Bichir *Hox*A Cluster Sequence Reveals Surprising Trends in Ray-Finned Fish Genomic Evolution

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The study of *Hox* clusters and genes provides insights into the evolution of genomic regulation of development. Derived ray-finned fishes (Actinopterygii, Teleostei) such as zebrafish and pufferfish possess duplicated *Hox* clusters that have undergone considerable sequence evolution. Whether these changes are associated with the duplication(s) that produced extra *Hox* clusters is unresolved because comparison with basal lineages is unavailable. We sequenced and analyzed the *Hox*A cluster of the bichir (*Polypterus senegalus*), a phylogenetically basal actinopterygian. Independent lines of evidence indicate that bichir has one *Hox*A cluster that is mosaic in its patterns of noncoding sequence conservation and gene retention relative to the *Hox*A clusters of human and shark, and the *Hox*Aα and *Hox*Aβ clusters of zebrafish, pufferfish, and striped bass. *Hox*A cluster noncoding sequences conserved between bichir and euteleosts indicate that novel *cis*-sequences were acquired in the stem actinopterygians and maintained after cluster duplication. Hence, in the earliest actinopterygians, evolution of the single *Hox*A cluster was already more dynamic than in human and shark. This tendency peaked among teleosts after *Hox*A cluster duplication.

[Supplemental material is available online at www.genome.org.]

Hox genes, which share sequence homology with the *Hom-C* genes of *Drosophila* and are clustered in the genome, form a distinct class of transcription factors that play an essential role in embryonic patterning (McGinnis and Krumlauf 1992). *Hox* clusters display the phenomenon of colinearity, in which the position of a gene in the cluster is related to its spatiotemporal pattern of expression along the anterior–posterior (A–P) axis (Lufkin 1996). *Hox* genes have a dynamic evolutionary history hallmarked by tandem (Kappen et al. 1989) and whole-cluster duplications (Holland and Garcia-Fernandez 1996; Ruddle et al. 1999). Whereas protostome taxa possess at most a single *Hox* cluster, the number of *Hox* clusters in different vertebrate lineages is varied (Ruddle et al. 1999). Vertebrate *Hox* clusters and their genes, therefore, are good models for identifying putative correlations between genomic and phenotypic evolution (Hughes and Kaufman 2002).

The ray-finned fishes (Actinopterygii; Fig. 1) are well suited to such studies. Teleost fishes (Nelson 1994) are the most diverse extant vertebrates, with >25,000 species. Moreover, the genomes of derived teleost fishes such as zebrafish, "euteleosts" *sensu* (Nelson 1994), contain duplicated genes of several different gene families (Postlethwait et al. 1998; Robinson-Rechavi et al. 2001; Taylor et al. 2001) and extra *Hox* clusters (Amores et al. 1998), as compared with their single orthologs in the genome of humans. The zebrafish (*Danio rerio*) possesses at least seven unlinked *Hox* clusters (Aα, Aβ, Bα, Bβ, Cα, Cβ, and D; Amores et al. 1998). Evidence for the presence of more than four *Hox* clusters also has been reported for additional euteleosts, including pufferfish (*Takifugu rubripes*; Aparicio et al. 2002; Amores et al. 2003), medaka (*Oryzias latipes*; Naruse et al. 2000), striped bass (*Morone saxatilis*; Snell et al. 1999), killifish (*Fundulus heteroclitus*; Misof and Wagner 1996), and tilapia (*Oreochromis niloticus*; Malaga-Trillo and Meyer 2001; Santini et al. 2003).

In our recent analysis of patterns of noncoding sequence evolution in the duplicated *Hox*Aα and *Hox*Aβ clusters of zebrafish and in the single *Hox*A clusters of horn shark (*Heterodontus francisci*) and mammals, we proposed that following *Hox* cluster duplication, the noncoding control elements of zebrafish undergo extensive remodeling (Chiu et al. 2002). But, as indicated in Figure 1, there are several basal and intermediate actinopterygian lineages for which we do not, at present, have definitive knowledge on *Hox* cluster number. Hence, major questions remain. When, during ray-finned fish phylogeny, did the duplications that produced extra *Hox* clusters occur? Is the loss of *Hox*

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Article and publication are at http://www.genome.org/cgi/doi/10.1101/gr.1712904.

