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Immunoglobulin Heavy Chain Constant and Heavy Chain Variable Region Genes in Phylogenetically Diverse Species of Bony Fish

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Summary. Genomic DNA from 18 phylogenetically diverse species of bony fish was hybridized with probes specific for the channel catfish immunoglobulin heavy chain constant (CH) gene, as well as with immunoglobulin heavy chain variable (VH) probes specific for five channel catfish VH gene families. The results showed that CH probes strongly hybridized only to genomic fragments from other catfish species. In contrast, restricted DNA from most other species hybridized with at least two channel catfish VH probes. In those species whose DNA hybridized with multiple VH probes, the restriction pattern of hybridizing fragments was probe-dependent. These studies suggest that (1) the CH gene defined in channel catfish appears to share similarity only with CH genes in other catfish species, (2) families of VH genes appear to have diverged in early phylogenetic lineages of teleosts, and (3) VH genes similar to those defined in catfish appear to be widely represented in phylogenetically diverse species of teleosts.

Key words: Immunoglobulin heavy chain — Immunoglobulin variable region — Immunoglobulin constant region — Fishes — Catfish — Phylogeny — Hybridization

The variable region (V) of immunoglobulin (Ig) heavy (H) chains is composed of three complementarity-determining regions (CDR), which serve as hypervariable binding sites for antigen, and four framework regions (FR). H chain diversity arises through a complex series of genomic rearrangements wherein selected individual variable (VH), diversity (DH), and joining (JH) gene segments undergo recombination events mediated by the recognition of specific signal sequences to form a functionally rearranged V region. This rearranged V region is then expressed in association with an H chain constant region (CH) gene (reviewed by Max 1989).

VH genes encode CDR1 and CDR2 of the H chain V region; hence, the structural diversity present within these regions is largely dependent upon the genomic diversity of different VH elements. Comparisons of the amino acid sequence of H chain V regions have shown that these sequences can be arranged into related groups (Kabat et al. 1987). Subsequent approaches comparing nucleic acid similarities of VH genes further extended these observations to define families of VH genes wherein members of the same VH family show greater than 80% nucleotide similarity, while the similarity among members of different VH families is generally less than 70% (Brodeur and Riblet 1984). Genomic hybridization analyses under appropriate stringency conditions have been used to estimate the genomic repertoire of VH members in each VH family. In addition Southern blot hybridization analyses have also been used to study the relationships of VH gene families in mammalian species (reviewed by Tutter and Riblet 1989).

The use of VH hybridization approaches to detect VH genes across distant phylogenetic lines has

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been demonstrated by the work of Litman and coworkers in their studies to define the genomic organization of immunoglobulin genes in the horned shark *Heterodontus francisci*. Probes derived from the murine S107 VH family were hybridized to shark genomic and cDNA libraries, and from these analyses shark VH and in turn shark CH genes were defined. These studies led to the finding that the genomic organization of Ig genes in the shark *Heterodontus* is quite different from that known in mammals. In this species there are at least 100 individual clusters of H chain genes, each cluster containing VH, DH, JH, and CH gene segments. However, the respective genes in these different clusters are highly related. For example, pairwise comparisons of nine *Heterodontus* VH segments indicate about 86% overall nucleotide similarity (Kokubu et al. 1988).

In contrast, the genomic arrangement of H chain genes in channel catfish appears to be quite distinct from that found in sharks. Gene titration experiments have shown that there is only a single copy of the CH gene expressed in characterized full-length heavy chain cDNA (Ghaffari and Lobb, 1989b). Phylogenetic lineages reflecting single-copy CH genes have also been identified in *Xenopus laevis* (Schwager et al. 1988) as well as in *Elops saurus*, another teleost fish (Amemiya and Litman 1990). This finding prompted our ongoing investigations to determine if structural diversity of V region genes coevolved with single-copy CH genes.

Earlier studies had shown that there was considerable FR1 diversity in the amino acid sequences of purified channel catfish H chains (Lobb and Olson 1988). This variation strongly suggested that there was likely to be extensive VH diversity. It has now been shown based upon sequence analysis of different channel catfish H chain cDNAs that five different groups of VH genes are present whose definition is consistent with the definition of five different VH gene families (Ghaffari and Lobb 1991). Recently a sixth channel catfish VH family has also been defined (Warr et al. 1991). Members of these different VH families likely recombine with putative DH segments and different JH segments, and these recombined elements are expressed with the same CH gene. A genomic cluster of JH gene segments has also been defined, and this JH gene cluster is closely linked to the CH gene known from cDNA analyses (Ghaffari and Lobb 1992).

VH genes have also been identified in other teleost species. These species include *Elops* (Amemiya and Litman 1990), the rainbow trout (*Oncorhynchus mykiss*, Matsunaga et al. 1990), the goldfish (*Carassius auratus*, Wilson et al. 1991), and the Atlantic cod (*Gadus morhua*, Bengtén et al. 1991). However, at this point, little is known about the immunoglobulins in most species of bony fish. Because of the vast number of species of bony fish, it is likely not feasible to study the immunoglobulins of each. For this reason it was important to determine if general methods could be used to address questions involving the diversity of Ig genes in bony fish. The experimental approach taken was to use DNA probes derived from defined regions of channel catfish H chain genes and by Southern blotting methods to determine under experimentally defined hybridization stringencies whether similar genes are likely present in other species. These results provide initial insight into CH and VH genes in phylogenetically diverse species of bony fish.

Materials and Methods

Animals and Purification of Genomic DNA. Channel catfish were obtained from nearby commercial processing plants (Delta Pride, Belzoni, MS) and maintained in recirculating holding facilities. Additional species of catfish were collected from nearby Ross Barnett reservoir. The other species of fish used in this study were either collected from feral populations or from animals maintained at the Mississippi Natural Science Museum. The common and scientific names for each of the species included in this study are listed in Table 1 of the Results section.

High molecular weight genomic DNA from peripheral blood erythrocytes was isolated according to methods previously described (Ghaffari and Lobb 1989b). Briefly, 10 volumes of 0.5 M EDTA pH 8.0, 0.5% *N*-lauroylsarcosine, and 100 μg/ml proteinase K were added to the suspended cells, and the suspension was incubated at 50°C overnight (Maniatis et al. 1982). After three or four extractions with equal amounts of equilibrated phenol, the samples were dialyzed against 10 mM Tris, 1 mM EDTA pH 8.0 at 4°C overnight. The genomic DNA was further purified by equilibrium centrifugation over a cesium chloride gradient (R = 1.399). Centrifugation was performed in a Beckman VTi-50 rotor at 45,000 rpm for 16 h at 15–20°C. The viscous fractions of DNA were collected and dialyzed at 4°C against 10 mM Tris, 1 mM EDTA pH 8.0.

