3. Results

3.1. Sequence analysis of *C. macrocephalus* C3 cDNA

The partial cDNA sequence for *C. macrocephalus* C3 was obtained by RACE-PCR and consisted of 4140 bp, comprising an incomplete open reading frame of 3933 bp and a 3′ UTR of 233 bp containing the poly A tail (Fig 1). The 3′ UTR contained a consensus polyadenylation signal (AATAAA) 18 bp upstream from the poly A tail. The incomplete open reading frame of *C. macrocephalus* C3 sequence encoded a part of protein consisting of 1311 amino acid residues. Three possible N-glycosylation sites were observed at amino acid position 507, 599 and 978 (Fig. 1).

The deduced 1311 amino acid sequence of the partial *C. macrocephalus* C3 was compared with the corresponding region of other known C3 sequences. As shown in Table 2, the partial *C. macrocephalus* C3 had greater similarity to the corresponding part of C3s from teleost fish (52-64% identity; 70-79% similarity) than to those of mammals (41-45% identity; 62-64% similarity). Fig. 2 shows amino acid sequence alignment of the partial *C. macrocephalus* C3 with the corresponding part of other known C3s using CLUSTAL X program, indicating the conserved amino acids and several functionally important sites. The alignment revealed that this part of *C. macrocephalus* C3 contained the partial β chain located at the NH2-terminal end and the complete α chain located at the COOH-terminal. The potential β-α processing signal of *C. macrocephalus* C3 (RKRR motif) was found to align perfectly with the homologous position of other C3s. The cystein residues related with disulfide bond between the β-α chains in human were conserved in *C. macrocephalus* C3. Likewise, *C. macrocephalus* C3 possessed the factor I and C3 convertase cleavage sites (Arg-Ser) as found in human, with the exception of the second factor I cleavage site (Arg-Thr). Moreover, the thiolester site (GCGEQ) of *C. macrocephalus* was completely identical to those of other C3s. The key amino acids for other complement proteins, such as factor B, and factor H as well as properdin binding were also conserved in *C. macrocephalus* C3.

Table 2 Amino acid comparison between *C. macrocephalus* C3 and other C3s.

Species	GenBank Accession no.	% identity/ similarity
Common carp, *Cyprinus carpio*	AB016210	64/79
Zebrafish, *Danio rerio*	XM002660575	61/78
Rainbow trout, *Oncorhynchus mykiss*	L24433	58/74
Japanese flounder, *Paralichthys olivaceus*	AB021653	54/71
Spotted wolffish, *Anarhichas minor*	AJ309570	53/69
Japanese medaka, *Oryzias latipes*	AB025576	52/70
Norway rat, *Rattus norvegicus*	NM016994	45/64
Cattle, *Bos taurus*	AM086793	45/63
Human, *Homo sapiens*	AY513239	41/62

