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Molecular diversity and genomic organisation of the α , β and γ eye lens crystallins from the Antarctic toothfish *Dissostichus mawsoni*

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Abstract

The eye lens of the Antarctic toothfish living in the -2 °C Southern Ocean is cold-stable. To investigate the molecular basis of this cold stability, we isolated, cloned and sequenced 22 full length crystallin cDNAs. We found two α crystallins ($\alpha A, \alpha B$), six β crystallins ($\beta A1, \beta A2, \beta A4, \beta B1, \beta B2, \beta B3$) and 14 γ crystallins ($\gamma N, \gamma S1, \gamma S2, \gamma M1, \gamma M3, \gamma M4, \gamma M5, \gamma M7, \gamma M8a, \gamma M8b, \gamma M8c, \gamma M8d, \gamma M8e, and <math>\gamma M9$). Alignments of α, β and γ with other known crystallin sequences indicate that toothfish α and β crystallins are relatively conserved orthologues of their vertebrate counterparts, but the toothfish and other fish γM crystalling genes screened from a toothfish BAC library indicated α crystalling genes occurred in a single genomic region of ~266 kbp, β crystalling genes in ~273 kbp, while the γ crystallin gene family occurred in two separate regions of ~180 and ~296 kbp. In phylogenetic analysis, the γM isoforms of the ectothermic toothfish displayed a diversity not seen with endothermic mammalian γ crystallins. Similar to other fishes, several toothfish γ crystallins are methionine-rich (γM isoforms) which may have predisposed the toothfish lens to biochemically attenuate γ crystallin hydrophobicity allowing for cold adaptation. In addition to high methionine content, conservation of $\alpha\beta$ crystallins could have allowed for greater evolutionary plasticity resulting in increased polydispersity of γ crystallins contributing to the cold-stability of the Antarctic toothfish lens.

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1. Introduction

The Antarctic toothfish *Dissostichus mawsoni* is a large predatory fish that is endemic to the waters of the Southern Ocean. It is a member of the teleost suborder Notothenioidei which includes about 45% of the fish species and accounts for over 95% of the fish biomass in the sub-zero (-2 °C) continental shelf waters of Antarctica. The Antarctic notothenioid fishes represent an important and diverse taxonomic group of cold adapted ectothermic vertebrates (Eastman, 1993; Parker et al., 2002; di Prisco et al., 2007). Many of the fishes which

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inhabit the perennially sub-zero seawater have undergone a myriad of physiological and biochemical adaptations (Detrich, 1991; Behan-Martin et al., 1993; Eastman, 1993; Cheng and Chen, 1999), most notable of which was the evolution of a blood borne antifreeze glycoprotein which prevents these fish from freezing (DeVries, 1988; Cheng and Chen, 1999). Adaptations in the visual system of these fishes have been recently described, specifically within the retinal organisation of several Antarctic species (Pointer et al., 2005) and in the eye lens of the Antarctic toothfish *D. mawsoni* (Kiss et al., 2004).

The eye lenses of endothermic vertebrates, as well as ectothermic tropical fishes display a phenomenon known as cold cataract (Delaye et al., 1982; Banh and Sivak, 2004; Kiss et al., 2004) which does not occur within the lenses of the Antarctic toothfish at their normal body temperature of -2 °C (Kiss et al., 2004). The phenomenon of cold-cataracts has been used to

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model cataracts of non-thermal aetiology as well as other related protein aggregation diseases (sickle cell anaemia, Alzheimer's dementia) in humans (Benedek, 1997). The common denominator in these seemingly unrelated pathologies is a protein instability caused by changes in the micro-environment of the particular proteins, which in the case of cataracts are the lens crystallins. Vertebrate eye lenses are composed of fibre cells arranged in an onion-like structure that are packed full of α , β and γ crystallins (Bloemendal et al., 2004). It is one or more of the γ crystallin proteins that are responsible for cold-cataracts in mammals (Broide et al., 1991). Cold-cataracts in endothermic mammals were well-documented with current opinion that coldcataracts can be attributed to a liquid-liquid phase separation of cold-sensitive crystallin proteins (Clark and Benedek, 1980; Delaye et al., 1982; Siezen and Benedek, 1985; Liu et al., 1996; Banh and Sivak, 2004). Because the Antarctic toothfish lens does not display a cold-cataract, it presents us with the inverse model system for understanding not only lens crystallin thermal stability, but possibly protein stability in general.

Previous work has suggested that a reduced proportion of hydrophobic residues in many proteins could be an adaptation to prevent cold denaturation at temperatures close to or below 0 °C (Privalov, 1990). Although this proposal is very attractive and has been convincing in limited *in vitro* biophysical studies, it remains to be conclusively demonstrated as a general strategy for cold adaptation of structural (non-enzymatic) proteins in cold stenothermal organisms such as the Antarctic toothfish.

Our previous results (Kiss et al., 2004) suggested that biochemical differences between γ crystallins of the toothfish, tropical fishes and endothermic mammals were the principal factors contributing to the cold stability of the toothfish lens. If biochemical differences of the toothfish γ crystallins are in fact responsible for the increased stability, these differences might be encoded at the level of the primary structure. Moreover, as the eye lens is incredibly dense in crystallins (close to 1000 mg mL^{-1} in fishes (Kroger et al., 1994)) contributions to cold stability undoubtedly arise from interactions between all three $(\alpha\beta\gamma)$ crystallin molecules. To address the hypothesis that biochemical properties encoded by the toothfish crystallins impart low temperature stability, we isolated, cloned and sequenced the toothfish lens crystallin cDNAs. To frame the evolution of the γ toothfish crystallins, we have analysed them in conjunction with the available sequences from the tropical zebrafish and a number of previously unidentified sequences from the temperate Tetraodon nigroviridis (spotted green pufferfish).

2. Materials and methods

2.1. cDNA library construction

Lenses of the Antarctic toothfish, *D. mawsoni* (Norman) were collected from live specimens caught in McMurdo Sound, Antarctica, and stored at -80 °C. Total lens RNA was isolated using Ultraspec RNA isolation reagent (Biotecx, TX), and the poly(A)⁺ fraction was isolated from the total RNA using oligo (dT)-cellulose (Collaborative Research, MA) following standard protocols (Sambrook and Russell, 2001). A cDNA library was

constructed from poly(A)⁺ RNA using the Universal Riboclone cDNA Synthesis System (Promega, WI) per manufacturer instructions except for the following modifications. In place of the *Eco*RI adaptors, *Bam*HI/*Xmn*I non-palindromic adaptor formed with complimentary oligonucleotides 5'-d(GATCCGAAGG-GGTTCG)-3' and 5'-d(pCGAACCCCTTCG)-3' (New England Biolabs, MA) was ligated to the blunt-ended doubled stranded cDNA, and then partially filled-in with dGTP and dATP. The cDNAs were then ligated to the phagemid pBK-CMV (Stratagene, CA) previously digested with *Xho*I and partially filled-in with dTTP and dCTP, transformed into XL1-BLUE Supercompetent cells as per manufacturer's protocol (Stratagene, CA).

2.2. Library screening for crystallin cDNA clones

2.2.1. αB and αB crystallins

Recombinant clones from the lens cDNA library were screened to identify α , β , and γ crystallin cDNA clones by a combination of PCR-amplification with crystallin primers and Southern analyses of restriction digested plasmid DNA. For α crystallin, clones of the αA isoform were inadvertently identified by PCRscreening of 96 clones using a primer pair that targeted the full length aB coding sequence - alpha5 (5'-ATGGAAATTTCTATC-CAGCATCCCTGG-3') and alpha3 (5'-TCCACAGATGATAG-GGATGCTGCG-3') based on reported zebrafish (Danio rerio) αB crystallin cDNA sequence (Posner et al., 1999). AlphaB crystallin was isolated using primers DRAB1 (5'-CCTGGATGT-GAAGCACTTCT-3') and DRAB1-2 (5'-CAACACGCCGT-CAGAGGATA-3') specific to sequence sites within the conserved α crystallin domain of zebrafish αB cDNA. An expected amplicon of ~250 bp was obtained from clones and subsequently sequenced to contain full length αB crystallin cDNA clone.

2.2.2. β and γ crystallins

Since β and γ crystallins are members of the same superfamily and they share sequence homology in their core structural regions (Slingsby and Clout, 1999), we were able to identify putative β/γ crystallin cDNA clones by low stringency hybridisation of a Southern blot of SstI & XhoI digested clones from the library using a ³²P-labeled 407 bp partial γ crystallin cDNA from D. mawsoni. This 407 nt partial y crystallin cDNA was obtained by RT-PCR amplification using previously reported primers CPG5 (5'-GAGGACAGGAACTTCC-AGGG-TCGC-3') and CPG3 (5'-GCCTCTGTAGTGGGGGCTGCTCA-TAC-3') designed to the Asian (or common) carp Cyprinus carpio yM2 crystallin (Chang et al., 1988); this partial cDNA was later found to correspond to nucleotide 46-453 of the toothfish yM8e. Initially, 50 randomly selected clones were restriction digested and screened in this low stringency manner. Of the 50 clones, 36 were identified by positive hybridisation which when sequenced gave five full length (3γ and 2β) and 11 partial unique β/γ crystallin cDNAs. Tentative identification of the sequences as β/γ crystallin isoforms came from nucleotide comparisons at GenBank/NCBI (BLASTN algorithm). These 11 partial β/γ cDNAs were used as templates to design isoform specific oligonucleotide primers ($T_{\rm m} \ge 65$ °C) to obtain full length cDNA sequences by 5' and 3' RACE.

Most of the known vertebrate β crystallin cDNA isoform sequences are conserved homologues, thus partial cDNA sequences that were not initially identified in the low stringency Southern blot were obtained by designing isoform specific primers based on an alignment of known mammalian and chicken β isoform sequences we obtained from GenBank/NCBI. Three toothfish β crystallins (β A2, β A4, β B3) were obtained by PCR screening of 288 clones in three 96-well plates using the following pairs of primers: β A2F 5'-CCCTCGCTACGAGGCCTGGAGCGGAAA-3', β A2R 5'-CGGATGGACTGCACCTGGTTGGTGTGAGCC-3'; β A4F 5'-CTGGAAGATCGTGGTGTGGGGATGA-3', β A4R 5'-CGTGCGTGTCGCTGTCCAG-3'; β B3F 5'-GCCCCCCTCCA-GATCGACAGCC-3', and β B3R 5'-GCGGCGCACCGACTG-CATCA-3'.

Sequences of several partial γ crystallins cDNA (γ S2, γ M7, γ M9) were used to design primers to obtain the full length crystallin cDNA sequences by PCR of clones from the cDNA library. For the γ S2 isoform we designed sense and antisense primers (γ S2F 5'-TGATAGCATCAAGTCATGCCGCTCTAT-CCAAAA-3', γ S2R 5'-AAATTCAGGGTTCTGCAGCAGGG-GG-3'). For the γ M7 and γ M9 we used isoform specific primers Dm γ M7-R 5'-TTCGTGCATCTGACCGACATGTCGGACGC-3' and γ M9-R 5'-TTAGCAGATTTTTGCAAACATTAGTTGC-3' paired with a vector (pBK-CMV) specific primer T7AS 5'-CAGTGAATTGTAATACGACTCACTATAGGGC-3'. A total of 384 clones in four 96-well microtitre plates were screened by this PCR method to obtain the full length cDNAs of these 3 isoforms.

The remainder of the γ cDNA clones reported herein were identified by Southern Blot screening of recombinant clones with the 407 nt γ M8e probe. Recombinant crystallin clones were sequenced in both directions using ABI Prism BigDye 3 Terminator reagent (Applied Biosystems, CA, USA) and the reactions analysed on a ABI3730*xl* sequencer (Applied Biosystems) at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois, Urbana-Champaign). Screening approximately 800 clones by Southern blot and PCR methods yielded two α , six β and fourteen γ crystallin cDNA sequences.

2.3. Genomic Southern Blots of D. mawsoni DNA

Approximately 20 µg of genomic DNA from *D. mawsoni* liver was digested overnight with 50 units of *Eco*RI. Digests were resolve on a 1% agarose gel, vacuum blotted to Hybond-N nylon membrane (Amersham Biosciences), UV-crosslinked and hybridised with ³²P-labeled probes (see figure legends) in PerfectHyb (Sigma–Aldrich, St Louis, MO, USA). The blot was washed to 55°C in 0.1XSSC/0.5%SDS, and autoradiographed on Kodak Biomax X-ray film.

2.4. Screening and analyses of BAC clones containing crystallin genes

A large-insert DNA bacterial artificial chromosome (BAC) library was constructed for *D. mawsoni* using red blood cells based on previously published methods (Miyake and Amemiya,

2004). A total of 67,584 recombinant clones, equivalent to about 6X genome (2C) coverage were robotically picked and archived in 384-well master culture plates, as well as printed on nylon hybridisation membranes as a macro-array for screening target genes. The α crystallin gene family was screened with full length $^{32}\text{P-labelled cDNAs}$ for αA and $\alpha B,$ and the β crystallin gene family with full length BA2 and BB2. Gamma crystallin gene regions were screened with the same 32 P-labelled γ M8e probe used in the initial identification (at higher stringency) of the 14 γ crystallin isoforms. Putative positive clones were then re-streaked from archived glycerol stocks to single colonies. Recombinant BAC plasmids were prepared from liquid cultures of single colonies, digested with NotI and electrophoresed on a pulsed field system (CHEF Mapper XA System, BioRad). Low range PFG (pulsed field gel) markers (NEB) were used to estimate the size of the BAC clone inserts. To confirm the clones were positive for crystallin genes, the NotI digested BAC DNA was vacuum blotted from the gel onto Hybond-Nylon membrane, and the membrane was hybridised with the same α,β or γ probes (as appropriate) used in the initial BAC library macro-array screening. The verified positive clones were then digested with HindIII, and separated on a 1% agarose gel (40 cm) along with DNA Marker II for Genomic DNA Analysis (Fermentas, MD), stained with SYBR Green I (Molecular Probes/Invitrogen) and photographed using Kodak 1D Image Analysis System (Eastman Kodak Company, New Haven, CT, USA). The BAC clone fingerprint (HindIII restriction fragment mobilities) images were edited with IMAGE v3.10 (www.sanger.ac.uk/Software/Image/), and imported into FPCv8.2 (Fingerprint Contig; www.agcol. arizona.edu/software/fpc/) for construction of clone order and overlap (Nelson and Soderlund, 2005).