Probes. Three different heavy chain cDNA fragments were used as CH probes in this study and were derived from previously isolated clones. From clone NG14 a 564 bp EcoRI-PvuII fragment was used as the CH1 and CH2 probe. CH3 and CH4 probes were generated from EcoRI fragments of 276 and 578 bp, respectively, from clone HG103. The CH4 probe encoded CH4 as well as the C-terminal region and the 3′ untranslated region (Ghaffari and Lobb 1989a,b).

Five different cDNA fragments were used as VH probes in these analyses; each was representative of one of the five VH gene families identified in the channel catfish and were derived from clones previously reported. Each VH probe coded for a region extending from the leader or FR1 through CDR2 or FR3. The VH1 and VH3 family probes were 300 and 266 bp PstI fragments isolated from clones NG70 and NG54, respectively. The VH2 probe was a 378 bp PvuII fragment isolated from clone NG41 while a PvuII fragment of approximately 450 bp from cDNA clone NG66 represented family VH5. The VH4 probe was a 260 bp PvuII-BstEII fragment derived from clone NG10 (Ghaffari and Lobb 1991). Each probe was radiolabeled with a random priming kit (Amersham, Arlington Heights, IL) to a specific activity of about 5×10^8 to 1×10^9 cpm/μg.

Dot-blot Hybridizations. Using a microsample filtration manifold (Minifold; Schleicher and Schuell, Keene, NH) the VH-containing plasmids (NG70, NG41, NG54, NG10, NG66) and pUC18 (a plasmid vector related to the pUC9 vector used for cloning the cDNA VH inserts) were transferred to nitrocellulose filters. Replicate dot-blots of the denatured DNA were hybridized with the various VH probes. The filters were prewashed in 6× SSC and 0.1% SDS at 65°C for 1 h, prehybridized in 3× SSC, 5× Denhart's, 25% formamide, 0.1% SDS, and hybridized in 3× SSC, 1× Denhart's, 25% formamide, 0.2% SDS, and 10% dextran sulfate overnight at 42°C. The filters were washed in 2× SSC and 0.1% SDS at 65°C, and autoradiography was done at −70°C with an intensifying screen. The resulting autoradiographs indicated that the VH probes did not cross-hybridize. These hybridization conditions were used in the genomic analyses presented in this study.

Analysis of Genomic DNA. Genomic DNA was restricted to completion with EcoRI. To verify complete digestion, 0.3 μg of lambda DNA was added to 0.5 μg of genomic DNA from representative samples (Klupt and Komro 1989). In each case restriction of lambda DNA indicated the absence of potential digestion inhibitors in the genomic samples. Ten micrograms from each of the restricted DNA samples in the absence of lambda DNA was electrophoresed in 0.8% agarose gels, and the DNA was transformed to either nitrocellulose or Nytran membranes (Schleicher and Schuell, Keene, NH). The filters were hybridized with the VH or CH probes according to the above conditions. Autoradiography was performed at −70°C with an intensifying screen. Some membranes were stripped of bound probe by shaking the filters in a boiling solution of 1 mM tetrapotassium pyrophosphate, 2.5 mM Tris, and 0.1 mM EDTA pH 8.0 for 15–30 min. Autoradiography of the stripped filters was developed, and if no signal remained, the filters were hybridized with another probe.

In general the known genomic equivalents from the erythrocytes of these fish ranged from approximately 1.6 to 5.8 pg/cell. The genomic equivalent of the bowfin (2.3 pg/cell) appears to be comparable to the genomic equivalents of the catfish (1.7–2.1 pg/cell) (Hudson 1976). Intermediate genomic equivalents are seen in a member of Acipenseriformes, the order in which the paddlefish is classified, as well as in members of the cyprinid family. An acipenseriform has a genomic equivalent of approximately 3.2 pg/erythrocyte while the range for cyprinids is generally from 3.2 to 3.5 pg/nucleus with an average of about 3.4 pg/nucleus. The highest-known genomic equivalents for the species used in this study are found in members of the salmonid family. In this family, the range is from 4.9 to 5.8 pg/cell with an average of about 5.3 pg/cell (Fasman 1976). Because of this wide variety in the DNA content of the species studied, Southern blot autoradiographs were developed at various time points (48–96 h). The autoradiographs in which the hybridizing fragments, if present, were most visible were used to assemble the data presented in Table 1 of the Results section.

Results

Ig CH Genes in Closely Related Species

To determine whether CH genes similar to the CH gene defined in the channel catfish were present in closely related species, genomic DNA from five additional species of catfish was digested with EcoRI and hybridized with probes representing the four CH domains of the known channel catfish C region

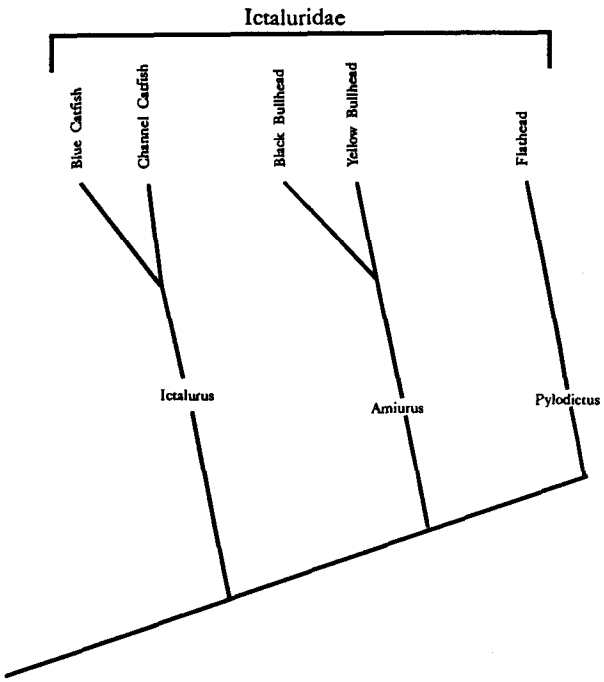

Fig. 1. Phylogenetic tree indicating the relative branching order of ictalurid species studied in this report. The information depicted is based on phylogenetic patterns of morphological features as reported by Lundberg (1982). The phylogenetic position of the family Ariidae is uncertain and is not shown (Lauder and Liem 1983).

gene. Four of the five species (the blue catfish, the yellow bullhead, the black bullhead, and the flathead) are from the same family as the channel catfish (*Ictaluridae*), while the fifth species (the sea catfish) is from the family *Ariidae*. The phylogenetic relationships of these ictalurids are depicted in Fig. 1.