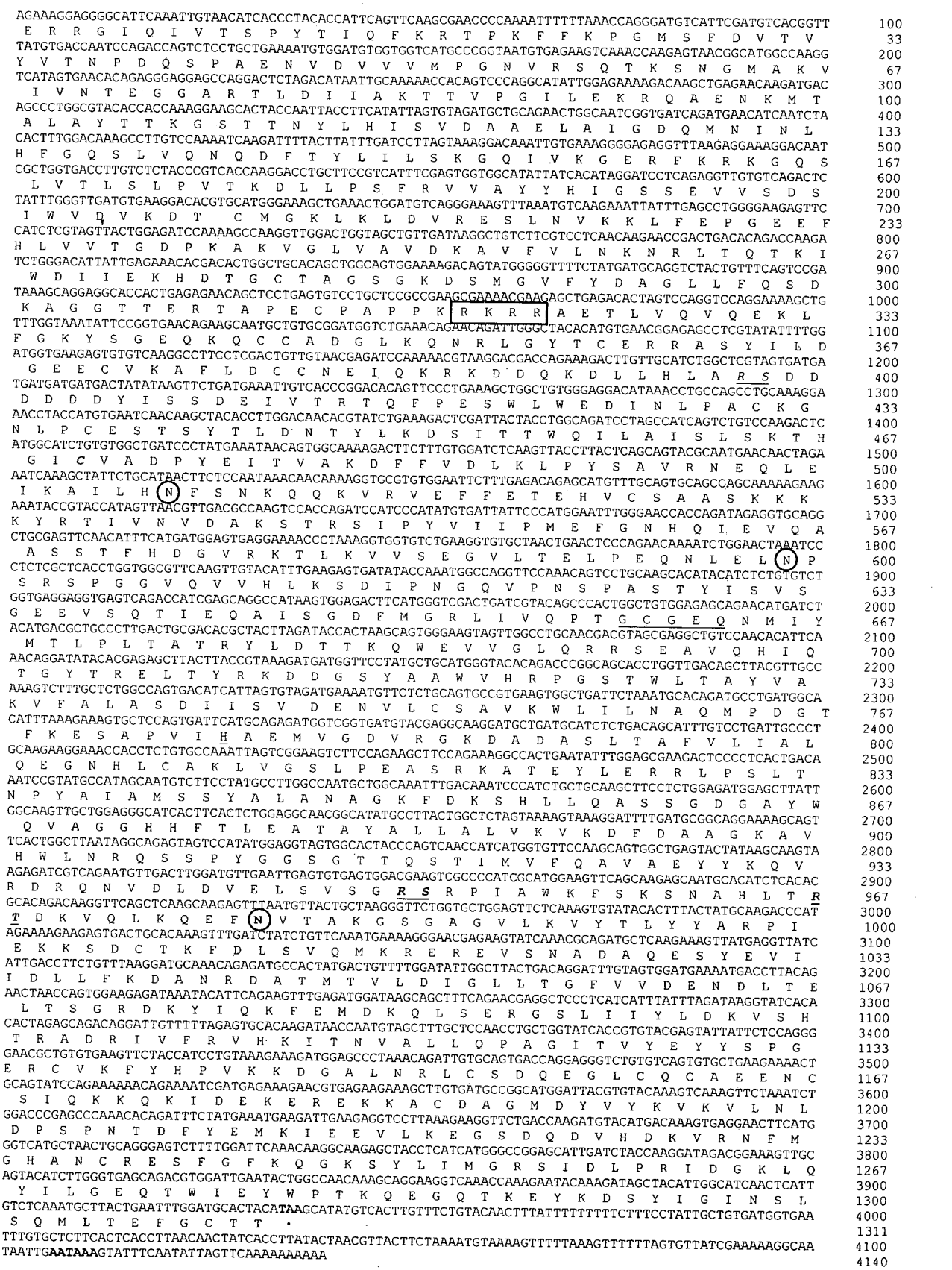


Fig. 1 Partial nucleotide and deduced amino acid sequences of *C. macrocephalus* C3. The predicted glycosylation sites are indicated by circles. The potential β-α chain processing signal is enclosed in square. The putative cleavage sites for C3 convertase and factor I are shown in underlined italic letters and bold italic letters, respectively. The thiolester site is underlined. The polyadenylation signal (AATAAA) is shown in bold letters at the end of 3' UTR.

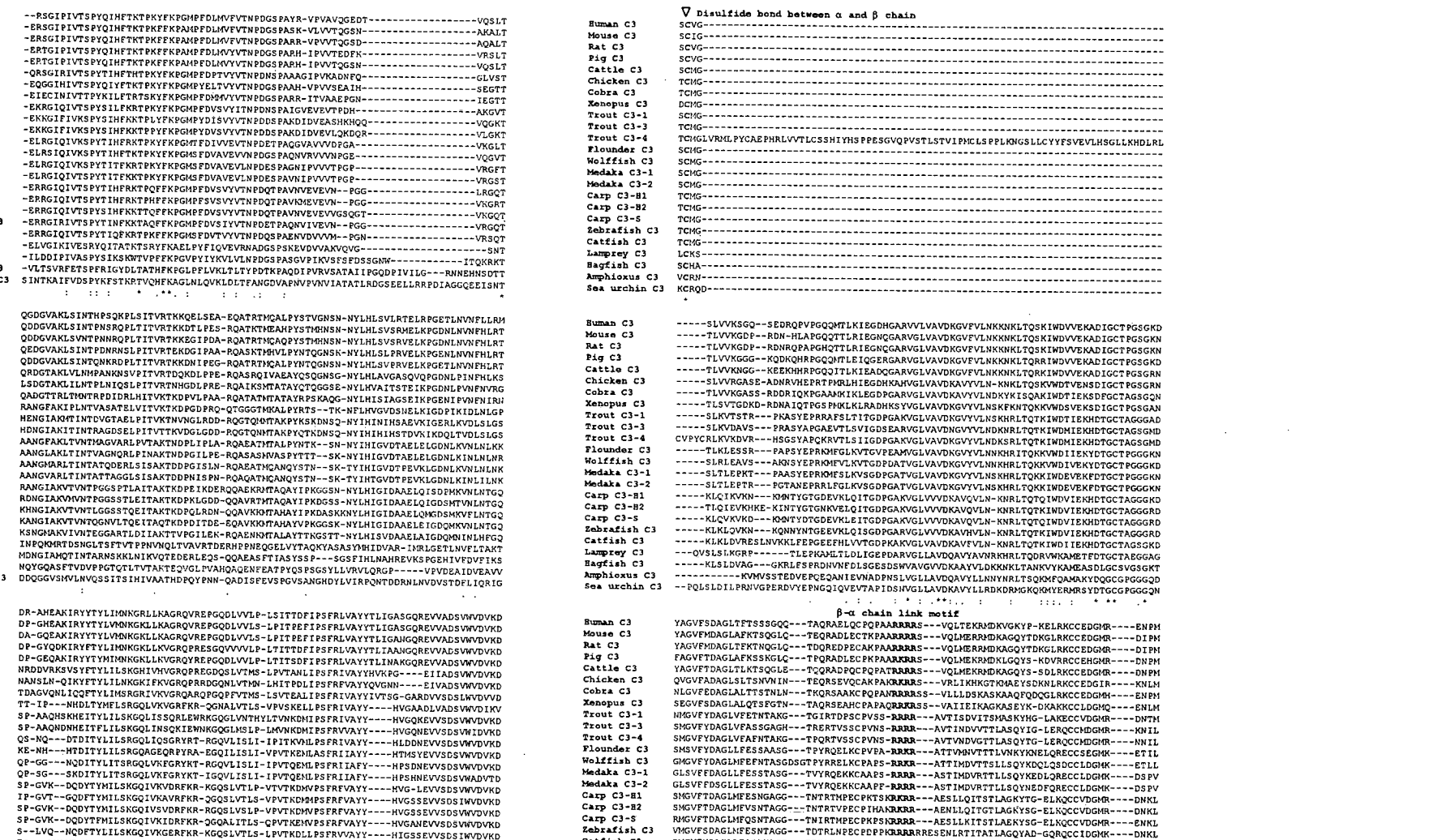


Fig. 2 Alignment of C3 amino acid sequences between *C. macrocephalus* and other known sequences. The 1311 amino acid sequence of C. macrocephalus C3 (Catfish C3, Genbank accession no. AB636135) was aligned with the corresponding part of *Homo sapiens* C3 (Human C3, Genbank accession no. K02765), *Mus musculus* C3 (Mouse C3, Genbank accession no. NM009778), *Rattus norvegicus* C3 (Rat C3, Genbank accession no. NM016994), *Bos taurus* C3 (Cattle C3, Genbank accession no. NM001040469), *Gallus gallus* (Chicken C3, Genbank accession no. NM205405), *Naja naja* (Cobra C3, Genbank accession no. Q0331), *Xenopus tropicalis* (Xenopus C3, Genbank accession no. BC168633), *Oncorhynchus mykiss* (Trout C3-1, Genbank accession no. P98093), *O. mykiss* (Trout C3-3, Genbank accession no. U 61753), *O. mykiss* (Trout C3-4, Genbank accession no. AF271080), *Paralichthys olivaceus* C3 (Flounder C3, Genbank accession no. AB021653), *Anarhichas minor* C3 (Wolffish C3, Genbank accession no. AJ309570), Oryzias latipes C3-1 (Medaka C3-1, Genbank accession no. NM_001105082), *O. latipes* C3-2 (Medaka C3-2, Genbank accession no. NM_001105083), *Cyprinus carpio* C3-H1 (Carp C3-H1, Genbank accession no. AB016210), *C. carpio* C3-H2 (Carp C3-H2, Genbank accession no. AB016212), *C. carpio* C3-S (Carp C3-S, Genbank accession no. AB016213), *Danio rerio* C3 (Zebrafish C3, Genbank accession no. XM002660575), *Lethenteron japonicum* C3 (Lamprey C3, Genbank accession no. AY359861), *Eutatretus burger* (Hagfish C3, Genbank accession no. P98094), *Branchiostoma belcheri* (Amphioxus C3, Genbank accession no. AB050668), and Strongylocentrotus purpuratus (Sea urchin C3, Genbank accession no. NM214521) using the CLUSTAL X program. Conservation of amino acid identity is shown with an asterisk '*' whereas ':' and '.' indicate high and low levels of amino acid similarity, respectively.

