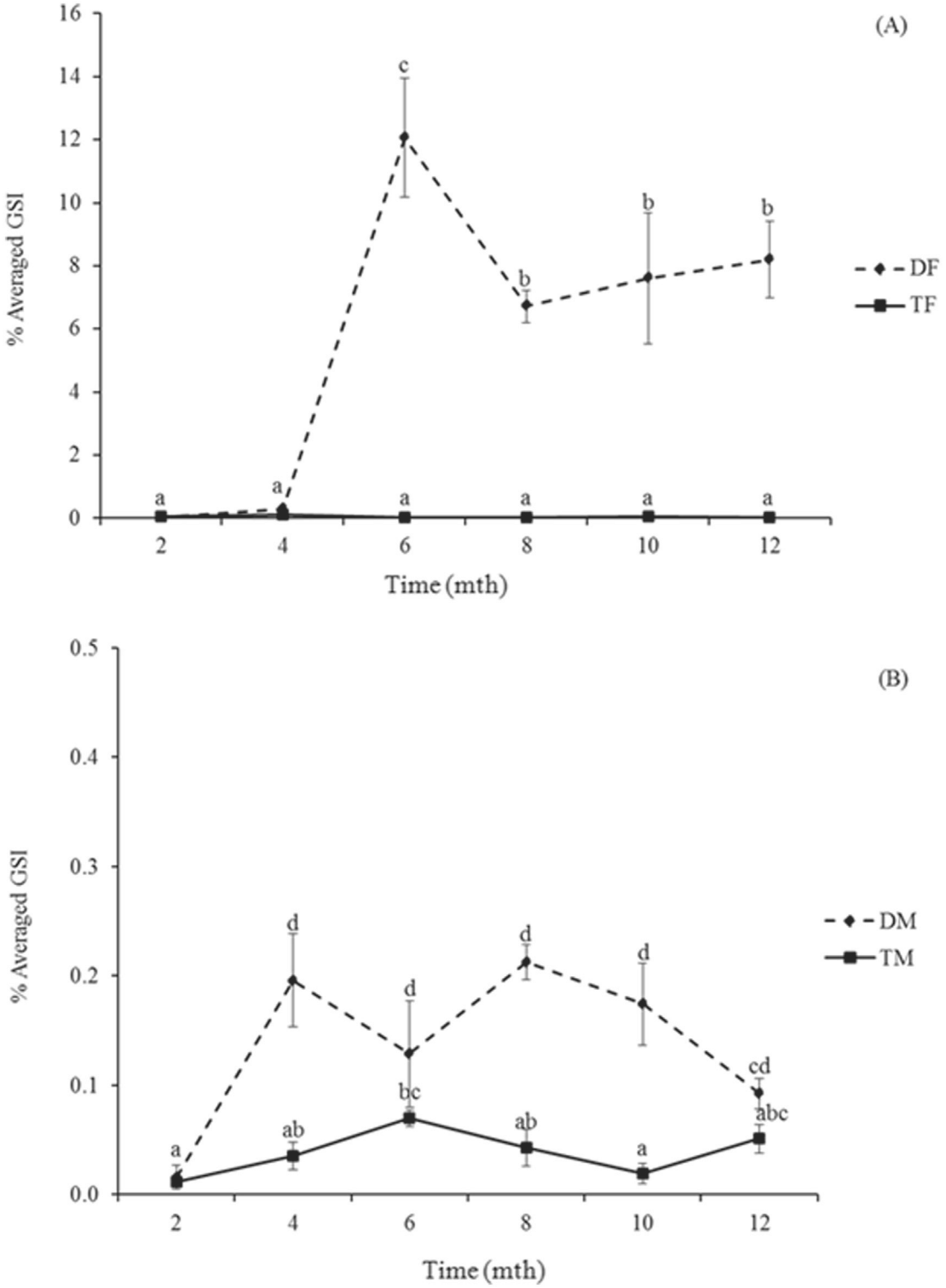

Fig. 2. Average GSI values of diploid and triploid females (A) and males (B) of *Clarias macrocephalus*. Each point corresponds to a particular catfish age group (2 mth, 4 mth, 6 mth, 10 mth and 12 mth). Vertical error bars show ± SD. Same lowercase letters above points reveal nonsignificant differences between groups of samples ($p > 0.05$). DF = diploid females, TF = triploid males, DM = diploid males, TM = triploid males.

Kozak's rule (Kozak, 1986) of A/GXXATGG was found in both transcripts. The deduced CmCcnb1 protein contained the PKA site, the cyclin destruction box and two cyclin domains typically found in the cyclin protein family. The deduced CmCdc2 protein contained an S_TKc domain, the conserved phosphorylation sites (Thr14, Tyr15 and Thr161 residues), the PSTAIRE variant and the ATP binding element (GEGTYGVV). The availability of a Kozak sequence and functional domains typically found in Ccnb1 and Cdc2 across various species strongly suggested that full-length cDNAs of these transcripts were readily isolated. Phylogenetic analysis revealed

