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# Developmental Roles of Pufferfish *Hox* Clusters and Genome Evolution in Ray-Fin Fish

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**The pufferfish skeleton lacks ribs and pelvic fins, and has fused bones in the cranium and jaw. It has been hypothesized that this secondarily simplified pufferfish morphology is due to reduced complexity of the pufferfish *Hox* complexes. To test this hypothesis, we determined the genomic structure of *Hox* clusters in the Southern pufferfish *Spheroides nephelus* and interrogated genomic databases for the Japanese pufferfish *Takifugu rubripes* (fugu). Both species have at least seven *Hox* clusters, including two copies of *Hoxb* and *Hoxd* clusters, a single *Hoxc* cluster, and at least two *Hoxa* clusters, with a portion of a third *Hoxa* cluster in fugu. Results support genome duplication before divergence of zebrafish and pufferfish lineages, followed by loss of a *Hoxc* cluster in the pufferfish lineage and loss of a *Hoxd* cluster in the zebrafish lineage. Comparative analysis shows that duplicate genes continued to be lost for hundreds of millions of years, contrary to predictions for the permanent preservation of gene duplicates. Gene expression analysis in fugu embryos by in situ hybridization revealed evolutionary change in gene expression as predicted by the duplication-degeneration-complementation model. These experiments rule out the hypothesis that the simplified pufferfish body plan is due to reduction in *Hox* cluster complexity, and support the notion that genome duplication contributed to the radiation of teleosts into half of all vertebrate species by increasing developmental diversification of duplicate genes in daughter lineages.**

**[Supplemental material is available online at www.genome.org. The sequence data from this study have been submitted to GenBank under accession nos. AY303229, AY303230, AY303231, AY303232, AY303233, AY303234, AY303235.]**

The relationship between genome complexity and phenotypic complexity has yet to be fully understood. In one model, gene number in a taxon correlates with morphological complexity and species diversity (Holland et al. 1994). Alternatively, novel interactions among existing genes may enhance developmental complexity (Wittkopp et al. 2002). Ray-fin fish are species rich and vary in gene number, including numbers of *Hox* clusters (Amores et al. 1998; Naruse et al. 2000; Malaga-Trillo and Meyer 2001; Scemama et al. 2002; C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al., in prep.). Some fish are secondarily simplified (Fig. 1a,b). Pufferfish, for example, have fused cranial skeletal elements, lack ribs and pelvis, and have the smallest number of vertebrae among fish (Brainerd and Patek 1998). Skeletal reduction in the pufferfish lineage accompanied genome diminution (Elgar et al. 1999), including seemingly fewer *Hox* clusters than other teleosts (Aparicio et al. 1997, 2002; Amores et al. 1998; Naruse et al. 2000; Malaga-Trillo and Meyer 2001; Scemama et al. 2002). These data raised the hypothesis that impoverished pufferfish *Hox* clusters are causally related to simplified morphology (Aparicio et al. 1997; Holland 1997; Meyer and Malaga-Trillo 1999; Snell et al. 1999; Aparicio 2000; Naruse et al. 2000). To test this hypothesis, we constructed a large-insert genomic library for the Southern pufferfish *Spheroides nephelus* (Amemiya et al. 2001), isolated genomic clones to map pufferfish *Hox* cluster organization, and compared results with sequences found in genomic databases for the Japanese pufferfish *Takifugu rubripes* (fugu) (Aparicio et al. 1997; Aparicio et al. 2002).

Results showed that the organization of pufferfish *Hox* clusters is similar to that of other teleosts (Amores et al. 1998; Naruse et al. 2000), refuting the hypothesis that morphological simplification is a direct result of the reduction in number of *Hox* clusters. Pufferfish have duplicate copies of *Hox* clusters that are present in single copy in tetrapods, including duplicate *Hoxa, Hoxb*, and *Hoxd* clusters. Initial analyses suggested that the duplicated pufferfish *Hoxa* cluster may have arisen in a whole-genome duplication event before the teleost radiation (Aparicio et al. 1997; Amores et al. 1998; Postlethwait et al. 1998, 2002; Wittbrodt et al. 1998; Gates et al. 1999; Meyer and Malaga-Trillo 1999; Meyer and Schartl 1999; Woods et al. 2000; Malaga-Trillo and Meyer 2001), and comparative analysis suggests that this event may have occurred over 300 million years ago (Mya; Taylor et al. 2001).

What processes may have preserved duplicated genes within these duplicated *Hox* clusters? The classical model for the evolution of gene duplicates suggests that one member of most duplicate pairs should mutate to a pseudogene within a few million generations (Haldane 1933; Ohno 1970; Nei and Roychoudhury 1973; Bailey et al. 1978; Kimura and King 1979; Takahata and Maruyama 1979; Li 1980; Watterson 1983; Ohta 1988; Clark 1994), and investigations of completely sequenced genomes suggests that duplicate genes usually become silenced within about 4 Myr (Lynch and Conery 2000). Many gene duplicates, however, remain for tens of millions of years after the duplication event (Allendorf et al. 1975; Ferris and Whitt 1979; Ahn and Tanksley

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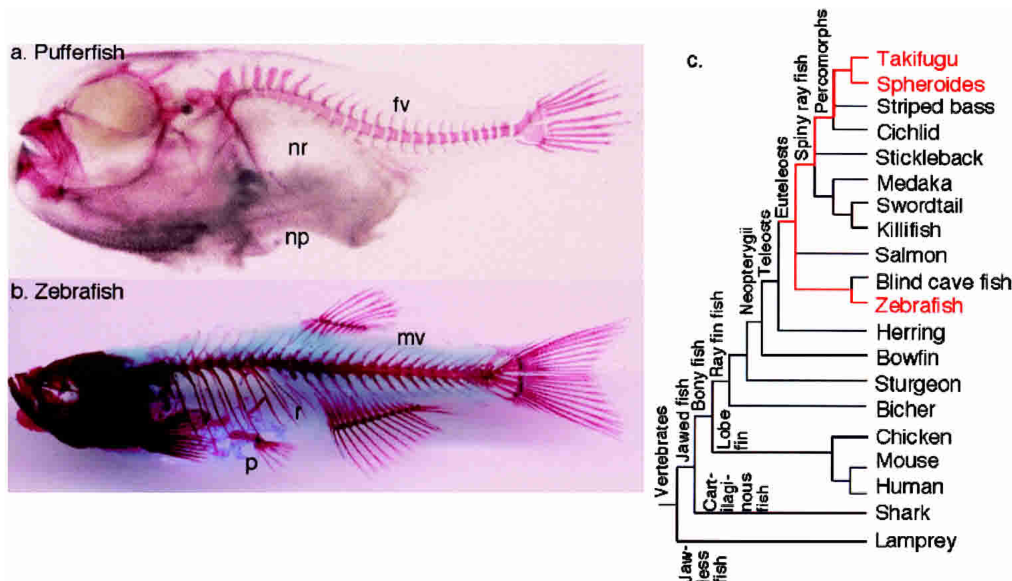