Table 1

Dissostichus mawsoni eye lens crystallin cDNA sequences and their GenBank accession numbers

Sequence name	cDNA length (bp)	Amino acid length (predicted)	GenBank accession		
αA	1012	176	DQ143965		
αΒ	1452	164	DQ147910		
β A 1	1301	196	DQ147911		
β A 2	850	196	DQ143966		
βΑ4	712	196	DQ143967		
β B 1	777	230	DQ143968		
β B 2	864	208	DQ143669		
β B3	1015	253	DQ143670		
γN	752	183	EU016230		
γS1	737	176	DQ143671		
γS2	891	175	DQ143672		
γM1	620	178	DQ143673		
γM3	603	176	DQ143675		
γM4	613	180	DQ143676		
γM5	765	176	DQ143678		
γM7	645	175	DQ143679		
γM8a	656	182	DQ268581		
γM8b	623	184	DQ143681		
γM8c	677	183	DQ143682		
γM8d	619	184	DQ143683		
γM8e	628	177	DQ143674		
γM9	672	178	DQ143677		

2.5. Nomenclature

Nomenclature of the α and β crystallins from *D. mawsoni* was based on homology with vertebrate crystallins. Gamma crystallin designations for toothfish were based on the previous nomenclature " γ M" of zebrafish and carp as much as possible (Chang et al., 1988; Wistow et al., 2005). Further designations were based on BLASTN (GenBank/NCBI), our phylogenetic reconstruction, identity matrices and manual inspection of aligned amino acid sequences.

2.6. Bioinformatics

DNA sequences were edited for quality and to remove vector and other non-cDNA portions. Amino acid translations of the crystallin cDNAs were aligned using ClustalW in BioEdit v7.0.5.2 (Hall, 1999), and manually checked for alignment quality. Amino acid identity matrices (IDENTIFY, BLOSUM62, GONNET) were generated using BioEdit v7.0.5.2.

For phylogenetic analyses, γ crystallins from *D. mawsoni* and other ectothermic species (see Table 1 Suppl. Tables 1 and 2 for GenBank accession nos.) were first aligned by the translated amino acid sequences. Nucleotide sequences were aligned based on this amino acid alignment using CodonAlign2.0 (Hall, 2004). All phylogenetic reconstructions were performed using DNA sequence alignments unless indicated otherwise. Tree construction was done by MrBayes3.1.1 using the evolutionary model GTR+I+ Γ (Waddell and Stell, 1997) as selected by Mr. Modeltest v2.0 (Nylander, 2004). Four runs using four chains and 4,000,000 generations were performed. All other parameters in MrBayes 3.1.1 were left at default. Stationarity was assessed using the "sump burnin=2500" command and examining the



Fig. 1. Alignment of the *in silico* translation of cDNAs of the Antarctic toothfish (Dm) αA (A) and αB (B) crystallins with zebrafish (Dr), Asian catfish (Cf, Cb), cow (Bt) and human (Hs). (A) The identity between the αA homologues is very high (black background) in the typically more divergent N-terminal domain, as well as the conserved α crystallin domain. (B) Although the α crystallin domain of the αB sequences is also highly conserved, the more divergent N-terminal domain and the C-terminal extension are more variable than in the αA crystallin. The domain region designations are based on previous reports (Caspers et al., 1995; Narberhaus 2002). Grey regions indicate \geq 50% similarity between sequences.

plot of the generation versus the log likelihood values. A 50% majority rule consensus tree was generated using the command "sumpt burnin=2500".

3. Results

3.1. a Crystallins

The *in silico* translation of the α A and α B lens crystallin cDNA sequences from the Antarctic toothfish *D. mawsoni* are shown in Fig. 1 (GenBank Accession nos. in Table 1). Toothfish sequences were aligned with zebrafish *D. rerio* (Dr) (Posner et al., 1999; Runkle et al., 2002), catfish *Clarias fuscus* (Cf) and catfish *Clarias batrachus* (Cb) (Yu et al., 2004), with cow *Bos taurus* (Bt) and human *Homo sapiens* (Hs) sequences (GenBank accession nos: NP_776714, NP_000385, NP_776715, AAP36581). Designations of the N-terminal region/domain, α crystallin domain and C-terminal extensions are based on previously published reports (de Jong et al., 1993; Caspers et al., 1995; Narberhaus, 2002).

The α A and α B alignments (Fig. 1) highlight the conservation of the α crystallin domain between these five fish and mammalian taxa. The amino acid identities between the α A sequences range from a low of 68.7% between toothfish and human, to a high of 94.2% between cow and human (Table 2). Among the fish species analysed, α B sequences are more divergent from each other than are the α A sequences (Table 2). The greatest variation is in the N-terminal region of the α B crystallin (Fig. 1B). In contrast, the N-terminal region of the α A crystallin is highly conserved between fish and mammals, thus accounting for the greater sequence similarity of α A between these phylogenetically distant species (Fig. 1A).

Southern blot of *Eco*RI digested *D. mawsoni* genomic DNA probed with ³²P-labeled full length *D. mawsoni* cDNAs for α A and α B resulted in hybridisation to three distinct bands (3, 1, 2) in decreasing intensity (Fig. 2). The presence of three bands is consistent with the presence of three α isoforms in zebrafish and catfish (Yu et al., 2004; Smith et al., 2006). It is tempting to suggest that the intensity of the three bands in the Southern blot is representative of the gene copy number, isoform type and

Table 2 Amino acid identities (percent identical) based upon the alignments of αA and αB crystallins presented in Fig. 1

Sequence name	Dm aA	$Dr \alpha A$	Cf αA	Bt αA	Hs αA
Dm αA	100	77.2	72.7	71.0	68.7
Dr aA		100	81.5	73.2	72.1
Cf αA			100	71.0	70.4
Bt αA				100	94.2
Hs αA					100
Sequence name	$Dm \ \alpha B$	$Dr \; \alpha B$	$Cf\alpha B$	Bt αB	Hs αB
Dm αB	100	47.0	51.4	48.2	47.7
Dr αB		100	56.5	60.5	59.4
Cb aB			100	62.5	61.9
Bt αB				100	97.1
					100



Fig. 2. Genomic Southern blot of *D. mawsoni Eco*RI digested DNA. Digested DNA (20 μ g) was probed with full length ³²P-labelled cDNA probes from α A and α B from cloned from *D. mawsoni*. The number arrows to the left of the blot indicate the three bands that hybridised to the probes. It is known that there are at least two (α A and α B) genes in toothfish (this study) and zebrafish (Dahlman et al., 2005), in addition we isolated a partial fragment of a third α B₂ (see Results section).

possibly expression levels. However, attempts to isolate a complete second α B cDNA sequence were unsuccessful. A partial second α B sequence was isolated giving an *in silico* translation tentatively designated α B_b "TKDGVVEITGKHEDRKDEHG-FVSRSFTRKYTLPSNTDVEKVNSSL", corresponding to position 86 to 130 of the full length *D. mawsoni* α B sequence (Fig. 1) which spans the conserved ' α crystallin' domain.

3.2. B Crystallins

The β crystallin protein sequences from the toothfish are presented in Fig. 3 and aligned with the zebrafish, cow and human sequences. The alignment highlights the highly conserved four domains (coloured underscores) which form the Greek-key fold motifs. Within the β crystallins, the β A3 and β A1 differ by their initiation codons; the translational start of the β A3 transcript is upstream of the β A1 (McDermott et al., 1997; Bloemendal et al., 2004). The six β crystallin isoforms isolated from toothfish are clear homologues of the β crystallin family. They are unambiguously identified when contrasted with each other (Table 3) and compared to mammalian orthologues (Fig. 3). The toothfish β A1, β A2, β A4 sequences show a high degree of sequence identity (100% identity – black background) or sequence similarity (\geq 50% similarity – grey background) to both the zebrafish and mammalian (cow and human) β crystallins (Fig. 3). The β B1, β B2, and β B3 toothfish sequences again show conservation within their domains (coloured underscores) to zebrafish, cow and human (Fig. 3). The proline– arginine rich repeat, designated a 'PAPA-arm' (Bloemendal et al., 1984; Hejtmancik et al., 1986; Coop et al., 1998) that is found N-terminal to the first domain of the β B1 mammalian crystallin, is similar to the PAPA domain found in the Cterminal domain of the toothfish β B3 crystallin. An analogous PNPN domain is also found in the C-terminal domain of zebrafish β B3 (Fig. 3).