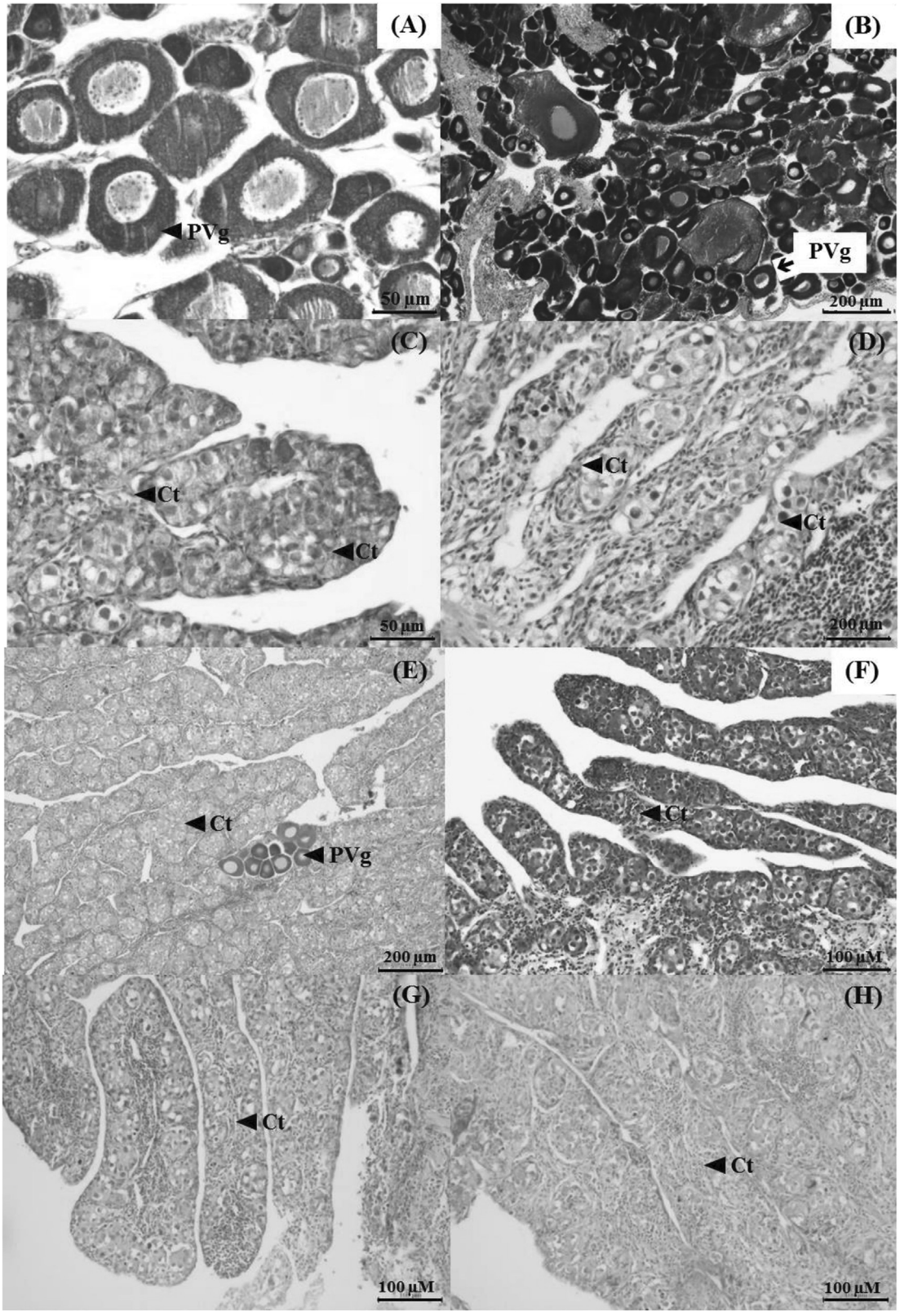

Fig. 3. Conventional histology showing ovarian development in different ages of diploidfemale catfish (A aged 2 mth and B aged 4 mth) and triploid female catfish (aged 2 mth, 4 mth, 6 mth, 8 mth, 10 mth and 12 mth for C–H, respectively). PVg = previtellogenic oocytes, Vg = vitellogenic oocytes, Ct = connective tissue.

