

Characterization of γ S-Crystallin Isoforms from a Catfish: Evolutionary Comparison of Various γ -, γ S-, and β -Crystallins¹

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Received August 3, 1998

γ S-Crystallin from catfish eye lenses, formerly designated β s-crystallin in mammalian lenses, is structurally characterized in this study by cDNA cloning and sequencing. To facilitate sequence characterization of γ S-crystallin with structural properties lying between β - and γ -crystallins, a cDNA mixture was constructed from the poly(A)⁺ mRNA isolated from catfish eye lenses, and amplification by polymerase chain reaction (PCR) was carried out to obtain nucleotide segments encoding multiple γ S-crystallin isoforms. Sequencing several positive clones revealed that at least two distinct isoforms exist in the γ S-crystallin class of this teleostean fish, similar to the authentic γ -crystallin family characterized previously in species of the piscine class. Comparison of protein sequences encoded by two representative catfish γ S1 and γ S2 cDNAs with the published sequences of β -, γ -, and γ S-crystallins from shark, carp, bullfrog, bovine, and human lenses indicates that there is about 20–50% sequence homology between catfish γ S-crystallins and various members of the related β/γ -crystallin superfamily from different evolutionary classes, with a higher sequence similarity being found between catfish γ S- and mammalian γ -crystallins than between catfish γ S- and bovine or carp γ S-crystallins. Phylogenetic trees constructed on the basis of the nucleotide and protein sequence divergence among various β -, γ -, and γ S-crystallins corroborate the closer relatedness of catfish γ S- to authentic γ -crystallin than to bovine and carp γ S-crystallins. The results suggest that evolution of catfish γ S-crystallins follows a different path from that of bovine and carp γ S-crystallins and may represent a more ancient offshoot from the an-

cestral γ/γ S coding gene than carp and bovine γ S-crystallins. © 1998 Academic Press

Fish represents the oldest and most diverse group of vertebrates (1,2). The modern fishes comprise two major classes of piscine species, *i.e.* *Osteichthyes* or teleostean (bony) fishes, and *Chondrichthyes* or cartilaginous fishes (sharks and skates). The study of lens crystallins from the piscine class is of special interest from the evolutionary point of view because they constitute the early protein forms of vertebrates and are thought to have been ancestral to those of land vertebrates. It is especially noteworthy that the abundant presence of various common and specific classes of structurally conserved proteins (crystallins) in eye lenses of different species of vertebrates constitutes a good model system to unravel the complex process of evolution in structurally homologous proteins (3-5).

Most previous studies on the characterization of crystallins were concerned with various species of higher vertebrates with relatively fewer reports on the lens crystallins from lower aquatic vertebrates, *i.e.* varied classes of fish. In this report we characterize two major γ S-crystallin isoforms with structural properties lying between the well-known β - and γ -crystallins. This class of crystallin, formerly called β S and now renamed γ S crystallin (6,7), exists as a monomeric protein which is similar to the major authentic γ -crystallins. However unlike γ -crystallins which possess a free N-terminal amino-acid residue, γ S-crystallin has a blocked amino terminus as most members of β -crystallin family.

In this report we have for the first time cloned and sequenced γ S-crystallins from one teleostean species, *i.e.* the catfish, which is commonly raised in local freshwater aquacultures of Taiwan. Most catfishes are mostly nocturnal scavengers with atrophied eyes, cast-

¹ The sequence data for the cDNAs of catfish γ S-crystallins have been deposited with the EMBL Data Library under the Accession Nos. X81458 and X81459 for γ S1 and γ S2, respectively.

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ing some great interest to study the evolutionary effects of atrophied eye lenses on the constituting lens-specific crystallins and their corresponding genes. The characterization of catfish crystallins would be of special interest to us in light of the recent elucidation of the complete sequences of γ -crystallins from several species of teleostean fishes (8-10) and γ S-crystallins from the cartilaginous fish of shark (11). We have amplified cDNAs constructed from the lenses of catfishes using PCR methodology to aid in the structural analysis of multiple isoforms of γ S-crystallins.