Dm_βA1 Dr_βA1 Bt_βA3 (βA1) Hs_βA3 (βA1)	10 20 30 40 50 60 70 80 90 100 110 120
Dm_βA2 Dr_βA2 Bt_βA2 Hs_βA2	-MA-TQQMEQMGQ FKITVWEBENFOCKROEBMLECONIMER-CFNKIRSIKVEN GEWVGYEYPEGOCOFILE
Dm_βA4 Dr_βA4 Bt_βA4 Hs_βA4	
Dm_βB1 Dr_βB1 Bt_βB1 Hs_βB1	MSQTAKSASSQGTDAKDKGAP-PAATSKATKTGEPGMGS FRMMMEDOBNFOGKMIEVQNECMNVCDR-CADRVRSIIVEC GPEVAFEOTNERGEMTIDE MSQTAKSATNQGTDAKEKGAPAPAATSKASKTGDEGFMGN VLIFLFDGDSNEOGRMMEVQMECMNVCSR-CADRVRSIIVEC GFFVAFEOTNERGEMT MSQPA-AKASATAAVNPGPDGKGKAGPPPGPAPGSGPAPAPAPAPAPAPAPAPAAKAELPPGS VLLVVPEOENFOGRRVEFSGECINLGDR-CFERVRSIIVTS GPWVAFEOSNERGEMFULE MSQAAKASASATVAVNPGPDTKGKGAPPAGTSPSPGTTLAPTTVPITSAKAAELPPGN VLLVVPELENFOGRRAEFSGECSNLADR-CFDRVRSIIVSA GPWVAFEOSNERGEMFULE
Dm_βB2 Dr_βB2 Bt_βB2 Hs_βB2	-MAADHQNPASKQQQPGTSA
Dm_βB3 Dr_βB3 Bt_βB3 Hs_βB3	-MSEQQSAPEQQAAGKSQGGAGASYKVFLPEPENFQGCKAEBSAEGKDVMEK-GLEKVGSVIVES GPWAGYDRHGFTGEQFILE
Dm_βA1 Dr_βA1 Bt_βA3(βA1) Hs_βA3(βA1)	130 140 150 160 170 180 190 200 210 220 230 240 KGDYPRPEMYSGSNSYRIERMISFRPI CCANHKE SRMTIPEKENMSGROFELC-DDYPSICAMGMINNEVCSMOIQG GAPVCYOPFGYRGYQYIMECDCRGGEYKCYREFGSHSOTPOIC FCDYPRMESMSGSNAYHIERIMSFRPI CCANHKE SKTTIPEKENMSGROFELC-DDYPSICAMGMENNEVCSMOIQG GAPVCYOPFGYRGYQYIMECDCHGGEYKCYREFGSHSOTPOIC FCDYPRMESMSGSNAYHIERIMSFRPI CSANHKE SKTTIPEKENTEGOWEIC-DDYPSICAMGMENNEVCSMOIQG GAPVCYOPFGYRGYQYIMECDHGGEYKHYREWGSHAOTFGVO RGEYPRWDAMSGSNAYHIERIMSFRPI CSANHKE SKTTIPEKENTEGROWEIC-DDYPSICAMGMENNEVGSMKIQC GAWVCYOYPGYRGYQYILECDHHGGDYKHWREWGSHAOTSGIO RGEYPRWDAMSGSNAYHIERLMSFRPI CSANHKE SKMTIPEKENTIGROWEIC-DDYPSICAMGMENNEVGSMKIQG GAWVCYOYPGYRGYQYILECDHHGGDYKHWREWGSHAOTSGIO
Dm_βA2 Dr_βA2 Bt_βA2 Hs_βA2	KGDYPRYEAWSGNSSYRTEHMLSFRPI KSANHSD SKVTMYDCEDFOGRKPEMC-DDYPSLOAMGMCSKEVPSIKUNS GAWVAYOFPGYRGYQYILERDRHOGEYRNYNEYSTOAHTNOVO KGDYPCYQAWSGNSSYRTEHMLSFRPI KCANHSD SKITMYECEDMLGRKPEMC-DDYPSLMAMGMCSKEVPSIKUNS GAWVGYQFPGYRGYQYIFERDRHOGEYRNYNEYSTOAHTNOVO KGDYPRMSAWSGSAGHHSDQULSFRFV LCANHSD SKVTLFEGENFOCORPELN-DDYPSLPSMGMASKDVGSLKVSS GAWVAYQYPGYRGYQYUERDHHSGEFRNYEPFCTOAHTGOLO KGDYPRMSAWSGSSSHNSNQLLSFRFV LCANHSD SKVTLFEGENFOCORPELN-DDYPSLPSMGMASKDVGSLKVSS GAWVAYQYPGYRGYQYUERDHHSGEFRNYEPFCTOAHTGOLO
Dm_βA4 Dr_βA4 Bt_βA4 Hs_βA4	RGEYPOCDAFGGSNAVHIERMTSFRPI ACANHRS CRMTIFERENFLORKGELS-DDYPSLOAMWNCNNEVGSLKTOS GAFVCYOYFGYRGYQYIMECDRHCGEFKHFREFCSHCOTFQIO RGEYPOCDSFGGSNAVHIERMTSFRPI SCANHRS CRMTIFERENTLGRKGELS-DDYPSLOAMGNCNNEVGSLRVOS GAFVCYOFFGYRGYQYIMECDRHCGEYKOFREFCSHSOTFQIO RGEYPSNDAWSGNTSYPAERLTSFRPV ACANHRD SRLTIFEQENFLGRKGELS-DDYPSLOAMGNDGNEVGSFHVHS GAWVCSOFFGYRGFQYVLECDHHSGDYKHFREWCSHAOTFQVO RGEYPSNDAWSGNTAYPAERLTSFRPV ACANHRD SRLTIFEQENFLGRKGELS-DDYPSLOAMGNDGNEVGSFHVHS GAWVCSOFFGYRGFQYVLECDHHSGDYKHFREWCSHAOTFQVO
Dm_βB1 Dr_βB1 Bt_βB1 Hs_βB1	KGEYPRWDWWSNSYRSDCIMSERPV RWDSL-5 HKICHPELSDEKONKWEIOEDDVFNIWNHGFCDR-VGSVRVPG GSWVGYOYPGYRGYQYLFECGDYKHYNDFSAYQPOMO KGEYPRWDWSNSYRSDCIMSERPF RWDPM-5 HKICHFELSDEKONKWEIOEDDVFNIWNHGFCDR-VGSVRVPG GAWVGYOYPGYRGYQYLFECGDYRHYNEFCAFOPONO KGEYPRWDWSSSYRSDRIMSFRFI KWDAQ-5 HKICHFEGANFKCNTMEIOEDDVPSLWVYGFCDR-VGSVRVSS GTWVGYOYPGYRGYQYLFECGDYRHWNEWGAFOPONO KGEYPRWDWSSSYRSDRIMSFRFI KWDAQ-5 HKICHFEGANFKCNTMEIOEDDVPSLWVYGFCDR-VGSVRVSS GTWVGYOYPGYRGYQYLFECGDYRHWNEWGAFOPONO
Dm_βB2 Dr_βB2 Bt_βB2 Hs_βB2	KGEYPRWDSWTNSRRSDTILAFRPI KVDSQ-D HKIVLYBNPSFACKITETIDDDVFSFHAHGYQEK-VSSVRVQS GTWVGYQYFGYRGYQYLFFXGEYNDSSEFGAEIFQIQ KGEYPRWDSWTNSRRSDCIVAFRPI KVDSQ-E HKIVLYBNPSFTGKXIETIDDDVFSFHAHGYHEK-VSSVRVQS GTWVGYQYPGYRGFQYLFFXGEFXBCSEFGALFQIQ KGEYPRWDSWTSSRRTDSISSIRFI KVDSQ-E HKITLYBNPNFTGKKMETIDDDVFSFHAHGYQEK-VSSVRVQS GTWVGYQYFGYRGLQYLFEXGDYNDSGDFGAFQFQVQ KGEYPRWDSWTSSRRTDSISSIRFI KVDSQ-E HKITLYBNPNFTGKKMETIDDDVFSFHAHGYQEK-VSSVRVQS GTWVGYQYFGYRGLQYLFEXGDYNDSSDFGAFQFQVQ
Dm_βB3 Dr_βB3 Bt_βB3 Hs_βB3	KGEYPRWDYWR - NSONSYSLFSLREL XVDGA-S HKLHLFENPGFTGREMEIVDDDVPSLWGHGFODR -VASVKALN GTWVGYLYFGYRGROFIFEK GDFKHWNDWE - APDFOIO KGEYPRWSTWT - NSONSFCLISIRPL RVDSA-D HKLHLFENCSFAGRKMEIVDDDIFSLWVHGFODR -VASAKAVN GTWVGYMYFGYRGROFVFEH GDYKHWNDWG - APEFOIO KGDYPRWDAWS - NSHHSDSLLSLRPL HIDGP-D HKLHLFENPAFGGRKMEIVDDDVPSLWAHGFODR -VASVKAIN GTWVGYEFFGYRGROYVFER GEYRHWNEWD - ANOFOLO KGDYPRWDAWS - NSRHSDSLLSLRPL HIDGP-D HKLHLFENPAFGGRKMEIVDDDVPSLWAHGFODR -VASVKAIN GTWVGYEFFGYRGROYVFER GEYRHWNEWD - ANOFOLO KGDYPRWDAWS - NSRHSDSLLSLRPL HIDGP-D HKLHLFENPAFGGRKMEIVDDDVPSLWAHGFODR -VASVKAIN GTWVGYEFFGYRGROYVFER GEYRHWNEWD - ANOFOLO

Fig. 3. Alignment of the *in silico* translation from cDNAs of the Antarctic toothfish (Dm) β crystallins against zebrafish (Dr), cow (Bt) and human (Hs). Immediately apparent is the extent of the identity of residues across the phylogenetically diverse taxa. Within the four conserved domains regions (alternating blue and green bars) there is high levels of identity across all of the β crystallins (black background) as well as substantial similarity (\geq 50% grey background). It is likely that only the β A1 is present for the toothfish (Dm_ β A1) as an upstream start codon (making an ' β A3' isoform) was not found in the cloned cDNA. The N-terminal regions of the toothfish β A1, β A2, β A4, β B2 and β B3 crystallins are highly conserved across all four taxa. Toothfish β B1 crystallin shows a high degree of conservation with zebrafish of the N-terminal linker element. Note the presence of a large poly-(PA) element in the C-terminal region of the toothfish β B3 crystallin, similar to the poly-(PN) element in the zebrafish β B3 crystallin.

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Fig. 3 (continued).

3.3. y Crystallins

A total of 14 distinct γ crystallin cDNAs from the lens of the Antarctic toothfish (Dm) were isolated, cloned and sequenced (Table 1). In silico translations of the γ cDNAs and their alignment with three γ crystallins from the Asian (common) carp C. carpio (Cc) (Chiou et al., 1986) and 16 γ crystallins from zebrafish D. rerio (Dr) (Wistow et al., 2005), 14 'unamed protein products' (which we propose are in fact γ ; see below and Fig. 4) crystallins from spotted green pufferfish T. nigroviridis, as well as several other γ crystallins from lipshark, blind Mexican cavefish, African clawed frog, Japanese firebelly newt, iguana and sea squirt are shown (Fig. 4). There is a high degree of sequence identity among the various toothfish isoforms (40.7% to 92.3%; Table 4) as well as high conservation between ectothermic species (black background in Fig. 4) and similarities $(\geq 50\%$ grey background Fig. 4). Of the fourteen Antarctic γ crystallin sequences, two (γ S1 and γ S2), are likely paralogues of the γS isoform and one was clearly an orthologue of a γN isoform (Pan et al., 1997; Bloemendal et al., 2004; Wistow et al., 2005). Based on alignment (Fig. 4), percent identities (Table 4) and phylogenetic analysis (Fig. 5), the remaining eleven

Table 3

Amino acid identities toothfish β crystallins (percent identical) based upon pairwise alignment against each other

Sequence name	β A 1	βΑ2	βA4	β B 1	β B 2	β B3
βA1	100.0	59.1	67.8	32.0	36.2	26.6
β A2		100.0	54.0	35.8	38.5	28.8
βA4			100.0	32.0	36.6	28.8
β B 1				100.0	48.2	40.2
β B2					100.0	42.6
β B 3						100.0

Antarctic γ crystallin cDNA sequences were designated (γ M) originally proposed for methionine rich γ crystallins from carp (Chang et al., 1988), but now adopted for other fishes as well as other aquatic animals such as some frogs (Lu et al., 1996). In naming the toothfish γ crystallins we used the orthologous name as assigned previously for carp and zebrafish (γ M1, γ M3, γ M4, γ M5, γ M7) where possible. In cases where there were not orthologues of toothfish γ crystallins, we continued to use the same γ M crystallin nomenclature, but used the next available " γ M#".

Five related γ crystallins from the toothfish, four of which all share a conserved nine amino acid N-terminal extension "MS (N/T)T(G/D)M(N/S)M(R/-)" and did not have clear homologues in zebrafish or carp, were assigned as $\gamma M8a \sim e$ (Figs. 4 and 5). These four $\gamma M8$ toothfish crystallins ($\gamma M8a \sim d$) consistently formed a distinct clade in phylogenetic trees, regardless of the method of tree construction (Fig. 5). In addition to the toothfish specific $\gamma M8$ isoforms there are clearly identified $\gamma M1$ crystallin with the three residue conserved "MMF" insertion between positions 102 and 104, which is also present in yM1 of zebrafish, carp, pufferfish and lipshark (Ccx_y1) (Fig. 4). No yMX sequence was identified from our toothfish lens cDNA library. However, given the resolution of our phylogram, we place the previously reported zebrafish yMX isoform (Wistow et al., 2005) very close to the γ S group indicating that this might be a γS 'caught in the act' of evolving into a more γM -like isoform (Fig. 5).

A phylogram of ectothermic and largely obligate aquatic γ crystallins is presented (Fig. 5) as a Bayesian 50% majority rule consensus tree. The evolutionary model used was the Generalised Time Reversible plus Invariant plus Gamma (GTR+I+ Γ) model of substitution as chosen by MrModelTest (Nylander 2004). We used the coding regions of the nucleotide sequences

of the cDNAs (*sans* 5'-UTRs, 3'-UTRs and stop codons) aligning the nucleotide sequences based on the amino acid alignments (Suppl. Fig. 1). The outgroup choice for this tree was urochordate sea squirt *Ciona intestinalis* because it represents an $\beta\gamma$ sequence from a basal chordate and an aquatic marine

ectotherm. (Franck et al., 2004; Shimeld et al., 2005; Riyahi and Shimeld, 2007).

In the construction of the phylogram we included the toothfish cDNAs cloned and sequences from this study as well as the recently reported zebrafish γ crystallins (Wistow et al., 2005).