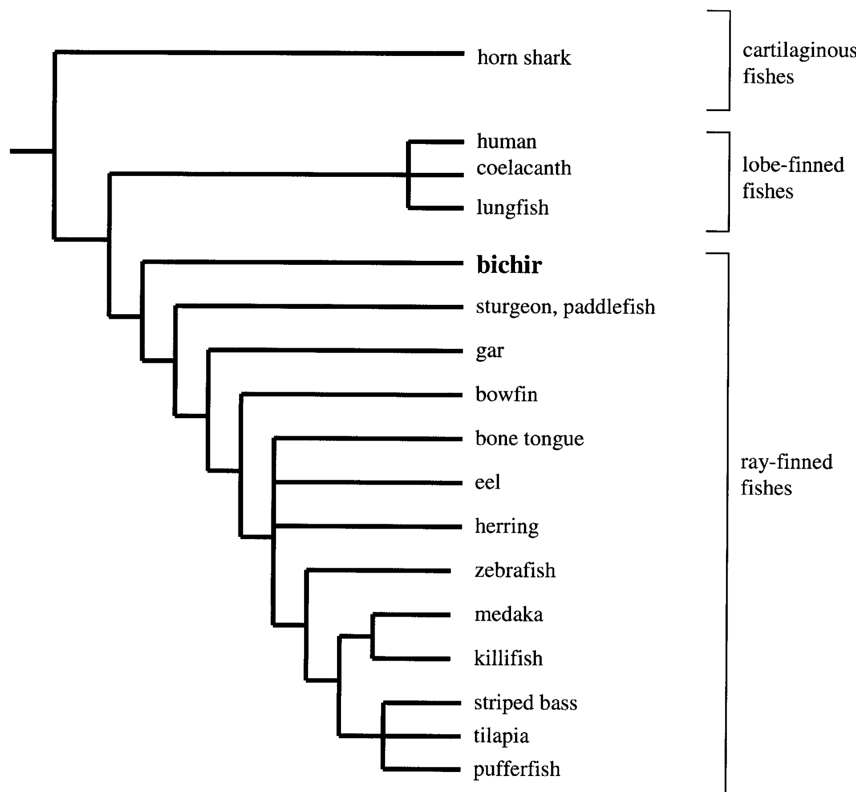


Figure 1 Overview of jawed-vertebrate phylogenetic relationships with focus on the ray-finned fishes (Actinopterygii; Patterson 1982; Nelson 1994; Bartsch and Britz 1997; Bemis et al. 1997). The other two living sister groups to the ray-finned fishes among gnathostomes are the cartilaginous (Chondrichthyes) and lobe-finned (Sarcopterygii) fishes, respectively. Note that only some euteleost clades are well represented to date, but remaining higher actinopterygian groups, Chondrostei (sturgeon and paddlefish), Neopterygii (gar, bowfin, and teleosts), and basal teleost clades are virtually unresolved. This general outline of actinopterygian phylogeny is supported in three of the most recent hypotheses based on molecular data (Le et al. 1993; Venkatesh et al. 2001; Inoue et al. 2003). But, as with some conflicting morphologically based hypotheses in the past, these also still indicate changing positions or lack of resolution among other basal actinopterygian fauna: sturgeon and paddlefish, gar, and bowfin.

cluster noncoding sequence conservation observed in euteleosts also present in primitive actinopterygians? That is, is this phenomenon a characteristic of all ray-finned fishes, independent of duplication? How have *Hox* clusters evolved after duplication? To address these questions, we sequenced and analyzed the complete *Hox*A cluster of the bichir (*Polypterus senegalus*), a representative of the most basal extant ray-finned fish lineage as inferred from independent molecular data sets (Le et al. 1993; Venkatesh et al. 2001; Inoue et al. 2003) as well as its possession of ancestral morphological characters (Patterson 1982; Nelson 1994; Bartsch and Britz 1997; Bemis et al. 1997).