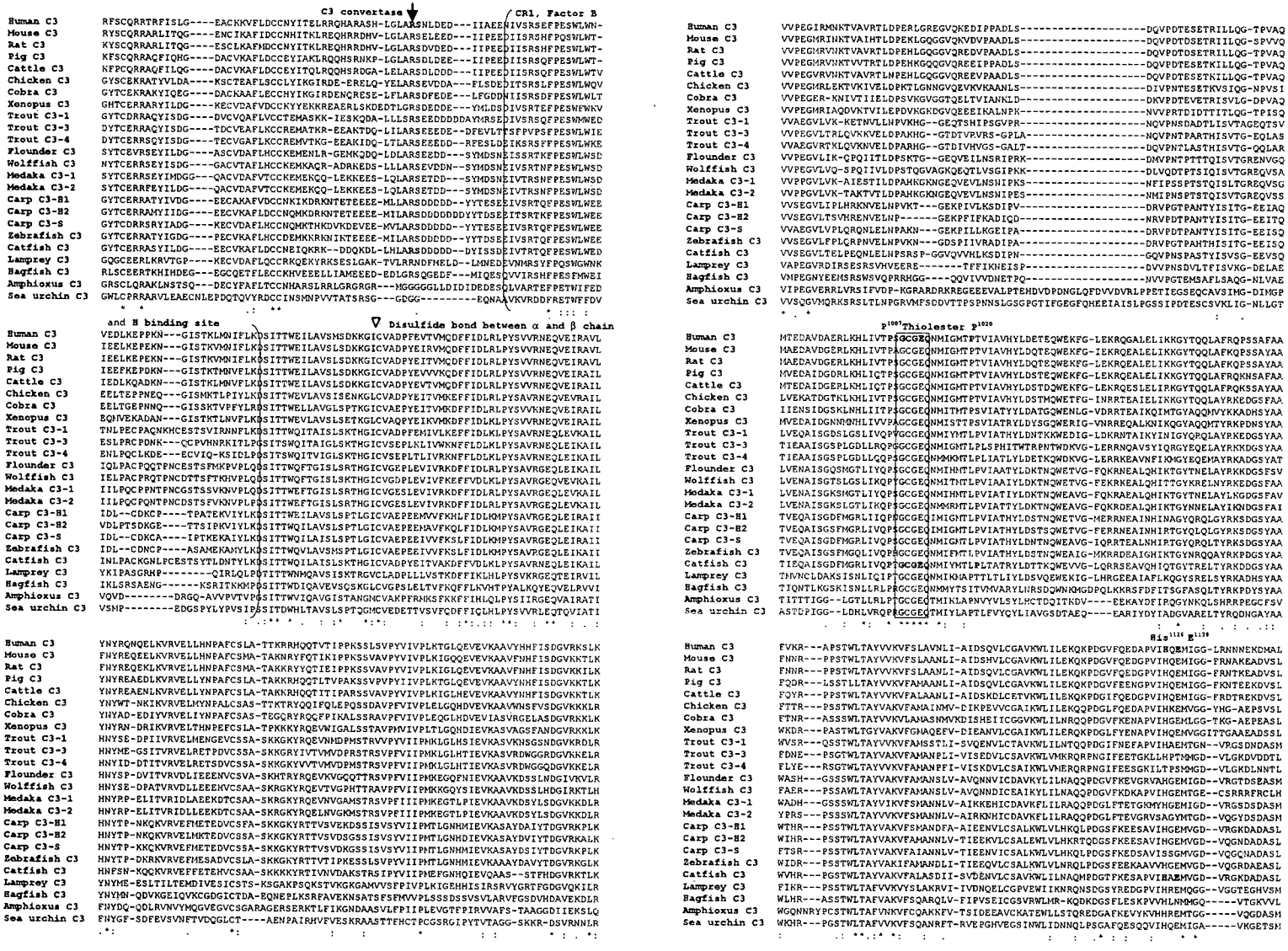


Fig. 2 (continued).