The results of the Southern blot analyses showed that at least two genomic fragments from each of these five other catfish hybridized with the channel catfish CH probes (Fig. 2). The comparison of the number and size of the hybridizing genomic fragments in these different species indicates that there is likely to be significant structural variation among catfish CH genes. EcoRI is known to cut within the coding region of the CH2, CH3, and CH4 domains of the channel catfish CH gene (Ghaffari and Lobb 1989a,b). EcoRI-restricted genomic channel catfish DNA yielded three fragments which hybridized with CH domain-specific probes in Southern blots: a 7.8 kb fragment which contains the CH1 and CH2 domains, a 0.7 kb fragment which contains most of CH3, and a 1.9 kb fragment which contains the CH4 domain. Another EcoRI genomic fragment of 5.2 kb is known to hybridize under high stringency conditions with probes derived from the CH1 and CH2 domains of the channel catfish CH gene. This latter fragment contains a putative second CH gene which, at the present time, is uncharacterized

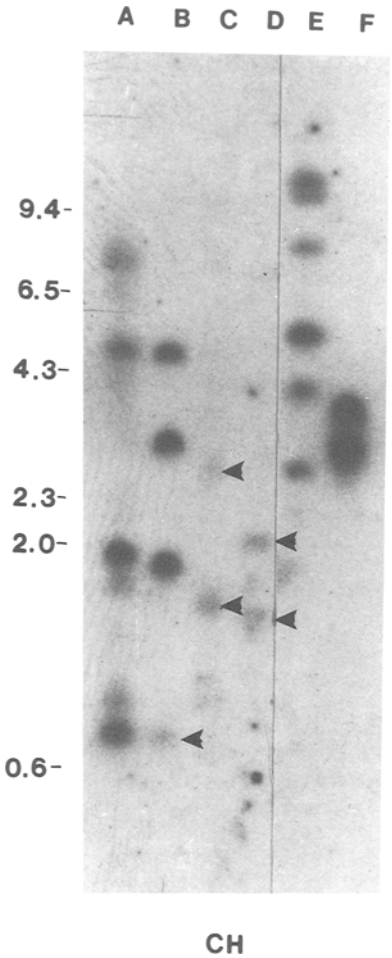

Fig. 2. Southern blot autoradiograph of EcoRI-restricted genomic DNA from six species of catfish hybridized with channel catfish CH probes. The lane designations are as follows: *A*, channel catfish; *B*, blue catfish; *C*, yellow bullhead catfish; *D*, black bullhead catfish; *E*, flathead catfish; and *F*, sea catfish. Arrowheads indicate the positions of weakly hybridizing fragments. The sizes shown in kilobases were determined from HindIII-digested lambda DNA.

(Ghaffari and Lobb 1989b). The blue catfish, which is the closest relative of the channel catfish (reviewed in Taylor 1969; and Lundberg 1982) had genomic EcoRI fragments that were approximately the size of some of the genomic fragments observed in the channel catfish (Fig. 2, lanes A and B). However, the lack of identical fragments suggests that CH structural differences exist.

In the more distant ictalurids as well as in the sea catfish, the size and number of the hybridizing fragments were quite different from those of fragments identified in either the channel or blue catfish. The yellow bullhead, the black bullhead, and the sea catfish had only two EcoRI fragments which hybridized with the channel catfish CH probes. The fragments from these three species were not similar in size to one another. In the flathead catfish, the channel catfish CH probes hybridized with six relatively large EcoRI fragments whose sizes differed from the CH-hybridizing fragments seen in the other catfish. Because of the differences in the size, number, and intensity of the hybridizing fragments, there are likely significant differences among the CH genes of these species. The results with the flathead catfish might suggest that there may also be differences in the number of CH genes which are present.

Ig CH Genes in Other Families of Bony Fish

To determine if the channel catfish CH gene was similar to CH genes in divergent species of bony fish, genomic DNA was obtained from 12 species representing 10 freshwater and saltwater families of bony fish. These species included some of the earliest bony fish still in existence, such as the paddlefish (family Polyodontidae), the spotted gar (family Lepisosteidae), and the bowfin (family Amiidae). Species were also collected to include modern teleosts such as the largemouth bass (family Centrarchidae) and the redfish (family Sciaenidae). The phylogenetic relationships of these bony fish are depicted in Fig. 3. These fish were bled, the genomic DNA was isolated, and the EcoRI-restricted DNA was hybridized with the channel catfish CH probes. The results from these hybridizations are indicated in Table 1. Figure 4 shows representative Southern blots for some of these species. In these combined studies only three species had restriction fragments which hybridized with the channel catfish CH probes. In these three species, the hybrid striped bass (lane F in Fig. 4), the coho salmon, and the highfin carpsucker (data not shown), a single restriction fragment of weak to modest intensity was observed. With the other nine species no fragments hybridized with the CH probes. These results are in striking contrast to the VH hybridization results described below. These analyses suggest that the CH gene defined in the channel catfish generally is not similar to the CH genes of other species of bony fish.

Ig VH Genes in Closely Related Species

Studies were undertaken to determine if VH genes identified in the channel catfish were likely represented in other catfish species. Prior to conducting genomic analyses, however, it was important to define hybridization conditions that would not permit cross-hybridization of channel catfish VH probes. Restriction fragments derived from prototype members of five channel catfish VH families were analyzed as potential probes; each of the probes were obtained from cDNA clones and extended from the