**Figure 1** Morphology and phylogeny. Pufferfish lack ribs and pelvis and have few vertebrae, as revealed by Alizarin red staining of the skeleton of the pufferfish *Takifugu rubripes* (*a*) and the zebrafish *Danio rerio* (*b*). (*c*) A phylogenetic tree for vertebrates (see Nelson 1994), times according to Hedges (2002), Hedges and Kumar (2002), and Santini and Tyler (1999). (fv) Few vertebrae; (mv) many vertebrae;(np) no pelvic appendage; (nr) no ribs; (p) pelvic apparatus; (r) ribs.

1993; Hughes and Hughes 1993; White and Doebley 1998), indicating that other mechanisms must exist to preserve genes, such as the rare evolution of novel positively selected gene functions (Ohno 1970), or the reciprocal sharing of gene subfunctions (Hughes 1994; Force et al. 1999; Stoltzfus 1999). Permanent preservation of gene duplicates is thought to be the outcome of these processes, but the time course of preservation is unclear. The comparison of *Hox* cluster content in the nested phylogenetic context of two species of pufferfish and zebrafish with tetrapods as outgroup (Fig. 1c) provides the opportunity to test how long this process of permanent preservation can take.

It is presumed that each member of a duplicated pair that has been retained for millions of years must now perform essential and unique functions. To test for function specialization in pufferfish *Hox* genes, we performed in situ hybridization to pufferfish embryos and compared patterns with those for zebrafish (Prince et al. 1998a,b; McClintock et al. 2002). The results showed evolutionary change in gene expression among teleosts. These experiments support the hypothesis that evolutionary divergence among duplicated genes arising from a genome duplication event in the ray-fin fish lineage contributed to the radiation of teleosts into half of all vertebrate species (Nelson 1994) by increasing developmental diversification of daughter lineages.

## RESULTS AND DISCUSSION

### Origin and Genomic Organization of Pufferfish *Hox* Clusters

We used degenerate PCR primers (Amores et al. 1998) to identify *S. nephalus* PACs (Amemiya et al. 2001) that contain *Hox* cluster genes, and sequenced *Hox* genes from each PAC to gene-identity *Hox* cluster gene content. Figure 2 shows PACs isolated and their gene content. We used *S. nephalus Hox* sequences to query the fugu database (Aparicio et al. 2002) to find fugu orthologs. Results showed that both species have at least seven *Hox* clusters (Fig. 2). *S. nephalus,* like medaka (Naruse et al. 2000), has at least two orthologs of mammalian *Hoxa*, *Hoxb*, and *Hoxd* clusters, but a single *Hoxc* cluster. In contrast, zebrafish (Amores et al. 1998) has two *hoxc* clusters and just one *hoxd* cluster (Fig. 3). The finding that pufferfish have the same *Hox* cluster complement as medaka, which is more closely related to pufferfish than to zebrafish (Fig. 1c), rules out the hypothesis that pufferfish skeletal simplification is due to a grand reduction in *Hox* cluster number.

At least three general models can explain duplicate teleost *Hox* clusters. First, four original clusters may have duplicated in an event before the divergence of the zebrafish lineage and the pufferfish-plus-medaka lineage. Second, all clusters within a lineage may have duplicated in a single event, with one such duplication event occurring in the pufferfish-plus-medaka clade and another event occurring independently in the zebrafish lineage. Third, *Hox* clusters may have duplicated individually at various times in different lineages. Most data support the first model. First, *Hox* cluster content is dramatically similar for the two cluster pairs present in both lineages, *Hoxa* and *Hoxb* duplicates (Fig. 3). For example, pufferfish and zebrafish *Hoxab*, *Hoxaa*, *Hoxba*, and *Hoxbb* clusters differ by zero, one, two, and two genes, respectively. What is the likelihood that such a pattern would occur if cluster duplication took place independently in the two lineages and if duplicates were lost at random? Assume that the last common ancestor of zebrafish and pufferfish had a *Hoxa* cluster with 11 genes (the sum of the *Hoxaa* plus *Hoxab* clusters from both species, Evx 13 11 10 9 7 5 4 3 2 1, see Fig. 3). After independent duplication, now assume that six specific genes were lost from one cluster in one of the two lineages to give the content of the *Hoxab* cluster in the zebrafish lineage. To calculate the probability that the other lineage had the same losses in one of the clusters, note that there is only one way to choose the same six locations, but 11 choose six ways of distributing six losses to 11 sites. So, the probability of this occurrence is 5!6!/11! = .0021645. The caveat to this calculation is that it groups zebrafish *Hoxaa* with pufferfish *Hoxaa*, and zebrafish *Hoxab* with pufferfish *Hoxab*, and if the original *Hoxa* cluster duplicated separately in each species, there is no such natural grouping. For the ancestral *Hoxb* cluster, assuming independent duplication and that the ancestral *Hoxb* cluster had 12 genes when it duplicated (Evx 13 10 9 8 7 6 5 4 3 2 1; see Fig. 3), then given one loss in the