RESULTS AND DISCUSSION

Bichir *Hox*A Cluster Architecture

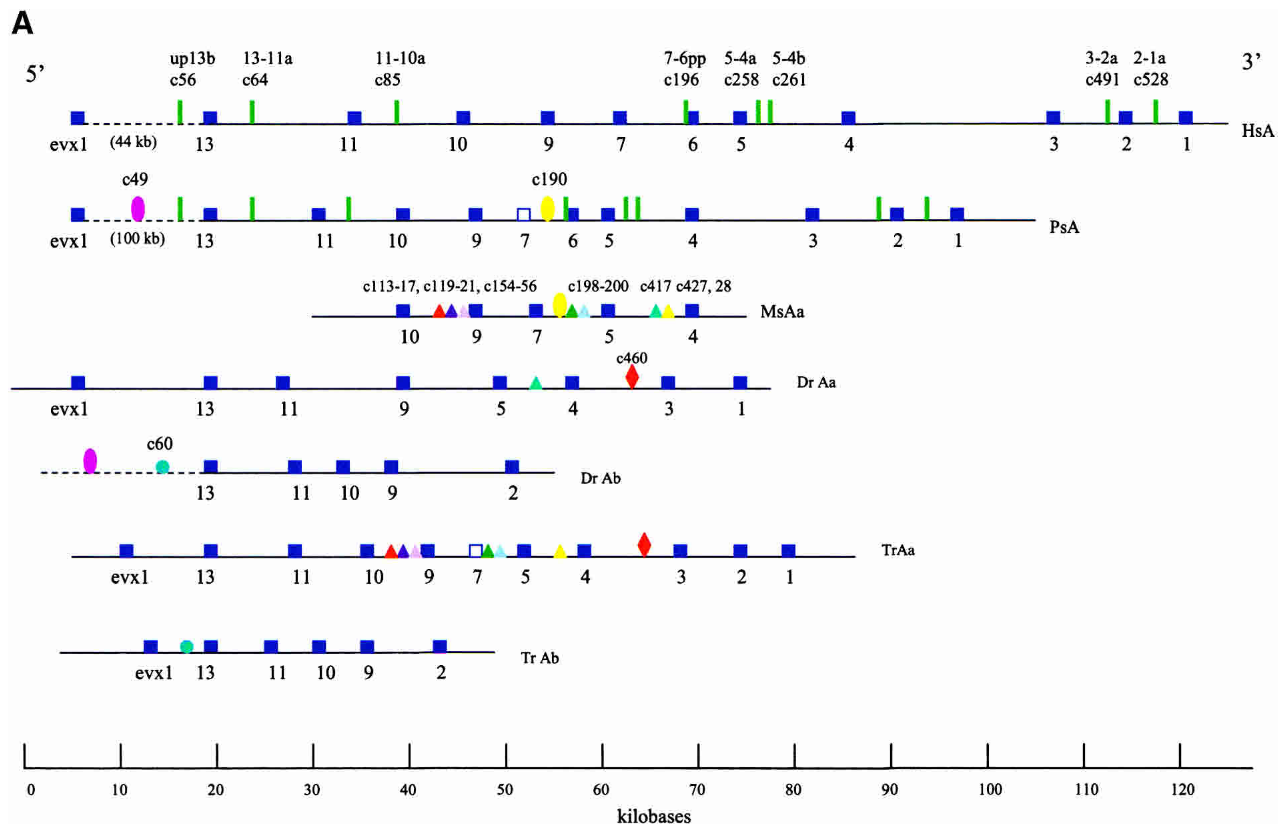
We screened a BAC genomic library from *P. senegalus* and isolated a 180-kb *Hox*A contig spanning *Evx*1 to *Hox*A1 (Fig. 2). An additional *Hox*A cluster was not identified in our screenings. The architecture and gene complement of the *Hox*A cluster closely resemble that of the single *Hox*A clusters of human and horn shark (Kim et al. 2000; Chiu et al. 2002) and of the hypothetical gnathostome ancestor (Stellwag 1999). Disparate patterns of retention were observed for two medial group genes, *Hox*A6 and *Hox*A7. The bichir *Hox*A cluster encodes an intact *Hox*A6 gene, which has not been found in any euteleost examined. Remnants of the *Hox*A7 gene were observed in the bichir (Fig. 2A), similar to the situation observed in pufferfish (Amores et al. 2003). No obvious traces of an *Hox*A7 ortholog have been found in either the zebrafish *Hox*Aα and *Hox*Aβ clusters (Amores et al. 1998) or the pufferfish *Hox*Aβ cluster (Fig. 2A; Aparicio et al. 2002; Amores et al. 2003). The *Hox*Aα clusters of striped bass (Snell et al. 1999) and tilapia (Santini et al. 2003), on the other hand, each house an intact *Hox*A7 gene. Thus the *Hox*A7 gene has been independently lost in at least three distinct actinopterygian lineages, at least once in a basal group (bichir) and at least twice in teleosts (zebrafish, pufferfish). These two genes, *Hox*A6 and *Hox*A7, underscore that parallel events of gene losses within actinopterygians cannot be inferred solely on the basis of character reconstructions using parsimony.