	10 20	1 20 40 50	C 0	
	10 20 	30 40 50	60	70 80
ci_γ1	M		HGFNDIVSSIIVES	GTWFVFDDEGFSGP
Cc_YM1 Dr_YM1		G-KIIFYEDRNFQGRSYDCMSDCSDISS G-KVIFYEDRNFQGRNYECMSDCSDISS	YLSRVGSIRVES YLGRVGSIRVES	GCFMVYERNSYMGN GCFIVYERNGFMGN
Ccx_y1		G-KITEVEDRNFOGRSVECMSDCSDUTT	YMSRCQSCRVES YLNRCQSCRVES	GCEMVYERPNEMGM
Tn_YM1			YLNRCQSCRVES	GCFMVYERPNFMGN
Tn_γM3 Tn γM11	M	G-KIIFYEDKNFQGRSYECMSDCSDMTS G-KIIFYEDRNFQGRSYETSSDCSELTS	YLNRCSSCRVES	GCFMVYDRPNFMGN GCFMVYDRPNFMGN
Tn YM13	MTTTDMSM	G-KIIFFEDRNFOGRSFEISSDCSEIS G-KIIFFEBRNFOGRSFECMSDCADMSS	YLSRCQSCRVES YLSRCQSCRVES	GCFMVYDRPNFMGN GCFMVYERPNFMGN
Tn_YM10	M	G-KIIFYEDRNFQGRSYETSSDCSDMTS	VI.SPCOSCPVES	GCVMVYERPNEMGN
Tn_YM12	MR		YLSRCQSCKVES YLSRCHSCRVES	GCFIVYDRPNFMGN GCFMVYDRTNYMGN
Tn_YM9 Dr_YM2b	MHG	G-KIIFYEDRNFQGRSYE <mark>TS</mark> SDCADMSS KVIFYEDRNFQGRSYEC <mark>M</mark> SDCAD <mark>MS</mark> S	VT. GDOHGODIEG	
Dr_YM2c	MHG	KVIFYEDRKFQGRSYECMSDCADMSS		GCFMMYDRPNYMGN GCFMMYDRPNYMGN GCFMMYDRPNYMGN
Dr_YM2a	MTSTSMNT	C-RVVFYEDRNFQGRSYECMSDVADMSP M-KVTFYEDRNFQGRSYDCMSDCADFSS	FLNRCHSCRVES YMSRCHSCRVHS	GCHIMMIDREIMIGR
Cc_γM2 Am_γ1		M-KVTFYEDRNFQGRSYDCMSDCADFSS IFYEDRNFMGRSWECSGDCADMSS	YMSRCHSCRVHS YMNRCHSFRVMS	GCWMMYDQPNYMGN GCWMFYDQPNYMGH
Cf_γM2-1	M	G-KIIFYEGRNFTGRSWECSGDCPNMSS	YMNRCHSFRVMS YLNRCYSCRVES	GEIMWYDRPNIAMGN
Cf_YM2-2	M	G-KVIFYEDRNFMGRSYECSSDCSDMSS G-KVIFYEDRNFMGRSYEC <mark>S</mark> SDC <mark>S</mark> DMSS	YLSRCHSCKVER	GCWMVYDRPNFMGN
Cf_YS2 Cf_YS1		G-KVIFYEDRNFMGRSYECSSDCSDMSS G-KVTFYEDRNFMGRSYECTGDCSDMHS	YLSRCHSCKVER YLSRCHSCKVGR YMSRCHSCRVES	GCWMVYDRPNFMGN GCWMVYDRTNFMGN
Tn_YM2	<u>V</u> i	S-KIIFYEERNFQGRSYECKSDCPDMSS	YLSRCHSCRVER	GCFVVYDRINYMGN
Dm_YM8c Dm YM8d	MSNTGMNMR	G-KITFYEBKNFQGRSYECMNDCSDMSS G-KITFYEBKNFQGRSYELMNDCSDMTS	SLSR <mark>SQSCRVES</mark> NLSRCQSCRVES	GCFMVYDRNNYMGN GCFMVYDRSNYMGN
Dm YM8b	MSNTGMNMR	G-KIIFYEEKNFQGRSYECMNDCSDISS	YLSRCQSCRVES YLSRCQSVRVES YLSR <mark>SQ</mark> SCRVES	GCHMVYDRNNYMGN
Dm_YM8a	MSTTDMSM-	G-KIIFYEERNFQGRSHECMSDCADMSS	YLSRSQSCRVES	GCFMVYDRNNYMGN
Dm_YM1	МТМ	G-KI <mark>VFYEEK</mark> NFQGRSYECMSDCSDMSS G-KIIFYEDRNFQGRSYE <mark>TS</mark> SDC <mark>S</mark> DMSS	YLSRCOSCRVES YLSRCHSCKVES	GCFMTYERPNYMGN GCFMVY <mark>DHN</mark> NYMGN
Dm_γM8e Dm γM3	MI		HLSRCHSCRVES HLSRCHSCRVES YL4RCHSCRVES	GYFMVYDRINYIGN
Dm_YM9	MTM		YLTRCHSCRVES	GNFMVCDRPNYMGN
Dr_үМЗ Сс_үМЗ	M	G-KIIFYEDRNFQGRSYEC <mark>ST</mark> DCADMST G-KIIFYEDRNFQGRSYEC <mark>SSDCS</mark> DMST	YLSRCNSCRVES	GCFVVYDRPNFMGN GCFVVYDVPNYMGN
Dr_YM4	M	N-KIVFYEDRNFQGRSYETSSDCDDMS1		GCFMIYDRSNFMGN
Dm_YM4	MD№	G-RITFYBEKNFQGRSYBTSNDCPELTS	YLSRCNSCRVES	GLFMVYEKPNFMGQ
Tn_γM5 Dr_γM5	MGIKVRVWLGKVPEQSGKELDTRM	G-RIIFYEDKNFQGRSYETSSDCABLTS G-KIIFYEDRNFQGRNFESSGDCPELTA	YLSRCNSCRVES YLSRCCSCRVES	GCFMVYERPNYMGH GCFMVYEHSNFIGH
Dm_YM5	MS	G-KIVFFEGRNFQGRSYECMSDCSEITS	HIGROSSCRVES	GTFMVYDQPNFTGQ
Dr_YM6	MAI		YFTQCNSIRVES YFNRCNSIRVES	GCFMVYE <mark>H</mark> PNYMG <mark>Q</mark>
$Dm_\gamma M7$ $Dr_\gamma M7$	N	G-KIIFYEDRNFQGRSHECSSDSADLHS G-KIIFYEDRNFGGRYHECMSDCADLHS	VENDOUSTRVES	GCEMHYERPNYIGN GCEMVYDRIINISMGR
Tn_YM14		G-KIIFYEDRNFQGRSYECSSECSDLHS	HFSRCNSIKVDS	GDWVVYEKPNYMGY
Dm_YS1 Tn_YS3	MS	S-KITFYEDRNFQGRSHECDTDCPDMHP S-KIIFYEDRNFQGRSYECATDCPDMHP	HFSRCNSIKVES HFSRCNSIKVES	GCWVLYEKPNYTGY GCWVLYEKTNYTGY
Dr_YMX	M3	S-KITFIEDKNFQGKBIECATDCFDMIF S-KITFYEEKNFQGRHYDCTGDCADMQS	HFNRCNSIRVDS	GSWVAYEKPNFSGY
$Tn_{\gamma M15}$	MAYNHSAATM	G-KIIFYEGRNFQGRHWECNTDCMDTFR	HFNCCNSIRVSG	GHWVAYEKPFYMG Y
Ср_ү1 Ср_ү2		G-KIIFYEDKNFLGRSYECSTECADLYL G-KIIFYEDKNFLGRSYECSTECADLTS	YFSRCNSIRVGS YFSRCNSIRVES	CHWILMEHPNFRGQ
x1_γb	M	G-KIFFYEDKNFQGRSYECNSDCSDLSS	YENRONSIRVES	GNWILYEQPSYRCH
x1_γ3	M	G-KIFFYEDKNFQGRSYECNSDCSDLSS G-KIFFYEDRNFOGRSYECSSECSDLSS	YFNRCNSIRVES YFNRCNSIKVEN	GNWILYEQPSYRGH
X1_γ1 X1 γ4	M	G-KIFFYEDRNFQGRSYECSSECSDLSS G-KIFFYEEKNFQGRHYECGSECSDLSS	YFNRCNSIRVEN	GNWILYEQPSYRGH GNWILYEHPSYRGH
x1_γ5		G-KIFFYEDRNFQGRCYECSSECSDLSS	YENRONSTRVEN	GNWILYEQPSYRGH
x1_γ2 Dr γSa	M	G-KIIFYEDRNFQGRSYEC <mark>NSECP</mark> DMSS G-RIIFFEDKNFQGRRHECDSDCSDFHT	NFRRCNSIRVES YLNRCNSIRVES	GDWILYEHPNYRGH GAWVVYERPNFIGY
Dr_YSb	M			
Ii_YS	MSKT	CNRTTEVECKNEOGREVESDEDCLDEHT	DLSRONSTRVEG	GAWWVVERDNEAGN
Dm_YS2 Tn YS2	М М	C-KIAFFEDKNFQGRSHECCTDCPDLRS C-KIVFFEDKNFQGRSYECATDTPDLRT C-KIVFYEDRNFQGRSFEC <mark>SLDC</mark> PELSS	MFGRONSVKVES	GCWVLYERPNYTGN GCWLLYEHPNYTGN
Dr_YSc	MKLAKNMDR	G-KIVFYEDRNFQGRSFECSLDCPELSS	HFTRCNSIRVEN	GAWVLYERPNYMGF
Tn_yS1	MDVV	C THEYEDRATEOCHOPECOCODET TH	HEDDONIOU/OT/EC	CADE VEDDIVE OV
Dr_ySd Dm_yN	MAT	E-SIVFIEDRAFQGSFECSDCFEII E-SIVFIEDRAFQGSYECKGDTSDLHS G-KIVFIEGKCFTGSKLEICSDCDAFQD G-KIVFFFGKCFTGSRLEVFGDCDAFQD	FFSKONSARVKG RGFMNRVNSVRVPS	GFWVLYERPNYMGY GAYVCYDHPDFKGQ
Dr_YN1	MSQYS	C-KIUFIGACHICKNEISVEGODNFQD C-KICFYBERCFIGRUEVEGODNFQD C-KICFYBERCFIGRUEVEGODNFQD C-KITFYBCKCFIGRUEVRGECDNFQD C-KIUFYBCKCFICKKLEISGFQDNFQE	RGFMNRVNSIRVES	GAWVCFDHPDFKGQ
Dr_YN2	MSQYS	G-KICEYEGROFTGROLEVYGDCDNFQD	RGFMNRVNSIRVES	GAWICYDHPDFKGQ GAWICYDHPDFKGQ
Tn_YN Ii_YN	MSQYS	G-KIVFYEGKCFTCKKLEISGPCDNFQE	RGFTNRVNSICVOS	GAWVGFSHADFRGQ

Fig. 4. Amino acid alignment of the 14 distinct γ crystallins from the Antarctic toothfish (Dm) with 51 other γ crystallin sequences from ectothermic animals. The four major Greek–Key fold motifs are indicated in alternating blue/green bars at the bottom of the figure. Boundaries between the domains are in red ()). The numbering (+1) of the crystallins begins where the first glycine (G) residue appears in the majority of sequences. The toothfish appears to have unique class of γ M8 crystallins which are absent in the other species. Identity (100%) between each species' γ crystallins is indicated by a black background, whereas similarity (\geq 50%) is indicated by grey background. Species are designated by a two (or three in the case of lipshark, i.e. *Chiloscyllium colax*=Ccx_ γ 1) letter abbreviation composed of *Genus species* name and one of the five different isoform classes (γ +#, M, S, N, MX).