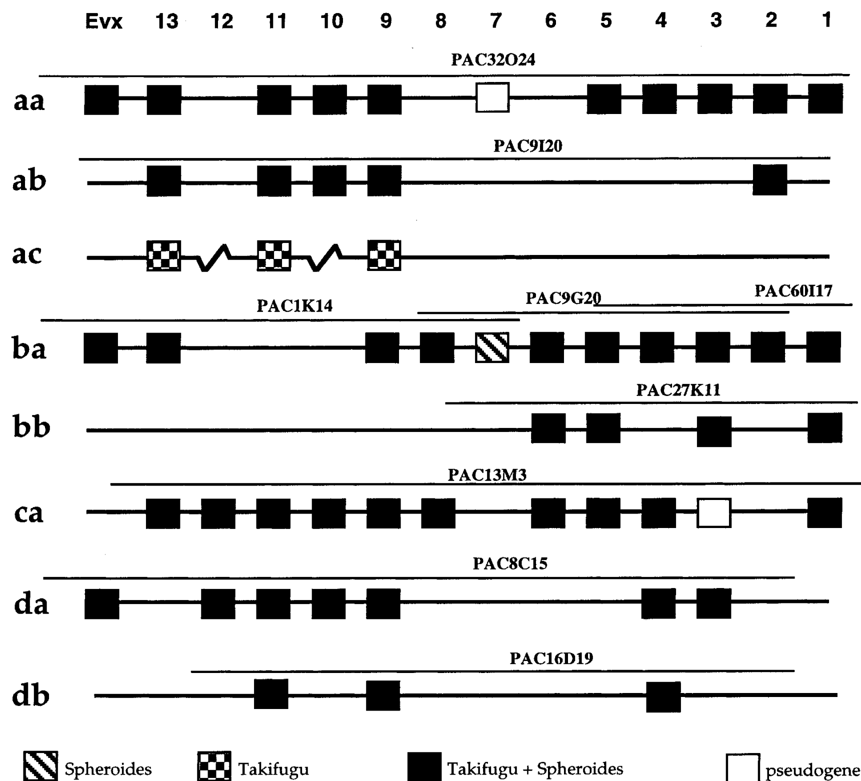


**Figure 2** Genomic organization of pufferfish *Hox* clusters. *Hox* paralog group shown along the *top* and cluster designation at *left*. Plate and well numbers of PACs as well as their genomic extent are sketched. (Filled squares) Genes present in both *S. nephalus* and *T. rubripes*. (Squares with diagonal lines) Genes found in *S. nephalus* but not *T. rubripes*. (Checkered squares) Genes found in *T. rubripes* but not *S. nephalus*. (Empty squares) Pseudogenes.

pufferfish lineage and one loss in the zebrafish lineage (the *Hoxba* clusters), the chance they would overlap is 1/12. Given eight losses in the pufferfish lineage and eight losses in the zebrafish lineage (the *Hoxbb* clusters), the chance that the losses would overlap seven or more times is [(8 choose 7)(4 choose 1) + 1]/(12 choose 8) = 0.067. These calculations show that the distribution of genes among these duplicated *Hox* clusters is unlikely to have occurred by chance. The most likely cause is that duplication and gene loss in the *a* and *b* copies occurred before lineage divergence, or alternatively, that the probability of *Hox* cluster gene loss deviates enormously from random between two initially identical duplicated clusters.

In addition to similar gene content, phylogenetic analysis also supports the duplication-first model. For six original genes, both lineages retain two copies (Fig. 4). Phylogenetic analysis of four of these (*Hoxa13*, *Hoxa9*, *Hoxb1*, and *Hoxb6*) supports the duplication-first model (Fig. 4A,D,E,H). In the fifth pair (Fig. 4C), *Hoxa11a* genes group as expected by the duplication-first model, but fugu *Hoxa11b* is an outgroup, perhaps due to long-branch attraction (Felsenstein 1978). In the final pair (Fig. 4F), zebrafish *Hoxb5a* and *Hoxb5b* branch as sisters, contrary to the duplication-first model, but pufferfish *Hoxb5a* and *Hoxb5b* do not branch as sisters, thus failing to support the other two models as well. Because most trees rule out the lineage-divergence-first model, but are consistent with the duplication-first model, we conclude that teleost *Hox* clusters duplicated before divergence of zebrafish and pufferfish lineages. Because these lineages separated at the base of the teleost radiation (Fig. 1b), at least euteleosts share these duplications, which probably occurred in a whole-genome duplication event that occurred sometime between divergence of bichir and Neopterygii and the divergence of pufferfish and zebrafish lineages (Amores et al. 1998; Postlethwait et al. 1998; Taylor et al. 2003; C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al. in prep.).

## Continuing Evolution of Pufferfish *Hox* Clusters

Although teleost *Hox* cluster duplicates probably arose in a single event, their structure and contents continue to evolve. Evidence for a large-scale change is found in Scaffolds (S007630, S011648, and S001602, Release 2) we identified in the fugu database that contains copies of *Hoxa9*, *Hoxa11*, and *Hoxa13*, tentatively called here the *Hoxac* complex (Fig. 2). The phylogenetic relationship of the three *Hoxa* clusters in fugu shows that *Hoxac* and *Hoxaa* clusters group as sisters (Fig. 4B,D). The *Hoxac* cluster is probably not an allele of *Hoxaa* because their nucleotide sequences differ by 14.1% in coding regions of *Hoxa9*, *Hoxa11*, and *Hoxa13*, far more than alleles in even widely divergent zebrafish populations, which is ~0.5% (Stickney et al. 2002). These three co-orthologs of the tetrapod *Hoxa* cluster should probably be called *Hoxaaa*, *Hoxaab*, and *Hoxab*, but that nomenclature seems too unwieldy for now. Assuming that *hoxac* is absent from zebrafish (Amores et al. 1998), at least a portion of the *Hoxaa* cluster was duplicated in the pufferfish lineage after it diverged from the zebrafish lineage. The most likely hypothesis is that after the duplication of all four original *Hox* complexes and the subsequent divergence of zebrafish and pufferfish lineages, a portion of the *Hoxaa* cluster reduplicated in the pufferfish lineage to produce the *Hoxac* cluster. We did not find the *Hoxac* cluster in *S. nephalus*, although the hox9 tree (Fig. 4D) tentatively suggests that it might have occurred before the divergence of pufferfish, medaka, and striped seabass.

Surprisingly, the *Hoxd11b* gene in both pufferfish species has a second intron with appropriate splice acceptor and donor sequences in the homeobox, in contrast to other vertebrate *Hox* cluster genes. Because this intron is absent from tetrapod *Hoxd11* and from teleost *Hoxd11a*, it is a new acquisition after *Hox* cluster duplication in ray-fin fish.

