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The evolution and maintenance of *Hox* gene clusters in vertebrates and the teleost-specific genome duplication

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ABSTRACT *Hox* genes are known to specify spatial identities along the anterior-posterior axis during embryogenesis. In vertebrates and most other deuterostomes, they are arranged in sets of uninterrupted clusters on chromosomes, and are in most cases expressed in a "colinear" fashion, in which genes closer to the 3′-end of the *Hox* clusters are expressed earlier and more anteriorly and genes close to the 5′-end of the clusters later and more posteriorly. In this review, we summarize the current understanding of how *Hox* gene clusters have been modified from basal lineages of deuterostomes to diverse taxa of vertebrates. Our parsimony reconstruction of *Hox* cluster architecture at various stages of vertebrate evolution highlights that the variation in *Hox* cluster structures among jawed vertebrates is mostly due to secondary lineage-specific gene losses and an additional genome duplication that occurred in the actinopterygian stem lineage, the teleost-specific genome duplication (TSGD).

KEY WORDS: *colinearity, two-round genome duplication, secondary gene loss*

Introduction

Hox genes are transcription factors that serve crucial roles during development in particular in embryonic anterior-posterior (A-P) patterning. In vertebrates and most other deuterostomes, *Hox* genes are arranged in sets of uninterrupted clusters on chromosomes. They specify the positional identities along the A-P axis and are in most cases expressed in a "colinear" fashion, i.e., genes closer to the 3'-end of the *Hox* clusters are expressed earlier and more anteriorly and genes close to the 5'-end of the clusters later and more posteriorly (Duboule, 1994; Kessel and Gruss, 1991; Lewis, 1978; McGinnis and Krumlauf, 1992). By now, it is understood that the multiple *Hox* gene clusters in the genomes of vertebrates are the remnants of an ancestral single homeobox gene cluster that was generated by successive rounds of tandem duplications early during metazoan evolution (reviewed in Garcia-Fernandez, 2005a). At least one (more or less complete and uninterrupted) *Hox* gene cluster is present in the genomes of almost all extant animal phyla, except for poriferans (Garcia-Fernandez, 2005b; Kamm, *et al*., 2006). The so-called colinear relationship between their genomic arrangement and their temporal and spatial expression remains one of the most interesting aspects of *Hox* clusters. It has been suggested that there is a link between this special genomic architecture and the origin of morphological novelties, such as modifications of axial segmental elements seen in the carapace of turtles (Ohya *et al*., 2005), loss of limbs in snakes (Cohn and Tickle, 1999), and the acquisition of jaws in gnathostomes (Cohn, 2002; Takio *et al*., 2004). In this review, we aim to summarize briefly the standing variation in the structures of *Hox* gene cluster architectures among vertebrates and attempt to reconstruct their evolutionary history. In light of known phylogenetic relationships we discuss alternative evolutionary processes that might have led to the clustered chromosomal arrangement of *Hox* genes. We also briefly survey the potential evolutionary forces that kept *Hox* genes clustered.

Early deuterostome orgins of the *Hox* cluster

Invertebrates typically, but not always, possess a single uninterrupted cluster of *Hox* genes while vertebrates have at least four such clusters (Fig. 1). There are no reports so far of invertebrates with more than one *Hox* cluster. In some invertebrate lineages,

Abbreviations used in this paper: TSGD, teleost-specific genome duplication.

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their *Hox* cluster was secondarily broken - as seen, for example, in the fly (*Drosophila melanogaster*; Von Allmen *et al.*, 1996) and the nematode (*Caenorhabditis elegans*; Van Auken *et al.*, 2000). This *Hox* cluster breakage is thought to have been caused by lineage-specific events that interrupted and dislocated an ancestrally intact *Hox* cluster (Akam, 1989; Aboobaker and Blaxter, 2003; Negre *et al.*, 2003). Future sequencing efforts will determine in how many animal phyla the *Hox* cluster is intact and how often during evolution it disassembled and partly relocated onto different chromosomes.

During the evolution of chordates from deuterostome ancestors, the genome was duplicated most likely twice consecutively in its entirety (Ohno, 1970; Lundin, 1993; Sidow, 1996; Dehal and Boore, 2005; McLysaght *et al.*, 2002; Kasahara, 2007). The evolution of *Hox* gene repertoires and their genomic structures in deuterostomes need to be reconstructed based on correctly inferred phylogenetic relationships between deuterostome phyla and major lineages within them (Fig. 1). Formerly, the hemichordata and echinodermata were placed basally, and the cephalochordata was regarded as the sister group of vertebrates ('Euchordata' hypothesis; Rowe, 2004; Schaeffer, 1987; Wada and Satoh, 1994). This relationship was, however, recently revised through the analysis of large amounts of sequence information for a larvacian tunicate (*Oikopleura dioica*; Delsuc *et al.*, 2006). These new data suggested a closer affinity of urochordates to vertebrates by excluding cephalochordata and clustered them with the echinodermata at the base of the deuterostomes ('Olfactore' hypothesis, after the name of clade combining urochordates and vertebrates; Jefferies, 1991). The Olfactore hypothesis has been supported by subsequent analyses that included the newly introduced phylum Xenoturbellida (Bourlat *et al.*, 2006) and genome-wide sequence data of the Florida lancelet (*Branchiostoma floridae*) (Putnam *et al.*, 2008), both of which placed cephalochordata at the basal lineage of chordates.