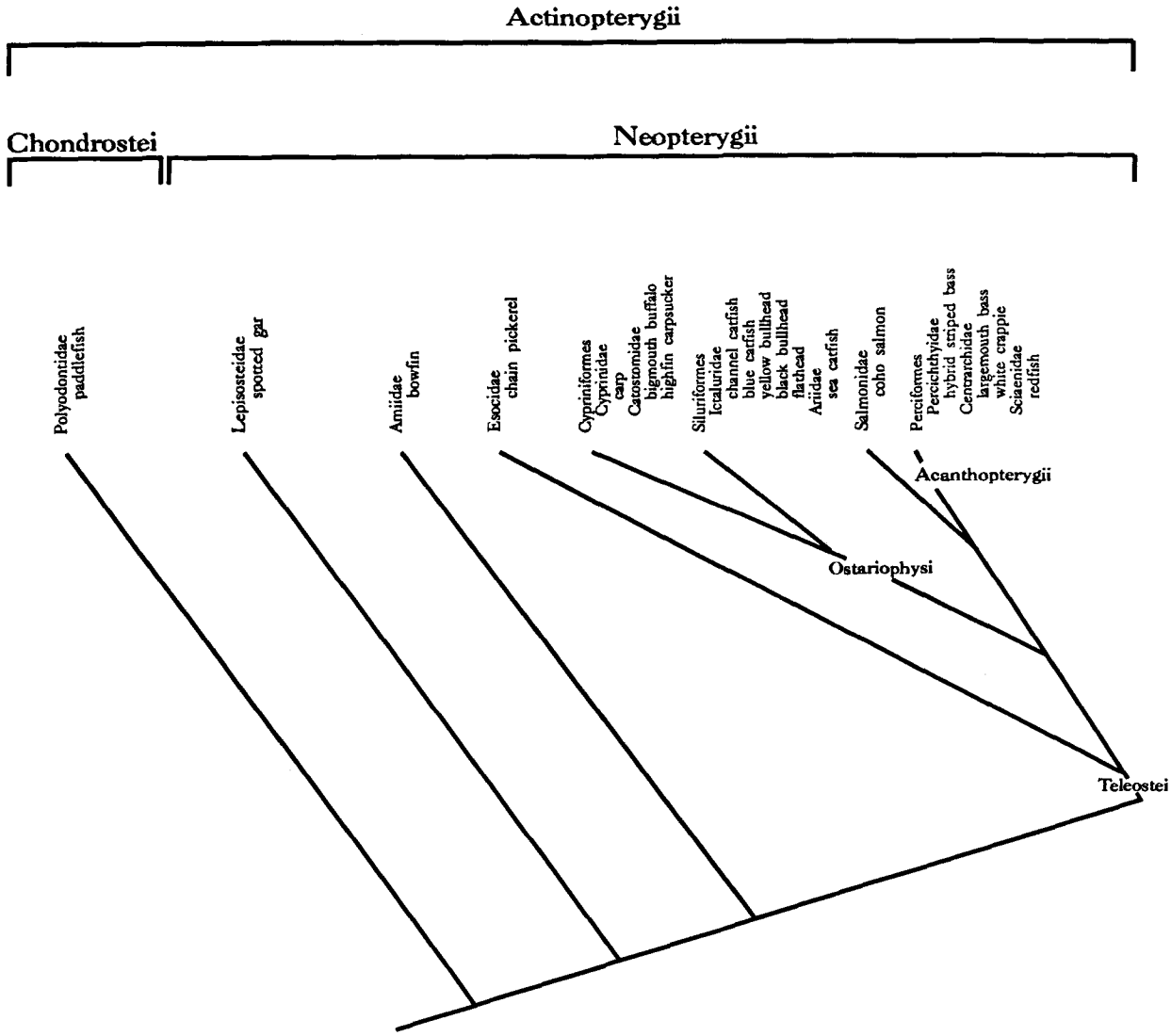

Fig. 3. Phylogenetic tree indicating the relative branching order of the bony fish studied in this report. The information depicted is based on phylogenetic patterns of morphological features as reported by Lauder and Liem (1983). The branching order shown is in agreement with the study of Carroll (1988); however, the figure incorporates Lauder and Liem's placement of Lepisosteidae (gars) within Neopterygii.

leader or FR1 and ended within CDR2 or FR3 of the V region. Plasmids containing the sequenced inserts were transferred to nitrocellulose filters in dot-blot experiments and replicate blots were probed with each of the labeled fragments under different hybridization conditions. These experiments defined stringency conditions which allowed hybridization to occur only within members of the same VH family (see Materials and Methods). Among the VH prototypes which were analyzed in these dot-blot studies, the highest nucleotide similarity (71%) was shared by NG10 (VH4) and NG66 (VH5) (Ghaffari and Lobb 1991). Because NG10 and NG66 did not appear to cross-hybridize in these experiments, these analyses suggest that for positive hybridization to occur, a recognized target sequence likely exceeds 71% nucleotide similarity with the VH probe.

With the above hybridization stringency conditions characterized, genomic DNA representing the five additional species of catfish that were used in the CH studies was analyzed. The genomic DNA was restricted with EcoRI, and the genomic Southern blots were hybridized with the different channel catfish VH family probes (Fig. 5). These analyses showed that multiple genomic fragments were detected in each catfish species with each of the five channel catfish VH family probes. The relative number of individual VH genes in these related species, as defined by the number of different hybridizing fragments, generally exceeded 15 and approached the number of different fragments observed in the channel catfish. Of general importance was that the stringency conditions defined in the dot-blot experiments and employed in these analyses gave the representative pattern of channel

Table 1. Summary of Southern blot analyses of EcoRI-digested genomic DNA from different species of bony fish hybridized with channel catfish CH and VH family probes[a]

	Hybridization probe					
Family (species)	CH	VH1 (NG70)	VH2 (NG41)	VH3 (NG54)	VH4 (NG10)	VH5 (NG66)
Sciaenidae						
Sciaenops ocellatus, red-fish	−	+/−	++	+/−	ND	+++
Centrarchidae						
Pomoxis annularis, white crappie	−	+	+++	++	++	ND
Micropterus salmoides, largemouth bass	−	++	+++	+++	+++	+++
Percichthyidae						
Morone saxatilis × *Morone chrysops*, hybrid striped bass	+	+	+++	+++	++	++
Salmonidae						
Oncorhynchus kisutch, coho salmon	+	+/−	++	+/−	ND	+++
Ariidae						
Arius felis, sea catfish	+	+++	+++	+++	+++	++
Ictaluridae						
Ictalurus punctatus, channel catfish	+	+++	+++	+++	+++	++
Ictalurus furcatus, blue catfish	+	+++	+++	+++	+++	++
Ictalurus natalis, yellow bullhead	+	+++	+++	+++	++	++
Ictalurus melas, black bullhead	+	+++	+++	+++	++	+++
Pylodictis olivaris, flat-head catfish	++	+++	+++	+++	+++	++
Catostomidae						
Ictiobus cyprinellus, big-mouth buffalo	−	++	+++	+	++	+++
Carpiodes velifer, highfin carpsucker	+	++	++	+	++	++
Cyprinidae						
Cyprinus carpio, carp	−	+++	++	+	++	++
Esocidae						
Esox niger, chain pickerel	−	+++	+++	+	+++	++
Lepisosteidae						
Lepisosteus oculatus, spotted gar	−	+/−	+/−	+/−	ND	+/−
Amiidae						
Amia calva, bowfin	−	+/−	+/−	+/−	ND	+/−
Polyodontidae						
Polyodon spathula, pad-dlefish	−	+/−	+/−	+/−	ND	+/−

[a] +++, more than 10 hybridizing fragments; ++, between 5 and 10 hybridizing fragments; +, less than 5 hybridizing fragments; +/−, positive hybridization but no individual fragments discernible; ND, not determined.

catfish genomic fragments that was observed when similar channel catfish genomic analyses were done under high stringency hybridization and washing conditions (see Fig. 4 in Ghaffari and Lobb 1991).