Further recent evolution in teleost *Hox* clusters is shown by *Hoxa7a*, which is a pseudogene in both pufferfish species, but has normal structure in striped bass and tilapia (Snell et al. 1999; Santini et al. 2003), which are more closely related to pufferfish than to either medaka or zebrafish (see Fig. 1c). The absence of *Hoxa7* from zebrafish (Amores et al. 1998) and bichir (C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al., in prep.) demonstrates multiple independent losses among ray-fin fish.

Teleost *Hox* clusters show that silencing of duplicated genes can continue far longer than thought previously. *Hoxb7a* is absent from fugu (Aparicio et al. 1997) but present in *S. nephalus* (Fig. 2). Both duplicate copies of *Hoxb7* must have remained intact from the time of the duplication event about 350 Mya (Taylor et al. 2001) until the divergence of *Spheroides* and *Takifugu* lineages only 5–35 Mya (Santini and Tyler 1999). Thus, both

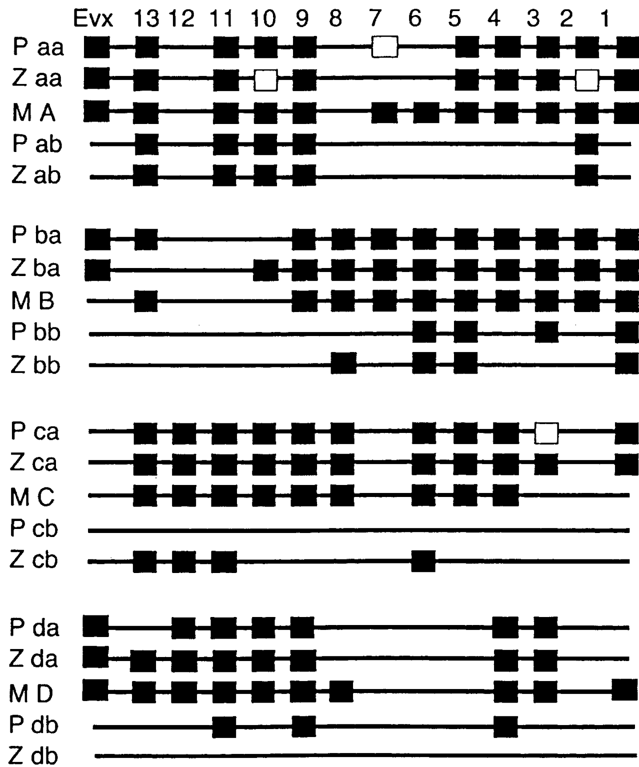


**Figure 3** Comparative genomics of pufferfish (P), zebrafish (Z), and mouse (M) *Hox* clusters. Paralog group shown along the *top* and cluster designation at *left*.

duplicate copies were maintained for about 300 Myr before the loss of one copy in the *Takifugu* lineage after it diverged from the *Spheroides* lineage. This is hundreds of millions of years longer than the permanent preservation of gene duplicates has been thought to take (Nei and Roychoudhury 1973; Kimura and King 1979; Li 1980; Watterson 1983; Ohta 1988; Clark 1994; Lynch and Conery 2000). In addition, at least four additional duplicates have one intact gene and one detectable pseudogene in teleost *Hox* clusters (Fig. 3; Aparicio et al. 1997; Amores et al. 1998), and these arose after pufferfish and zebrafish lineages diverged. Because molecular clock estimates suggest that the ray-fin genome duplication occurred about 350 Mya (Taylor et al. 2001), and that zebrafish and pufferfish lineages diverged about 284 Mya (Kumazawa et al. 2000), genes that have recently become pseudogenes must have been retained for tens or hundreds of million years without being lost or permanently preserved. Theory and analysis of sequenced genomes suggest that one copy of a duplicate usually becomes nonfunctional within a few million generations (Li 1980; Lynch and Conery 2000), and that duplicates retained longer probably become essential either by evolving novel, positively selected functions (Ohno 1970) or by partitioning ancestral vital subfunctions (Force et al. 1999). The long persistence of both duplicates, but eventual loss of one copy discovered here suggests either that fully redundant duplicates can be retained far longer than thought previously, or that evolutionary mechanisms that initially preserved both duplicates within a few million years of the duplication event later changed so that one copy secondarily became nonessential.

## Expression of *Hox* Cluster Genes in Fugu Embryonic Development

Expression patterns of pufferfish *Hox* genes support suggestions that gene duplication may facilitate the evolution of new functions and the reciprocal retention of ancestral functions (Ohno 1970; Hughes and Hughes 1993; Force et al. 1999). Our results showed that pufferfish have two orthologs of mammalian *HOXD4*, but zebrafish has just one (Fig. 3). In fugu, *Hoxd4a* is expressed with a sharp anterior boundary at hindbrain rhombomeres r6/r7 (Fig. 5h) as it is in tetrapods, zebrafish, and flounder (van der Hoeven et al. 1996; Prince et al. 1998b; Suzuki et al. 1998; Nolte et al. 2003). The duplicate gene *Hoxd4b*, however, is expressed with a more caudal anterior border (Fig. 5i). The anterior boundary of *Hoxd4* expression in mouse is regulated by elements conserved between zebrafish and mouse (Morrison et al. 1996; Nolte et al. 2003). This suggests that fugu *Hoxd4b* has lost some ancestral subfunctions. *Hoxd4a* is expressed weakly in hindbrain and neural crest, whereas *Hoxd4b* is expressed strongly in crest and hindbrain, suggesting quantitative subfunction partitioning (Force et al. 1999) in this pair (Fig. 5j–m). Further analysis is necessary to identify the molecular genetic mechanism for the evolved differences in gene expression for *Hoxd4* duplicates, and whether the differences in expression of the single *hoxd4* gene in zebrafish and the differently evolved duplicate *Hoxd4* genes in pufferfish are related to the evolution of skeletal differences between the species.