	90	100 110	120	130	140	150 160
Ci γ1	CWWITTDOWNDCCW			· · · · · · · · · · ·	NDDELSS	
Cc_YM1	OFFLRRGEVHDMORM-	MSMGMMFDTIRSCRMI	PPYRCS	YRMRIYERDTFGG	OMHISVMDDCDNI	
Dr_YM1	OFFLRRGEYHDIORM	MSMGMMFDTIRSCRMI	PSYRCS	FRMRIYERDNFGG	OMYELMDDCESI	~
Ccx y1	OFFLRRGEYHDMORM	MSMGMMFDSIRSCR			QMSELMGDCDSI	
Tn_YM1	QYFLRRGEYNDMORL	FSMGMMFDSIRSCRLI	PHHRGQ	FRMRIYERENFSG	QMHELMDDCDNI	QDRYRMSD-CLSS
Tn_YM3	QYFLRRGEYSDY	ISFGMS-DSIRSCRLI	PQHRGT	YRLRIYERENFQG	QSQELMDDCDNI	~
Tn_YM11	QYF <mark>MK</mark> RGEYADYMSM	MGLTGGIRSCRMI		FRMRIYERENFSG	QMNELTDDCDSI	QDRYRMSD-CLSS
Tn_YM13	QYFLRRGEYSDYMSM	101		FRMRIYERENFSG	QMNELTONCONI	QDRYRMSD-CLSS
Tn_YM10	QYFMRRGEYADYMSM ONFMKRGDYADNMSM	MGMRDC IRSCRMI MGMRDY IRSCRMI			QMHEVMEDCGNI	
Tn_γM12 Tn γM9	QFFVRRGEYSDYQ-H	MGMRDY IRSCRMI MGMSDC IRSCRMI	~	FRMRIYERENFSG FRMRIYERENFSG		~
Dr_YM2b	OVEERKGDYADYMSM	FGMNDCIRSCRII			- OMYELMDDCDNN	
Dr YM2c	OYFFRKGDYADYMSM	FGMNDCIRSCRMI	PMHRGS	YRLKIYERENFGG	QMYELMDDCDNI	
Dr YM2a	QYFFRRGDYADYMSM	FGMNDCIRSCRMI			QMHEMMDDCDNI	MDRYRMSH-CQSC
Cc_YM2	QYFFRRGEYADYMSM	FGMSNCIRSCRMI	PMHRGS	YRMRIYERENFMG	QMYEMADDCDSI	MDRYRMPH-CQSC
Am_71	QYCF <mark>RRGEY</mark> SDYMSM	WGSSSWVRSCRMI		YRMRMYERDNYMG		MNRYNWSHGCQSC
Cf_γM2-1	QYFLKKGEYNDYIGT	WGMNGWIRSCRWI		hkmrlyprenfmg-		MDRYNRSHGCMSW
Cf_YM2-2	QYFIKRGEYPDYMSM OFFIRRGEYPDYMSA	WGWGNNCIRSCRMI WGWGNNCIRSCRMI	· · · · · · · · · · · · · · · · · · ·	YTTTMYRRENSMA	QMMDVIEDCDSI QMMEISDDCDSI	
Cf_γS2 Cf_γS1	OFFIRRGEYPDYMSA OYFMRRGEYADYMSM	-WGWGNNCIRSCRMI -WGWGNNCIRSCRMI	· · · · · · · · · · · · · · · · · · ·	YRMKIYERENFLG		
Tn_γM2	QYFLRRGDYADYMSM	MGMSDCIRSCRMI	100 00000	YRMRIYERENFGG	OMSELMEDCONV	
Dm YM8c	OYFMKRGEYSDYMSM	MGMR DCIKSCRMI		FRMKIYEKENFDG	OSHDMMEDCDNI	
Dm_YM8d	QCFMKRGEYSDYMSM	MGMR DCIKSCRMI	PMHRGQ	YRMKIYEKENFGG	QSHELMEDCONI	MDRYRMNE - CQSC
Dm_YM8b	QYFMRRGEYSDYMSM	MGMR ENIKSCRMI		FRMKIYERENFGG	QSHEMMDDCENI	
Dm_YM8a	QFFMRRGEYSDYMSM	MGMRESIKSCRMI			QSHEMMEDCDNI	
Dm_YM1 Dm_YM8e	OFFMRKGEYODMORM OYFVRKGEYSDYOR	MSMGMMFDTIRSCRMI MGMSDCIKSCRMI		FRMRIHEKENFGG FRMRIHEKENFGG	QMNELMDDCDNI	~
Dm_YM3	OYFMKRGEYSDYORM	MGFGDCIRSCRMI	PMHKGQ	YKLRIYERENFGG	OMNEVNEDCONI	
Dm YM9	QYFMKRGEYSDCMSM	MGMSDCIRSCRTI		YKMRIYEKENFGG	OMNEMNEDCDNI	~
Dr_YM3	QFFMRRGEYADYMRMGN	ISDGIRSCRVV		YRMRIYERDNFGG	QMYELTDDCDSF	MDRYRISD-CQSC
Сс_үмз	QFFMRRGEYADYMRMGN			YRMRIYERENFGG	QMYDLTDDCDSF	
Dr_YM4	QFFLRRGEYADCKRMGI			FRMRIYEKENFGG	MSYELTEDCEST.	
Dm_YM4	OMLVRRGEMPDNORL OMLVRRGEMPDNORL	MGMT-TSDCIRS <mark>S</mark> RMI MGMS-MSDCIRSCRMI		FRMKIYEKENFGG FRMRIYEKENFGG	QMHELMDDCDNM	
Tn_γM5 Dr_γM5	OMLVRRGDYHDNCRL	MGMS-MSDCIRSCRMI		FRMRIFEKENFGG	OKYELMDDCDNI	
Dm jM5	OYLLTRGEYPEYONT	IGFNECIOSCRMV		FKMRIYERANFEG	OMOELTDDCDSI	
Dr_YM6	OFFLRRGDYSDCORM	IGFSNSIRSCRLI		YKMRLYDQADMGG	QMIEVTEDCPNI	MDRFHTSD-IHSC
$Dm_\gamma M7$	QYYLRRGEYSDNQRA	IGLNDCVRSCRMI		YKMRLYERSDMSG	QMHEMLDDCPN1	-
Dr_YM7	QYFLRRGEYPDYMRT	MGMNDCVRSCRMI		FKMRLYEHSDMGG	RMMELMDDCPNL	
Tn_YM14	QYFLRKGEYSDYQRW QYVLTRGEYPDYQRW	MGFNDCVRSCRMI MGFNDTIRSCRTF		HKMMLYERPEFGG YRMRIYERPNFQG	-RVMELMEDVPSL -OMMPFSEDCESV	YEHFHSTD-VHSC
Dm_γS1 Tn γS3	OYVLTRGEYPDYORW	MGFNDTIRSCRTF MGYNDTICSCRTF		YRMRIYERPNFOG	OMMERSEDCESV	~
Dr_YMX	QYMLFKGEYPDFQHW	AGFNDCIRSCRVV		YRMKIFERADFGG	QAMELNEDCPDL	
Tn_YM15	QYILGPGEYPDYHSW	MGFNNCVRSCQML	SPYRGS	YKMRIYNRPDLMG	NMMEFSDDCPNV	
Cp_71	QYYLRRGEYPDFQHW	MGFNDSIRSCRLT	PQHRGS	YRIRVCERDNFGG	QMMEFSEDCPHV	and the second se
Cp_γ2	QYYLRRGEYPDFQHW	MGFNDSIRSCRLT	~	YRIRVYERDNFGG	OMMEFSEDCPHV	
X1_γB	QYYLWKGEYPDFQRW	MGFNDSIRSCRMS		YKMRIYERGDEQG	-ONMEFFEDCPNT ONMEFFEDCPNT	
X1_γ3 X1 γ1	QYYLWKGEYPDFQRW QYYLWKGEYPDFQRW	MGFNDSIRSCRMS MGFNDSIRSCRMS		YKMRISERGDEQG	OMMEFFEDCPNT	
x1 y4	QYYLWQGEYPDFQRW	MGFN DNIRSCRFI	~ ~	YKMRIYEKGDYQG	OMMEFFDDCPNT	
x1_γ5		MGFNEYIRSCRFI	PQHHGQ			
x1_γ2	QYYLRRGEYPDFQQW	MGFNDCIKSCRLS	PQHQGS	FRMKIYEREDFRG	QMMEFTEDCPNV	YERFNFQD-IHSC
Dr_YSa	QYVLTRGEYPDYQRW	MGLNDRLCSCKMI	HF-VSGSE	HKIQLYDKGDFAG	QVYETTEDCPSV	VERFRTRE - VHSC
Dr_YSb	RYLLTRGEYPEYLGW	MGLNDCLSSCKII	RF-TSGMQ	YKVQLYDKPDFTG	QAIESIEDCPSV	QERFRLRE - VNSC
Ii_γS Dm_γS2	MYVITHGEYPEYORW	MGLN DRLSSCKFI	QL-SGGCK	MQIQFYEKGDFGG- WKIDEYENKDEEG-	OMYESTEDCPSV.	MEQYHFRE-IHSC
$Tn_{\gamma S2}$	OYILSHGEYPDHOOW	MGFNDSIKSCRAI	KN-VYGKS	WKIRFYDKODFGG	OTAPCVVDCPSV	YETLKLRE-FHSC
Dr_YSc	QYILTRGEYPDYQRW	MGYNDTIRSCRMV	RN-HTG-S	FRIRLYERPDFOG	QTMESSEDWPSL	YDRFRQRE-VHSC
Tn_YS1	QYVLTQGEYPDYQHW	MGYNDSVRSCRLI	RN-TSS-V	FKIRVYERPDFSG	QMLESTEDLRDL.	adywhrhe - vhsa
Dr_7Sd	QYILGPGEYPDYQHW	IGFNDCVRSCRLV	RH-VIG-D	LKLKLFERPNFDG	OTWEVTESTPSI	QERFLCRE-INSC
Dm_YN	QYILEHGEYPEFORWN-	-AHNDHMGSCKPI	RMHGEH	YRMELFEGENFSG	QCVELCEDCPFL	QARGLTKSCVNSV
Dr_γN1 Dr_γN2	OVILLERGEVPDFORWN-	- SHN DHMGSCKPI	RMHGEQ	YRMELFEGONYTC	OCMELCODOPFL	OSBGENTNOVNSV
DI_γN2 Tn_γN	QYILEHGEYPEFORWN-	-SHNDHMGSCKPT	RMHGEH	YRIELFDACNESG	OCVEICDDTPFL	QSRGLSKNCINSV
li_γn	QYLLWKEYPFORW QYLLWKEYPFOW QYLLRGEYPYQRW RYLLTRGEYPYQRW QYLLSSGEYPFOW QYLLSSGEYPFO QYLLSEGEYPFO QYLLTRGEYPFO QYLLGPGEYPFORW - QYLLGPGEYPFORW QYLLEGEYPFORW QYLLEGEYPFORW QYLLEGEYPFORW	- GHN DRMGSCKPV	GMHGEH	YRIELYEGKYFSG	RSQEFTKDCSFL	SRQGWAKNWINAI

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Fig. 4 (continued).

In addition, we included a number of other fishes and ectotherms, specifically; *C. fuscus*, whitespotted clarias; *Chiloscyllium colax*, lipshark; *Astyanax mexicanus*, blind Mexican cavefish; *C. carpio*, Asian (common) carp, *Xenopus laevis*, African clawed frog; *Cynops pyrrhogaster*, Japanese firebelly newt and *Iguana iguana*, iguana (Suppl. Table 2). Furthermore, there are 15 previously 'unidentified proteins' from *T*. *nigroviridis* (spotted green pufferfish) used in this analysis which were deposited into the GenBank/NCBI database by Genoscope (France) and the Whitehead Institute (Massachusetts Institute of Technology) during the course of the *Tetraodon* genome project (www.genoscope.cns.fr/externe/English/ Projets/Projet_C/organisme_C.html). We have identified these 15 sequences as γ crystallins and thus propose isoforms names

	1	170 180 190 200 21 	
Ci γ1			
Cc YM1	HVM-D	GHWLFYEQPHYRGRMWYFRPGEYRSFRDMG-YSNMRFMSMRRI GHWLMFEQPHYRGRMI <mark>YFRPGEYRSFRDMG-FSNMRFIS</mark> MRRI	TDMC
Dr_YM1	HVM-D	GHWLMFEQPHYRGRMIYFRPGEYRSFRDMG-FSNMRFISMRRI	TDMC
Ccx_71	HVM-D QVM-D NVL-D	GHWLMEQFHIRGKMIIFRPGEIRSFROMG-FSMARFIOWRI GHWLMYEQAHYRGKMMYLRPGEYRSFROMG-MSGMRFMSMRRI GHWLLYEQPHFRGRMMYLRPGEYRSFROQG-FSGMRFMSMRRI	TDMC
Tn_YM1	QVM-D	GHWLLYEQPHFRGRMMYLRPGEYRSERDQG-FSGMRFMSMRRI	TDIC
Tn_үM3	NVL-D	GHWLLYEQPHFRGRMMYLRPGEYRSIRDVG-FSGMRLSSIRRI	MDS
Tn_YM11	QVM-D QVM-D	GHWLCTSSP	
Tn_YM13	QVM-D QVL-D	GHWDDYEQPHFRGRMIYDRPGEYRSFRDDG-VSGMRFMSMRRI	MDMC
Tn_γM10 Tn_γM12	OWM-D	GHWLLYEQPOYRGSMIYLRPGEYRNFRDLG-VSGRAFMOWRRI GHWLLYEQPOYRGSMIYLRPGEYRNFRDLG-VSSSRFMSMRRI GHWLLYEQPHFRGRMMYLRPGEYRSFRDLG-FSGMRFMSMRRI	MDPCS
Tn YM9	QVM-D HVM-D	GHWLLYEOPHFRGRMMYLRPGEYRSFRDLG-VNGMRVMSMRRI	MDSCY
Dr_YM2b	- HVM-D	GHWLMYEQPQYRGRMMYLRPGEYRSEREMGGTRFMSMRRI	IDSMY
Dr_YM2c	HVM-D	GHWLMYEQPQYRGRMMYLRPGEYRSFREMGGTRFMSMRRI	IDSMY
Dr_YM2a	HVM-E	GHWLVYEQPQYRGRMMYMRPGEYRSPREMGGMRFLSMRRI	NDSFY
Cc_γM2	H V M−D	CHWLLYEQPHERGEMMYLRPGEYRSFRDLC-FSGREFMSNRRI GHWLLYEQPHERGEMMYLRPGEYRSFRDLG-VNGREVMSMRRI GHWLYEQPQYRGEMMYLRPGEYRSFREMGGTRFMSWRRI GHWLYEQPQYRGEMMYLRPGEYRSFREMGGTRFMSWRRI GHWLYEQPQYRGEMMYRPGEYRSFSNMGGREFLSMRRI GHWLYEQPEYRGEMWYFRPGEYRSFSNMGGREFMSWRRI	MDSWY
Am_y1	HVM-D	GHWLDYEQPEYRGRMWYFRPGEYRSFSN MG GMRFMSWRRI GHWLDYEQPEYRGRMWYFGPGEYRNFSNYG NNRFMSWRRI GHWLMYEPHYKGRMWYFGPG2YRSYRHMMGMS GMRFQSMRRI GHWLMYEQPEYRGRMWYFRGEYRSFRETMGMS GMRFMRWRRI AHWLMYEHPHYRGRMWYFRGEYRSFRE FG- NTNFMSMRRI AHWLMYEHPHYRGRMWYFRGEYRNFRE YG- GMRFMSMRRM GHWLMYEQPEYRGRMMYWRPGEYRNFRE - YG- GMRFMSMRRI GHWLMYEQPEYRGRMMYWRPGEYRNFRE - CCRFMSMRRI GHWLMYEQPEYRGRMMYWRPGEYRNFRE - CCRFMSMRRI	
Cf_YM2-1	PRD-G	CHWLMYEFPHYKGRMWYFGPCOYRSYRHMMGMSGMRFQSMRRI	MDSWY MDSWY
Cf_γM2-2 Cf γS2	HVM-D HVT-G	GHWDMTEOPHYRGRUWIFRPGETRSNREIMGMSGMRFMRMRRI GHWDMYFODHYDCPMWYFDCFYDSFDDFCNTNFMSMDDT	MA
Cf YS1	NVM-D	AHWLMYEHPHYRGRMWYFRPGEYRNERD YG GMRFMSMRRM	VA
Tn YM2	NVM-E	GHWLMYEOPHYRGRMMYVRPGEYRNEMS-TIGSNMRVISMRRI	TDSCQ
Dm_YM8c	NVM-D	GHWLMYEQPOFRGKMMYMRPGEYRNEKDMG-MSCQRFMSMRRI	TDSC
Dm_YM8d	NVM-D	GHWLMYEQPQFRGKMMYMRPGEYKNEMDMG-MSGQRFMSMRRI	TDSCN
Dm_YM8b	NVM-D NVM-D NVM-D NVM-D	GHWLMYEQPNFRGKMMYMRPGEYRSEREMG-MSGIKFMSMKRI	TDSCY
Dm_YM8a	NVM-D	GHWLMYEQPOFRGNOMIOFGEINNHOMG-NSCORFMGNRI GHWLMYEQPOFRGNMYMRPGEYNNFMDMG-MSGORFMGNRI GHWLMYEQPOFRGNMYMRPGEYRNFREMG-MSGIKFMSMRRI GHWLMYEQPOFRGNMYMRPGEYRNFREMG-MSGORFMSMRRI GHWLMYEQPOFRGNMYMRPGEYRNFRDMG-MSGORFMSMRRI GHWLMYEQPOFRGNMYMRPGEYRNFRDMG-MSGORFMSMRRI	TDMC
Dm_YM1	NVM-E NVM-D	GHWLMYEQPQFRGKMMYMRPGEVRNEKDMC-MSGQRFMSMRRI	TDMC
Dm_YM8e	NVM-D NVM-D	GHWLMYEQPQYKGKMMYLRPGEYKSERDMG-MSGQRFMSMKRI	MDSCY
Dm_γM3 Dm_γM9	NWM-D	GHWLMYEQPQFRGKMMYLRPGEYKSFRDMC-YD-AMRFSSIRRI CNWLMYEQPNFRGKMMYMRPGEYKSFREMC-MSGQRFMSMRRI	MDSCN
Dr_YM3	HVM-D HVM-D	GHWLMYEQPHYRGRMIYFRPGEYRSFRDMG-YSNVRFSSVRRI GHWLMYEQPHYRGRIVYFRPGEYRSFRDMG-YSNVKFSSVRRI	VDLC
Сс үМЗ	HVM-D	GHWLMYEQPHYRGRIVYFRPGEYRSFRDMG-YSNVKFSSVRRI	MDLC
Dr_YM4	HVM-D	CHWLIVEOPHYPSPMLYLPPCFYPSPDMC-TSPPFSSLPPT	MEDON
Dm_YM4	NVM-E	GHWLMFEQPNYRGMMYLRPGEYRNLRETG-MSNMTK-FSSMRRI GHWL <mark>LYEQPHFRGRMMYLRPGEYRSMRDMG-MGPMDMR</mark> IGSIRRI	MDSC
Tn_γM5	HVM-D	GHWLLYEQPHFRGRMMYLRPGEYRSMRDMG-MGPMDMRIGSIRRI	MDSC
Dr_YM5	NVT-H	GHWLLYERPNFEGRMIYIRPGEYSTESDMG-LGSLKIASVRRI	MESC TESV
Dm_YM5	NVM-E QVM-D	GHWLMFEQPNFEGRMSYTKPGKYRNLKEMN-SEDMKFNSIRRI GHWLLYEQPNYRGRMFYLGPGEYRKYSDMGGMAPRIGSURRI	TESV
Dr_γM6 Dm_γM7	NVM-E	GHWLIT DOPNYKGRAYYMRPGRYRRESDWGGVSPRYGSLRRT	SDLN
Dr YM7	HVM-D	GHWLLYDOPNYKGRAYYMRPGEYRRESDWGGVSPRVGSIRRI GHWLVYEOPNYTGROFYLRPGEYRSYNDWGGVTSRMGSIRRI	TDL
Tn_YM14	NVI-G	GHWDYEGENYIGKOFTIKEGENKEIMWEGETSAMGETKEI GHWIEYEHPHYRGROYLMGPGOYRRENEWGELSPRVGSIRHI GYWTLYEOPNYRGROYFMRPGEYRKESDWGATCATTGSFRRI	vc
Dm_YS1	NVM – E	GYWTLYEQPNYRGRQYFMRPGEYRKFSDWCATCATTGSFRRI	TEF
Tn_YS3	NVM-D	GYWTLYEHSNYRGROYFMRPGEYRKESDWGATCATTGSFRRI	TDF
Dr_YMX	N <u>VM</u> -E	CYWILBHPNYKGROFFLR9GYRYIEWGSQSPTIGSLRRV CYWILBHPNYKGROFFLR9GYRACGDWGCINPTIGSLRRV GFWIFYBHPNYKGROYFLR9GEYRACGDWGCINPMVGSFRRI GHWVFYEBPNYRGROYYLR9GEYRRYSDWGASSPKVGSFRRV GHWVFYEBPNYRGROYYLR9GEYRRYSDWGASSPKVGSFRRV	тррк
Tn_γM15	NIL-E	GFWIFYEHPNYKGRQYFLRPGEYRACGDWCCHNPMVGSFRRI	RTLM
Cp_y1	NEQ-D NVQ-D	GHWVFYDEDNYRGROYYLFPGEYRRYSDWCASSPKVGSFRRV	
Ср_ү2 х1_үв	NVQ-D NVF-D	GAWFIEBENIRGROIIBRGERRIDWCASSFRVGERRV CNWMFYEBENYRGROYYLRPGEYRRYSDWCASSARIGSFRRV CNWMFYEBENYRGROYYLRPGEYRRYSDWCASSARIGSFRRV	HHMF
x1_γ3	NVF-D	GNWMFYEEPNYRGROYYLRPGEYRRYSDWCASSARIGSFRRV	HHIF
x1_γ1	NVS-D	GYWMFYBBPNYRGROYYLRPGOYRRYNDWGASS SRIGSFRRV GHWMFYBBPNYRGROYYLRPGBYRKYSDWGASS PRIGSFRRV	RQMF
X1_γ4	NVF-D	GHWMFYEEPNYRGRQYYLRPGEYRKYSDWGASSPRIGSFRRV	YHKFKSTQTFTNTFVLVQ
x1_γ5	NVS-D	GHWMFYEEPNYRGRQYYLRHGEYRRESDWGASSARIGSFRRV	HHMF
x1_γ2	QVL-D	GHWMFYBEPNYRGROYYLREGEYRRESDWCASSARIGSFRRV GYWMFYBEPNYRGROYYLRPGEYRRYDWCAMNPRIGSFRHV GIWIFYEHPNYRGROYLLOKGEYRKPVDWCAVCPTVOSFKRL	YHR
Dr_YSa	KVL-D	GYWMFYBERNYRGROYLHROESRRYTWCANNPRIGSFRHV GIWIFYBHPNYRGROYLLORGEYRRPVDWCAVCPTVOSFRRL GYWIFYBHPNYRGHOYFMERGNYRRPVDWCAICPSVOSFRRF CAWVFYBHPNFRGROYFMERGEYSRPMEWCAASPVVOSFRRV	TE
Dr_γSb Ii γS	KVL-D	GIWIFYEHPNYRCHQIFIERCENYRKFYUWCAICPSVQSFRRF GAWVFYEHPNFRGRQYFLERCEYSKPMEWCAASPVVQSFRRV	AF
Dm YS2		CANVITEDINFRONDIFIERGEVINYTDWCATSPAVGSFRMU	TKF
Tn_YS2	VVM-D	CAWUYYEHPARKGROYFLERGEYNNYDWGAASPAVOERNRY CAWUYEOPNYCGHOYFLERGEYNNYDWGATSPAAGSRMI CAWUYEOPNYHGHOYFLERGEYNNYDWGATSPAAGSRMI GAWIFFEHPNYRGROYLLERGEYRCFDWNAMHPTVGSIRRI CAWVFHELSNFHGROYLLERGEYRRFTEWAAMNPTVGSIRCA APCVFFEHANYRGROYFLERGEYRHTEWCAMHPTVGSIRQI	TDF
Dr_YSc	NVL-D	GAWIFFBHPNYRGRQYLLEKGEYRCFTDWNAMH PTVGSIRRI	QDF
Tn_ys1	QVL-D	GAWVFHELSNFHGRQYLLEKCKYRRFTEWAAMNPKVGSFRCA	V
Dr_ysd	KVH-E	APCVFFEHANYRGRQYFUEKGEYRRHTEWGAMHPTVGSIRQI	TTD
Dm_YN	KVYGD	GAWVMYBEPNYRGHMYIVERRNYSTHTEWQAENPSIQSVRRV	ANYF
Dr_YN1	KVYGD	APCVFMBHANYRGEQYFIEKGEYRHTHWGAHH - PTVGBIRGI GAWAYYBBPNYRGEMYIVERRNYSTHTEWQAEN - PSIQSVRRV GAWAYYBBPNYRGEMYIVERGNYCAFTEWQSEN - PNIQSIRRI GAWAYYBBPNFRGRMYIVERGNYCAFTEWQAQN - PNIQSIRRI GAWAYYBBPNFRGRMYVVERGDYCSHNEWQAQN - PNIQSIRRV GAWAYYBBPNFRGMYVVERGDYSSCNEWQASN - PANIQSIRRV	VNYF
Dr_YN2 Tn_YN	KVFGD	CAMVMYDEDNFRCRMYTYVERCDYCSHNEWQAQNPHIQSIRRI CAWVMYDEDNFRCRMYVVERCDYCSHNEWQAQNPHIQSIRRI	VN1F
II_γN II_γN	KVYGD	GAWVLYBEPNYRGOMYVVERCDYSSCNEWOASN ANIOSIRRV	INYF