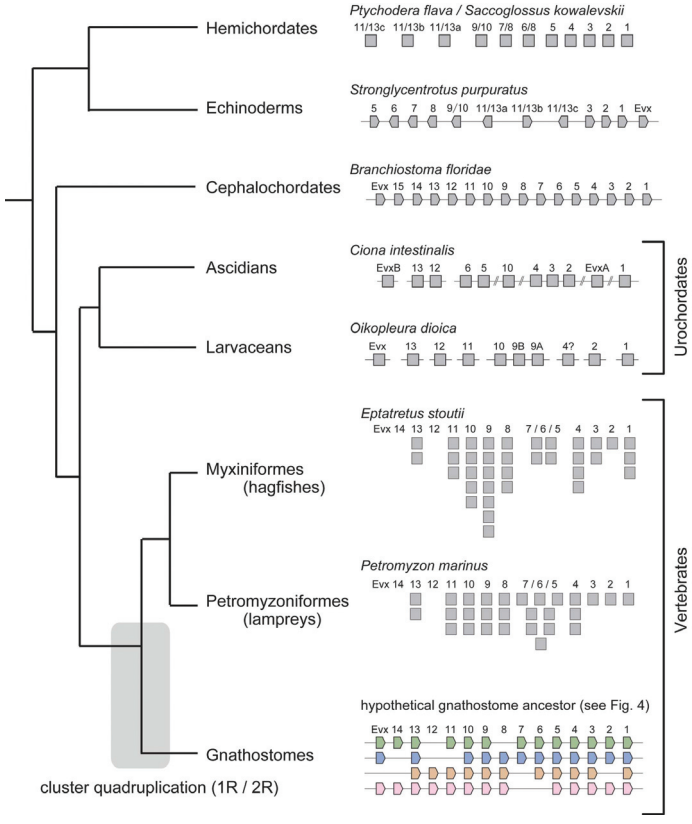

Fig. 1. Evolution of *Hox* clusters in deuterostomes. *The relationship between urochordates, cephalochordates, and vertebrates is depicted as a trichotomy, based on a conservative view between the conflicting reports of Delsuc et al. (2006) and Bourlat et al. (2006). The relationships between hagfishes, lampreys, and gnathostomes are based on references included in Kuraku and Kuratani (2006). The information for hagfish and lamprey are based on previous analyses (Force et al., 2002; Irvine et al., 2002; Stadler et al., 2004). See Fig. 4 for details of the hypothetical gnathostome ancestor. Direction of pentagonal boxes indicates transcriptional orientation, where this information is available. For hemichordates, Hox cDNAs isolated both from Saccoglossus kowalevski and Ptychodera flava are shown as in Monteiro and Ferrier (2006).*

Among all deuterostome invertebrates, the genomic analyses of *B. floridae* provided the first report of what was interpreted to be the ancestral condition of vertebrate *Hox* clusters (Garcia-Fernandez and Holland, 1994). Together with *Hox14* (Ferrier *et al.*, 2000) and *Hox15* found more recently (Holland *et al.*, 2008), the amphioxus *Hox* genes constitute an uninterrupted array of 15 *Hox* genes (*AmphiHox1-15*) and one *Evx* gene (Fig. 1; Amemiya *et al.*, 2008; Holland *et al.*, 2008). By contrast, in the sea urchin *Strongylocentrotus purpuratus*, only eleven *Hox* genes plus one *Evx* gene are aligned within 700 kb in a single genomic contig (Cameron *et al.*, 2006). Notably, the gene order and orientation of transcription are quite scrambled in this echinoderm lineage (Fig. 1). For the phylum hemichordata, so far only cDNA sequences are available for *Saccoglossus kowalevskii* and *Ptychodera flava* (Lowe *et al.*, 2003; Peterson, 2004), and the genomic structure of their *Hox* genes remains still unknown (Fig. 1). Within urochordates, the ascidian species *Ciona intestinalis* exhibits a broken *Hox* cluster with scrambled gene order and transcriptional orientation (Ikuta and Saiga, 2005), while the *Hox* cluster was disassembled secondarily in another urochordate, a larvacean *O. dioica* (Seo *et al.*, 2004; Fig. 1). This is interpreted as an aberration, and might be related to an altered upstream machinery, where major components of retinoic acid (RA) signaling, retinoaldehyde dehydrogenase (*Aldh1a1*), cytochrome P450 family-26 (*Cyp26*), and the retinoic acid receptor (*RAR*), that normally specifies the spatial identities along the anterior-posterior axis, seems to be absent from its genome (Canestro and Postlethwait, 2007; Canestro *et al.*, 2006). Strangely, however, the spatial expression patterns of *Hox* genes in this species still exhibit "colinearity", in spite of the atomized cluster organization and a lack of what heretofore was seen as crucial signaling cascade (Seo *et al.*, 2004; reviewed in Duboule, 2007). This surprising example poses the question of which kind of constraints keep multiple *Hox* genes clustered (reviewed in Duboule, 2007; also see Hoegg and Meyer, 2007 and Siegel, *et al.*, 2007, for examples in other conserved gene clusters).