A novel expression pattern of one *Hox* gene is tantalizingly related to pufferfish puffing. Fugu *Hoxa2b* is expressed in hindbrain rhombomeres r2–r5 with sharp borders (Fig. 5b) like *hoxa2b* in zebrafish and *Hoxa2* in mouse (Prince and Lumsden 1994; Prince et al. 1998b; Hunter and Prince 2002). The duplicate *Hoxa2a* gene, however, is a pseudogene in zebrafish (Amores et al. 1998) but an expressed gene in pufferfish (Fig. 5). Fugu *Hoxa2a* has a striped expression pattern in r1 and r2 (Fig. 5a), a pattern not reported previously in r1, although in zebrafish, *hoxa1a* is expressed in small bilateral cell clusters in r1–r3 (McClintock et al. 2001). In fact, ectopic expression of *Hoxa2* in r1 of chicken embryos causes motor neuron development in r1, which normally has no motor neurons (Jungbluth et al. 1999). One can speculate that the evolutionary invention of the buccal pump in pufferfish that puffs the stomach with water (Brainerd and Patek 1998) might have involved the origin of new motor neurons stimulated by novel expression of *Hoxa2a* in r1.

**Figure 4** Phylogenetic relationships of pufferfish *Hox* clusters. Neighbor-joining trees are based on amino acid sequences as described (Amores et al. 1998). (*A*) Paralog group 13, exon 1, and exon 2. The tree is as expected if *Hox* cluster duplication occurred before the divergence of pufferfish and zebrafish lineages. (*B*) Paralog group 13, exon 2 only (due to limited sequence availability). The tree shows that *Hoxa13c* is the sister group to *Hoxa13a*. (*C*) *Hoxa11a* of pufferfish and zebrafish group as sisters, but *Hoxa11b* orthologs do not. (*D*) *Hoxa9* tree strongly supports duplication before lineage divergence, and the close similarity of the *Hoxaa* and *Hoxac* clusters. (*E,F*) The *Hoxb1* and paralog group-5 trees support duplication before lineage divergence. (*G*) The paralog group-4 tree shows rapid evolution of *Hoxd4b*. (*H*) The *Hoxb6* tree supports duplication before lineage divergence. Alignments and accession numbers are available as Supplemental material. Numbers are bootstrap values per 1000 runs. (Cca) *Carassius carassius*, crucian carp; (Dae) *Danio aequipinnatus*, giant danio; (Bfl) *Branchiostoma floridae*, amphioxus; (Dre) *Danio rerio,* zebrafish; (Gga) *Gallus gallus*, chicken; (Hfr) *Heterodontus francisci*, horned shark; (Hsa) *Homo sapiens*, human; (Mmu) *Mus musculus* mouse; (Msa) *Morone* saxatilis, striped bass; (Ola) *Oryzias latipes*, medaka; (Pma) *Petromyzon marinus*, lamprey; (Pol) *Paralichthys olivaceus,* Japanese flounder; (*Sne*) *Spheroides nephalus*, Southern pufferfish; (Tru) *Takifugu rubripes,* Japanese pufferfish.