Phylogenetic Analysis Supports a Single *Hox*A Cluster in the Bichir

Phylogenetic analyses of *Hox*A cluster coding sequences of human, shark, bichir, and euteleosts are consistent with the inference that the bichir has a single *Hox*A cluster (Figs. 3A–E). We examined trees for *Hox*A13, *Hox*A11, *Hox*A10, and *Hox*A2 amino acid sequences of shark (Kim et al. 2000), human (Venter et al. 2001), coelacanth (Koh et al. 2003; C.T. Amemiya and T. Powers, unpubl.), bichir (this study), zebrafish (Amores et al. 1998), and pufferfish (Fig. 3; Aparicio et al. 2002). The topology of a neighbor-joining tree of concatenated *Hox*A11 and *Hox*A13 exon 1 coding sequences indicates that the bichir and teleosts last shared a common ancestor prior to the duplication event that gave rise to the duplicated *Hox*A clusters in zebrafish and pufferfish (Fig. 3A). This leaves open the possibility that bichir has independently acquired an *Hox*A cluster duplication. To address this possibility, we examined the rate of nonsynonymous substitutions (Figs. 3B–E) because our earlier findings for the duplicated *Hox*A11α and *Hox*A11β paralogs of zebrafish (Chiu et al. 2000a) showed that gene duplication is associated with an increased rate of replacement substitutions. Amino acid character reconstructions of *Hox*A13 (Fig. 3B), *Hox*A11 (Fig. 3C), and *Hox*A2 (Fig. 3E) confirm a higher rate of nonsynonymous substitutions for the duplicated paralogs of zebrafish and pufferfish. The orthologous bichir sequences accumulated significantly fewer amino acid changes (Figs. 3A–E). This finding is consistent with our library screening results and gene tree reconstructions indicating only a single *Hox*A cluster in bichir.

Noncoding DNA Sequence Comparisons Suggest the Single *Hox*A Cluster in Bichir Is Mosaic Between Human and Derived Ray-Finned Fishes

We next examined the evolution of noncoding sequences to determine whether the single *Hox*A cluster of bichir exhibits the dramatic loss of noncoding sequence conservation observed in the duplicated *Hox*A clusters of euteleosts (Chiu et al. 2002). We