In spite of the observed variation of gene repertoires and their genomic organization among deuterostome invertebrates (Monteiro and Ferrier, 2006), the apparent similarity between the amphioxus *Hox* cluster and the gnathostome clusters seems to support the notion that it represents the ancestral structure of the vertebrate and also chordate *Hox* cluster. It should be noted again, however, that this inference strongly depends on the

phylogenetic position of cephalochordates. For the time being, we still favor the traditional hypothesis that the last common ancestor of cephalochordates and vertebrates is expected to have already possessed a single"*Hox* cluster containing up to 14 *Hox* genes plus one *Evx* gene that were all transcribed in the same direction (Fig. 1).

How many *Hox* genes made up the ancestral vertebrate *Hox* cluster?

A related question is how many *Hox* genes made up the ancestral vertebrate *Hox* cluster. The answer to this question hinges on some difficult issues that are specific to *Hox* genes. Molecular phylogenetic analyses of *Hox* genes usually can only provide phylogenetic trees with limited confidence (Malaga-Trillo and Meyer, 2001; Meyer, 1998). This is mainly due to the conserved nature of the homedomain that is only 60 amino acids in length. Regions outside of the homeobox cannot be aligned reliably across large evolutionary distances and therefore are not available for phylogenetic inferences. This is particularly problematic for the posterior *abdominal* genes (*Hox9-13*), since it is often difficult to determine paralogy group relationships, (posterior flexibility; Ferrier *et al*., 2000). This is also reflected in the uncertain nomenclature for posterior *Hox* genes, for example in hemichordate and echinoderms (Fig. 1).

The situation is further complicated by the recent discovery of the *Hox14* genes located between the *Hox13* and *Evx* gene in vertebrate *Hox* clusters. This paralogy group was first reported from rather basal vertebrate lineage – from horn shark and the coelacanth (Powers and Amemiya, 2004a,). This has been followed by the discovery in the elephant fish (Venkatesh *et al*., 2007) and lamprey (Kuraku *et al*., 2008). Interestingly, the molecular phylogenetic tree including available posterior genes did not suggest orthology of the *Hox14* genes between amphioxus and vertebrates, whereas the position of the intron ('split homeobox') and gene location (between *Hox13* and *Evx*) supported a single origin of *Hox14* in the common ancestor of cephalochordates and vertebrates (Powers and Amemiya, 2004a; also see Ferrier, 2004; Garcia-Fernandez, 2005b, for reviews). Especially, when a single origin of *Hox14* shared by both amphioxus and vertebrates is assumed, the Euchordata hypothesis described above seemed to fit this scenario better (Fig. 2A). However, under the Olfactore hypothesis that is currently supported by large-scale molecular phylogenetic data, the scenario is not so clear-cut. If the *Hox14* of amphioxus and vertebrates are orthologous to each other, a loss of *Hox14* should be assumed in the urochordate lineages, because none of species in this group has been shown to possess a *Hox14* homolog (Fig. 2B). As mentioned above, in fact, molecular phylogenetic analysis has never supported this orthology (Powers and Amemiya, 2004a; Kuraku *et al*., 2008; Holland *et al.,* 2008). Moreover, it has been shown that an intron in the homeodomain does not serve as an unambiguous marker of *Hox14* – a lamprey *Hox13* homolog (*LjHox13β*) also has this feature (Kuraku *et al*., 2008). This accumulating evidence favors paralogy of *Hox14* between amphioxus and vertebrates. So far, molecular phylogenetic trees have strongly suggested that amphioxus *Hox13* and *Hox14* duplicated in the cephalochordate lineage (Fig. 2C and 2D; Powers and Amemiya, 2004a; Kuraku *et al*., 2008). For the origin of vertebrate *Hox14*, however, two scenarios, shown in Fig. 2C and 2D, have been supported with relatively high confidence (Kuraku *et al*., 2008). In fact, the origin of *Hox14* at the Olfactore ancestor (Fig. 2D), which compels a secondary loss of *Hox14* in the urochordate lineage, was favored over other scenarios that are more parsimonious (Kuraku *et al*., 2008).

In vertebrates, *Hoxd14* is retained by horn shark and elephant fish, whereas the coelacanth possesses *Hoxa14* (Powers and Amemiya, 2004a; Venkatesh *et al*., 2007). Therefore, it is likely that *Hox14* was gained before the quadruplication (1R/2R genome duplications) of *Hox* clusters (Powers and Amemiya, 2004a; also see Ferrier, 2004; Garcia-Fernandez, 2005b; Hoegg and Meyer 2005; Powers and Amemiya, 2004b for reviews). Based on a parsimony reconstruction of the *Hox* cluster evolution, it is likely that the *Hox14* paralogy group is the only one that might have undergone two independent gene losses during two subsequent rounds of whole genome duplications (Fig. 3). To date, *Hox14* genes have been found neither in any tetrapod nor any teleost genome in spite of relatively abundant sequence information for