Fig. 4 (continued).

based on the γM nomenclature used in this study in Suppl. Table 1.

The γ crystallins can be divided into three major isoform groups (γN , γS , and γM which includes amphibian γ) largely based on the phylogram (Fig. 5). The γN isoforms form a distinct clade (green, Fig. 5) indicating their ancestral position and high conservation across species. Most of the γ S isoforms (blue, Fig. 5) are basal to the γ M isoforms (purple, Fig. 5) with exception of the γ S1 and γ S2 from *C. fuscus* whitespotted clarias. The γ crystallins included from the amphibians (*X. laevis* and *C. pyrrhogaster*) form a clade nested within the γ M crystallins of the fishes (red, Fig. 5). From the Bayesian tree

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Amino acid identi	ities (perce	ent identica	(I) based u	pon pairwi	se alignme	ent of tooth	fish γ crys	tallins						
Sequence name	$\gamma M1$	γM3	$\gamma M4$	$\gamma M5$	$\gamma M7$	γM8a	γM8b	γM8c	γM8d	γM8e	γМ9	$\gamma S1$	γ S2	γN
γM1	100.0	69.8	69.6	56.9	58.4	76.8	73.2	76.3	75.9	76.7	69.6	51.1	42.7	49.6
γM3		100.0	64.4	61.9	61.3	69.9	69.0	70.4	72.2	75.2	76.4	53.6	45.7	50.2
γM4			100.0	61.1	55.0	63.4	65.2	65.5	66.8	71.2	66.2	49.1	40.8	53.2
γM5				100.0	53.9	55.1	57.6	55.1	54.3	55.0	55.6	51.4	42.3	51.9
γM7					100.0	55.7	55.4	53.0	52.1	58.4	60.1	58.1	50.8	53.9
γM8a						100.0	87.5	86.3	83.6	73.3	72.8	47.8	40.7	49.9
γM8b							100.0	85.3	83.6	73.9	71.7	48.1	41.0	50.1
γM8c								100.0	92.3	73.9	69.5	47.8	41.3	49.1
γM8d									100.0	76.0	72.8	47.0	41.0	48.9
γM8e										100.0	75.2	48.0	42.4	50.5
γM9											100.0	48.6	41.8	52.7
γS1												100.0	59.6	51.9
γS2													100.0	49.0
γN														100.0

(Fig. 5) the phylogenetic distance (expected changes per site) between the γ crystallin cDNA coding sequences can be inferred from the branch lengths in the tree (Fig. 5), thus it appears that the amphibian γ crystallins are more closely related to the γ S isoforms than to some of the more derived γ M fish crystallins. The remaining γ M crystallins show clustering based on their relatedness to the previously named carp and zebrafish sequences.

A Southern blot of *Eco*RI digested toothfish genomic DNA probed with a conserved centre section (407 nt of γ M8e) of a toothfish γ crystallin revealing 17 bands (Fig. 6). The number of distinct bands in the Southern blot is within the magnitude of number of γ cDNAs isolated and sequenced.

3.4. Genomic organisation

Table 4

A BAC library macro-array was screened with ³²P-labeled toothfish α (α A and α B), β (β A2 and β B2) and γ (γ M8e) crystallin probes. Seventeen BAC a clones were initially identified from the macro-array and further re-analysed by HindIII digest Southern blot (Suppl. Fig. 2). Thirteen of these clones (a1, a3~a11, a13~a15, a 17) were found to be overlapping and form one contig 46 consensus band CB units long (Fig. 7A). Each CB unit was estimated to be ~5800 bp long based on the mean restriction fragment sizes in the HindIII DNA fingerprint analysis (Suppl. Figs. 2, 3 and 4), therefore 46 CB units would cover a region of 266.8 kbp (Castellarin et al., 2006). Clones a12, a16 did not overlap with the single contig. Clone a2 was also not in the contig and was not analysed further as it had only two bands at ~8000 and 5700 bp in the HindIII DNA fingerprint and very weak Southern Blot hybridisation (Suppl. Fig. 2). Southern blot analysis of the other 16 α clones indicated strong hybridisation for all the clones except weak hybridisation for clone a16. Hybridisation patterns were similar between the clones, except for clones a5 and a12 which also had different HindIII DNA fingerprint patterns (Suppl. Fig. 2). Only clone a12 showed both a different fingerprint/hybridisation pattern than that of the other α BAC clones.

Six β crystallin clones identified in the initial macro-array screen had extremely similar *Hin*dIII fingerprints and almost identical hybridisation patterns by Southern blot analysis (Suppl.

Fig. 3). These data were confirmed in FPC analysis which placed all the β BAC clones within the same 47 CB (272.6 kbp) contig (Fig. 7B).

There were twenty seven positive clones found for γ crystallins in the BAC macro-array screen. Based on our FPC analysis the γ crystallin BAC clones divide up into three groups. The first group forms a contig of 31 CB units (179.8 kbp) and includes clones g1, g3-5, and g12 (Fig. 7C and Suppl. Fig. 4) and have similar Southern Blot hybridisation (Suppl. Fig. 4). The second group forms a separate contig of 51 CB units (295.8 kbp) and includes clones g6-11, g13-20 and g22 (Fig. 7D and Suppl. Fig. 4). All of these clones within this second contig group also have a similar Southern Blot hybridisation, which differs from the first γ contig group. Two clones that did not contig with either of the two previous groups (g2 and g21, Suppl. Fig. 4) also shared similar Southern Blot hybridisation patterns to each other. These two clones failed to contig even after re-analysis with adjusted Tolerance and Cut-Off parameters in the FPC analysis program (Soderlund et al., 2000).

4. Discussion

The Antarctic toothfish *D. mawsoni* lives in the perennially sub-zero seawater (-2° C) of the Southern Ocean at the lower thermal limit of marine vertebrate ectotherms. At this subzero temperature, the toothfish has a completely transparent lens composed of α , β and γ lens crystallins that are similar to other mammalian vertebrates (Kiss et al., 2004). To investigate the possible molecular basis of the cold stable toothfish lens, we have obtained the crystallin sequences by cDNA cloning and sequencing.

There were two complete α crystallin (α A and α B) cDNAs isolated from toothfish lens. Toothfish α A crystallin show high sequence identity to α A from both fishes and mammals in contrast to α B sequences which are more divergent (Table 2). The homology of the toothfish α A amino acid sequence with other vertebrates suggests that α A crystallin is under greater functional constraint than the α B isoform, therefore maybe acting as the primary sHSP within the eye lens as has been previously hypothesized (Bova et al., 1997; Rajaraman et al.,





Fig. 6. Genomic Southern blot of *D. mawsoni Eco*RI digested DNA. DNA was probed with 407 nucleotide ³²P-labeled PCR amplicon from the coding region of γ M8e (*D. mawsoni*). Labeled arrows to the left of the blot indicate bands that hybridised to the toothfish DNA. There were 17 positive bands.