Within a species the genomic EcoRI fragments which hybridized with the different VH probes were generally of dissimilar size. There were, however, some fragments of similar size which were detected by different VH probes. While the dot-blot experiments suggest that cross-hybridization of VH probes should not occur, if there was comigration of similar-sized fragments or if VH genes representing different VH gene families were interspersed within the genome, then fragments of similar size might be detected by different VH family probes. Thus these hybridization results are consistent with the inter-

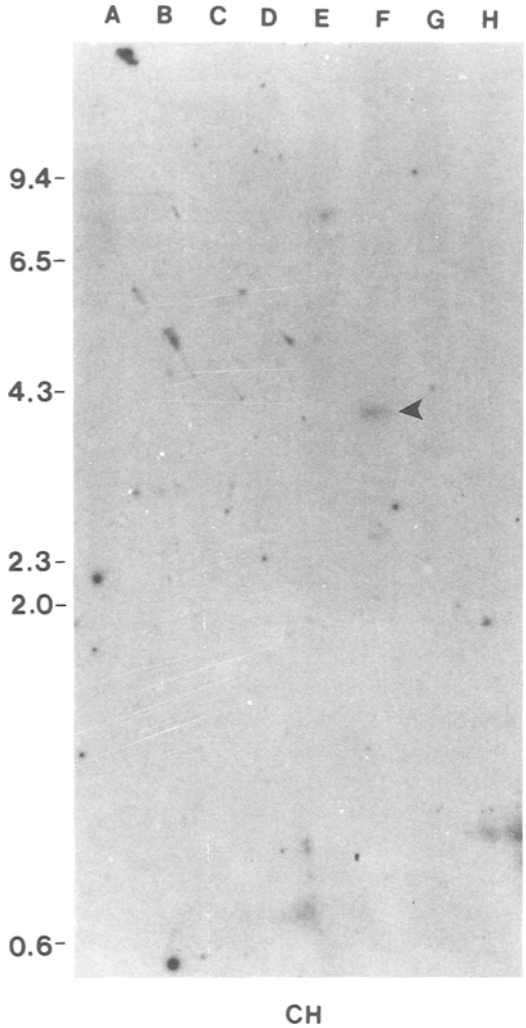

Fig. 4. Representative Southern blot autoradiograph of EcoRI-restricted genomic DNA from eight species of bony fish hybridized with channel catfish CH probes. The lane designations are as follows: *A*, chain pickerel; *B*, Asiatic carp; *C*, bowfin; *D*, spotted gar; *E*, largemouth buffalo; *F*, hybrid striped bass; *G*, largemouth bass; and *H*, white crappie. The position of the hybridizing fragment in the restricted DNA of the hybrid striped bass is indicated by the arrowhead. The sizes in kilobases were determined from the products of HindIII-digested lambda DNA.

pretation that VH genes similar to those defined in the channel catfish appear to be present within these related species.

Earlier work had shown that restricted genomic DNA from different individual channel catfish gave similar hybridization patterns when probed with a particular VH family probe (Ghaffari and Lobb 1991). To determine if the VH hybridization pattern was similar among individuals of other catfish species, the genomic DNA from two individual channel, blue, and flathead catfish from feral populations was compared by Southern blot analysis. Representative results with two of the channel catfish VH probes are shown in Fig. 6. These results showed that the genomic EcoRI hybridization pattern was similar, although not identical, between individuals of the same species. In addition these results showed that each hybridization pattern was distinct for each species. These combined studies suggest that genomic VH analyses should be useful to distinguish closely related species of bony fish and that VH probes might be used to define restriction-length polymorphisms between genetically different populations of the same species.

Ig VH Genes in Other Families of Bony Fish

To determine if VH genes similar to those defined in channel catfish are represented in divergent species of bony fish, EcoRI-restricted genomic DNA from the 12 species used in the CH analyses was hybridized with channel catfish VH probes under the same stringency conditions used above. Southern blot results showed that multiple genomic DNA restriction fragments from each of these representatives of bony fish except the paddlefish, gar, and bowfin hybridized with the channel catfish VH probes. Representative results of the hybridization reactions for seven of these different species with three of the catfish VH probes are shown in Fig. 7. The results for all experiments are summarized in Table 1. Although not all of the VH probes hybridized with each of the species which were examined, the general conclusion was that for those species which had restriction fragments which hybridized with multiple VH probes, the patterns of hybridizing fragments were different; the observed restriction pattern was dependent upon which catfish VH probe was used for hybridization. Generally different EcoRI fragments were detected with each of the different VH probes, but like the hybridization results observed with the catfish, there were restriction fragments of the same relative size which hybridized with different VH probes. This may be due to reasons which were discussed above.