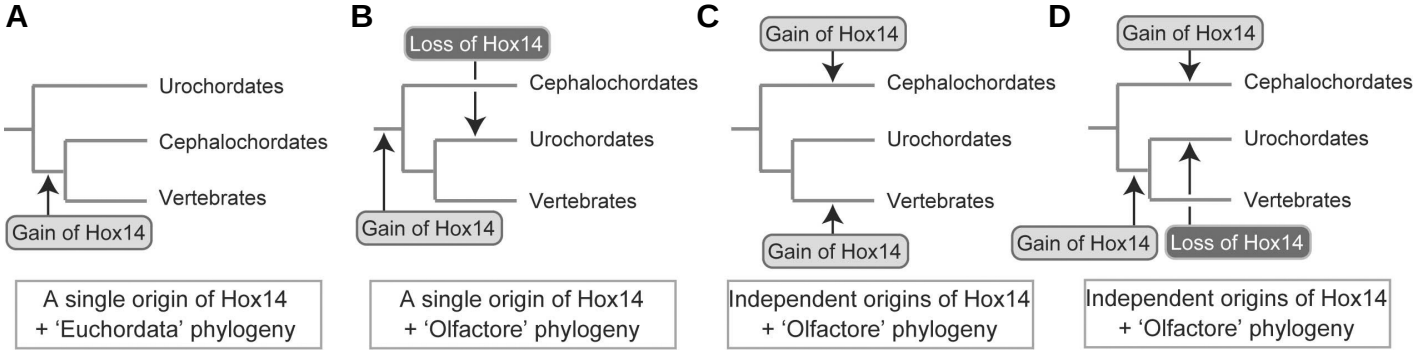

Fig. 2. The phylogenetic timing of the origin(s) of *Hox14* genes. *Possible scenarios for gain(s) of Hox14 were inferred. Under the assumption of the 'Euchordate' tree, if Hox14 of amphioxus and vertebrates shared a common ancestry, the presence of Hox14 can be interpreted as a synapomorphy for the Euchordata* **(A)**. *Under the 'Olfactore' tree, and if Hox14 of amphioxus and vertebrates are orthologous, it can be assumed that a secondary loss of Hox14 gene occurred in the urochordate lineage* **(B)**. *If Hox14 of amphioxus and vertebrates are paralogous, the vertebrate Hox14 originated after the divergence from the urochordate lineage* **(C)**, *or before (that is, at the common ancestor of vertebrates and urochordates)* **(D)**. *In (D), the absence of the Hox14 gene in the urochordates is regarded as a result of a secondary gene loss in the urochordate lineage.*

these lineages. It is suggested that less functional constraint, suggested by loss of expression in axial elements where the Hox-code is normally functioning, permitted the secondary losses of *Hox14* genes in these lineages (Kuraku *et al.*, 2008). Data from other taxa, such as hagfishes, non-teleost actinopterygians, non-tetrapod sarcopterygians will provide further information about the history of gains and losses of *Hox14*.

The example of *Hox14* potentially cautions that gene order in *Hox* clusters does not necessarily imply orthology. Early in the evolution of bilateral body plans, the *Hox* cluster was generated as the result of successive tandem duplications (reviewed in Garcia-Fernandez, 2005a). Moreover, secondary shuffling of gene order in a *Hox* gene cluster is frequently observed in invertebrate deuterostomes (Fig. 1; Monteiro and Ferrior, 2006). Thus, relative positions of genes within a cluster itself, which have been the basis of gene annotation especially in studies of *Hox* genes, cannot alone serve as sole and unequivocal criterion for postulating homology relationships among *Hox* genes and their assignment to paralogy groups. This circumstance combined with the difficulty of reliable phylogeny reconstruction renders orthology statements tenuous. This makes it difficult to reconstruct unequivocally the evolutionary history of posterior *Hox* genes - at least between some deuterostome lineages (Fig. 1).

Agnathans: how many *Hox* clusters?

Agnathans are jawless fishes that branched off the chordate stem lineage early during vertebrate evolution. Extant agnathans are grouped into hagfishes (Myxiniformes) and lampreys (Petromyzoniformes), and the phylogenetic relationships of these two lineages with gnathostomes (jawed vertebrates) remained a controversial issue for over a century. However, recent molecular phylogenetic analysis using a large number of genes suggested that hagfishes and lampreys form a monophyletic group, the Cyclostomata (Fig. 1; Blair and Hedges, 2005; Delarbre *et al.*, 2002; Delsuc *et al.*, 2006; Furlong and Holland, 2002; Kuraku *et al.*, 1999; Mallatt and Sullivan, 1998; Stock and Whitt, 1992; Takezaki *et al.*, 2003; also see Kuraku and Kuratani, 2006; reviewed in Meyer and Zardoya, 2003).