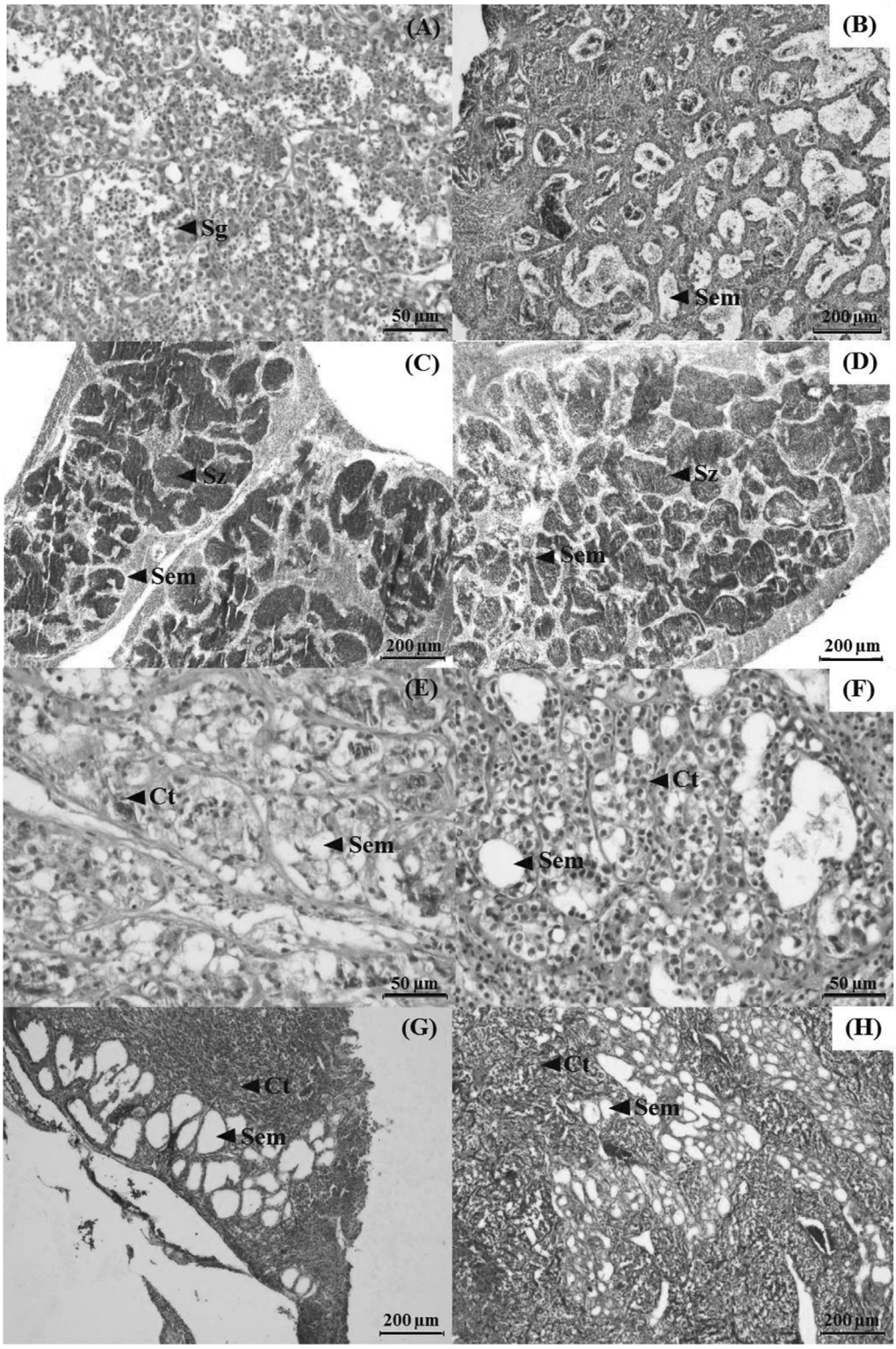

Fig. 4. Conventional histology showing testicular development at different ages of diploid male *Clarias macrocephalus* (2 mth, 6 mth, 8 mth and 10 mth for A–D, respectively) and triploid female *C. macrocephalus* (2 mth, 4 mth, 8 mth and 10 mth for E–H, respectively). Ct = connective tissue, Sem = seminiferous tubules, Sg = spermatogonia, Sz = spermatozoa.

that *CmCcnb1* was closely related with Cyclin B1 of *Carassius gibellio*, *C. auratus*, *C. carpio* and *D. rerio* while the newly isolated *CmCdc2* clustered with *I. punctatus* and they should be regarded as new members of *Ccnb1* and *Cdc2*.

The amplification products of 498 and 614 bp fragments were obtained using degenerate primers for *Ccnb* and *Cdc2*. Nucleotide sequences of these gene segments significantly matched *Ccnb* of *C. auratus* (AAF82780.1; *E*-value = 2e-104) and *Cdc2* of *I. punctatus*

(NP_001187396, *E*-value = 2e-143). The 5′ and 3′ RACE-PCR of each gene was further carried out and generated 740 and 941 bp fragments for the former and 347 and 775 bp fragments for the latter.

After sequence assembly, cDNAs of *CmCcnb1* and *CmCdc2* transcripts were obtained. *CmCcnb1* was 1539 bp in length containing an open reading frame (ORF) of 1194 bp corresponding to a polypeptide of 397 amino acids with the poly An additional signal (AAATAA) was located at nucleotide positions 1489–1494 (GenBank accession no. KR674084, Fig. 5). The calculated molecular mass and p*I* of the deduced CmCcnb1 protein were 45.88 kDa and 8.44, respectively. The deduced *CmCcnb1* protein contained the PKA site (RRxSK, amino acid positions 273–277), a characteristic of B-type cyclins and the cyclin destruction box (RxALGxIxN, positions 35–43). Two cyclin domains were found at positions 173–257 (*E*-value = 6.3e-26) and 270–351 (*E*-value = 3.1e-17) of the deduced CmCcnb1 protein (Fig. 5). The deduced CmCcnb1 protein showed the greatest similarity to Cyclin B1 of *D. rerio* (*E*-value = 0.0).

The characterized *CmCdc2* mRNA was 1355 bp in length with an ORF of 909 bp deducing to a polypeptide of 302 amino acids with the poly An additional signal (AAATAA) was located at nucleotide positions 984–989 (GenBank accession no. KR674083, Fig. 6). The calculated molecular mass and p*I* of the deduced CmCdc2 protein were 34.41 kDa and 7.10, respectively. The phosphorylation sites of Thr14, Tyr15 and Thr161 residues and the catalytic domain of serine/threonine kinases (S_TKc, positions 4–287, *E*-value = 2.19e-102) were also found. The PSTAIRE variant (PSTAVRE) and DFG sequences which are related to the cyclin binding, and GxGxxGxV (GEGTYGVV) elements which are involved in ATP binding (De Bondt et al., 1993), were found at amino acid positions 45–51, 146–148 and 11–18, respectively (Fig. 6). The potential *N*-linked glycosylation site was not found in the deduced CmCdc2 protein. Its closest similar sequence was Cdc2 of *I. punctatus* (*E*-value = 0.0).

Phylogenetic analysis of CmCcnb1 and CmCdc2

A bootstrapped neighbor-joining tree clearly indicated monophyletic differentiation between *Ccnb1* and *Ccnb2*. *CmCcnb1* was more closely related with the *cyclin B1* than *cyclin B2* of various fish species (Fig. 7A). A phylogenetic tree of *Cdc2* from various taxa was also analyzed. *CmCdc2* was phylogenetically clustered with that of *I. punctatus* (Fig. 7B).

Expression of CmCcnb1 and CmCdc2 in different ages of diploid and triploid C. macrocephalus

In females, the expression level of *CmCcnb1* in diploids was significantly greater than that of triploids at ages 2 mth (513.43 ± 83.22) and 8 mth (2430.87 ± 900.06). Within the diploids group, its expression at age 2 mth was significantly greater than that at age 6 mth but not in catfish aged 4 mth. The expression level peaked at age 8 mth before sharply decreasing ($p < 0.05$) to the threshold level at ages 10 mth (25.60 ± 6.91) and 12 mth (2.24 ± 0.55). In the triploid group, the expression level of *CmCcnb1* was not significantly different across different age groups (0.004 ± 0.002 to 0.047 ± 0.033; Fig. 8A).