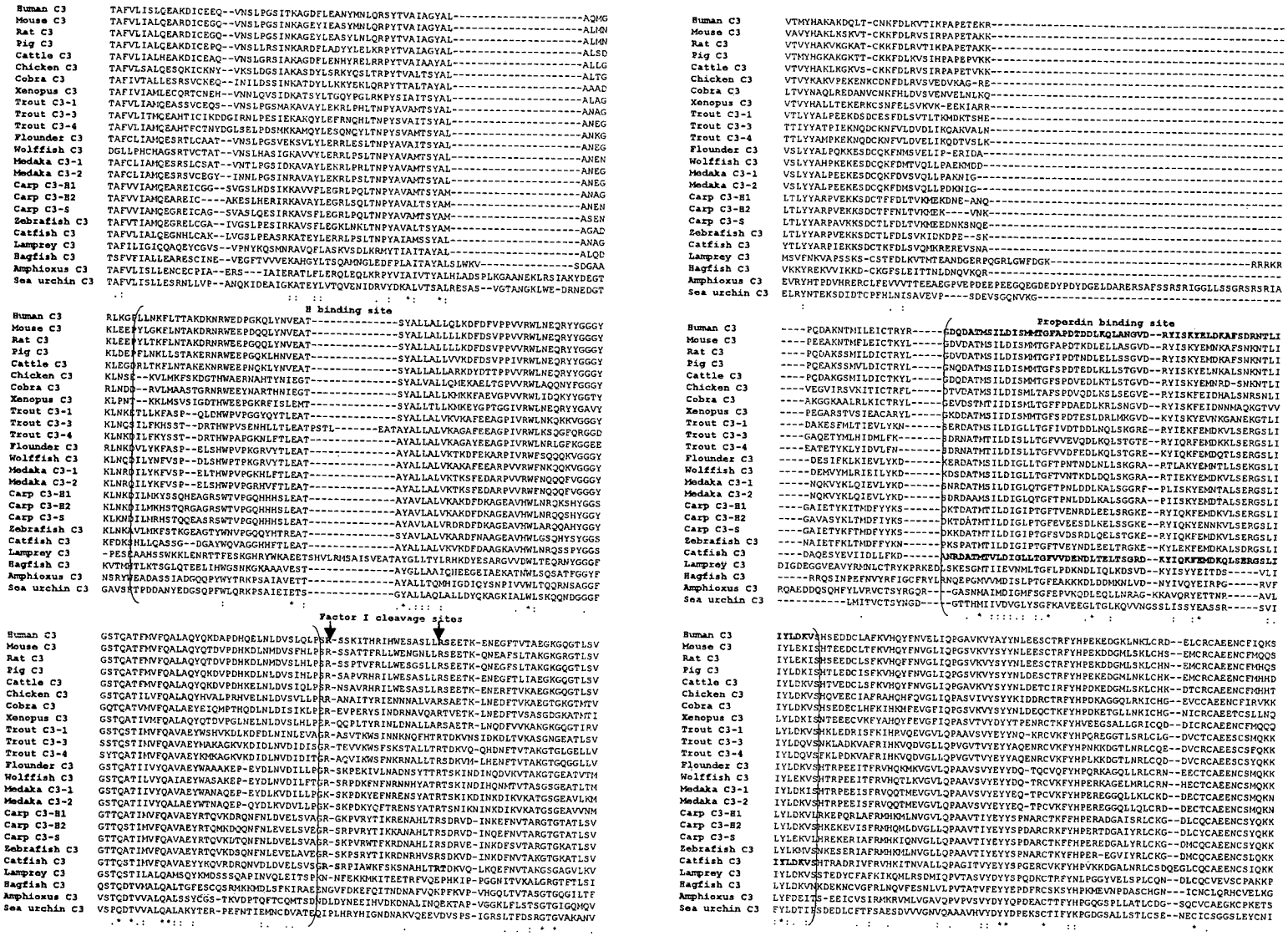


Fig. 2 (continued).

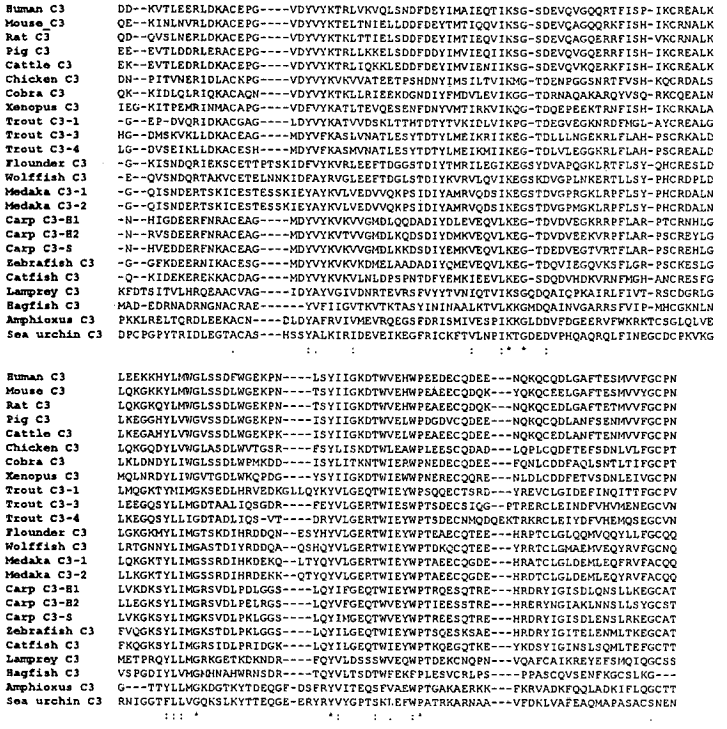


Fig. 2 (continued).

3.2. Sequence analysis of *C. macrocephalus* C8γ cDNA

The full-length of C8γ cDNA consisted of 886 bp comprising a 5′ untranslated region (UTR) of 100 bp, an open reading frame of 633 bp, a stop codon of 3 bp and a 3′ UTR of 150 bp containing the poly A tail. The 3′ UTR contained a consensus polyadenylation signal (AATAAA) 16 bp upstream from the poly A tail (Fig. 3). The open reading frame encoded a protein consisting of 211 amino acid residues. N-terminal segment included a high proportion of hydrophobic amino acid residues and therefore the first 24 amino acid residues were predicted to be a signal peptide by the SignalP program. The mature *C. macrocephalus* C8γ consisted of 187 amino acids with molecular mass of 21.64 kDa. No potential *N*-glycosylation site was observed in *C. macrocephalus* C8γ after the NetNGlyc program analysis.

A BLASTX homology search revealed high similarity to C8γ from fish and mammals, and much lower to other known lipocalins (Table 3). Multiple alignment of amino acid sequence alignment of *C. macrocephalus* C8γ and other known C8γ indicated three conserved cystein residues that involved in formation of disulphide bond in most C8γ proteins (Fig 4).