Although several attempts have been made to determine the *Hox* repertoires and their genomic organization of lamprey by cDNA isolation (Pendleton *et al.*, 1993; Sharman and Holland, 1998; Takio *et al.*, 2007; Takio *et al.*, 2004) and genomic sequencing (Force *et al.*, 2002; Irvine *et al.*, 2002), not all *Hox* genes have been discovered so far and their genomic organization remains uncertain. In addition to a high GC-content in protein coding regions (Kuraku and Kuratani, 2006), available cDNA sequences are usually incomplete and often derived from multiple closely related species. These factors prevented a precise categorization of available sequences into paralogy groups. Targeted genomic sequencing of *Hox* clusters in a sea lamprey *Petromyzon marinus* succeeded in the identification of regions containing multiple *Hox* genes, but they did not encompass entire *Hox* clusters (Force *et al.*, 2002; Irvine *et al.*, 2002). A phylogenetic analysis using available sequences suggested that lamprey has at least three or four *Hox* clusters (Force *et al.*, 2002; Irvine *et al.*, 2002; Fig. 1), which might, at least partly, be the result of cluster duplications specific to the cyclostome or lamprey lineage (Fried *et al.*, 2003). In contrast, a PCR survey of genomic sequences in the Pacific hagfish *Eptatretus stoutii* detected at most nine genes in a single paralogy group (Fig. 1), suggesting again the possibility of independent duplication(s) of *Hox* clusters in the cyclostome or hagfish lineage (Stadler *et al.*, 2004). Since hagfishes and lampreys diverged apparently relatively early (more than 400 million years ago) during cyclostome evolution–(Blair and Hedges, 2005;

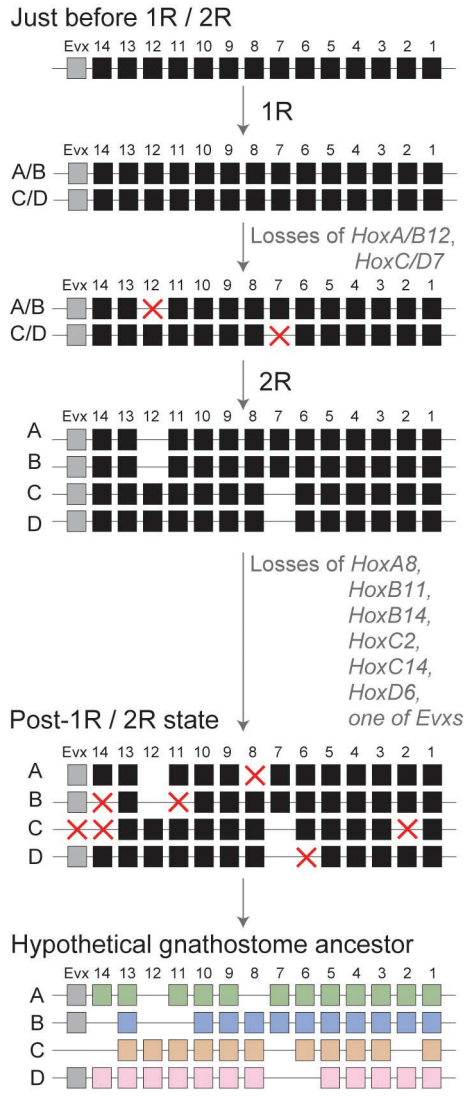

Fig. 3. Processes of *Hox* cluster quadruplication based on currently available data. *A possible scenario of quadruplication of Hox clusters was reconstructed based on parsimony criteria. For the gene repertoires of the hypothecial gnathostome ancestor (bottom), see Fig. 4. The cluster structure at the pre-1R/2R state (top) represents that of a hypothetical ancestor just before the cluster quadruplication that had already gained Hox14. It is notable that Hox14 is the only paralogy group of Hox genes that might have undergone two gene losses in the course of cluster quadruplication. The pattern of Hox cluster relationships we assume here is that the A and B and the C and D clusters are "sister clusters", that is, [(A,B),(C,D)] (see Meyer, 1998). This assumption is confirmed by our parsimony reconstruction of patterns of cluster duplications using the hypothetical gnathostome ancestor: each of three possible scenarios of cluster duplications, that is, [(A,B),(C,D)], [(A,C),(B,D)], and [(A,D),(B,C)], requires nine, eleven, and ten steps of gene losses, respectively.*

Kuraku and Kuratani, 2006), it would therefore not be surprising if these two major groups of extant jawless fishes would turn out to possess different organizations of *Hox* clusters.

The incomplete knowledge of the organization of *Hox* clusters in cyclostomes has implications for the understanding of the phylogenetic timing of the genome expansion event, the so-called two-round (2R) whole genome duplications (Ohno, 1970; Lundin, 1993; Sidow 1996, Dehal and Boore, 2005; McLysaght *et al.*, 2002). In these two rounds of whole genome duplications, a single gene or gene array of a pre-vertebrate ancestral genomes, would have been duplicated into multiple (up to four) copies in derived vertebrates. If the quadruplication of *Hox* clusters was caused by this event (Fig. 3), it is expected that the size of other gene families as well should provide clues on how many *Hox* clusters cyclostomes should possess. The same line of reasoning was used in the initial investigations of the teleost-specific genome duplication (TSGD or 3R), which will be discussed later (Vandepoele *et al.*, 2004; Hoegg *et al.*, 2004; Meyer and Schartl, 1999; Taylor *et al.*, 2001b, Wittbrodt *et al.*, 1998). However, attempts to analyze the phylogenetic position of cyclostome genes have so far failed to provide a reliable timing of genome duplications in early vertebrate evolution (e.g, Escriva *et al.*, 2002; also see Kuratani *et al.*, 2002 for review), probably due to the lack of strong phylogenetic signal that would be required for resolving multiple events (two rounds of genome duplications and the cyclostome/gnathostome divergence) that occurred within a short period of time (Horton *et al.*, 2003). In order to infer the timing of this genome expansion event reliably, larger numbers of gene families would need to be characterized in the future (see Kuraku *et al.*, 2009 for a recent study on this issue). The ongoing genome sequencing project for *Petromyzon marinus* (URL: http://genome.wustl.edu/genome.cgi?GENOME=Petromyzon%20marinus) is expected to provide additional information about the timing of the 2R genome duplications and the evolution of the genomic organization of *Hox* clusters in different lineages of deuterostomes.