MATERIALS AND METHODS

Catfish classification and description. Catfish (*Clarias batrachus*), one species of the common edible fishes, belongs to one of the teleostean fishes of the order or suborder *Nematognathi* (or *Siluroidei*). Most of catfishes are nocturnal scavengers and inhabit under fresh water. It is characterized by barbels around the mouth and has a very small atrophied eye lens as compared to that of bony fishes such as common carps. It spends some of its life cycle under the mud all-year around. The catfish of Southeast Asia such as the species studied here is sometimes called "walking catfish" due to its ability of moving across land (between bodies of water) by a slithering motion combined with a thrashing of its tail.

Isolation of mRNA from catfish lenses. The walking catfishes of less than 1-year-old were obtained from a local aquarium shop under a special contract for scientific research. Lenses were removed and stored in liquid-nitrogen container immediately after they were dissected and before the processing for mRNA isolation. Two deep-frozen lenses from one catfish were homogenized and RNA was extracted according to the standard cloning manual of Maniatis et al. (12). To obtain a full-length crystallin cDNA, poly(A)⁺RNA was purified using QuickPrep mRNA preparation kit (Pharmacia, Uppsala, Sweden) and then subjected to the synthesis of cDNA mixture by cDNA Synthesis System/Plus kit (Amersham, England).

PCR amplification, cloning, and sequencing of γ S-crystallin isoforms. Two oligonucleotide primers of sense and antisense orientations, covering 5'- and 3'-nucleotide coding regions for N- and C-terminal 4-6 amino-acid segments of the previously determined cDNA sequence for one carp γ S-crystallin (13), with the forward sequence, 5'-CATGGGCAAG(A/G)TCA(T/C)CTT(C/T)-3' (19-mer) and the reverse sequence, 5'-CATCACGCCA(T/C)(C/A)ATGCG-3' (17-mer) (with slant lines indicating use of degenerate codons in the primers) were synthesized. The conditions for PCR reactions were similar to the previous reports for cDNA amplification of teleostean and shark lenses (9-11), *i.e.* subjecting to 40 cycles of heat denaturation at 94 °C for 2.5 min, annealing the primers to the DNAs at 48 °C for 1 min and 20 sec and running DNA chain extension with *Taq* polymerase at 72 °C for 3 min, followed by a final extension at 72 °C for 10 min. Products were treated with Klenow Fragment and T4 polynucleotide kinase, and separated on a 1.2 % agarose gel and electroeluted according to standard procedures. The DNA fragments were subcloned into pUC18 previously digested with *Sma*I/BAP, and then transformed into *E. coli* strain JM 109. Plasmids purified from positive clones were prepared for nucleotide sequencing by dideoxynucleotide chain-termination method (14). The DNA sequences were determined by automatic fluorescence-based sequencing of templates amplified by PCR using model 373A DNA sequencing System (Applied Biosystems Inc., CA, USA) with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc.).

Sequence comparison of catfish γ S-crystallins and other crystallins. A commercially available software (DNASTAR Inc., Madison, WI, U.S.A.) was used for the estimation of DNA and protein sequence homology based on percent sequence identity (9).

Hydropathy profile analysis. A computer-based program analysis of the overall surface distribution of hydrophilic amino acids in five γ S- and γ -crystallins, based on the Kyte-Doolittle hydropathy scale (15) was performed using the MacVector sequence analysis software (International Biotechnologies, Inc., New Haven, CT). The signs of the values have been reversed in order to plot the hydrophilicity instead of hydrophobicity profile. A window of size N=7 was run along the amino-acid sequence length of each crystallin; for each window, the hydropathy values of the 7 amino acids were summed and divided by 7 to obtain the average hydrophilicity per residue for the window. Values above the axis denote hydrophilic regions which may be exposed on the outside of the protein molecule whereas those values below the axis indicate hydrophobic regions which tend to be buried inside the protein.