3.3. Phylogenetic analysis of *C. macrocephalus* C3 and C8γ

Phylogenetic analysis of *C. macrocephalus* C3 with C3 molecules from other species was performed using the Clustal software, based on the deduced amino acid sequence alignment of the C3 α-chains. The resulting tree showed that all the bony fish formed a cluster (Fig. 5). *C. macrocephalus* C3 were grouped with zebra fish and carp C3 indicating that the walking catfish (belong to order Siluriformes) and these two fish species (belong to order Cypriniformes) were phylogenetically close species. However, among these three species, zebra fish and carp, which belong to the same order, are strongly genetically related.

The amino acid sequence alignment of full-legth *C. macrocephalus* C8γ with other members of lipocalin family was used to generate a phylogenetic tree. The unrooted phylogenetic tree obviously indicated that *C. macrocephalus* C8γ is more closely related to C8γ of fishes and mammals than to other lipocalins (Fig. 6).

Table 3 Amino acid comparison between *C. macrocephalus* C8γ and other known lipocalin proteins.

BLAST match	GenBank Accession no.	% identity/ similarity
C8γ (Zebrafish, *Danio rerio*)	NM200863	50/68
C8γ (Rainbow trout, *Oncorhynchus mykiss*)	NM001124408	44/68
C8γ (Human, *Homo sapiens*)	NM000606	36/59
C8γ (House mouse, *Mus musculus*)	NM027062	36/56
α-1-Macroglobulin (Cattle, *Bos taurus*)	BC102637	30/54
α-1-Macroglobulin (Human, *H. sapiens*)	NM001633	25/48
Prostaglandin D-synthase (Zebrafish, *D. rerio)*	NM213634	22/46

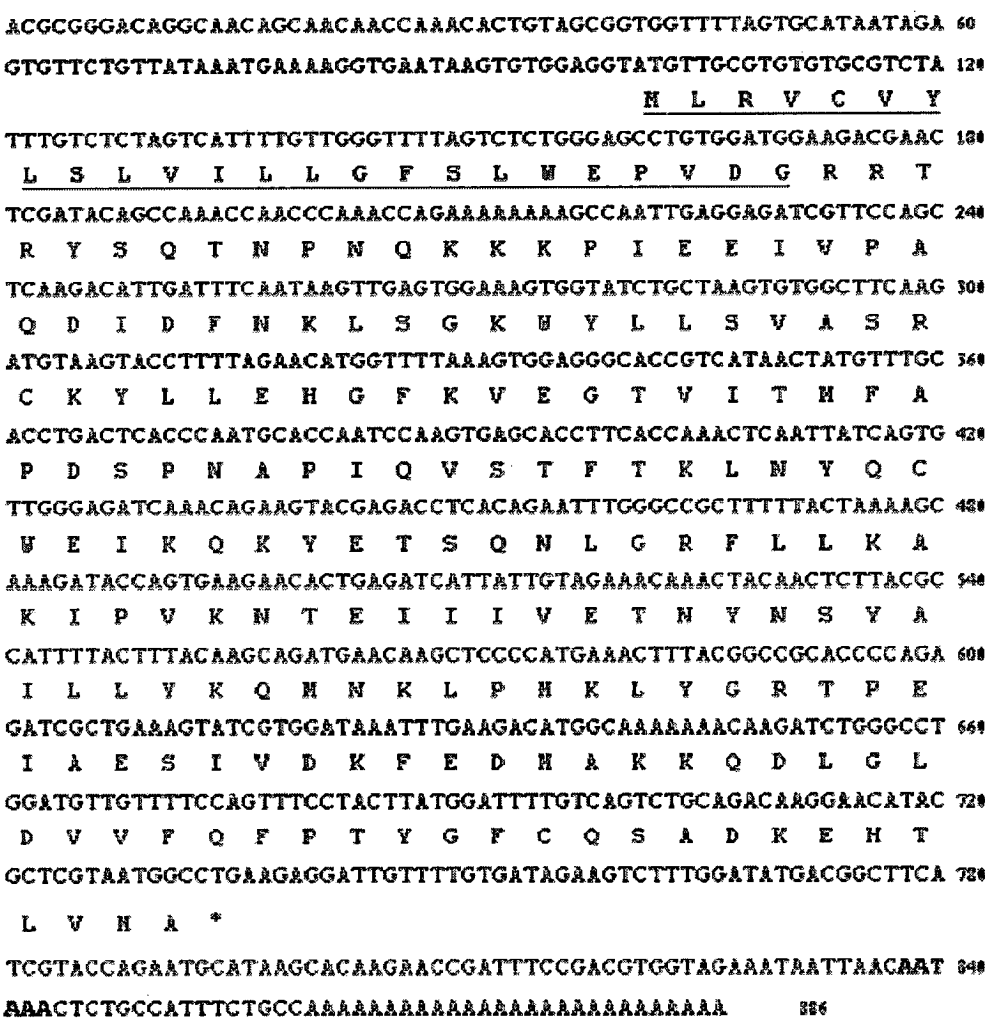


Fig.3 Full-length nucleotide and deduced amino acid sequences of C. *macrocephalus* C8γ cDNA

Fig. 4 Amino acid sequence alignment of *C. macrocephalus* C8γ (Catfish C8γ, Genbank accession no. AB474962), *Danio rerio* C8γ (Zebrafish C8γ, Genbank accession no. NM200863), rainbow trout C8γ (trout C8γ, Genbank accession no. NM001124408) and *Homo sapiens* (Human C8γ, Genbank accession no. NM027062). Conserved Cys residues are indicated with arrows.