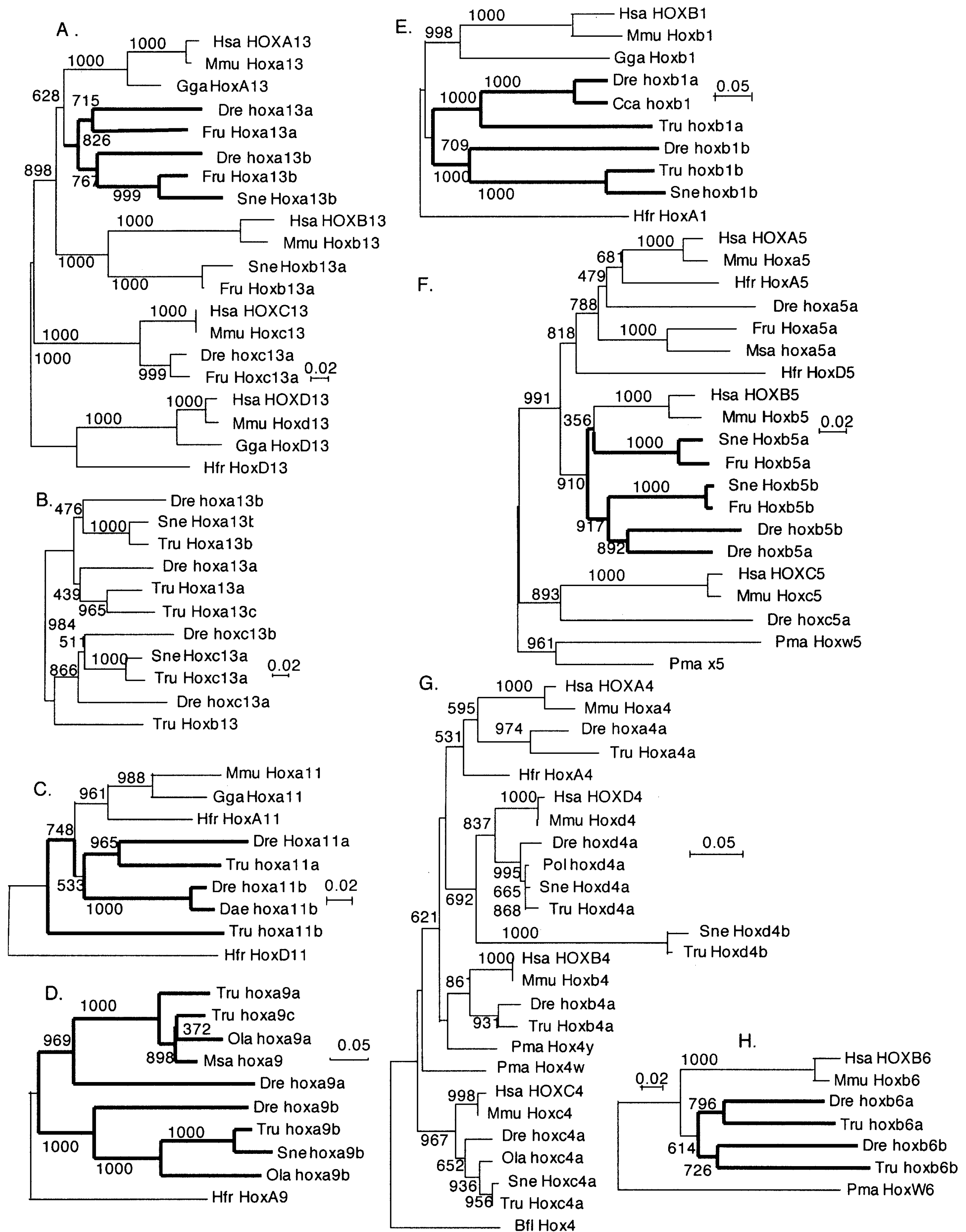

**Figure 4** (Legend on facing page)

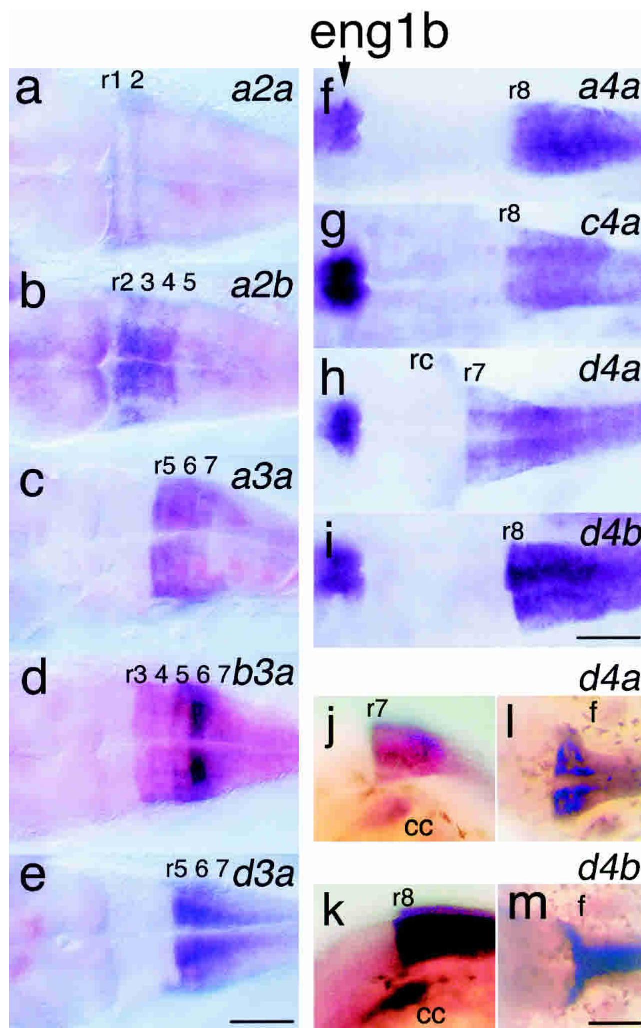


**Figure 5** Expression of *Hox* genes in 5-d fugu embryos. (*a*) *Hoxa2a* is expressed in an apparently novel striped pattern in r1 and r2. (*b*) *Hoxa2b* is expressed in r2–r5 as in other vertebrates. (*c*) *Hoxa3a* has an anterior boundary at the r4/r5 border as in other vertebrates. (*d*) *Hoxb3a* has strong expression in r5 and r6 and weak expression in r4 as in zebrafish, but with weak expression extending into r3 in fugu and not in zebrafish (Prince et al. 1998b). (*e*) *Hoxd3a* expression mimics that of zebrafish, with an anterior limit at the r5/r6 border, and a small lateral/ventral group of r5 cells (Prince et al. 1998b). Expression of *eng1b* at the midbrain/hindbrain border serves as a marker for rhombomere position in *f–i*. (*f*) *Hoxa4a* is expressed with a diffuse anterior expression boundary near the r7/r8 border as in zebrafish (Prince et al. 1998b). (*g*) *Hoxc4a* is expressed with a diffuse anterior boundary, and as in zebrafish, the border lies within r7, at least medially, and interneurons express *eng1b* as in zebrafish (Force et al. 1999). (*h*) *Hoxd4a* transcript tissue distribution as in other vertebrates. (*i*) *Hoxd4b* has a more caudal anterior expression border than *Hoxd4* in other vertebrates. (*j*) *Hoxd4a* in lateral view. (*k*) *Hoxd4b* in lateral view. (*l*) *Hoxd4a* in dorsal view showing fin buds. (*m*) *Hoxd4b* in dorsal view showing fin buds. Scale bar, 100 µ. (cc) Cranial crest; (f) fin buds.

## Evolution of the Genomic Organization of Vertebrate *Hox* Clusters

The results of these experiments and those of the accompanying paper (C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al., in prep.) allow us to paint a more complete picture of vertebrate *Hox* cluster evolution (Fig. 6). The ancestral vertebrate *Hox* cluster possessed by the last common ancestor of surviving vertebrates probably consisted of 13 *Hox* genes and an *Evx* gene (Fig. 6, #1), even though amphioxus, a cephalochordate representing the sister group of the vertebrates has 14 *Hox* cluster genes (Ferrier et al. 2000). Evidence from lamprey, a jawless vertebrate, suggests that the ancestral vertebrate *Hox* cluster probably duplicated once before the lamprey lineage diverged (Fig. 6, #2), and once again after divergence (Fig. 6, #3; Escriva et al. 2002; Force et al. 2002; Irvine et al. 2002). After the initial *Hox* cluster duplication event (Fig. 6, #2), the paralogy group-12 gene was lost from one copy, which became the proto-ab cluster, and group-7 and group-2 paralogs were lost from the other copy, which became the proto-cd cluster. These two clusters probably duplicated in an ancestor of jawed fish (Fig. 6, #4), because the horned shark *Heterodontus francisci*, a cartilaginous fish, has orthologs of mammalian *Hoxa* and *Hoxd* clusters (Kim et al. 2000; Chiu et al. 2002). In our phylogenetic trees, the horned shark *Hoxa* paralogs generally group with the tetrapod clade as a sister to the ray-fin clade (Fig. 4C,D,F, but not G), although bootstrap values are not high. As yet, there is no information about the predicted *Hoxb* and *Hoxc* clusters in the shark; they may be missing or more likely have not yet been identified.