2001; Dahlman et al., 2005). The converse, that α B likely has a diversity of non-lenticular roles (Bennardini et al., 1992; Piatigorsky 1998; Dahlman et al., 2005) is also supported by our toothfish α B crystallin sequence data. Interestingly, we had some difficultly obtaining α B cDNA from the toothfish lens, which may reflect the low expression levels of the α B message (mRNA). Proteomic analysis of α A and α B crystallins from toothfish as well as detailed chaperone-like assays of recombinant toothfish α A and α B crystallins is in progress and will be published elsewhere.

Beta crystallin protein sequences from toothfish lens are aligned with zebrafish, cow and human (Fig. 3). They display a high degree of conservation both for each β isoform and between each species analysed. We did not isolate a BA3 isoform, which in mammals has a longer N-terminal extension that normally 'incorporates' the BA1 isoform by means of an earlier, upstream translational start codon (Bloemendal et al., 2004). There are three basic β crystallin isoforms (β B1, β B2, β B3) in toothfish, as in mammals. The 'A or 'B' designation after the ' β ' referring to either an acidic (βA #) or basic (βB #) class of the β crystallin isoform (Bloemendal et al., 2004). The N-terminal PAPA-arm of β B1 (Fig. 3) of cow and human lenses has been shown to be strongly associated with the membrane (aqueous insoluble) portion of the lens (Bloemendal et al., 1984; Hejtmancik et al., 1986; Coop et al., 1998; Bateman et al., 2001). We did observe a significant proportion of β crystallins associate with the membrane (insoluble) component of the toothfish lens during isolation of the lens proteins (Kiss, 2005). Although the N-terminal extension in the toothfish BB1 does not have a PAPA-arm, there is a long PAPA-arm in the Cterminal of the toothfish β B3 crystallin. Interestingly, this toothfish C-terminal PAPA-arm appears to have a counterpart C-terminal 'PNPN-arm' in zebrafish BB3 crystallin (Fig. 3). Although the hydrophobic PAPA-arm of bovine BB1 is thought to insert itself into the lipid membrane, the substitution of asparagine (N) for alanine (A) in the zebrafish PNPN-arm would make the PNPN-arm very polar and thus its function could be quite different than what is hypothesized for the mammalian and possibly toothfish PAPA-arms.

Recent structural studies of recombinant mammalian β crystallins (Bateman et al., 2001; Bateman et al., 2003; Van Montfort et al., 2003) have suggested that linker regions as well as the extensions (N- and C-terminal) are involved in oligomerisation of the β crystallins. While it is clear that within recombinant experiments this is the case, it remains unclear what are the native function(s) of N-terminal and C-terminal extensions in an intact lens.

The 14 distinct γ crystallin isoforms found in the Antarctic toothfish lens fall into three isoform groups. We found a single γN isoform, which based on gene structure is believed to be an evolutionary bridge between the β and γ isoforms (Wistow et al., 2005). Two more cDNAs belonged to the γS (formerly β_S (Björk, 1961)) group and the remaining eleven were γM isoforms (Chang et al., 1988; Wistow et al., 2005) so named originally for their high methionine content. The very high methionine content in the γM crystallins of the toothfish and other fishes is a curious adaptation (Chang et al., 1988), and so far remains unexplained. Unlike other non-polar residues, methionine has special properties which may in fact contribute significantly to the stability of fish lenses, both in terms of their

Fig. 5. Phylogram of ectothermic γ crystallins from the Antarctic toothfish, zebrafish, pufferfish and select other fishes, amphibians and iguana. Tree was constructed from the aligned nucleotides of the coding region from the cDNAs (Suppl. Fig. 1). The 50% majority-rule consensus tree was generated by the program Mrbayes, implementing a GTR+I+ Γ evolutionary model. Each major isoform class in the tree is colour coded (γ N=green, γ S= blue, amphibian γ = red, γ M= purple). Only the γ N isoform is monophyletic. The cross-species conserved γ S isoforms are polyphyletic in this analysis. The amphibian γ crystallins (red) form a clade nested basally within the γ M isoform group, close to the γ S isoform class indicating that they may be intermediate between the ancestral γ S and the fish γ M isoforms. Numbers at branch points are Bayesian consensus values (similar to bootstrap values) and the scale bar indicates 0.2 expected changes per site. Images of species used are included (but are not to relative scale).



Fig. 7. Genomic organisation of the α , β and γ lens crystallin genes from the Antarctic toothfish as assayed by BAC library analysis. (A) The single contig of the α crystallin gene region found to be 46 CB units (266.2 kbp) long. All but three clones (a7 and a12) were part of the contig. (B) Single contig region of β crystallin genes inclusive of all positive BAC clones was 47 CB units (272.6 kbp) long. (C) Two contigs of 31 CB units (contig group 1; 179.8 kbp) and 51 CB units (contig group 2; 295.8 kbp) encompassed all but g2 and g21 γ crystallin clones. Designations following the clone name indicate that: '*' clone was buried, '=' same bands as a parent clone, '~' approximately same bands as a parent clone.

high density and in terms of their ability to readily adapt to a range of temperatures. Methionine has both a structural plasticity as well as the ability to have its hydrophobicity altered by reversible oxidation of its thioether (Gellman, 1991). In the context of the extremely dense fish lens where protein concentrations are upwards of 1000 mg mL⁻¹ (Kroger et al., 1994), the multiple isoforms of γM crystallins would undoubtedly contribute to the protein stability by increasing the crystallin polydispersity, thus preventing catastrophic crystallization at such high concentrations. Furthermore, γM crystallins would impart 'flexibility', or sponginess to the yM crystallin surface which would further discourage crystal lattice formation. Additionally, the abundance of methionines may have biochemically predisposed the vM crystallins to cold adaptation by allowing the fish lens to reversibly oxidize their thioethers via enzymatic means (Marchetti et al., 2005; Sagher et al., 2006) thereby affording a mechanism to attenuate the hydrophobicity of these γ crystallins. Consequently, a primary adaptation of toothfish lenses by increasing the protein stability at high density by generating polydisperse γM crystallin isoforms, could have leant itself to a secondary adaptation of cold stability.

Phylogenetic analysis of the γ crystallin isoforms was done using a Bayesian approach employing a GTR+I+ Γ model of molecular evolution (Waddell and Stell, 1997). This model of evolution is particularly well-suited for ancient protein coding sequences as it acknowledges that (i) there has been reversion, (ii) that there are invariant sites and (iii) that not all sites are under the same selective pressures. Furthermore, using the nucleotides that code for the amino acids aligned as codon units may facilitate greater resolution depth in the tree (Simmons et al., 2002). Phylogenetic reconstruction of the toothfish γ

crystallins in comparison with other ectothermic species shows that the γN isoforms form a monophyletic clade, whereas conserved yS crystallins are polyphyletic. The placement of the recently discovered γN class of crystallins ancestrally to the γS clade suggests that they pre-date the evolution of γS , as has been suggested from comparative analysis of their gene structure in D. rerio (Wistow et al., 2005). Based on phylogenetic analysis, there is a range of γS isoforms with a decreasing gradient of similarity from γN to γM . The two C. fuscus γ S isoforms (Cf_ γ S1 and Cf_ γ S2, Figs. 4 and 5) could very well be misnamed as they seem to be well-situated with the γ M group (specifically γ M2) in both our alignment and Bayesian phylogenetic analysis. The multiplicity of the toothfish γM crystallin isoforms are emphasized in the phylogram, many of which are distinct from the zebrafish and pufferfish (Fig. 5). In our previous study (Kiss et al., 2004), we proposed that the γ crystallin component of the toothfish lens as the most likely candidate for the transparency of the toothfish lens at -2° C. However, while there are several yM crystallins isoforms unique to the toothfish, comparative hydrophobicity plots did not suggest major differences from other fish crystallins. It is possible that a few select amino acid changes, in addition to post-translational modifications of the γ crystallins could have profound implications on the cold stability of the lens. Regions between the domains of the γ crystallins termed 'linker' regions, and N-terminal extensions (such as the long methionine rich ones in the γM class) may also have significant influence on cold stability by affecting the solution dynamics (Wu et al., 2005). To fully address these issues, extensive biochemical analysis and proteomics experiments would likely be informative.

An outline of the genomic organisation of the α , β and γ crystallin genes was obtained by screening a BAC library and analyses of the DNA fingerprints by FPC. Using this approach we found that α and β crystallin separately formed a single α or β contig each whereas the γ genes formed two contigs (Fig. 7). The two γ contigs differ both in *Hin*dIII fingerprint pattern as well as Southern blot hybridisation patterns. The second γ contig appears to have a Southern blot hybridisation pattern similar to that of the β contig Southern blot (Suppl. Fig. 3). This similarity is not unexpected as both β and γ crystalling share conserved domains and are members of the same gene superfamily (Bloemendal et al., 2004; Wistow et al., 2005). The probe used to hybridize to the γ gene regions did have variable hybridisation strength to some BAC clones, of which most are represented in the second contig. Consequently, these results suggest that some β and γ genes are in spatial proximity, or alternatively that the second contig of γ BAC clones could very well be β crystallins. Two γ clones (g2 and g21) do not form a contiguous group with either of the γ gene contigs even though their Southern blot hybridisation pattern is similar to the β and second γ gene contig groups. Within mouse, rat and humans, the γ crystallin genes are clustered as $\gamma A \sim F$ with $\gamma S (\beta_S)$ a short distance apart, interspersed with highly repetitive sequences (Willard et al., 1985; den Dunnen et al., 1987; Skow et al., 1988; den Dunnen et al., 1989). Based on annotation in NEIBank, zebrafish vS isoforms are present on two different chromosomes (16 and 9) (Wistow, 2002). Without more information at the DNA sequence level, it is difficult to state with certainty how the $\beta\gamma$ gene organisation in the Antarctic toothfish is arranged. Current and proposed BAC projects argue persuasively for the sequencing of the Antarctic toothfish which will enable questions regarding γ crystallin evolution to be addressed more fully (NRC, 2003; Clark et al., 2004; Cheng et al., 2007).

Comparison of toothfish $\alpha\beta\gamma$ crystallin sequences with other vertebrates indicates that α and β isoforms are well-conserved both in number and isoform type. Our current work illustrates the homology of α and β toothfish crystallin sequences to other phylogenetically distant vertebrate species (Bloemendal et al., 1984; Hejtmancik et al., 1986; Behrens et al., 1998; Chen et al., 2001; Runkle et al., 2002) reiterating the likely conservation of function of these two types of crystallins. Conversely, toothfish γ crystallins exist as multiple polydisperse isoforms, some of which appear to be paralogues and some unique. Given this nature of the toothfish γ crystallins, it is attractive to suggest that they are more evolutionarily plastic without the functional constraints of the $\alpha\beta$ isoforms. Thus, the combination of an increased methionine content allowing for attenuation of protein hydrophobicity, along with increased polydispersity of γM isoforms in the dense eye lens may have predisposed the toothfish lens for biochemical adaptations to the extreme cold.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbd.2008.02.002.

References

- Banh, A., Sivak, J.G., 2004. Laser scanning analysis of cold cataract in young and old bovine lenses. Mol. Vis. 10, 144–147.
- Bateman, O.A., Lubsen, N.H., Slingsby, C., 2001. Association behaviour of human betaB1-crystallin and its truncated forms. Exp. Eye Res. 73, 321–331.
- Bateman, O.A., Sarra, R., van Genesen, S.T., Kappe, G., Lubsen, N.H., Slingsby, C., 2003. The stability of human acidic beta-crystallin oligomers and hetero-oligomers. Exp. Eye Res. 77, 409–422.
- Behan-Martin, M.K., Jones, G.R., Bowler, K., Cossins, A.R., 1993. A near perfect temperature adaptation of bilayer order in vertebrate brain membranes. Biochim. Biophys. Acta 1151, 216–222.
- Behrens, M., Wilkens, H., Schmale, H., 1998. Cloning of the alphaA-crystallin genes of a blind cave form and the epigean form of *Astyanax fasciatus*: a comparative analysis of structure, expression and evolutionary conservation. Gene 216, 319–326.
- Benedek, G.B., 1997. Cataract as a protein condensation disease: the Proctor Lecture. Invest. Ophthalmol. Vis. Sci. 38, 1911–1921.