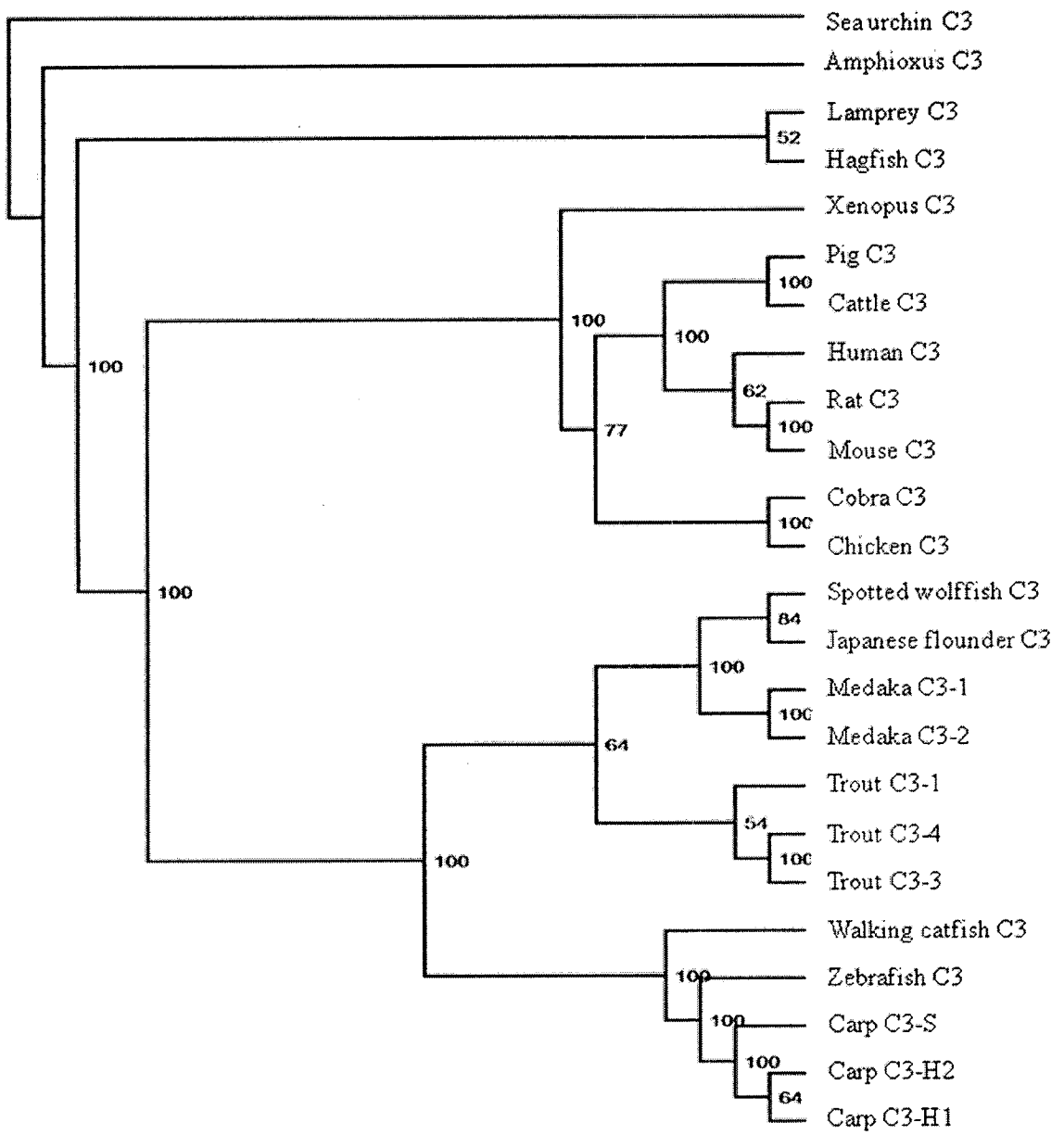


Fig. 5 Phylogenetic relationships of C3 proteins from different species. The tree was conducted by the neighbor-joining method based on the deduced amino acid sequences of C3s aligned in Fig. 2.

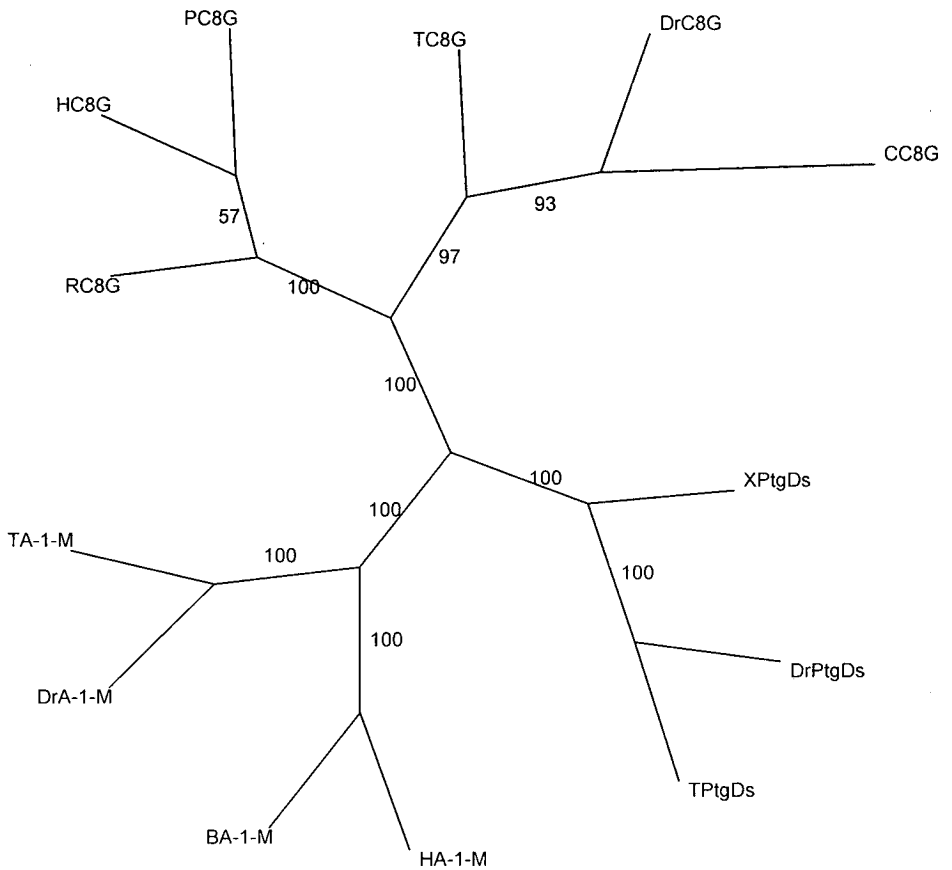


Fig. 6 Unrooted phylogenetic tree various lipocalins. Amino acid sequence aligments of *Bos taurus* α-1-microglobulin (BA-1-M), *Danio rerio* α-1-Microglobulin (DrA-1-M), *Homo sapiens* α-1-microglobulin (HA-1-M), *Oncorhynchus mykiss* α-1-microglobulin (TA-1-M), *D. rerio* prostaglandin D-synthase (DrPtgDs), *Oncorhynchus mykiss* prostaglandin D-synthase (TPtgDs), *Xenopus laevis* prostaglandin D-synthase (XPtgDs), *Clarias macrocephalus* C8γ (CC8G), *D. rerio* C8γ (DrC8G), *H. sapiens* C8γ (HC8G), *Sus scrofa* C8γ (PC8G), *Orytolagus cuniculus* C8γ (RC8G), *Oncorhynchus mykiss* C8γ (TC8G) were used to generate the tree by the Phylip program.