Apart from genomic structure of *Hox* clusters, recent studies of lamprey *Hox* genes provided an overview about the colinearity in gene expression (Takio *et al.*, 2007; Takio *et al.*, 2004). Based on the expression patterns of paralogy group 2-8, the lamprey exhibits spatial colinearity in the central nervous system and pharyngeal arches, whereas no significant temporal colinearity could be detected. This observation will have to be investigated by more solid genomic linkage data and the collection of expression patterns of more gene repertoires. There are no reports at all about expression patterns of *Hox* genes in hagfish, so far. The recent success of hagfish embryology is expected to change this in the near future (Ota *et al.*, 2007).

Below, we discuss the evolutionary processes during the evolution of *Hox* clusters in gnathostomes by reconstructing ancestral cluster structures. We follow the methodology of parsimony reconstructions as was previously done (Hoegg and Meyer, 2005; Malaga-Trillo and Meyer, 2001; Meyer, 1998).

Hox clusters in chondrichthyans

Cartilaginous fishes (chondrichthyes) diverged first among extant gnathostome lineages from the gnathostome stem lineage (Nelson, 1994; Janvier, 1996; also see Kikugawa *et al.*, 2004 for recent molecular phylogenetic evidence). They are divided into elasmobranchii (sharks and rays) and holocephali (chimaeras; Fig. 4). For elasmobranchii, data of the horn shark *Heterodontus francisci* are available (Kim *et al.*, 2000, Prohaska *et al.*, 2004), and those of the elephant fish *Callorhinchus milii* are available for holocephali (Venkatesh *et al.*, 2007). One of the interesting genomic aspects of this group is the retention of *Hox14* genes (Powers and Amemiya, 2004a; Venkatesh *et al.*, 2007). In the horn shark, *Hoxa14* was found to be a pseudogene. It can safely be presumed that this gene was present still in the chondrichthyan ancestor (Fig. 4). It is also notable that in the holocephalan lineage as much as five genes were secondarily lost mainly in the *Hox* D cluster. Based on updated information for horn shark presented by Powers and Amemiya (2004b), in which the *Hox* C cluster is not fully reported, the structure of *Hox* A, B and D clusters of the horn shark is identical to that of the genomic reconstructions of the chondrichthyan and gnathostome ancestors (Fig. 4), except for the pseudogenization of *Hoxa14* gene. This possible retention of the ancestral gnathostome state by elasmobranchs needs to be confirmed by additional studies on the *Hox* C cluster and on other representative species in these lineages.

The actinopterygian lineage: Pre-3R and Post-3R modes

Almost 10 years ago extranumeral *Hox* clusters were first reported in teleosts (Amores *et al.*, 1998; Prince *et al.*, 1998; also see Hoegg and Meyer, 2005; Meyer, 1998; Meyer and Malaga-Trillo, 1999; Wittbrodt *et al.*, 1998 for reviews). We now know that the *Hox* cluster duplicates arose by a whole genome duplication that occurred early in the evolution of teleost fishes (VandePoule, *et al.* 2004; Christophels and Venkatesh, 2004; Crow *et al.*, 2006). This was previously also shown by the analyses of non-*Hox* gene families (Hoegg *et al.*, 2004; Taylor *et al.*, 2003; Taylor *et al.*, 2001a). This event was formerly termed the "fish-specific genome duplication" (FSGD; reviewed in Meyer and Van de Peer, 2005). However, this event has been proven to be shared by teleost fish lineages only and excludes more basal actinopterygian fish lineages (Hoegg *et al.* 2004), therefore the term "teleost-specific genome duplication (TSGD)" more accurately describes this important evolutionary event.

In recent years, information on *Hox* gene cluster architecture became available for more teleost species. In the order Cypriniformes, the data of blunt snout bream *Megalobrama amblycephala* was published (Zou *et al.*, 2007) and it closely resembles those of the zebrafish *Danio rerio* (data not shown in Fig. 1). For example, these two cypriniform species share the loss of the *Hox* Db cluster. Interestingly, all available data for the more derived Neoteleostei, namely, two pufferfishes (*Tetraodon nigroviridis* - Jaillon *et al.*, 2004; *Takifugu rubripes* - Aparicio *et al.*, 2002), and stickleback (*Gasterosteus aculeatus*; Hoegg *et al.*, 2007), medaka (*Oryzias latipes*; Kasahara *et al.*, 2007; Kurosawa *et al.*, 2006), and a haplochromine cichlid fish (*Astatotilapia burtoni*; Hoegg *et al.*, 2007; also see Thomas-Chollier and Ledent, 2008), showed that they shared the loss of the *Hox* Cb cluster, as described previously (Hoegg and Meyer, 2005). In addition to sporadic small-scale gene losses that occurred recently in each lineage (e.g., loss of the *hoxB7a* gene in the medaka lineage), massive gene losses seem to have occurred just after the origin of Neoteleostei (Fig. 4).