The number of potential VH genes, as judged by the number of different restriction fragments which hybridized with each probe, varied among the represented species of bony fish. As shown in Table 1, species within the catfish families appear to have the largest number of genomic fragments which were recognized by the channel catfish VH probes. In the other families of bony fish, the number of hybridizing fragments detected with each VH probe was usually less than observed in the catfish. There also appeared to be variability in the number of hybridizing fragments observed within different species of the same family of bony fish. For example, in the family Centrarchidae, the white crappie had

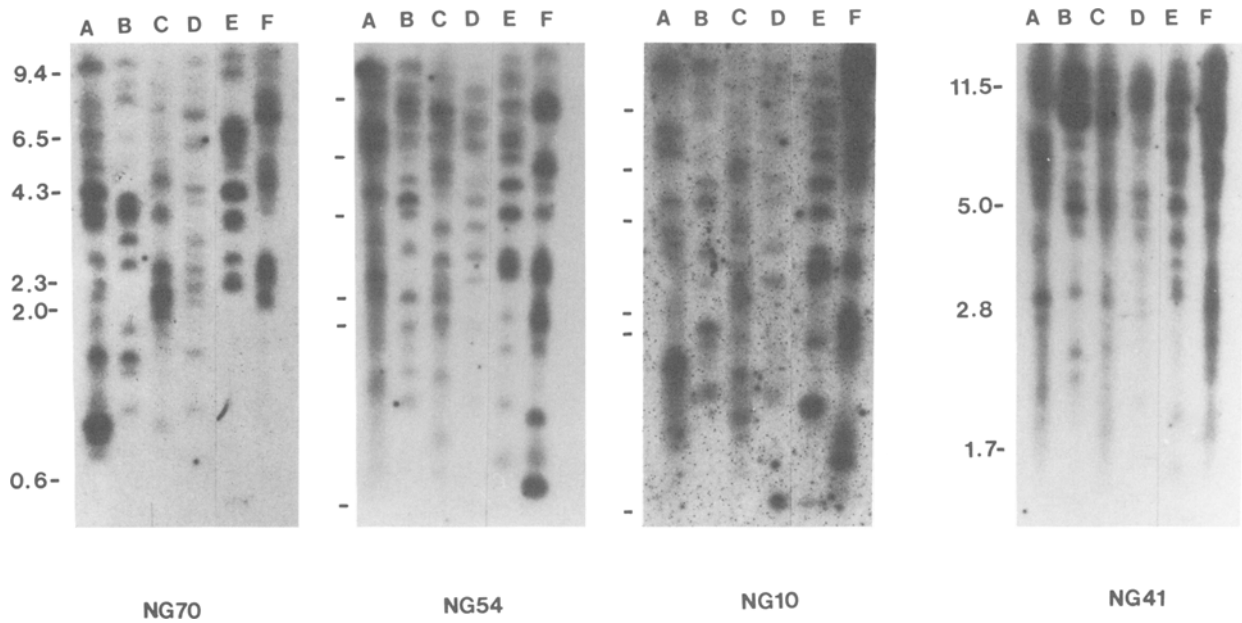

Fig. 5. Southern blot autoradiograph of EcoRI-restricted genomic DNA from six species of catfish hybridized with the indicated channel catfish VH family probes. The lane designations are the same as those in Fig. 2. The sizes in kilobases were determined from the products of HindIII- or PstI-digested lambda DNA.

fewer genomic fragments which reacted with the VH1, VH3, and VH4 probes than did the largemouth bass.

Restricted DNA from the paddlefish, spotted gar, and bowfin yielded no discernible bands when hybridized with the channel catfish VH probes. Strong hybridization of the VH probes occurred with the restricted DNA from these three fish, but no distinct bands were observed above the intense background. These results were not due to partial restriction of the genomic DNA as judged by the complete digestion of exogenous lambda DNA. To determine if increasing the hybridization stringency would allow distinct fragments to be observed, the stringency of the hybridization wash was increased from 2× SSC at 65°C to 1× SSC at 65°C. Strong hybridization without clearly defined bands was again observed with the restricted DNA from the paddlefish and bowfin. In contrast, when genomic DNA from the chain pickerel and redfish was reanalyzed under these increased stringency conditions, distinct restriction fragments were again observed. Hence a moderate increase in hybridization stringency did not appear to prevent VH probe recognition of target sequences. Thus in the analyses of the DNA from the paddlefish, gar, and bowfin, it is not clear if VH genes similar to those defined in the channel catfish are present.

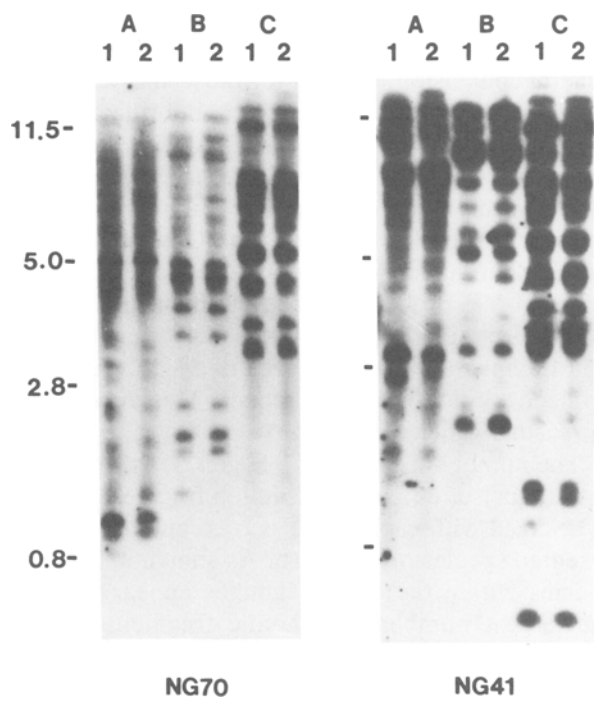

Fig. 6. Southern blot autoradiograph of EcoRI-restricted genomic DNA from different individual catfish hybridized with the indicated channel catfish VH probes. The lane designations are as follows: *A*, channel catfish; *B*, blue catfish; *C*, flathead catfish. The numbers 1 and 2 refer to different individuals of the same species. The sizes in kilobases were determined from the products of PstI-digested lambda DNA.

Discussion

CH Genes in Phylogenetically Diverse Species of Bony Fish

When probes specific for each of the four channel catfish CH domains were used in Southern blot analyses, only the DNA from species within the