2.5. Tissue distributions of *C. macrocephalus* C3 and C8γ mRNA

Tissue expression profiles of *C. macrocephalus* C3 and C8γ mRNA were studied by RT-PCR. As shown in Fig. 7 and Fig. 8, *C. macrocephalus* C3 and C8γ mRNA expression was detected mainly in liver (lane 4). Expression was also observed at low level in brain (lane 1), heart (lane 2) and muscle (lane 7) for *C. macrocephalus* C3 and in kidney (lane 3), spleen (lane 5), intestine (lane 6) and muscle (lane 7) for C8γ. Expression of β-actin used as internal control was observed in all tested tissues.

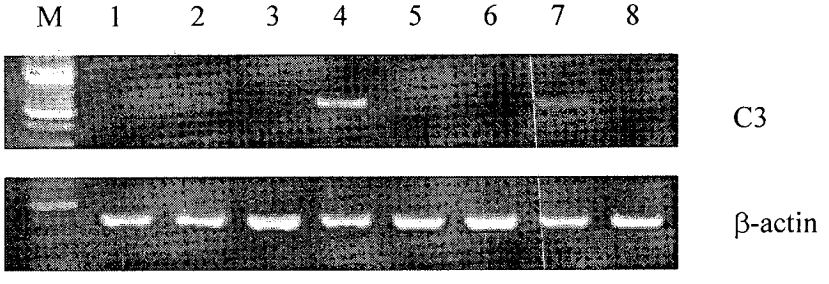


Fig. 7 Expression of *C. macrocephalus* C3 gene in various tissues. Lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, spleen; lane 6, intestine; lane 7, muscle; lane 8, ovary. M indicates the molecular weight marker (100 bp. ladder).

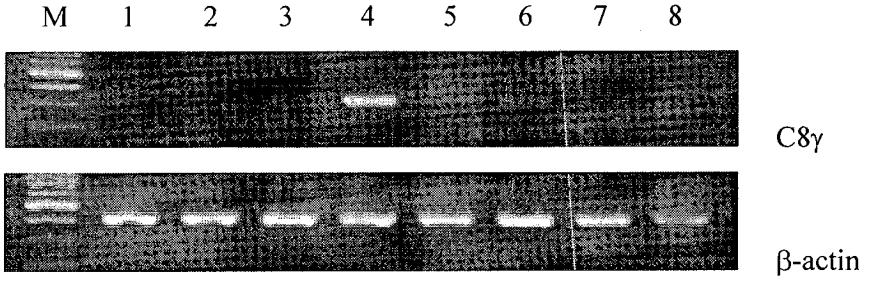


Fig. 8 Expression of *C. macrocephalus* C8 gene in various tissues. Lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, liver; lane 5, spleen; lane 6, intestine; lane 7, muscle; lane 8, ovary. M indicates the molecular weight marker (100 bp. ladder).

2.6. Developmental expression of *C. macrocephalus* C3 and C8γ mRNA

The expression levels of *C. macrocephalus* C3 and C8γ mRNA were determined during catfish development at 1, 3, 5, 10, 20 and 30 days after hatching. *C. macrocephalus* C3 and C8γ expressions were detected at all tested ages. Both *C. macrocephalus* C3 and C8γ were found to be expressed at a low level at 1, 3 and 5 days post hatching. Strong expressions were observed at 20 and 30 days after hatching. Over all, transcripts of *C. macrocephalus* C3 and C8γ gradually increased as development progressed (Fig. 9).

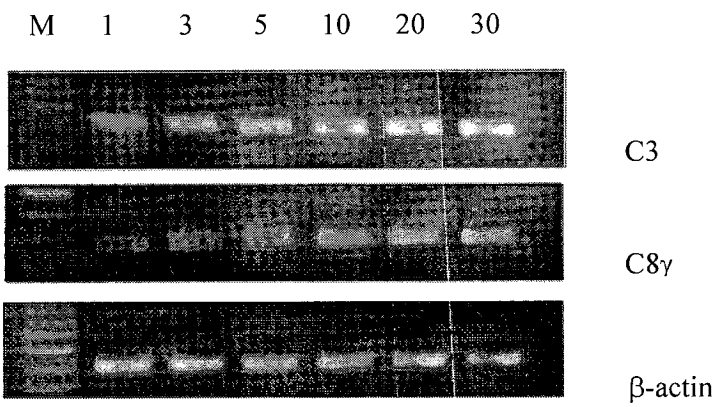
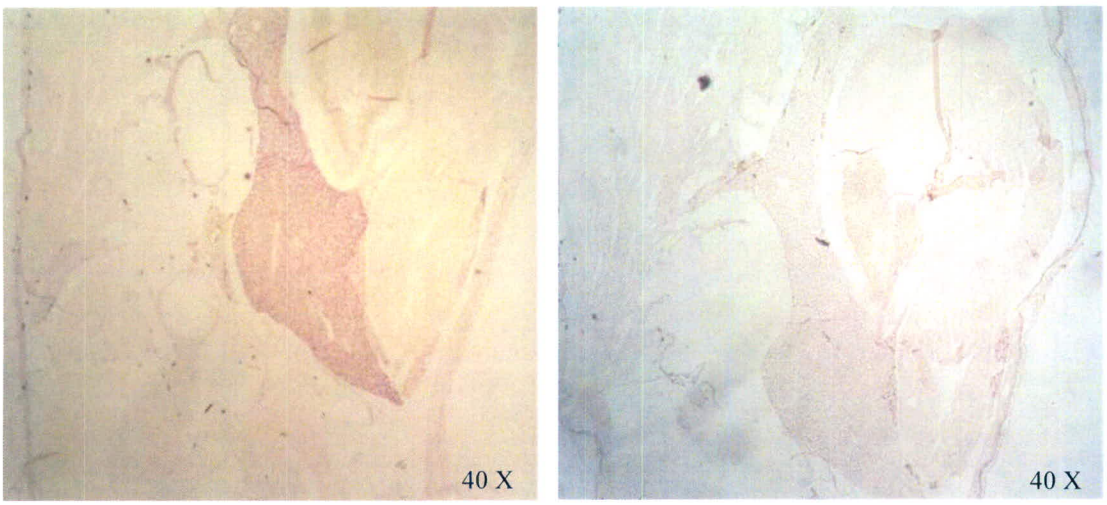