Construction of a phylogenetic tree for β -, γ -, and γ S-crystallins. A software package of LaserGene for the Apple Macintosh computer from DNASTAR, Inc. was used for the estimation of sequence homology based on percent similarity and divergence among different cDNA and protein sequences of β -, γ - and γ S-crystallins. Percent divergence is calculated by comparing sequence pairs in relation to the phylogenetic tree. On the other hand the percent similarity is estimated by comparing sequences directly without accounting for phylogenetic relationships. Phylogenetic tree was then constructed using the algorithm of Hein (16), which was included in the MegAlign programs of the package. It is a multiple-sequence alignment program that builds trees as it aligns DNA or protein sequences using a combination of distance matrix and approximate parsimony methods. This method constructs multiple-alignment by imposing restrictions based on evolutionary relatedness of the aligned sequences, which is useful to align highly evolved gene families that have clear evolutionary relationships.

RESULTS AND DISCUSSION

Understanding the mechanism for the evolution of functionally related proteins from different species remains a major theme of current research in protein chemistry and molecular biology. The structural and genetic basis for the generation of multiple γ S-crystallin isoforms in the shark lens (11) contrasting with only one γ S-crystallin found in most mammalian class is of significant interest, which provides the motivation to study and compare the primary structure of this unique crystallin class from one teleostean fish we previously characterized (10), *i.e.* catfish. Especially noteworthy is our recent findings that catfishes with atrophied eye lenses appear to possess several mutated or different crystallin isoforms from the homologous crystallin class found in varied species of the mammalian class. Therefore a more extensive characterization from an evolutionarily or developmentally unique animal species such as the catfish presented here may eventually provide some insight into the phenomenon of species diversification and the accompanying molecular origin of various crystallins.

Characterization of γ S-Crystallins from a Catfish of the Teleostean Class

Previous studies have suggested the distinct difference in structural characteristics between shark γ -crystallin (17,18) and those homologous crystallins obtained from