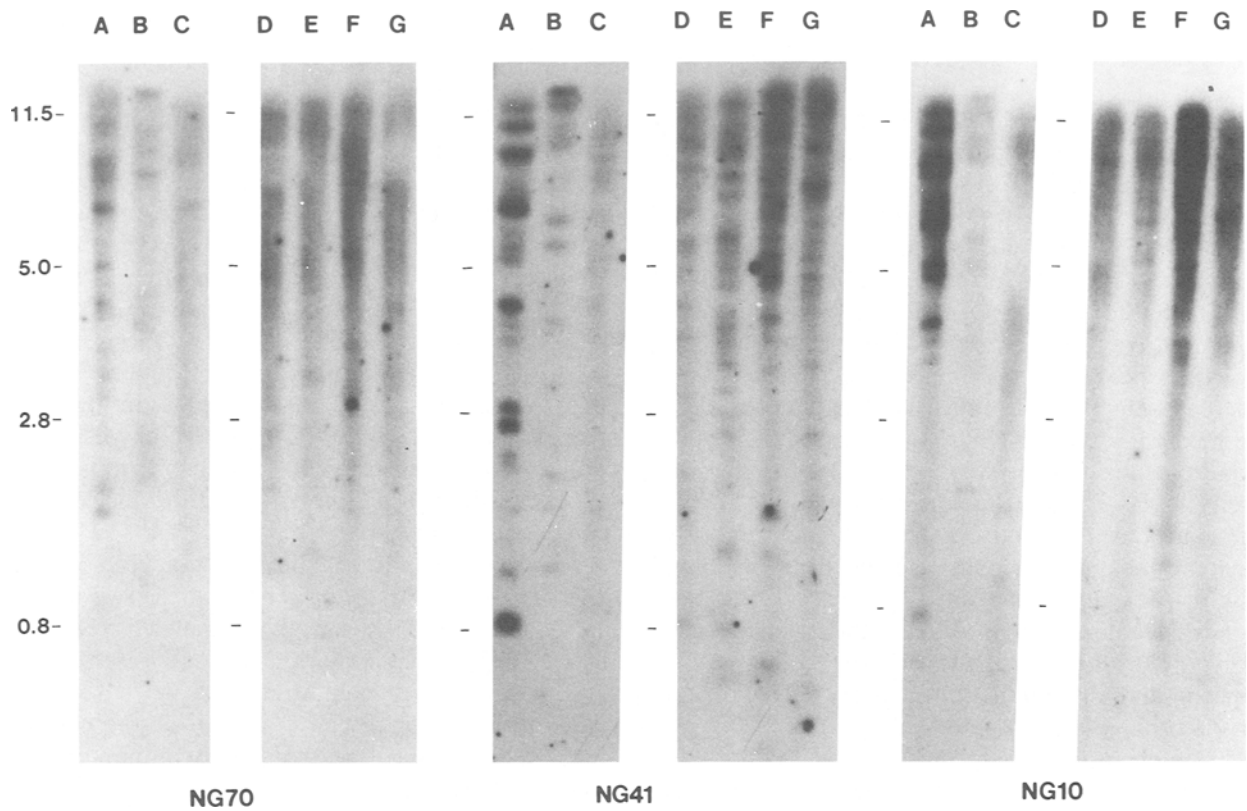

Fig. 7. Southern blot autoradiograph of EcoRI-restricted genomic DNA from seven species of bony fish hybridized with the indicated channel catfish VH family probes. The lane designations are as follows: *A*, chain pickerel; *B*, Asiatic carp; *C*, highfin carpsucker; *D*, largemouth buffalo; *E*, hybrid striped bass; *F*, largemouth bass; *G*, white crappie. The sizes in kilobases were determined from the products of PstI-digested lambda DNA.

catfish families hybridized strongly. The six species of catfish analyzed in this study have a relatively close ancestral history (see Fig. 1) wherein these species likely diverged from a common ancestor during the Eocene (reviewed by Taylor 1969). Because these common species were historically limited to the greater Mississippi Valley region, it might be suggested that similar evolutionary pressure might have been applied to these species and that a CH gene derived from an ancestral form may have been conserved. The experimental results from this study would agree with this supposition; however, more intriguing is the readily interpreted observation that the CH gene known in the channel catfish must be structurally different from that observed in these other catfish species. Even in the most closely related species to the channel catfish, the blue catfish, there were differences in the size and intensity of some of the hybridizing fragments. Comparison of the more distant ictalurids showed even greater variation in the number and size of hybridizing fragments. Only two genomic fragments were observed in the DNA from the two bullhead species as well as the sea catfish, whereas six fragments were observed in the flathead catfish. Such findings indicate that there are likely structural differences within the CH genes of these related catfish species.

The CH gene of the channel catfish does not appear to be similar to the CH genes of most other species of fish. This finding coupled with the above relationships observed with CH genes in various catfish would be consistent with the hypothesis that CH genes in different fish will likely exhibit significant structural variability. There are at present few studies on the diversity of CH genes in bony fish, but at this point these studies appear to strongly support this conclusion. The complete nucleotide sequences of CH genes from three species of bony fish have been derived. The other two species are the ladyfish (*Elops saurus*, Amemiya and Litman 1990) and the Atlantic cod (*Gadus morhua*, Bengtén et al. 1991). The comparison of the predicted amino acid sequences of the CH genes from these species shows only a similarity of about 30% when aligned sequences are compared with one another. These analyses indicate that the CH genes in these divergent species are structurally distinct.

VH Genes in Phylogenetically Diverse Species of Bony Fish

In order to discuss the results of the VH analyses, an overview of the bony fish examined in this study is required. The phylogenetic information that is discussed is derived from Colbert (1969), Carroll (1988), and Lauder and Liem (1983). Bony fish (class Osteichthyes) are divided into two subclasses: Sarcopterygii (lobe-finned fish) and Actinopterygii (ray-finned fish). In this study only the actinopterygians were examined, and this subclass was represented by two lineages: the chondrosteans and the neopterygians (Fig. 3). The ancestors of the chondrosteans diverged early during bony fish phylogeny, likely in the Paleozoic era within the Devonian and Carboniferous periods. In contrast the neopterygians, which comprise the overwhelming majority of existing bony fish, appeared after the chondrosteans. The neopterygian ancestral lineages evolved near the end of the Paleozoic era and underwent diversification during the Mesozoic and early Cenozoic eras.

The most primitive species represented in this study are the one chondrostean, the paddlefish, and two neopterygians, the spotted gar and the bowfin. Because of the early divergence of these species, it was of interest to determine if these fish had VH genes similar to those identified in the channel catfish. When restricted DNA from these three fish was hybridized with catfish VH probes, strong hybridization resulted, but no distinct bands were seen. Although hybridization lacking distinguishable fragments could result from nonspecific binding, the same experiment performed under moderately higher stringency conditions showed no observable changes in the results. In addition this intense "background" was not observed when the DNA from these species was hybridized with catfish CH probes. Hence there might be alternative explanations for these results. One possibility is that these species might have an extensive repertoire of VH genes which share partial similarity with different catfish VH genes. In this regard if a VH gene family underwent diversification to give rise to divergent VH gene families, probes derived from VH genes representing these divergent VH families might cross-hybridize with ancestral VH genes. However, at this point the lack of distinct fragments does not allow any conclusions to be made about VH genes in these species.