The data for bichirs are only available for the most basal

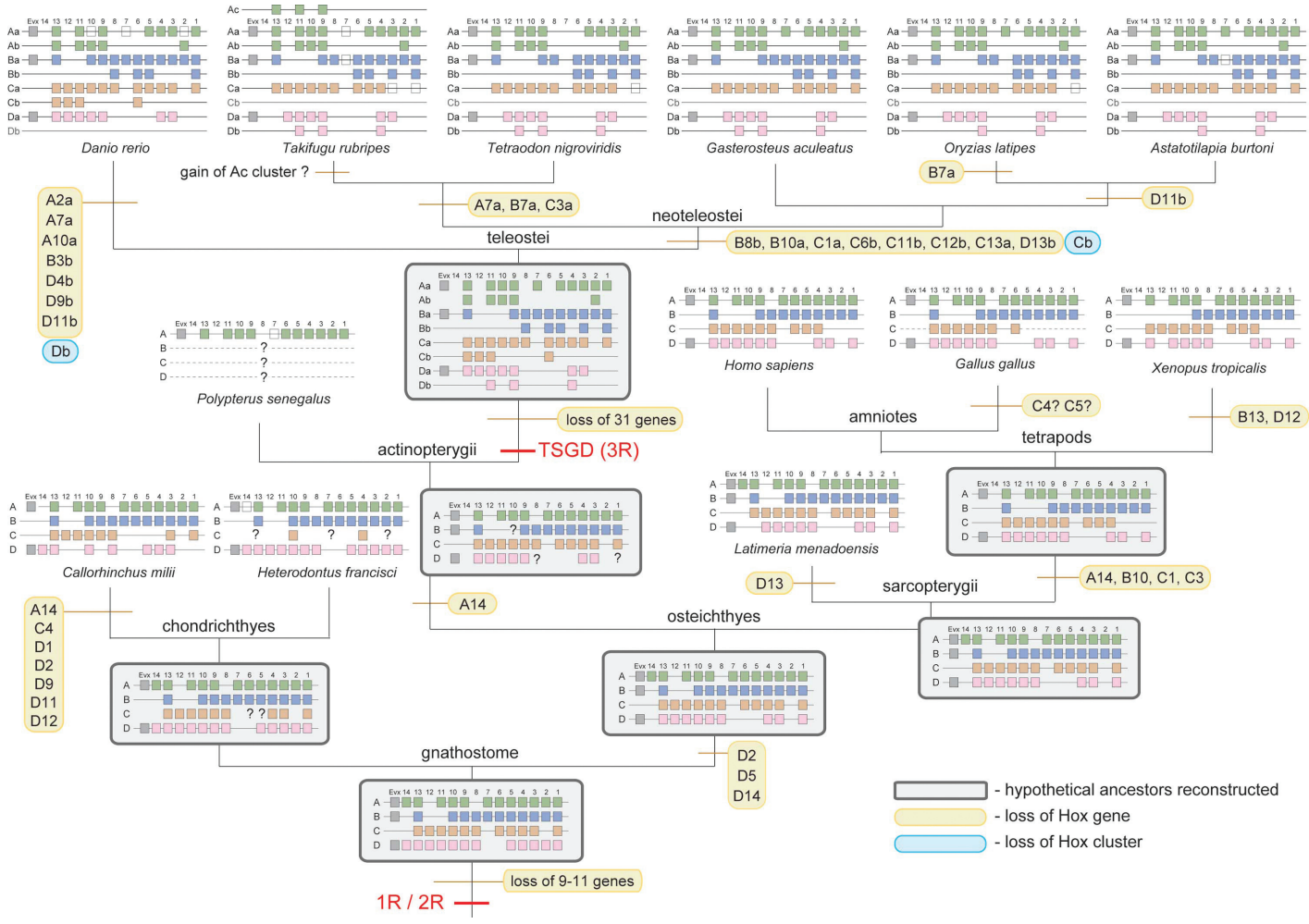

Fig. 4. Reconstructed evolutionary history of *Hox* cluster evolution within the jawed vertebrates. *Closed squares indicate genes that have been previously described. Open squares are reported pseudogenes. Shaded squares are genes that have not been sequenced yet, but probably are present in the cluster(s). Hypothetical organizations of Hox clusters, shown in grey boxes, in the hypothetical ancestors of various evolutionary lineages were reconstructed based on parsimony principles. Secondary losses of Hox gene and entire clusters are shown in yellow and blue boxes, respectively. See text for the original literature that reported the organization of Hox clusters for particular species. Possible genome duplication events are indicated with red bars. Genomic organization of Hox clusters in the horn shark and the coelacanth were based on unpublished data reported by Powers and Amemiya (2004b). Possession and pseudogenization of teleost hoxC1a are based on a recent report by Thomas-Chollier and Ledent (2008). Abbreviations: TSGD, teleost-specific genome duplication; 1R/2R, first- and second-round genome duplications.*