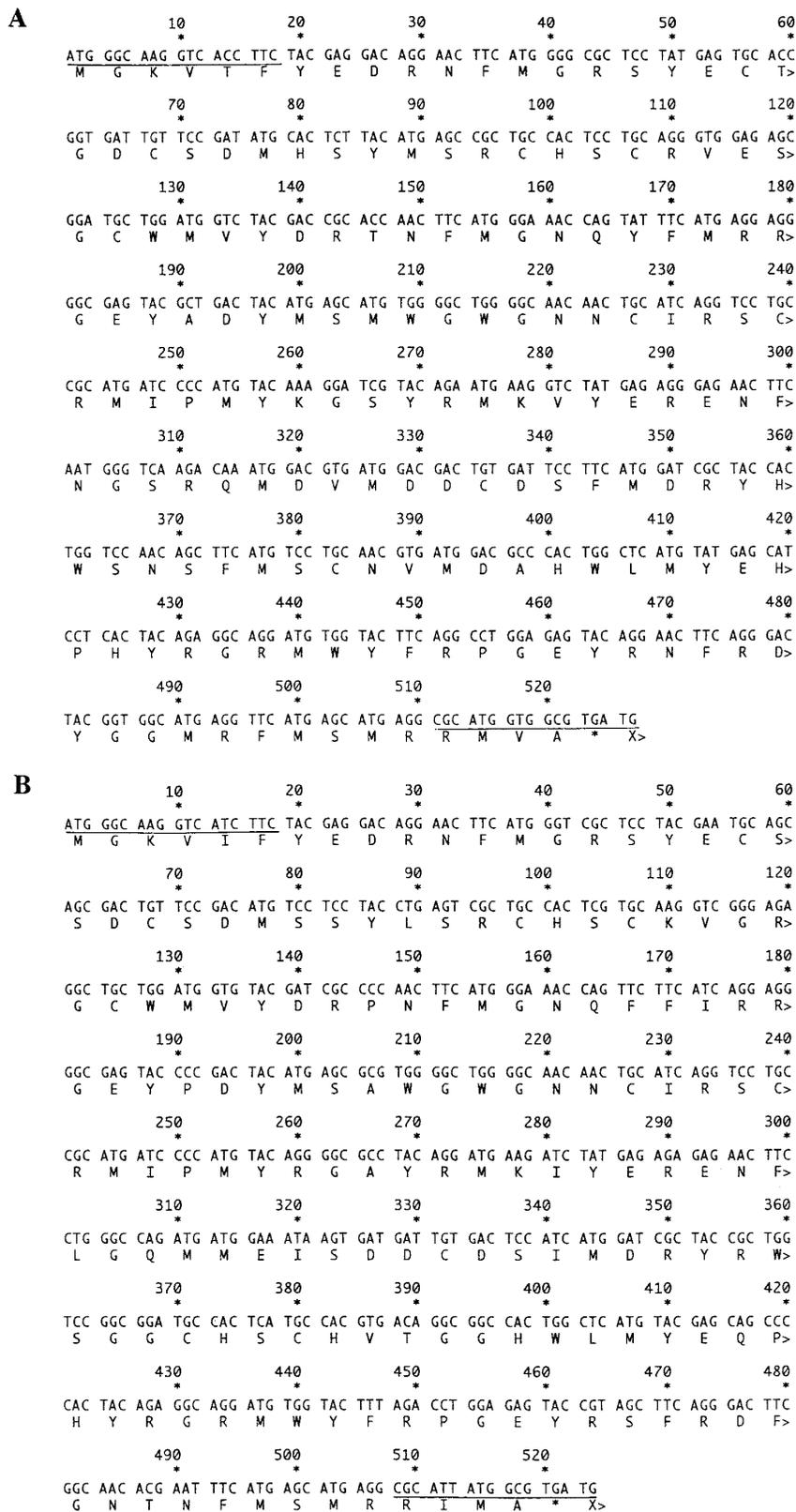


FIG. 1. Nucleotide and deduced protein sequences of catfish γ S-1 (**A**) and catfish γ S-2 (**B**) crystallins. In (**A**) the nucleotide sequence of 527 bp is shown above the amino acid sequence of 174 residues, including the translation initiation methionine. In (**B**) the nucleotide sequence comprises 524 bp encoding a protein sequence of 173 amino acids. Asterisks (*) are indicated in every 10-nt segment for easy tracing of sequence contents. Amino acids are denoted by one-letter symbols. The 5' and 3' nucleotide segments used as primers for PCR reactions are underlined.

A						
% homology	Bovine γ II	Carp γ S	Bovine γ S	Bullfrog β	Shark γ S	Human γ 5
Catfish γ S-1	50.8	44.5	44.5	28.4	54.7	52.3
Catfish γ S-2	53.2	47.8	44.0	33.1	56.2	53.0

B						
% homology	Bovine γ II	Carp γ S	Bovine γ S	Bullfrog β	Shark γ S	Human γ 5
Catfish γ S-1	41.0	35.5	30.7	20.4	43.0	42.1
Catfish γ S-2	46.4	37.8	34.9	24.0	48.7	45.9

FIG. 2. Pair-wise comparison of nucleotide (**A**) and protein (**B**) sequence homology between two catfish γ S-crystallins and various β -, γ -, and γ S-crystallins from species of different vertebrate classes. Analysis of sequence homology was carried out using the software package (DNASTAR Inc., Madison, WI) on the published sequences of carp γ S (13), bovine γ S (6), bullfrog β 2 (28), bovine β 2 (30), bovine γ II (31), and human γ 5 (32) crystallins.

lenses of teleostean fishes such as carp (8,13). The structural analysis of shark cDNAs encoding γ -crystallins by means of PCR technique has also revealed two cDNAs encoding two γ -crystallins supposedly to be uniquely expressed only in teleostean or mammalian classes alone (19). Especially interesting is the finding that the amino acid compositions of γ -crystallins seem to lack the unique characteristic of high methionine content (> 10%) as commonly observed for that of teleostean fishes (9,10). Shark γ -crystallin showed a much more complex pattern in the multiplicity of isoforms (17,20) than that of teleostean crystallins. Similarly γ S-crystallin of shark lens was also found to be present in multiple isoforms (11).