Fig. 9 Semi-quantitative RT-PCR analysis of C3 and C8 in the different larval stages of *C. macrocephalus*. M indicates the molecular weight marker (100 bp Ladder).

2.7. Expression of *C. macrocephalus* C3 and C8γ mRNA in fish larvae

Localization of *C. macrocephalus* C3 and C8γ mRNA expression in fish larvae were determined by *in situ* hybridization. Fish larval sections were prepared and hybridized with the sense and anti-sense probes for *C. macrocephalus* C3 and C8γ mRNA. Hybridization performed with the *C. macrocephalus* C3 anti-sense probe showed staining of hepatocyte cells (dark purple hepatocytes) (Fig. 10), whereas no hybridization was observed in any

sections using the *C. macrocephalus* C8γ anti-sense probe. There was no staining of the control sections hybridized with the *C. macrocephalus* C3 and C8γ sense probes.



(A) (B)

Fig. 10 Expression of *C. macrocephalus* C3 mRNA in liver hepatocytes performed by *in situ* hybridization with anti-sense probe (A) and sense probe (B).

2.8. The relative expression levels of *C. macrocephalus* C3 and C8γ mRNA after β-glucan administration

The expression of *C. macrocephalus* C3 and C8γ transcripts was examined in catfish fingerlings fed with β-glucan for 1, 3, 7 and 14 days. Semi-quantitative PCR analysis showed significant up-regulation of *C. macrocephalus* C3 mRNA in all β-glucan feeding groups (Fig. 11), compared to the 0 day unstimulated group ($P<0.05$). The highest level of C3 gene expression was observed in catfish fingerlings fed with β-glucan for 7 days. There was no difference of the expression levels of *C. macrocephalus* C8γ mRNA between β-glucan feeding groups and the 0 day unstimulated control (data not shown).

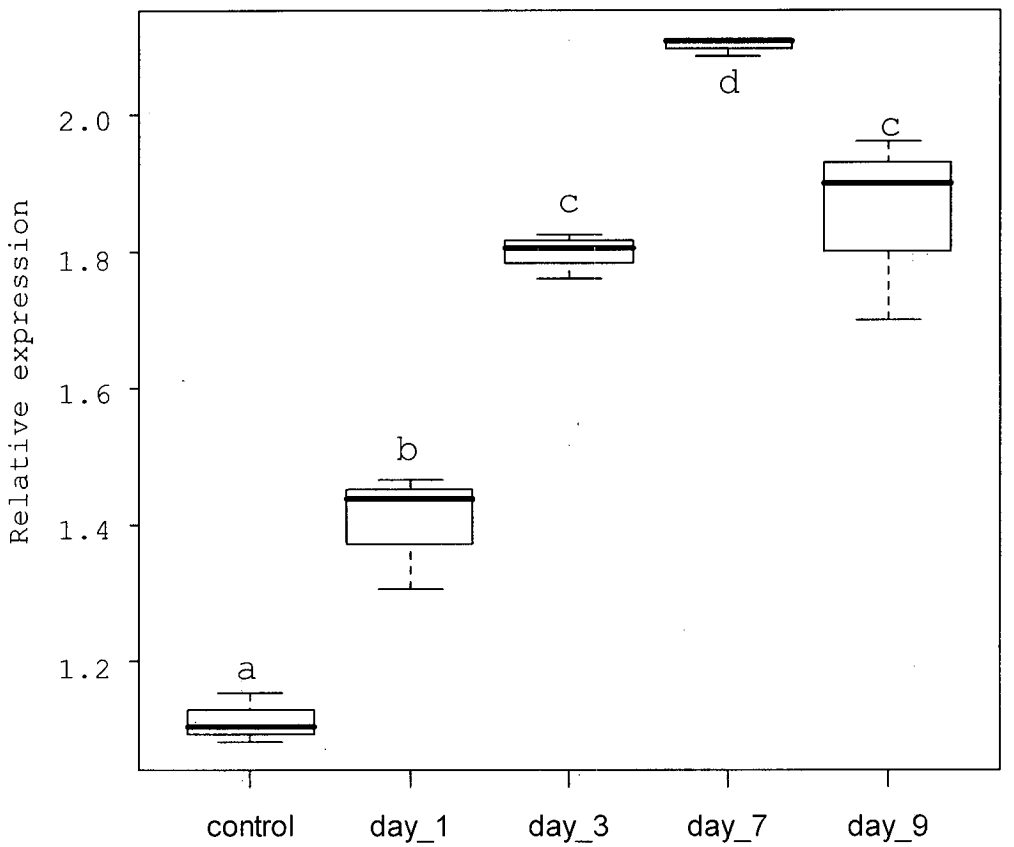


Fig. 11 Relative expression of *C. macrocephalus* C3 in liver from *C. macrocephalus* administered with β-glucan. Fish were collected at 1, 3, 7 and 9 days post β-glucan feeding. *C. macrocephalus* mRNA levels were determined by semi-quantitative RT-PCR and standardized according to the respective β-actin mRNA levels. Statistically significant differences are indicated by different letters ($p < 0.05$).