In males, the expression level of *CmCcnb1* in diploids was significantly greater than that of triploids only at age 2 mth (928.85 ± 208.72). Within diploid males, its expression was significantly decreased from age 2 mth in catfish aged 4–12 mth (0.12 ± 0.03, 90.85 ± 10.94, 14.16 ± 1.36, 0.084 ± 0.027 and 0.0005 ± 0.0002, respectively). In triploid males, the expression level of *CmCcnb1* was not significantly different among different age groups (0.007 ± 0.0005 to 0.34 ± 0.10; Fig. 8B).

The expression level of *CmCdc2*, in diploid females was significantly greater than that of triploid females at ages 2 mth, 4 mth and 8 mth (7.66 ± 3.42, 59.41 ± 10.46 and 42.74 ± 8.36 fold, respectively). Within diploid females, its expression was significantly increased from age 2 mth (7.66 ± 3.42) to the peak level at age 4 mth (59.41 ± 10.46) before significantly reducing in catfish aged 6 mth (0.52 ± 0.22). Its expression then rapidly increased again at age 8 mth (42.74 ± 8.36) before subsequently reducing to the threshold level in catfish aged 10 mth (6.11 ± 1.18) and 12 mth (1.48 ± 0.47) as shown in Fig. 8C. In triploid females, the expression level of *CmCdc2* was not significantly different between different age groups (0.12 ± 0.05 to 3.21 ± 0.35).

In males, the expression level of *CmCdc2* in diploids was significantly greater than that of triploids aged 6 mth and 8 mth (58.61 ± 34.64 and 72.70 ± 4.36 fold, respectively). Within diploid males, its expression was significantly increased from ages 2 mth and 4 mth (3.48 ± 1.32 and 0.62 ± 0.38, respectively) and in catfish aged 6 mth and 8 mth (58.61 ± 34.64 and 72.70 ± 4.36, respectively) and significantly reduced to the previous level in catfish aged 10 mth and 12 mth (0.82 ± 0.42 and 0.63 ± 0.09, respectively; Fig. 8D). In triploids, the expression level of *CmCdc2* was not significantly different throughout the rearing period (0.10 ± 0.04 to 3.42 ± 0.54).

Discussion

Ovarian and testicular development in diploid and triploid C. macrocephalus

Conventional histology revealed that the ovaries of diploids were in previtellogenic (immature) stages during age 2–4 mth and they developed to postvitellogenic (mature) stages when the catfish were aged 6 mth onwards. The histological results conformed to the GSI values where the GSI peaked at age 6 mth and extended during age 8–12 mth. The gonadal maturation of female and male *C. macrocephalus* based on their GSI values were not synchronous, as the peak maturation period in males based on the GSI was observed in fish aged 4 mth and continued until the end of the present study (12 mth). However, histology showed that spermatozoa were fully produced in males aged 8 mth. In practice, males and females aged 8 mth are a priority for farmers undertaking commercial production of fry (Ingthamjitr, 1997).

The GSI values of triploid female and male *C. macrocephalus* were very low throughout the year and were lower than those of diploid catfish in most developmental stages (>150 fold and >1.8 fold in females and males, respectively). Both the GSI and conventional histology confirmed that triploids should be sterile as has been previously reported (Na-Nakorn and Lakhaanantakun, 1993; Fast et al., 1995). It was noteworthy that the gonads of triploid *C. macrocephalus* were completely suppressed as shown by a lack of oocytes in the previtellogenic, vitellogenic and mature stages while in male triploids, no developing spermatogenic cells were observed. In some fish species triploid gonads have gone through certain gonadal development; for example, a few spermatozoa were observed in the testes of triploid Thai silver barb, *Barbodes gonionotus* (previously *Puntius gonionotus*) (Koedprang and Na-Nakorn, 2000), grass puffer, *Takifugu niphobles* (Hamasaki et al., 2013) and sea bass, *Dicentrarchus labrax* L. (Felip et al., 1999) while oocyte development was arrested at the previtellogenic or vitellogenic stage (Felip et al., 1999; Hamasaki et al., 2013; Koedprang and Na-Nakorn, 2000).

Among catfish species, triploid *C. macrocephalus* (this study) showed severe testes retardation similar to *C. batrachus* (Siraj et al., 1993) and channel catfish (Chrisman et al., 1983), where the testes contained only spermatogenic epithelium. In females, the current results were similar to only the triploid female channel catfish wherein ovaries were filled with connective tissues with rarely