We question whether such multiplicity of isoforms for shark γ S-crystallin may be also present in catfish lenses, which is a favored species for us to study the evolutionary effects of atrophied eye lenses on their lens-specific crystallin gene expression. We have hence used the recent rapid method of cloning and sequencing by means of PCR methodology for the determination of cDNA sequences of catfish γ S-crystallin(s). PCR amplification of total lens cDNA mixtures prepared from lenses of at least five catfishes with the designed and degenerate primers based on partial DNA coding sequences of carp γ S-crystallin (13) achieved the isolation of one major PCR fragment corresponding to the complete open reading frame encoding γ S-crystallin isoforms from catfish lenses. The size determination of PCR-amplified cDNA coding for γ S crystallin was estimated to be about 520 bp, similar to that of shark γ S-crystallin and in agreement with protein species of about 170-180 amino-acid residues for mammalian γ - and γ S-crystallins.

Sequence Analysis of cDNA Encoding Catfish γ S-Crystallins

Several positive clones have been identified, with their 5' and 3' nucleotide sequences being determined to be essentially identical to those predicted by degenerate primers, indicative of the existence of multiple

isoforms for catfish γ S-crystallin, which is similar to shark and in contrast to bovine (6,7) and human (21) γ S-crystallins with only one sequence being identified. The deduced protein sequences together with their genetic coding sequences of two clones, designated as catfish γ S-1 and γ S-2 are shown in Fig. 1A and 1B. The cDNA sequences encoding catfish γ S-1 and γ S-2 were both found to consist of 522 and 519 nucleotides respectively, each of which covering a full-length protein of 174 and 173 amino-acid residues including the initiating methionine. They are close to carp γ S (174 a.a.) and slightly lower than bovine γ S (177 a.a.). In order to avoid sequencing errors, sequence accuracy was doubly checked and confirmed by automatic fluorescence-based DNA sequencing technique. The only uncertainty may lie in the first and last few nucleotides present in the 5' and 3' region of the PCR fragment even though we have used some degenerate codons in the primers. Recently we have used 3'- and 5'-RACE (Rapid Amplification of cDNA Ends) protocols of PCR to further validate these ambiguous short segments with comfort and gratification.

Sequence Alignment and Comparison of β -, γ -, and γ S-Crystallins

In the pair-wise sequence homology comparison of various nucleotide (Fig. 2A) and deduced amino-acid sequences (Fig. 2B) from species of different classes using commercial software package (DNASTAR program), it is found that catfish γ S-1 and γ S-2 crystallins show 50-53% and 41-47% DNA and protein sequence homology to bovine γ II crystallins respectively whereas only 44-45% and 30-35% DNA and protein sequence homology respectively are found between catfish and bovine γ S-crystallins, indicating that catfish γ S is evolutionarily more related to authentic mammalian γ - than γ S-crystallins and may represent an "off-shoot" crystallin form from the divergent evolution of ancient γ -crystallin gene family. On the other hand, it