After the second round of *Hox* cluster duplication in early vertebrates (Fig. 6, #4), but before the divergence of cartilaginous and bony fish, there were cluster-specific gene losses, including the loss of paralogy group-8 from the *Hoxa* cluster, group-11 from the *Hoxb* cluster, *Evx* from the *Hoxc* cluster, and group-6 from the *Hoxd* cluster (Fig. 6, #4). The next event was the divergence of ray-fin and lobe-fin fish. The common ancestor of lobe-fin fish had four *Hox* clusters (Koh et al. 2003), including *Hoxc1* (Fig. 6, #5), which is present in the coelacanth but was lost in the tetrapod lineage (Fig. 6, #6). The ancient *Evx* gene adjacent to the *Hoxb* cluster was also lost in the lobe-fin, but not the ray-fin lineage.

Among ray-fin fish, the bichir is the only basally diverging lineage that has yet been investigated (Fig. 6, #7), and results show a single *Hoxa* cluster, suggesting that the last common ancestor of ray-fin fish had just four *Hox* clusters (C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al., in prep.). More work needs to be done on this and other basally diverging ray-fin fish, because five group-9 genes have been identified in the bichir (Ledje et al. 2002), which may indicate either tandem duplication or allelic variation of a group-9 gene, or maybe single cluster duplication, or loss of several duplicated clusters in the bichir lineage, leaving a single *Hoxa* cluster.

Here, we show that two species of pufferfish have at least seven *Hox* clusters, and because other teleost fish also have duplicates of tetrapod *Hox* clusters (Misof and Wagner 1996; Amores et al. 1998; Snell et al. 1999; Naruse et al. 2000; Malaga-Trillo and Meyer 2001; Aparicio et al. 2002), there must have been a duplication of all four clusters originally possessed by the last common ancestor of extant bony fishes (Fig. 6, #8). The duplication of at least the *Hoxa* cluster occurred after the divergence of the bichir and other ray-fin fish (C.H. Chiu, K. Dewar, G. Wagner, K. Takahashi, F. Ruddle, C. Ledje, P. Bartsch, J.L. Scemama, E. Stellwag, C. Fried, et al., in prep.), suggesting that the genome duplication event happened in the time period denoted by the thick red lines in Figure 6.

The time at which *Hox* clusters duplicated in ray-fin phylogeny has not yet been determined precisely, and the estimate currently spans several hundred million years. It is important to define more accurately the timing of this duplication event, because zebrafish genetic mapping experiments showed that *Hox* cluster duplication occurred in the context of an ancient whole-genome duplication event (Aparicio et al. 1997; Amores et al.