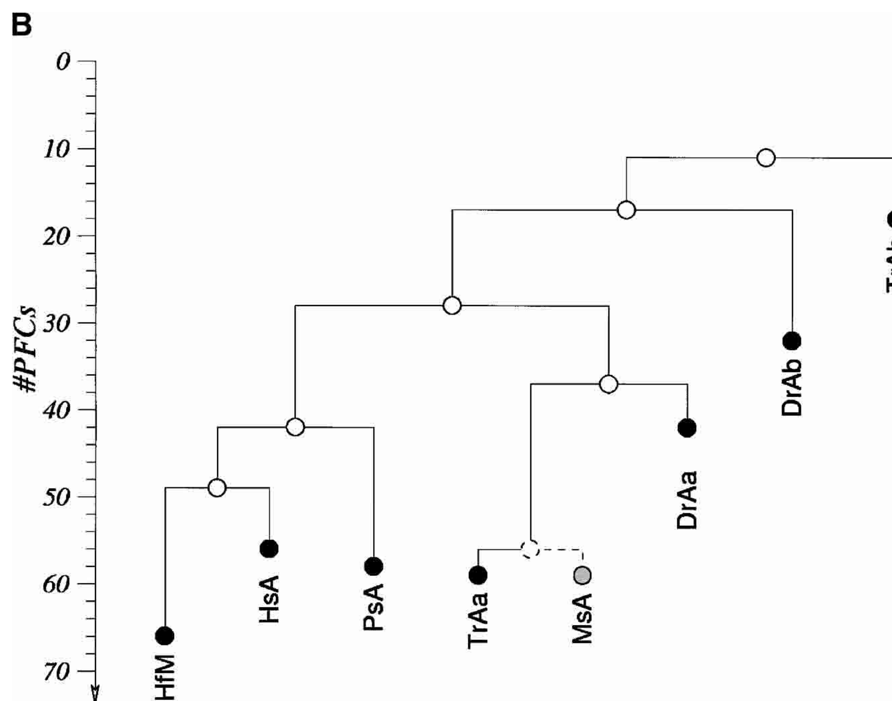


Figure 2 *Hox*A clusters and PFCs in human, shark, bichir, and euteleosts. (Hs) *Homo sapiens*; (Hf) *Heterodontus francisci*; (Ps) *Polypterus senegalus*; (MsAa) *Morone saxatilis Hox*Aα; (DrAa) *Danio rerio Hox*Aα; (DrAb) *D. rerio Hox*Aβ; (TrAa) *Takifugu rubripes Hox*Aα; (TrAb) *T. rubripes Hox*Aβ. (*A*) *Hox* genes are indicated by blue rectangles. PFCs shared exclusively between human and bichir are indicated by green bars. PFCs shared exclusively between bichir and euteleosts are indicated by colored ellipses. PFCs shared between the *Hox*Aα clusters of striped bass, zebrafish, and/or pufferfish are indicated by colored triangles. The PFC shared between only zebrafish and pufferfish *Hox*Aα clusters is indicated by a red diamond. The PFC shared between zebrafish and pufferfish *Hox*Aβ clusters is indicated by an aqua blue circle. See text for description. (*B*) The co-occurrences of the PFCs in different clusters (Supplemental Table 1) can be represented as a tree. The height of an internal node is the average number of PFCs shared by two clusters in the two different subtrees. The position of the tips gives the total number of PFCs in the segment of the *Hox*A cluster that spans from *Evx*1 to *Hox*A1. The nonduplicated *Hox*A regions of human, shark, and bichir form one group. The second significant group consists of the *Hox*Aα sequences of the euteleosts. The position of the incomplete striped bass (*Morone saxatilis*) sequence is estimated by assuming that in a complete sequence we would have found roughly the same number of PFCs as in pufferfish (*T. rubripes*). The *Hox*Aβ clusters are much further diverged and do not appear to group together because euteleost-specific PFCs in the *Hox*Aβ cluster are very rare (Supplemental Table 1). The smaller rate of PFC loss in the zebrafish *Hox*Aβ cluster is the dominating effect.