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- Bennardini, F., Wrzosek, A., Chiesi, M., 1992. Alpha B-crystallin in cardiac tissue. Association with actin and desmin filaments. Circ. Res. 71, 288–294.
- Björk, I., 1961. Studies on g-crystallin from calf lens: I. Isolation by gel filtration. Exp. Eye Res. 1, 145–154.
- Bloemendal, H., Berbers, G.A., De Jong, W.W., Ramaekers, F.C., Vermorken, A.J., Dunia, I., Benedetti, E.L., 1984. Interaction of crystallins with the cytoskeletal-plasma membrane complex of the bovine lens. Ciba Found. Symp. 106, 177–190.
- Bloemendal, H., De Jong, W., Jaenicke, R., Lubsen, N.H., Slingsby, C., Tardieu, A., 2004. Ageing and vision: structure, stability and function of lens crystallins. Prog. Biophys. Mol. Biol. 86, 407–485.
- Bova, M.P., Ding, L.L., Horwitz, J., Fung, B.K., 1997. Subunit exchange of alphaA-crystallin. J Biol Chem 272, 29511–29517.
- Broide, M.L., Berland, C.R., Pande, J., Ogun, O.O., Benedek, G.B., 1991. Binary-liquid phase separation of lens protein solutions. Proc. Natl. Acad. Sci. U. S. A. 88, 5660–5664.
- Caspers, G.J., Leunissen, J.A., de Jong, W.W., 1995. The expanding small heatshock protein family, and structure predictions of the conserved "alphacrystallin domain". J. Mol. Evol. 40, 238–248.
- Castellarin, S.D., Di Gaspero, G., Marconi, R., Nonis, A., Peterlunger, E., Paillard, S., Adam-Blondon, A.F., Testolin, R., 2006. Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. BMC Genomics 7, 12.
- Chang, T., Jiang, Y.J., Chiou, S.H., Chang, W.C., 1988. Carp gamma-crystallins with high methionine content: cloning and sequencing of the complementary DNA. Biochim. Biophys. Acta 951, 226–229.
- Chen, J.-Y., Chang, B.-E., Chen, Y.-H., Lin, C.J.-F., Wu, J.-L., Kuo, C.-M., 2001. Molecular cloning, developmental expression, and hormonal regulation of zebrafish (*Danio rerio*) [beta] crystallin B1, a member of the superfamily of [beta] crystallin proteins. Biochem. Biophys. Res. Commun. 285, 105–110.
- Cheng, C.H., Chen, L., 1999. Evolution of an antifreeze glycoprotein. Nature 401, 443–444.
- Cheng, C.-H.C., Nicodemus, J., Silic, S., Ghigliotti, L., Pisano, E., 2007. Genomic analysis of the evolution of antifreeze glycoprotein genes in antarctic notothenioid fish. Joint Genomics Institute User Meeting, vol. 2. US Department of Energy, Mariott Hotel, Walnut Creek, California.
- Chiou, S.H., Chang, T., Chang, W.C., Kuo, J., Lo, T.B., 1986. Characterization of lens crystallins and their mRNA from the carp lenses. Biochim. Biophys. Acta 871, 324–328.
- Clark, J.I., Benedek, G.B., 1980. The effects of glycols, aldehydes, and acrylamide on phase separation and opacification in the calf lens. Invest. Ophthalmol. Vis. Sci. 19, 771–776.
- Clark, M.S., Clarke, A., Cockell, C.S., Convey, P., Detrich III, H.W., Fraser, K.P.P., Johnston, I.A., Methe, B.A., Murray, A.E., Peck, L.S., Römisch, K., Rogers, A.D., 2004. Antarctic genomics. Compar. Funct. Genom. 5, 230–238.
- Coop, A., Goode, D., Sumner, I., Crabbe, M.J., 1998. Effects of controlled mutations on the N- and C-terminal extensions of chick lens beta B1 crystallin. Graefe Arch. Clin. Exp. Ophthalmol. 236, 146–150.
- Dahlman, J.M., Margot, K.L., Ding, L., Horwitz, J., Posner, M., 2005. Zebrafish alpha-crystallins: protein structure and chaperone-like activity compared to their mammalian orthologs. Mol. Vis. 11, 88–96.
- de Jong, W.W., Leunissen, J.A., Voorter, C.E., 1993. Evolution of the alphacrystallin/small heat-shock protein family. Mol. Biol. Evol. 10, 103–126.
- Delaye, M., Clark, J.I., Benedek, G.B., 1982. Identification of the scattering elements responsible for lens opacification in cold cataracts. Biophys. J. 37, 647–656.
- den Dunnen, J.T., Szpirer, J., Levan, G., Islam, Q., Schoenmakers, J.G., 1987. All six rat gamma-crystallin genes are located on chromosome 9. Exp. Eye Res. 45, 747–750.
- den Dunnen, J.T., van Neck, J.W., Cremers, F.P., Lubsen, N.H., Schoenmakers, J.G., 1989. Nucleotide sequence of the rat gamma-crystallin gene region and comparison with an orthologous human region. Gene 78, 201–213.
- Detrich III, H.W., 1991. Cold-stable microtubules from Antarctic fish. In: di Priscu, G. (Ed.), Life Under Extreme Conditions: Biochemical Adaptations. Springer-Verlag, Berlin, pp. 35–49.

- DeVries, A.L., 1988. The role of antifreeze glycopeptides and peptides in the freezing avoidance of antarctic fishes. Comp. Biochem. Physiol. B 90, 611–621.
- di Prisco, G., Eastman, J.T., Giordano, D., Parisi, E., Verde, C., 2007. Biogeography and adaptation of Notothenioid fish: hemoglobin function and globin-gene evolution. Gene 398, 143–155.
- Eastman, J.T., 1993. Antarctic Fish Biology: Evolution in a Unique Environment. Inc., New York, Academic Press.
- Franck, E., Madsen, O., van Rheede, T., Ricard, G., Huynen, M.A., de Jong, W.W., 2004. Evolutionary diversity of vertebrate small heat shock proteins. J. Mol. Evol. 59, 792–805.
- Gellman, S.H., 1991. On the role of methionine residues in the sequenceindependent recognition of nonpolar protein surfaces. Biochemistry 30, 6633–6636.
- Hall, T., 1999. BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
- Hall, B.G., 2004. Phylogenetic Trees Made Easy: A How-to Manual. Sunderland, Mass, Sinauer Associates.
- Hejtmancik, J.F., Thompson, M.A., Wistow, G., Piatigorsky, J., 1986. cDNA and deduced protein sequence for the beta B1-crystallin polypeptide of the chicken lens. Conservation of the PAPA sequence. J. Biol. Chem. 261, 982–987.
- Kiss, A.J., 2005. Functional, biochemical and molecular analyses of the cold stable eye lens crystallins from the Antarctic toothfish *Dissostichus mawsoni*. (Dissertation). Ecology, Ethology & Evolution. University of Illinois. Urbana-Champaign.
- Kiss, A.J., Mirarefi, A.Y., Ramakrishnan, S., Zukoski, C.F., Devries, A.L., Cheng, C.H., 2004. Cold-stable eye lens crystallins of the Antarctic nototheniid toothfish *Dissostichus mawsoni* Norman. J. Exp. Biol. 207, 4633–4649.
- Kroger, R.H.H., Campbell, M.C.W., Munger, R., Fernald, R.D., 1994. Refractive index distribution and spherical aberration in the crystalline lens of the African cichlid fish haplochromis burtoni. Vis. Res. 34, 1815–1822.
- Liu, C., Asherie, N., Lomakin, A., Pande, J., Ogun, O., Benedek, G.B., 1996. Phase separation in aqueous solutions of lens gamma-crystallins: special role of gS. Proc. Natl. Acad. Sci. U. S. A. 93, 377–382.
- Lu, S.F., Pan, F.M., Chiou, S.H., 1996. Characterization of gamma-crystallin from the eye lens of bullfrog: complexity of gamma-crystallin multigene family as revealed by sequence comparison among different amphibian species. J. Protein. Chem. 15, 103–113.
- Marchetti, M.A., Pizarro, G.O., Sagher, D., Deamicis, C., Brot, N., Hejtmancik, J.F., Weissbach, H., Kantorow, M., 2005. Methionine sulfoxide reductases B1, B2, and B3 are present in the human lens and confer oxidative stress resistance to lens cells. Invest. Ophthalmol. Vis. Sci. 46, 2107–2112.
- McDermott, J.B., Cvekl, A., Piatigorsky, J., 1997. A complex enhancer of the chicken beta A3/A1-crystallin gene depends on an AP-1-CRE element for activity. Invest. Ophthalmol. Vis. Sci. 38, 951–959.
- Miyake, T., Amemiya, C.T., 2004. BAC libraries and comparative genomics of aquatic chordate species. Comp. Biochem. Physiol. C 138, 233–244.
- Narberhaus, F., 2002. Alpha-crystallin-type heat shock proteins: socializing minichaperones in the context of a multichaperone network. Microbiol. Mol. Biol. Rev. 66, 64–93.
- Nelson, W., Soderlund, C., 2005. Software for restriction fragment physical maps. In: Meksem, K., Kahl, G. (Eds.), The Handbook of Plant Genome Mapping: Genetic and Physical Mapping, vol. 1. Wiley-VCH, Hoboken, NJ, pp. 285–306.
- NRC, 2003. Frontiers in Polar Biology in the Genomic Era. Washington, D.C, National Academies Press.
- Nylander, J.A.A., 2004. MrModeltest. vol. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
- Pan, F.-M., Chuang, M.-H., Chiou, S.-H., 1997. Characterization of gS-crystallin isoforms from lip shark (*Chiloscyllium colax*): Evolutionary comparison between gS and b/g crystallins. Biochem. Biophys. Res. Commun. 240, 51–56.
- Parker, R.W., Paige, K.N., DeVries, A.L., 2002. Genetic variation among populations of the Antarctic toothfish: evolutionary insights and implications for conservation. Polar Biol. 25, 256–261.

- Piatigorsky, J., 1998. Multifunctional lens crystallins and corneal enzymes. More than meets the eye. Ann. N. Y. Acad. Sci. 842, 7–15.
- Pointer, M.A., Cheng, C.-H.C., Bowmaker, J.K., Parry, J.W.L., Soto, N., Jeffery, G., Cowing, J.A., Hunt, D.M., 2005. Adaptations to an extreme environment: retinal organisation and spectral properties of photoreceptors in Antarctic notothenioid fish. J. Exp. Biol. 208, 2363–2376.
- Posner, M., Kantorow, M., Horwitz, J., 1999. Cloning, sequencing and differential expression of alphaB-crystallin in the zebrafish, *Danio rerio*. Biochim. Biophys. Acta 1447, 271–277.
- Privalov, P.L., 1990. Cold denaturation of proteins. Crit. Rev. Biochem. Mol. Biol. 25, 281–305.
- Rajaraman, K., Raman, B., Ramakrishna, T., Rao, C.M., 2001. Interaction of human recombinant alphaA- and alphaB-crystallins with early and late unfolding intermediates of citrate synthase on its thermal denaturation. FEBS Lett. 497, 118–123.
- Riyahi, K., Shimeld, S.M., 2007. Chordate betagamma-crystallins and the evolutionary developmental biology of the vertebrate lens. Comp. Biochem. Physiol. B 147, 347–357.
- Runkle, S., Hill, J., Kantorow, M., Horwitz, J., Posner, M., 2002. Sequence and spatial expression of zebrafish (*Danio rerio*) alphaA-crystallin. Mol. Vis. 8, 45–50.
- Sagher, D., Brunell, D., Hejtmancik, J.F., Kantorow, M., Brot, N., Weissbach, H., 2006. Thionein can serve as a reducing agent for the methionine sulfoxide reductases. Proc. Natl. Acad. Sci. U. S. A. 103, 8656–8661.
- Sambrook, J., Russell, D.W., 2001. Protocol 3: selection of poly(A)+ RNA by oligo(dT)-cellulose chromatography. In: Irwin, N., Janssen, K.A. (Eds.), Molecular Cloning: A Laboratory Manual, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 7.13–17.17.
- Shimeld, S.M., Purkiss, A.G., Dirks, R.P., Bateman, O.A., Slingsby, C., Lubsen, N.H., 2005. Urochordate betagamma-crystallin and the evolutionary origin of the vertebrate eye lens. Curr. Biol. 15, 1684–1689.
- Siezen, R.J., Benedek, G.B., 1985. Controlled modulation of the phase separation and opacification temperature of purified bovine gamma IV-crystallin. Curr. Eye Res. 4, 1077–1085.

- Simmons, M.P., Ochoterena, H., Freudenstein, J.V., 2002. Amino acid vs. nucleotide characters: challenging preconceived notions. Mol. Phylogenet. Evol. 24, 78–90.
- Skow, L.C., Donner, M.E., Huang, S.M., Gardner, J.M., Taylor, B.A., Beamer, W.G., Lalley, P.A., 1988. Mapping of mouse gamma crystallin genes on chromosome 1. Biochem. Genet. 26, 557–570.
- Slingsby, C., Clout, N.J., 1999. Structure of the crystallins. Eye 13, 395-402.
- Smith, A.A., Wyatt, K., Vacha, J., Vihtelic, T.S., Zigler Jr., J.S., Wistow, G.J., Posner, M., 2006. Gene duplication and separation of functions in alphaBcrystallin from zebrafish (*Danio rerio*). FEBS J. 273, 481–490.
- Soderlund, C., Humphray, S., Dunham, A., French, L., 2000. Contigs built with fingerprints, markers, and FPC V4.7. Genome Res. 10, 1772–1787.
- Van Montfort, R.L., Bateman, O.A., Lubsen, N.H., Slingsby, C., 2003. Crystal structure of truncated human betaB1-crystallin. Protein Sci. 12, 2606–2612.
- Waddell, P.J., Steel, M.A., 1997. General time-reversible distances with unequal rates across sites: mixing gamma and inverse Gaussian distributions with invariant sites. Mol. Phylogenet. Evol. 8, 398–414.
- Willard, H.F., Meakin, S.O., Tsui, L.C., Breitman, M.L., 1985. Assignment of human gamma crystallin multigene family to chromosome 2. Somat. Cell Mol. Genet. 11, 511–516.
- Wistow, G., 2002. A project for ocular bioinformatics: NEIBank. Mol. Vis. 8, 161–163.
- Wistow, G., Wyatt, K., David, L., Gao, C., Bateman, O., Bernstein, S., Tomarev, S., Segovia, L., Slingsby, C., Vihtelic, T., 2005. gN-crystallin and the evolution of the bg-crystallin superfamily in vertebrates. FEBS J. 272, 2276–2291.
- Wu, Z., Delaglio, F., Wyatt, K., Wistow, G., Bax, A., 2005. Solution structure of (gamma)S-crystallin by molecular fragment replacement NMR. Protein Sci. 14, 3101–3114.
- Yu, C.M., Chang, G.G., Chang, H.C., Chiou, S.H., 2004. Cloning and characterization of a thermostable catfish alphaB-crystallin with chaperone-like activity at high temperatures. Exp. Eye Res. 79, 249–261.