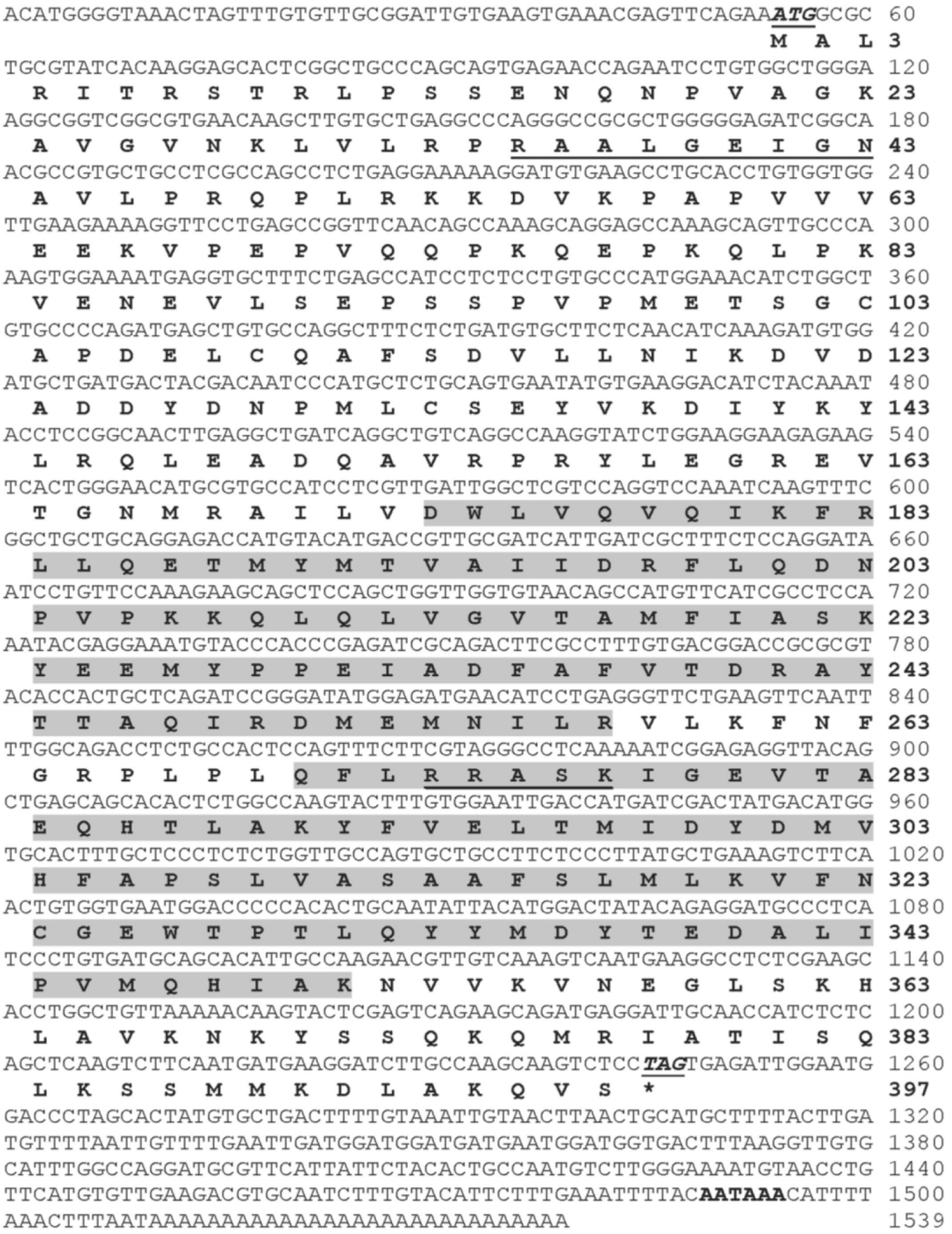

Fig. 5. Full-length cDNAs and deduced amino acids of *CmCcnb1*. The start and stop codons are boldfaced, italicized and underlined. The poly A additional signal is illustrated in boldface. The PKA site (RRxSK, positions 273–277) and the cyclin destruction box (RxALGxIxN, positions 35–43) are underlined and double-underlined. Two cyclin domains (positions 173–257 and 270–351) are highlighted and underlined.

found previtellogenic oocytes (Chrisman et al., 1983). However, triploid of other catfish species seemed to have more advanced gonads, for example, triploid female Indian catfish (*Heteropneustes fossilis*) showed retarded ovaries at the previtellogenic oocyte stage; additional atretic advanced oocytes were occasionally found in *C. batrachus* (Siraj et al., 1993) and *C. gariepinus* (Karami et al., 2011). Likewise, the testes of triploid *H. fossilis* developed to advanced stages with developing spermatocytes and spermatids although without spermatozoa (Tiwary et al., 2000). The discrepancies in the triploidy effects on gonadal development may vary with species and hence may possibly reflect different mechanism responsible for sterility.

Molecular characterization and expression levels of CmCcnb and CmCdc2 mRNAs during ovarian and testicular development

Meiotic maturation of oocytes is regulated by the maturation promoting factor (MPF), a complex of Cdc2 (Cdc2) and cyclin B; MPF controls eukaryotic cell cycles at the checkpoint from G2 to M-phase in all animals examined so far (Katsu et al., 1995; Ledan et al., 2001; Okano-Uchida et al., 1998; Voronina and Wessel, 2003; Voronina et al., 2003; Yamashita, 1998). However, there has been no report on isolation and characterization of these transcripts in *C. macrocephalus*.

In oviparous animals, a wide variety of maternal mRNA is generally transcribed at the early oogenesis stage, then stored in oocytes and carried into fertilized eggs (Nishimura et al., 2009). Qiu et al. (2008) examined the expression level of *Ccnb1*, *Ccnb2* and *Cdc2* mRNAs during oogenesis and spermatogenesis in rainbow trout (*O. mykiss*). The amount of *Ccnb1* and *Ccnb2* mRNAs was higher at the previtellogenesis and late vitellogenesis stages, but lower in the early vitellogenesis stage and during early embryogenesis (days 0–5). A similar expression pattern was also observed for *CmCcnb1* in diploid females in the current study. In contrast, *Cdc2* mRNA in rainbow trout was constitutively present throughout the processes of oogenesis and early embryogenesis but it decreased in early vitellogenesis while *CmCdc2* in diploid females was up-regulated only in the previtellogenic stages at ages 2 mth and 4 mth and at age 8 mth in post-vitellogenic stages.

Typically, the expression level of *CmCcnb1* was greater than that of *CmCdc2* in each developmental stage of ovarian and testicular development of *C. macrocephalus* ($p < 0.05$). In diploid females, abundant expression levels of ovarian *CmCcnb1* and *CmCdc2* at age 8 mth (postvitellogenic stage) primarily suggested the involvement of these transcripts in the mature stages of ovarian development of *C. macrocephalus*. Generally, these two genes are responsible for the synthesis of proteins which will combine into MPF, an essential factor for triggering oocyte maturation (Katsu et al., 1995; Kishimoto, 2003; Nagahama and Yamashita, 2008; Voronina and Wessel, 2003). However, at the previtellogenic (immature; ages 2 mth and 4 mth) stages, significant expression levels of these two genes were also observed implying a certain role in the early developmental period, probably during oogonial proliferation in the mitotic division (Fang and Qiu, 2009; Kotani and Yamashita, 2002; Xie et al., 2001) and also the involvement in transition of oogonia toward the G2/M stage during the first meiotic division (Basu et al., 2004; Katsu et al., 1995; Kotani and Yamashita, 2002; Qiu et al., 2008). Roles of *Cdc2* in mitotic division were shown in sea urchin embryos (Salaün et al., 2004). The surge of *CmCdc2* mRNA in ovaries of females aged 4 mth suggested the involvement of *CmCdc2* in vitellogenesis. However, it is also possible that *CmCdc2* mRNA may be synthesized and accumulated to maintain multiple times of its translations throughout oogenesis (Chesnel and Eppig, 1995).