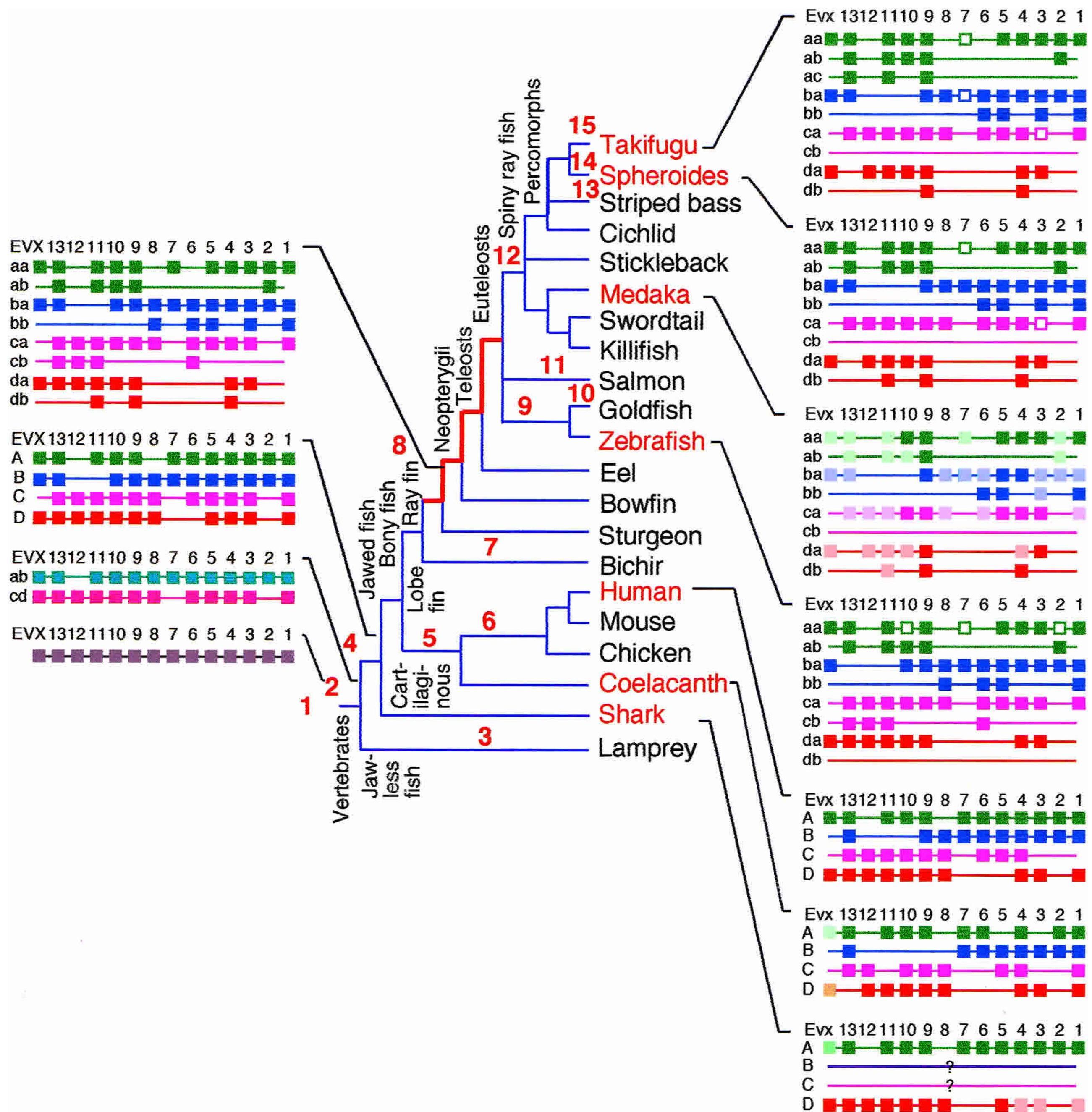


**Figure 6** The evolution of vertebrate *Hox* cluster genomic organization. See text for explanation.

1998; Postlethwait et al. 1998; Gates et al. 1999; Meyer and Malaga-Trillo 1999; Meyer and Schartl 1999; Woods et al. 2000; Taylor et al. 2001, 2003; Van de Peer et al. 2001, 2002; Postlethwait et al. 2002). It has been hypothesized that the genome duplication event contributed to the vast radiation of teleosts (Amores et al. 1998; Postlethwait et al. 1998). A theoretical reason for thinking that the ray-fin genome duplication could have played a major role in the teleost radiation is the differential resolution of duplicated genes, as evidenced here by the lineage-specific loss of *Hox* cluster genes and entire *Hox* clusters, and by lineage-specific gene expression patterns, as with *Hoxd4* genes. Such reciprocal loss of duplicated genes or duplicated gene subfunctions in different lineages could act as reproductive isolating mechanisms by decreasing the fitness of hybrids when separately evolving populations come into recontact (Lynch and Conery 2000; Lynch and Force 2000). If future phylogenetic studies show that the ray-fin genome duplication occurred hundreds of millions of years before the teleost radiation, then it is unlikely to have played a major role in spurring lineage divergence among teleost fish. Key lineages to query regarding the timing issue include the Japanese eel, a basally diverging teleost amenable to analysis of embryonic development (Kurokawa et al. 2002), and the bowfin, a nonteleost ray-fin lineage diverging just before the teleost radiation.

Genomic analysis of pufferfish, zebrafish, and medaka *Hox* clusters permits inferences regarding *Hox* clusters present in the

last common ancestor of euteleosts (Fig. 6, #8). First, it is clear that this organism had two copies of each tetrapod cluster, because the cluster missing in pufferfish and medaka is present in zebrafish, and the cluster missing in zebrafish is present in pufferfish and medaka. Second, at least one gene may have been lost in the ray-fin lineage after the divergence of bichir, and that is *Hoxa6*, which is present in bichir, but absent from examined teleosts, although this *Hoxa6* might also have been lost independently in *Hoxaa* and *Hoxab* clusters after duplication Fig. 6, #7 and #8). Third, several additional genes dropped out probably before the ray-fin genome duplication, including *Hoxd1*, *Hoxd5*, and *Hoxd8*.

As teleost lineages diverged, so did their *Hox* cluster content. In the zebrafish lineage (Fig. 6, #9), the entire *hoxdb* cluster appears to have been lost. We have identified and mapped to LG6 the zebrafish ESTs orthologous to human loci that directly flank the *HOXD* cluster, and are currently determining the genomic footprint left by the missing zebrafish cluster. In addition, *hoxa10a*, *hoxa7a*, and *hoxa2a* have all become pseudogenes in the zebrafish lineage, and *hoxb13a* and *hoxb3b* have been lost. Some Cyprinids other than zebrafish, including goldfish and some carp species (Risinger and Larhammar 1993; Larhammar and Risinger 1994), have undergone tetraploidization events after divergence from the zebrafish lineage (Fig. 6, #10). Independent additional tetraploidization has occurred in salmonds (Fig. 6, #11) (Allendorf and Thorgaard 1984), catostomids (Uyeno and Smith 1972), and other lineages. These more recent tetraploids we predict to have up to 14 *hox* clusters, each more depauparate than their single-copy orthologous cluster in zebrafish due to additional reciprocal gene losses between the new duplicates.

Pufferfish and medaka belong to the Acanthopterygii or spiny-ray fish, a group rich in species and morphologically diverse (Nelson 1994). From available data, the loss of the *Hoxcb* cluster appears to be a shared derived feature of this group (Fig. 6, #12). In the lineage giving rise to pufferfish (Fig. 6, #14 and #15), *Hoxa7* become a pseudogene because striped bass has a good *Hoxa7* gene (Fig. 6, #13) (Snell et al. 1999). *Hoxc3* became a pseudogene sometime after the divergence of medaka and pufferfish lineages, and *Hoxd13a* dropped out. Finally, several changes occurred after the divergence of the two pufferfish lineages, including the acquisition of an intron in *Hoxd11b*, and the mutation of *Hoxb7a* to a pseudogene.

The continuing evolution of *Hox* cluster genomic organization chronicled in Figure 6 raises the question of the roles of gene and genome duplication in the evolution of novel, lineage-specific morphologies. The pufferfish skeleton is highly derived, and many of these features are adaptations for puffing, the expansion of the gastrointestinal tract with water, which enlarges the fish and makes body spines more formidable defensive armamentation (Brainerd 1994; Wainwright et al. 1995; Wainwright and Turingan 1997). The molecular genetic basis for the evolution of morphologies and behaviors essential for puffing may have now become tractable given the availability of a draft genome sequnce (Aparicio et al. 2002) and the demonstration here that fugu embryos are amenable to developmental genetic investigation.

## METHODS

The *S. nephalus* PAC library was constructed as described (Amemiya et al. 2001). PACs were screened by PCR using a hierarchical pooling protocol (Amores et al. 1998; Amemiya et al. 2001). Degenerate primers for screening the *S. nephalus* genomic library included posterior *Hox* forward primers for paralogy groups 9–13 [CGAAAGAAG(C/A)G(I/C)GT(I/C)CC(I/C)TA(T/C)AC], anterior *Hox* forward primer for paralogy groups 1–9 [GAATTC CACTTCAAC(C/A)(G/A)(C/G)TACCT], and the universal reverse primer (CATCCTGCGGTTTTGGAACCAIAT). PACs shown to contain *Hox* cluster genes were screened individually by the above primers to amplify *Hox*-containing PCR fragments, which were then sequenced. From the sequenced fragments, gene-specific primers were designed for sequencing portions of each PAC, and in addition, PACs were subjected to shotgun sequencing. For the phylogenetic analyses, neighbor-joining trees were constructed from unambiguously aligned sequences as described (Amores et al. 1998). The alignments are available as Supplemental data available online at www.genome.org. Probes for in situ hybridization experiments were constructed from *S. nephalus* genomic DNA and used for in situ hybridization experiments on *T. rubripes* embryos collected as described (Suzuki et al. 2002), and using the zebrafish protocol (Force et al. 1999). To assign rhombomere identity, we used krox20, which is expressed in rhombomeres three and five.

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