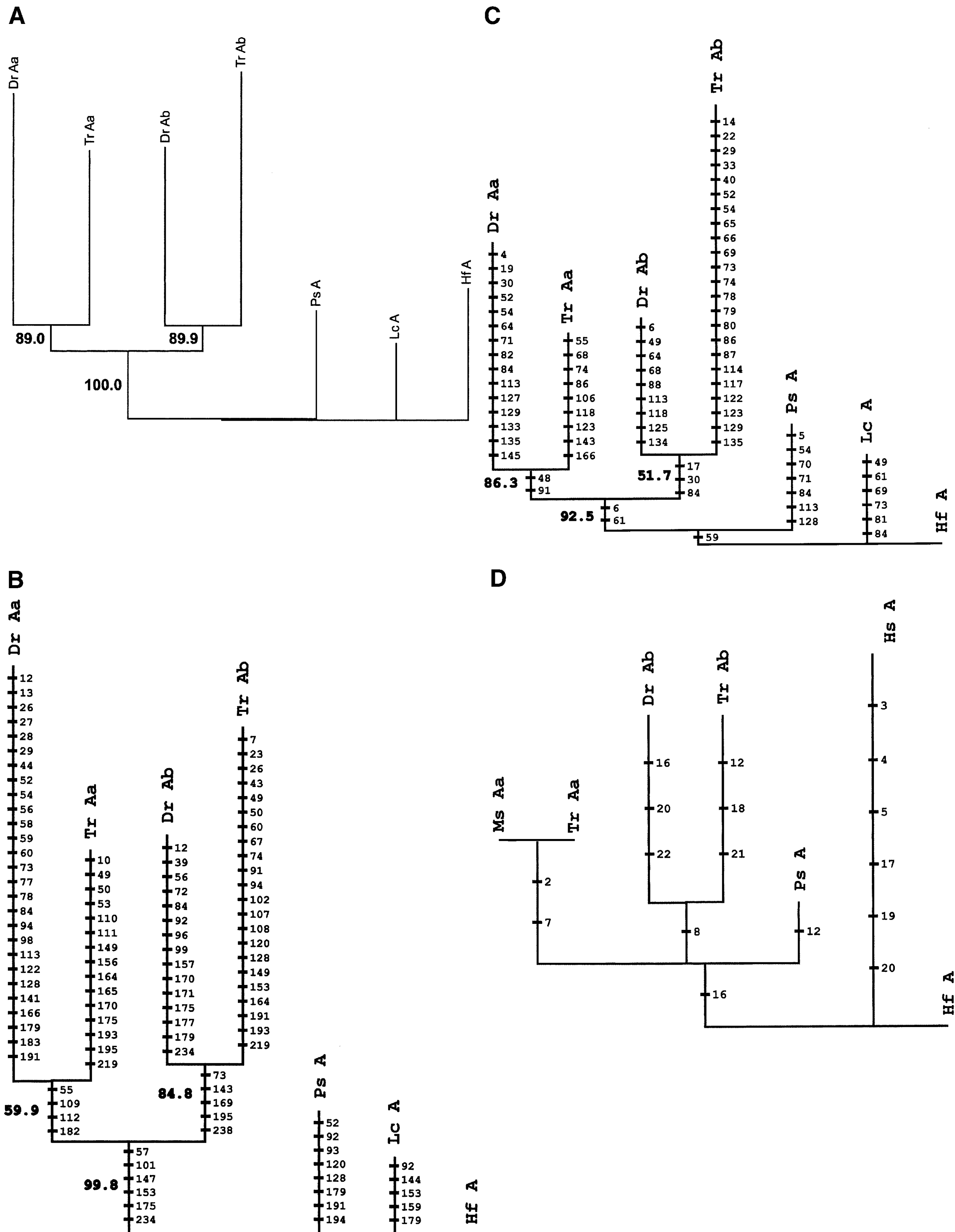


Figure 3 (*Continued on next page*)

used a new software package, Tracker (Prohaska et al. 2003), to identify conserved *Hox* noncoding sequence tracts (potential *cis*-regulatory elements) in all the taxa shown in Figure 2A. Here, we report phylogenetic footprint clusters (PFCs; Chiu et al. 2002) of each *Hox*A cluster spanning from *Evx*1 to *Hox*A1 (Fig. 2A) counted by Tracker in pairwise sequence alignments of intergenic sequences between orthologous *Hox*A gene pairs (Supplemental Table 1). Figure 2B diagrammatically illustrates the number of PFCs that each pair of sequences (e.g., human, bichir) has in common. Several notable patterns of conservation of PFCs are evident. First, the *Hox*A clusters of human and horn shark share the most PFCs, consistent with our earlier findings (Chiu et al. 2002). Second, the bichir *Hox*A cluster shares 44 PFCs with horn shark and 40 PFCs with human; these numbers are much higher than the number of PFCs shared between any of the euteleosts and human and/or shark. A subset of these PFCs conserved between bichir and human are completely absent in euteleosts (Fig. 2A). Two extensive PFCs, of 11 total, shared exclusively between bichir and euteleosts (c49, c190 of Supplemental Table 1 available online at www.genome.org) are illustrated in Figure 2A. These observations indicate that the bichir exhibits a mosaic pattern of conservation of *Hox*A noncoding sequence tracts with human and derived actinopterygians. Third, the common ancestor of bichir and teleosts possessed *cis*-sequence elements that were largely acquired before the duplication and were retained in the duplicated *Hox*Aβ clusters only. Most of the changes are located between *Evx*1 and *Hox*A13 (Fig. 2A; c36,37,38,47,49,50,54 of Supplemental Table 1), indicating that this modification was a single event. Fourth, 24 PFCs are shared only among the different euteleosts examined (Fig. 2A; Supplemental Table 1). Based on parsimony, these PFCs were uniquely derived in the stem lineage of euteleosts prior to the *Hox*A cluster duplication. We conclude that the loss of conservation in zebrafish, bass, and pufferfish is a derived state for teleost fishes and that the bichir is already more derived in its noncoding sequence than human and shark.