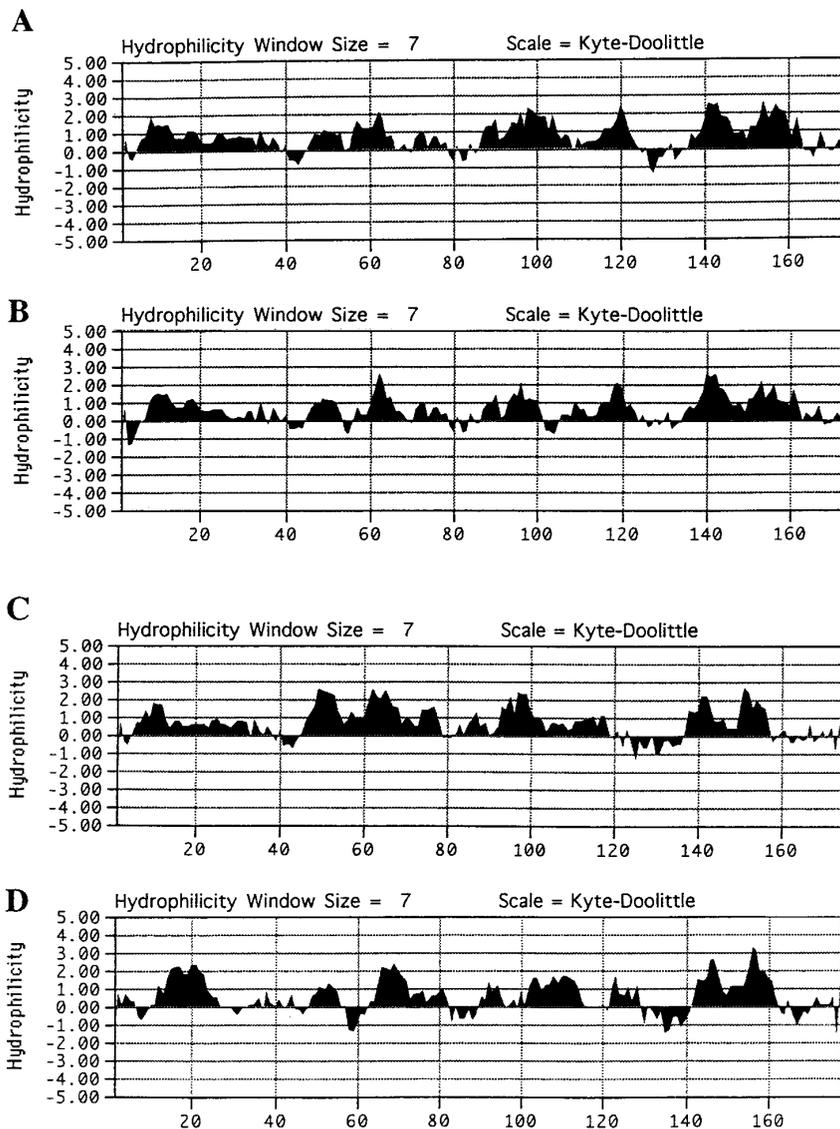


FIG. 4. Hydropathy profile prediction patterns of catfish γ S-1 (A), catfish γ S-2 (B), bovine γ II (C), and bovine γ S (D) crystallins based on primary amino acid sequences. The analysis of the distribution of surface hydrophilicity along each full sequence was based on the method of Kyte and Doolittle (15). It is noteworthy that the patterns for two catfish γ S-crystallins and bovine γ II are very similar, while that of bovine γ S shows a distinct difference from the other three at the N-terminal region (residues 1–40) and the middle region (residues 90–120).

and calf are aligned jointly for structural comparison. It is noteworthy that the overall hydropathy profiles along the full length of primary sequences for catfish γ S-1 and γ S-2 crystallins (Fig. 4A, 4B) are very similar, which are also fairly similar to that of bovine γ II (Fig. 4C) and in great contrast with the dissimilar pattern for bovine γ S-crystallin (Fig. 4D). These profiles exemplify very similar surface distributions of hydrophilic amino-acids in the two catfish γ S-1 and γ S-2 crystallins and may suggest a resemblance in the secondary structure between the two crystallins. It appears that distinct difference found in the distribution of polar or hydrophobic amino-acid residues is somewhat greater between catfish γ S and bovine γ S than that of catfish

γ S and bovine γ II crystallins, which is also reflective of the difference found in the pair-wise comparison of sequence homology between these crystallins.

Construction of Phylogenetic Trees

In our systematic pair-wise sequence comparison of crystallin genes and their deduced protein sequences from varied species of vertebrates, higher sequence homology is generally found between cDNA sequences than their deduced protein sequences. Two phylogenetic trees based on nucleotide (Fig. 5A) or protein (Fig. 5B) sequence alignment of eight β -, γ - and γ S-crystallins are constructed using a combination of dis-