The current results implied that mechanisms for meiotic maturation of eggs and sperm in *C. macrocephalus* may be different. In males, early up-regulation of *CmCcnb1* was observed in immature *C. macrocephalus* diploids (aged 2 mth) followed by down-regulations in catfish aged 4–12 mth. This suggested that *CmCcnb1* may be functionally involved in mitotic cell division at the early stage of spermatogenesis of *C. macrocephalus*. In mammals, MPF is essential for spermatogenic cells to undergo the G2/M transition of first meiosis (Zhang et al., 2008). It is possible that *CmCcnb1* may be early transcribed and stored in testes for multiple translation during spermatogenesis in catfish aged 4–12. *In situ* hybridization of medaka (*O. latipes*) testes provided supporting evidence in that *Ccnb1* mRNA was detected in all stages of spermatogenic cells except spermatozoa (Mita et al., 2000) while in rainbow trout, the Ccnb1 protein was detected in spermatogonia and spermatocytes (Qiu et al., 2008). However, in the current results, *CmCcnb1* showed an abundant expression level during the early spermatogenic stage (aged 2 mth). This implied a different mechanism regulating the early spermatogenesis of *C. macrocephalus* compared with that of mammals.

The expression level of *CmCdc2* was up-regulated at the mature stage (ages 6 mth and 8 mth) in diploid males when the testes were filled with spermatozoa and thus may suggest its functional involvement in the maturation of spermatogenic cells. However, a controversial report in mouse illustrated that *Cdc2* mRNA was not observed in spermatozoa and spermatids by *in situ* hybridization (Zhang et al., 2008) while it was detected in all stages of spermatogenesis including spermatids and spermatozoa in rainbow trout (Qiu et al., 2008). Contradictory results from qRT-PCR and by *in situ* hybridization on this issue may result from the detection sensitivity where qRT-PCR detects gene expression with much greater sensitivity than by *in situ* hybridization.

The low abundance expression level of *CmCcnb1* and *CmCdc2* in the immature ovaries and testes of triploid catfish was consonant with the results from GSI values and conventional histology. An absence of up-regulation of *CmCcnb1* and *CmCdc2* in male and female triploids compared with that of their respective counterparts, together with the suppression of the triploid gonad from the early stages of development, support the important role of *CmCcnb1* and *CmCdc2* in ovarian and testicular development of *C. macrocephalus*.

The expression profiles of CmCdc2 in the ovaries of diploid females at the transcriptional and translational levels were different. At the mRNA level, *CmCdc2* in fish aged 2 mth was slightly (but significantly) greater than that of fish added 6 mth, 10 mth and 12 mth and its expression peaked at age 8 mth. In contrast, the immunological results against anti-rCmCdc2 PAb indicated that different forms of the CmCdc2 (both non-phosphorylated and phosphorylated) protein seemed to be comparably expressed throughout the ovarian developmental period of *C. macrocephalus*. The circumstance suggested that the CmCdc2 mRNA is accumulated at the early stage and rapidly transcribed during the mature stage (i.e. age 8 mth) but it was translated at an approximately comparable rate during reproductive development and maturation of *C. macrocephalus*.

The results of the current study showed that *CmCcnb1* and *CmCdc2* mRNAs remarkably expressed during ovarian and testes development of diploids, whereas their expression was extremely low in triploids, which coincided with suppressed gonadal development in both male and female triploids. These results are consistent with the results of Dheilly et al. (2014) and Xu et al. (2015) who, based on transcriptomic profiling, showed down-