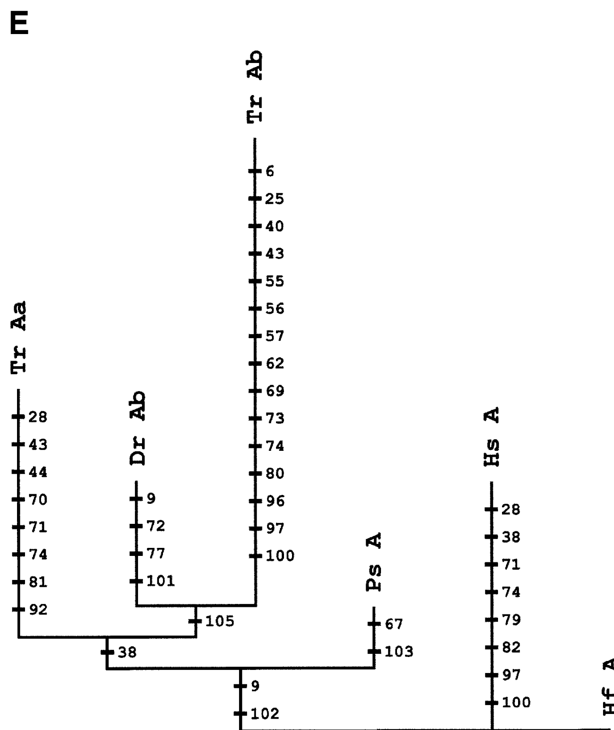


Figure 3 Phylogenetic analysis and character reconstructions of *Hox* coding sequences. (*A*) Neighbor-joining tree of concatenated *Hox*A13 and *Hox*A11 exon 1 coding sequences. Bootstrap support (1000 replications) for the euteleost nodes are shown. (Hf) *Heterodontus francisci*; (Lc) *Latimeria chalumnae*; (Ps) *Polypterus senegalus*; (TrAb) *Takifugu rubripes Hox*Aβ; (DrAb) *Danio rerio Hox*Aβ; (TrAa) *T. rubripes Hox*Aα; (DrAa) *D. rerio Hox*Aα. (*B–E*) Character reconstructions of exon 1 coding sequences under constraint trees for *Hox*A13, *Hox*A11, *Hox*A10, and *Hox*A2, respectively. Taxa abbreviations are as in *A* above, including (Hs) *Homo sapiens*. The indicated substitutions are only those that map unambiguously to the branches of the trees. The numbers of analyzed amino acid residues and assumed unambiguous changes for each gene are (*B*) *Hox*A13, 244 amino acids and 196 steps; (*C*) *Hox*A11, 192 amino acids and 151 steps; (*D*) *Hox*A10, 24 amino acids and 35 steps; (*E*) *Hox*A2, 105 amino acids and 59 steps.

Conclusions

In this study we found independent lines of evidence indicating that the bichir has one *Hox*A cluster that is mosaic in its patterns of noncoding sequence conservation and gene retention relative to the single *Hox*A cluster of human and shark, and the duplicated *Hox*Aα and *Hox*Aβ clusters of zebrafish, pufferfish, and striped bass. Our findings show that the duplication that produced additional *Hox*A clusters in derived actinopterygians occurred after the bichir diverged from the rest of the ray-finned fishes (Fig. 1). Hence, it is important to investigate the genome situation in additional basal ray-finned fishes such as the paddlefish, bowfin, sturgeon, and gar, as well as basal teleosts such as the eel (Fig. 1). Some of the changes that distinguish zebrafish and fugu from human, however, were acquired prior to the teleost *Hox*A cluster duplication, as shown in the pattern of *cis*-sequence conservation between teleosts and bichir. Hence the divergence of actinopterygian *Hox* clusters from the gnathostome archetype already began before the duplication that produced duplicate *Hox* clusters in teleost fishes.

In this genome-enabled era, two major problems remain: how evolutionary forces such as mutation, duplication, and selection shape genomes, and how genomic variation relates to phenotypic alterations in different lineages. This study reinforces the view that the ray-finned fishes provide good opportunities for addressing these challenges. Comparisons on a genome-wide basis are urgently needed to understand the complex interactions between evolution of genomes and body plans in this speciose assemblage.

METHODS

Construction and Screening of the Bichir BAC Genomic Library

A 5× coverage BAC genomic library, with an average insert size of 130 kb, was constructed for the bichir (*P. senegalus*) as described (Strong et al. 1997; Osoegawa et al. 1998). High-density 5 × 5 arrayed filters were made by the RZPD (http://www.rzpd.de). Hybridization using nonradioactive DIG-labeled probes was done following methods described in Chiu et al. (2000b). The first screen of this library was carried out using a pool of bichir (gift of C. Ledje and F. Ruddle; Ledje and Ruddle 2002) and coelacanth (gift of T. Powers [Benaroya Research Institute at Virginia Mason, Seattle, WA] and C. Amemiya) homeobox sequences isolated in genome-wide PCR surveys. This screen identified nine BAC clones of *P. senegalus*. A PCR survey of each BAC clone was done using a degenerate homeobox primer pair (334, 5′-GARYTIGARAARGARTTY-3′; 335, 5′-ICKICKRTTYTGRAACAA-3′). One clone containing the posterior part of the *Hox*A (*Evx*1, *Hox*A13, *Hox*A11, *Hox*A10) cluster was identified. Bichir *Hox*B, *Hox*C, and *Hox*D cluster sequences are presently being analyzed (C.-H. Chiu, K. Dewar, and P.F. Stadler, unpubl.).