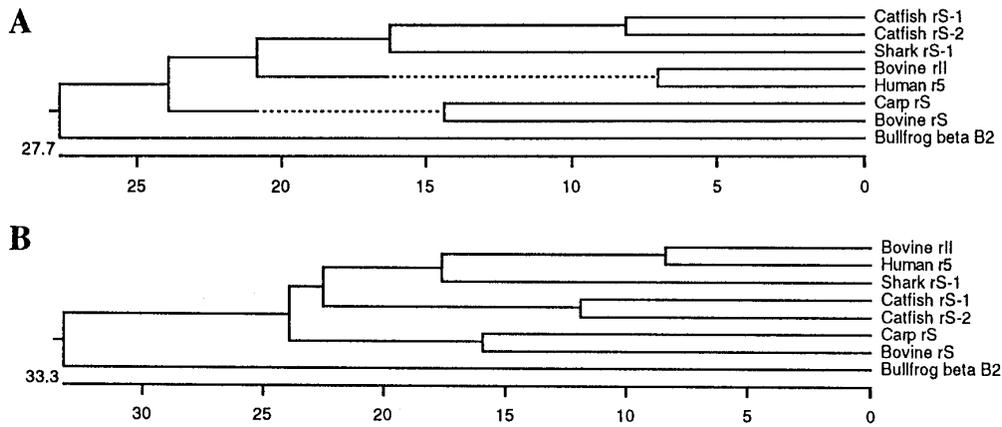


FIG. 5. Construction of phylogenetic trees of eight β -, γ -, and γ S-crystallins from species of different vertebrate classes based on nucleotide (A) and protein (B) sequences. Analysis of sequence data was carried out using the software package LaserGene on an Apple Macintosh computer (DNASTAR Inc., Madison, WI). Percentage divergence is calculated by comparing sequence pairs in relation to the relative positions in the phylogenetic tree, in contrast to the percentage similarity, which is estimated by comparing percentage sequence identity directly without accounting for phylogenetic relationships. A phylogenetic tree was then constructed based on the percentage divergence between nucleotide and protein sequences using a combination of distance matrix and approximate parsimony methods using the phylogeny generation program of Hein (16). This algorithm carries out multiple alignment by imposing restrictions based on evolutionary relatedness of the aligned sequence pairs. The tree was built using the clustal method and weighted residue-weight table. The length of each pair of branches represents the sequence distance between aligned pairs. The scale beneath the tree measures the distance between sequences (in millions of years). The dotted lines shown in (A) illustrate the fact that the sequence distance based on cDNA comparison is not proportional to the scale.

tance matrix and approximate parsimony methods (16). Similar to our previous phylogenetic analysis of various crystallins from invertebrate and vertebrate species (25-29), the overall patterns of the mutual phylogenetic interrelationship among these crystallins are fairly similar, attesting to the general applicability of the tree construction based on cDNA or protein sequence comparison. However detection of sequence divergence based on protein sequences rather than cDNA sequences appears to be more sensitive when comparing highly homologous protein families such as β/γ crystallins shown here. It is noteworthy that the phylogenetic tree based on the sequence divergence among these protein sequences indeed exemplifies the close relatedness of catfish and shark γ S-crystallins to γ -crystallins from bovine and human lenses. On the other hand, carp γ S-crystallin is grouped with bovine γ S-crystallin, in agreement with the percent homology shown in Fig. 2. Especially notable is the observation that β 2-crystallin sequence from bullfrog is correctly placed at a different branching point of the tree from that of γ - and γ S-crystallins, indicative of two distinct evolutionary pathways leading to β - and γ/γ S crystallins from the ancestral β/γ protein family.

CONCLUSION

The abundant presence of various common and specific classes of structural proteins, *i.e.* lens crystallins, in different species of vertebrates constitutes a good model system to unravel the complex process of evolu-

tion in structurally homologous proteins (3-5). Extensive protein and cDNA sequence data on various lens crystallins have been obtained from various species of vertebrates, allowing evolutionary relationships of these highly evolved and related crystallin families to be derived. The present sequence characterization of catfish γ S crystallins and phylogenetic comparison of various β -, γ - and γ S-crystallins suggest that evolution of catfish γ S-crystallins follows a different path from that of bovine and carp γ S crystallins and may represent a more ancient offshoot from the ancestral γ/γ S gene than carp /bovine γ S-crystallins.

ACKNOWLEDGMENTS

This work was supported by Academia Sinica and the National Science Council (NSC Grants 83-0203-B-001-086, 83-0418-B-001-020BA, 84-2311-B-001-050-BA, and 86-2311-B-002-031-B15), Taipei, Taiwan.

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