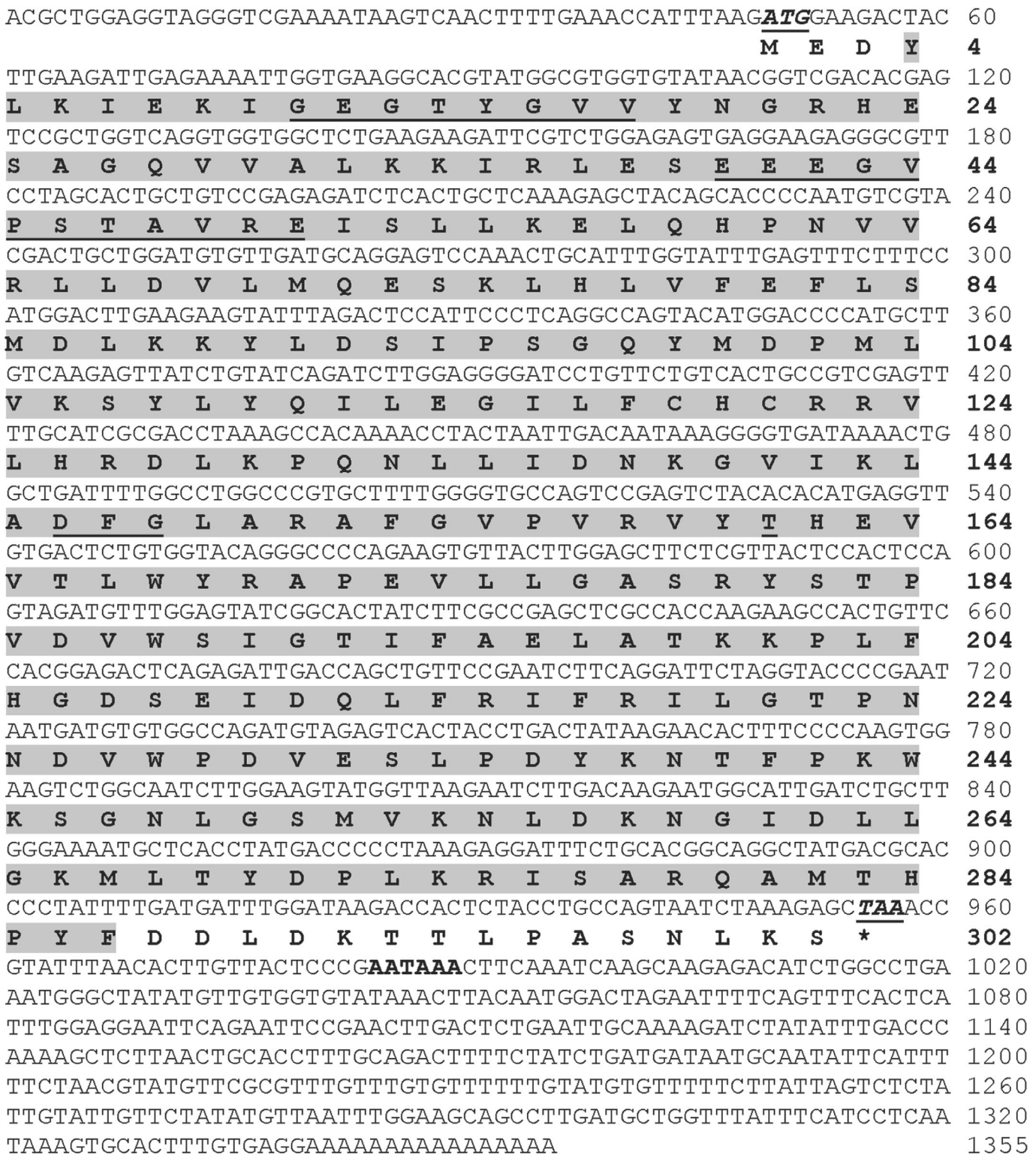

Fig. 6. Full-length cDNAs and deduced amino acids of *CmCdc2*. The start and stop codons are boldfaced, italicized and underlined. The poly A additional signal is illustrated in boldface. The conserved phosphorylation sites (Thr14, Tyr15 and Thr161) were found. The catalytic domain of serine/threonine kinases (S_TKc, positions 4–287) is highlighted. The PSTAIRE variant (PSTAVRE), DFG sequence and GxGxxGxV (GEGTYGVV) element (positions 45–51, 146–148 and 11–18, respectively) are underlined.

regulation of genes associated with cell division cycles in triploid Pacific oyster and with sperm development, assembly and motility in triploid cyprinid fish, respectively. The basic knowledge obtained in the current study paves the way for further studies to understand the molecular mechanisms for the resumption of oocytes/sperm maturation in *C. macrocephalus*. Furthermore, the important roles of *CmCyB1* and *CmCdc2* in gonadal development suggest that knocking down of these genes may lead to sterility of the fish and