To find overlapping BAC clone(s) that contain the rest of the *Hox*A cluster, PCR primers specific to bichir *Hox*A10 exon 1 were designed (PseA10F, 5′-ATGTCATGCTCAGATAGCCCGG-3′; PseA10R, 5′-TGATGTTTTGTATAAGGACATCG-3′). Using these

primers, exon 1 of *Hox*A10 was amplified from whole-genomic DNA of the bichir (*P. senegalus*), cloned, and five independent colonies were sequenced on both strands. All five clones from genomic DNA were identical in sequence to each other and to the *Hox*A10 sequence on the BAC clones isolated using hybridization (described above). The PseA10F/R PCR primer pair was then used to PCR-screen DNA pools of the bichir BAC library (the library consists of 216 pools, with each pool equivalent to one 384-well plate). The screen with *Hox*A10 primers yielded two clones, the original BAC clone described above (containing *Evx*1 to *Hox*A10) and one overlapping clone, spanning *Hox*A10 to *Hox*A1. These two clones overlap ~4.0 kb encompassing the entire *Hox*A10 locus with 5′- and 3′-flanking sequences. Concurrent with PCR screening of the library pools, the 5 × 5 high-density filters of the bichir BAC library were also screened by hybridization, using bichir-specific *Hox*A10 exon 1 (from genomic DNA) as probe. Hybridization yielded the identical two *Hox*A cluster clones identified above that overlap over *Hox*A10. Finally, bichir *Hox*A2 exon 1 was amplified from genomic DNA (*P. senegalus*) using universal primers we designed (PseA2UF, 5′-AATAG TCAGCCR TCGCTYGCTGAG-3′; PseA2UR, 5′-CTTGGAHGCYTT TTTCTCKTTC-3′). The bichir *Hox*A2 exon 1 PCR product was cloned, sequenced, and used to screen the bichir BAC library filters by hybridization. Only one strong hybridization signal was detected, corresponding to the same BAC clone (spanning *Hox*A10 to *Hox*A1) identified above.

Phylogenetic Analyses

Phylogenetic reconstructions were conducted using the neighbor-joining method (Saitou and Nei 1987). Character reconstructions were done using MacClade Version 4.03 (Maddison and Maddison 2001) under constraint trees that retain bony fishes (ray-finned fishes and lobe-finned fishes) as monophyletic to the exclusion of horn shark. The indicated substitutions are only those that map unambiguously to the branches of the trees. Supplemental Table 2 contains the alignments used for phylogenetic tree reconstructions.

Noncoding Sequence Analyses

The program Tracker is based on BLAST (Altschul et al. 1990) searches of all possible sequence pairs. The resulting list of pairwise sequence alignments is then assembled into groups of partially overlapping regions that are subsequently passed through several filtering steps. There are three levels of resolution of conserved noncoding sequences that are analyzed. (1) Individual phylogenetic footprints (PFs) are blocks of 6 bp or more of DNA sequence 100% conserved in taxa that have an additive evolutionary time of 250 million years (Tagle et al. 1988). PFs are considered to be putative transcription-factor-binding sites. (2) Cliques (CCs) are groups of contiguous footprints (Prohaska et al. 2003). (3) Phylogenetic footprint clusters (PFCs; Chiu et al. 2002) are composed of at least two PFs that are separated by <100 nt. CCs and PFCs are considered to be putative enhancer/promoter regions.

ACKNOWLEDGMENTS

We are grateful to April Cook and the Whitehead Institute Center for Genome Research sequencing teams for the complete sequencing of the bichir *Hox*A cluster; and Tom Powers and Allan Force for discussion of portions of this manuscript. C.-H.C. and G.P.W. thank Austin Hughes for providing the SCR3 software. Funding for this research is gratefully acknowledged from the Busch Biomedical Research Fund to C.-H.C.; NSF IBN-9905403 to F.R. and G.P.W.; IBN-9905403 and NIH R24-RR14085 to C.T.A.; and DFG Bioinformatics Initiative BIZ 6/1-2 and FWF P#15893 to P.F.S.

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Received July 1, 2003; accepted in revised form October 30, 2003.