The other neopterygians examined in this study are classified as teleosts (Fig. 3). Among the teleosts included in this study is the chain pickerel, which diverged early in teleost phylogeny. The other teleosts examined in this study are thought to have emerged after the arrival of the pike/pickerel family Esocidae. Because the channel catfish is a member of Ostariophysi and thus developed from an ancestor that likely emerged after the chain pickerel, the chain pickerel served as a key representative species in this study. Southern blot analysis of chain pickerel DNA showed that each of the five channel catfish VH probes reacted with the restricted pickerel DNA and that the hybridization pattern for each VH probe was different. The results suggest that VH genes similar to those identified in the channel catfish are represented in the chain pickerel; i.e., VH genes representing five channel catfish VH families appear to have similar counterparts in an early teleost whose existence was established before the arrival of the ancestor of the channel catfish.

With the exception of the salmonid family the other teleosts in this study represent the two major lineages, Ostariophysi and Acanthopterygii (Fig. 3). In these experiments Ostariophysi is represented by the minnow and sucker families, Cyprinidae and Catostomidae, respectively, as well as the catfish families, Ictaluridae and Ariidae. Acanthopterygii, which includes some of the most advanced bony fish, is represented by the temperate bass family (Percichthyidae), the largemouth bass family (Centrarchidae), and the drum family (Sciaenidae). The results showed that the restricted DNA from the species representing these lineages contained fragments which hybridized with different channel catfish VH probes. Moreover, the observed restriction patterns differed according to which VH probe was used. These hybridization analyses indicate that VH genes which appear to share similarity are also found in the minnow, sucker, and catfish families where the relative number of hybridizing fragments appears the greatest. In addition restriction fragments from the DNA obtained from the represented acanthopterygians hybridized with channel catfish VH probes in a probe-specific pattern. Together these results are consistent with the interpretation that VH genes similar to those defined in the channel catfish are likely present in diverse lineages of teleosts. This conclusion, however, should not be interpreted to imply that the VH families represented in the channel catfish exist within the entire teleost lineage. Formal proof of this latter hypothesis would by necessity require extensive sequence data to establish sequence identities between multiple but related VH genes in different species.

The comparisons of recent nucleotide sequence information indicate that related VH genes are present in divergent teleost species. Nucleotide sequence comparisons of two VH genes isolated from a cDNA library of *Elops*, a primitive teleost

(Amemiya and Litman 1990), with the five channel catfish VH family prototypes showed that *Elops* 14501 is most similar to channel catfish VH gene NG54 (72%) while *Elops* 14515 is similar to channel catfish VH genes NG41, NG10, and NG66 (67%, 70%, 70%, respectively). Two VH genes from the goldfish, a member of the minnow family, have also been sequenced (Wilson et al. 1991). The goldfish VH gene 99A shares 83% similarity with NG70 whereas the nucleotide sequence of the VH pseudogene 99B shares about 68% similarity to NG54. From another teleost, the rainbow trout, the genomic VH gene RTVH431 (Matsunaga et al. 1990) was compared with the five channel catfish VH family prototypes. RTVH431 appears to be most similar to NG54; the nucleotide sequences are about 71% similar. Based on these sequence comparisons, VH genes from one teleost species can share significant nucleotide sequence similarity with VH genes isolated from phylogenetically more distant species of teleosts. This apparent relationship is quite different from that observed when the five catfish VH families were compared with VH genes representing ten murine VH families. In these comparisons the average total nucleotide similarity was less than 55%; the highest total nucleotide similarity observed was 62% (Ghaffari and Lobb 1991).

In an earlier report the sequences of the prototype VH genes representing the five catfish VH families were subjected to multiple sequence alignments with representative VH genes from the horned shark (*Heterodontus*), the clawed toad (*Xenopus*), and the mouse (Ghaffari and Lobb 1991). For each possible pair of aligned VH sequences the accepted point mutations per 100 amino acid residues (PAM) values were determined and phylogenetic trees were constructed using the unitary matrix as well as by an alternative approach wherein measurements of difference were determined using the log odds matrix. These results showed that the branching order of the VH families in the phylogenetic trees determined by both approaches was the same. These analyses indicated that the catfish VH family that was most related to the VH genes in the mouse and toad was the catfish VH3 family. Important to the present discussion, however, is that these analyses indicated that the two major groups of catfish VH families (VH1 and VH3 vs VH2, VH4, and VH5) diverged from an ancestral animal which phylogenetically preceded the radiation of the channel catfish and the horned shark.

In conclusion, it is important to summarize the evidence which supports the hypotheses presented here. As determined by dot-blot experiments, the stringency conditions used in these experiments did not allow the channel catfish VH family probes to cross-hybridize. Of equal importance was that the genome of the channel catfish served as an internal control to evaluate the hybridization conditions employed in these experiments. Specifically, using the hybridization conditions defined in the dot-blot analyses, the observed number and pattern of channel catfish VH-hybridizing fragments were representative of the results observed in experiments that were conducted under high stringency conditions (see Fig. 4 in Ghaffari and Lobb, 1991). These controls indicate that nonspecific binding of VH probes was limited. The CH hybridization results also indicate that nonspecific binding was limited. Although the restriction fragments used to generate both VH and CH probes were approximate in size, the total length of cDNA used to derive CH probes was three to five times greater than that used to derive VH probes. Yet in these experiments there was essentially no background CH hybridization signal with any of the analyzed DNA samples. Lastly, as shown by the studies of Litman and coworkers, VH probes derived from distantly related species can be successfully utilized under relaxed stringency conditions to isolate VH genes. Probes derived from murine VH S107 have defined VH genes in the reptile *Caiman* (Litman et al. 1985a), the shark *Heterodontus* (Litman et al. 1985b), and the teleost *Elops* (Amemiya and Litman 1990). Based on the results presented here, fish VH probes may have similar applications in future studies to isolate undefined VH genes.

In summary, these studies provide initial insight into CH and VH genes of phylogenetically diverse species of bony fish. First, CH genes appear to have undergone significant divergence during bony fish phylogeny. The CH gene defined in the channel catfish appears to share strong similarity only with CH genes of other catfish. However, even within these related catfish species, it appears that significant CH structural differences are present. Second, families of VH genes appear to have diverged in early lineages of teleosts. Lastly, VH genes similar to those identified in catfish appear to be widely represented in phylogenetically diverse species of teleosts.

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