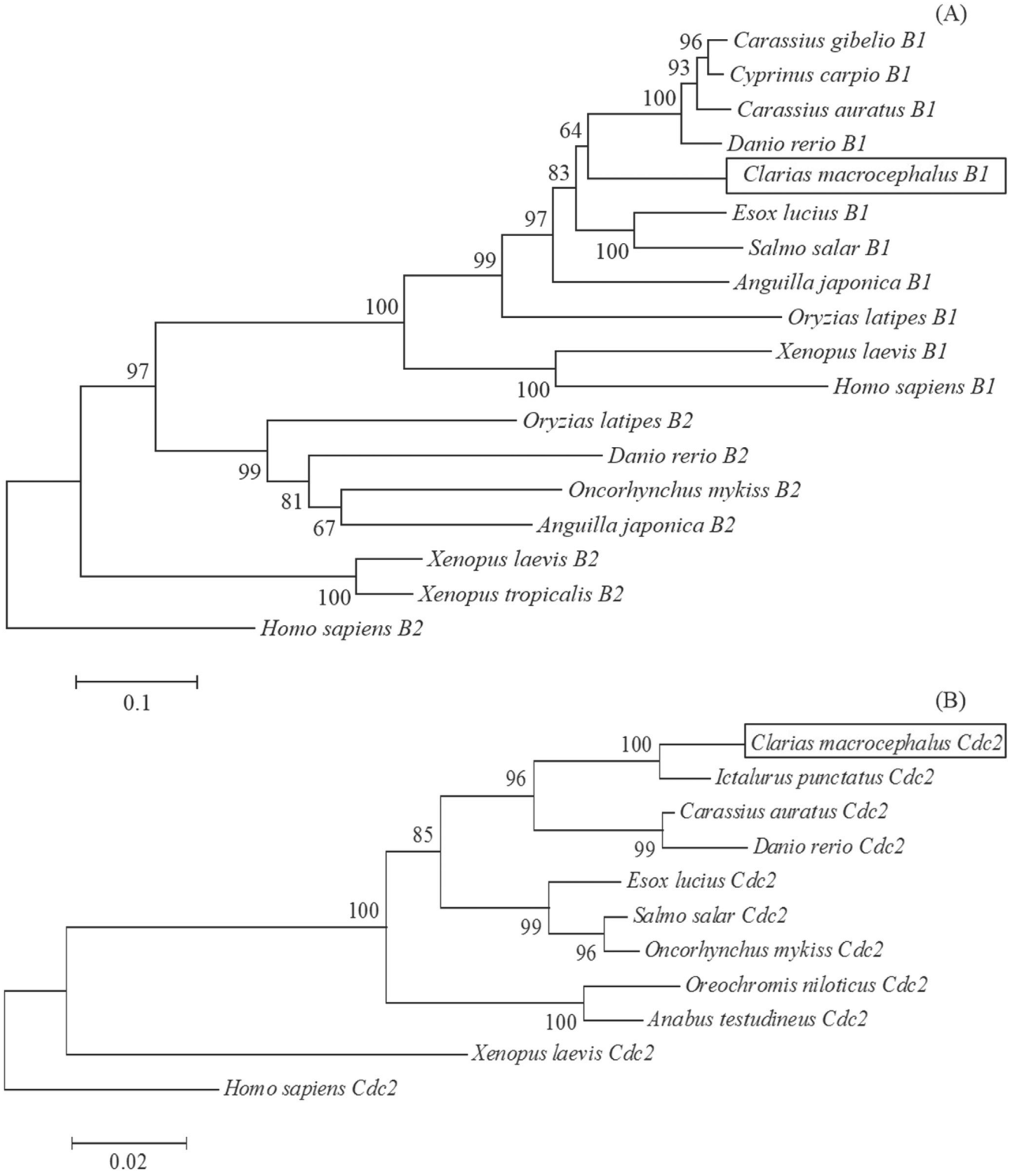

Fig. 7. Bootstrapped neighbor-joining trees illustrating phylogenetic relationships of *CmCcnb1* (A) and *CmCdc2* (B) with *Ccnb* and *Cdc2* of various taxa. Values at a node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original aligned sequences.

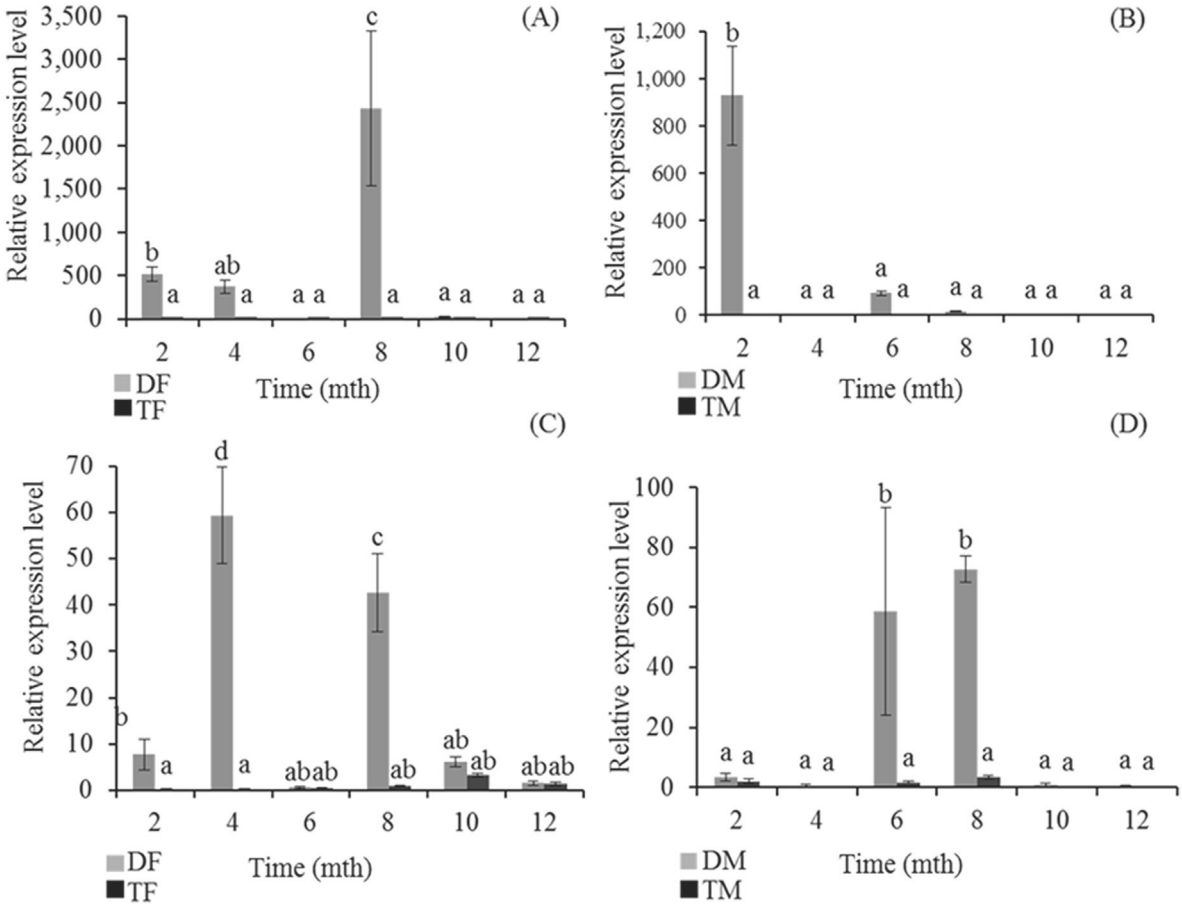

Fig. 8. Time-course relative expression levels of *CmCcnb1* (A and B) and *CmCdc2* (C and D) in ovaries (A and C) and testes (B and D) of different aged (2 mth, 4 mth, 6 mth, 8 mth, 10 mth and 12 mth) diploid and triploid *Clarias macrocephalus*. The expression levels were measured as an absolute copy number of *CmCcnb1* or *CmCdc2* and normalized by that of *18S rDNA*. Each histogram corresponds to different groups of samples. Vertical error bars indicate ± SD. Data were analyzed separately in females (A and C) and males (B and D). Same lowercase letters above bars indicate that the expression levels were not significantly different ($p > 0.05$). DF = diploid females, TF = triploid males, DM = diploid males, TM = triploid males.

thus can be used for biocontrol of unwanted reproduction, for example in the control of transgenic fish and introduced fish.

Conflict of interest

There is no conflict of interest.

Acknowledgments

This research was supported by the Kasetsart University Research and Development Institute (KURDI), through the project "A study on the role and function of reproduction-related genes/proteins in gonads of diploid and triploid walking catfish (*Clarias macrocephalus* Günther)" awarded to the third author (grant No. KURDI-64.57). The first author was funded by a student grant from The Program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission (OHEC), Thailand. The authors thank the National Center for Genetic Engineering and Biotechnology (BIOTEC) and the National Science and Technology Development Agency (NSTDA) for providing facilities for the molecular biology research in this study.

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