actinopterygian lineage that represents the pre-3R (TSGD) state (Chiu *et al.*, 2004; Ledje *et al.*, 2002). For *Polypterus palmas*, only cDNA isolation was performed (Ledje *et al.*, 2002), while the genomic sequences of the entire *Hox* A cluster is determined for *Polypterus senegalus* (Chiu *et al.*, 2004). Only very few gene losses seem to have occurred around the actinopterygii/sarcopterygii split, though this might be partly due to insufficient information for non-teleost actinopterygians. Although the structure of the *Hox* B, C, and D clusters remain to be determined for this lineage, it seems probable that bichirs have retained a similar set of *Hox* gene repertoires to that of the hypothetical ancestor of the Actinopterygii, Euteleostomi, and Sarcopterygii. Currently, there is no information available yet about *Hox* genes in Acipenseriformes (sturgeons), Amiiformes (bow fins), and Semionotiformes (gars). Knowledge about the genomic situation of *Hox* genes in these basal fish lineages would provide valuable information about the transition from the pre-3R state (before the TSGD and the evolutionary diversification of teleosts) to the post-3R genomic architecture.

Structural stability in the sarcopterygian lineage

For the sarcopterygian lineage including tetrapods, there are some reports for early-branching taxa. In the Australian lungfish *Neoceratodus forsteri* and the Indonesian coelacanth *Latimeria menadoensis*, only fragmental data have been reported so far based on cDNA isolation (Koh *et al.*, 2003; Longhurst and Joss, 1999). Recently, an overview of coelacanth *Hox* clusters was reported (Powers and Amemiya, 2004b), and its genome was found to contain highly similar *Hox* gene repertoires to those of tetrapods (Fig. 4). In the tetrapod lineage, four *Hox* genes including *HoxA14*, that is still present in the coelacanth, have been lost

secondarily (Fig. 4). Especially, it is curious to see whether the lungfishes possess *Hox14* genes in their genomes. In the lineages leading to western clawed frog (*Xenopus tropicalis*), two genes (*HoxB13* and *HoxD12*) are thought to have been lost (Fig. 4), based on an *in silico* survey by Hoegg and Meyer (2005). For the chicken (*Gallus gallus*), a recent report on genomic annotation of *Hox* clusters describes that the assembly is not complete, and thus entire or partial coding regions of some *Hox* genes (*HoxB2, HoxC4, HoxC5, HoxC13, HoxD1, HoxD8, HoxD9 and HoxD10*) are still missing (Richardson *et al*., 2007). Among these genes, entire coding regions of two genes (*HoxC4* and *HoxC5*) have not been found by our survey in available genomic and cDNA sequences (Fig. 4). These potential losses have to be confirmed with more complete genomic assembly in the future.

It remains an open question if phenotypic evolution and genomic evolution march to the beat of the same drummer. Interestingly, the hypothetical ancestral organization of tetrapods reconstructed from currently available information is identical to that still found today in the human genome (Fig. 4). In general it would appear that during almost 400 million years of tetrapod evolution only very few gene losses seem to have occurred. It appears as if the diversification of body plans in land vertebrates and a possible macroevolutionary trend towards increased complexity during the evolution of vertebrates was not accompanied by any (at least obvious major) changes in their *Hox* cluster architecture. Based on our preliminary analysis, however, the grey short-tailed opossum *Monodelphis domestica* might be an exception. In an *in silico* survey of its still somewhat preliminary genomic sequences (version MonDom5; URL, http://www.broad.mit.edu/mammals/opossum/; Mikkelsen *et al*., 2007), only a single *Hox* gene (*HoxC6*) has been found so far in the *Hox* C cluster. But, the *Hox* A, B, and D clusters are almost identical to the situation in the human genome. We also investigated the genomic sequences of the duck-billed platypus *Ornithorhynchus anatinus* (Ensembl database: URL, http://www.ensembl.org/Ornithorhynchus_anatinus/), but it would be premature to derive any conclusions based on this incomplete data set.

It is possible, however, that the TSGD is not only temporally correlated but even causally linked to the significantly accelerated rates of diversification/speciation and increased levels of phenotypic complexity in teleost fish (e.g. Wittbrodt *et al*., 1998; Meyer and Schartl, 1999; for more detailed discussion). Future work on a possible relationship of *Hox* cluster architecture and phenotypic diversification and increased complexity should also attempt to quantify changes in regulatory regions as well as protein coding regions and numbers of *Hox* gene repertoires.

Conclusions

In contrast to invertebrates, all vertebrates have four or more *Hox* gene clusters. Vertebrate *Hox* clusters are peculiar in that the linkages of genes in clusters are never broken and that all genes in a cluster are transcribed in the same direction (Duboule, 2007). A possible exception is the opossum *Hox* C cluster, but more data are needed to confirm this hypothesis. Also for agnathans, more reliable information is eagerly awaited since it might shed light on the phylogenetic timing of the 1R and 2R genome duplication events during chordate evolution. For the gnathostomes our reconstruction of the genomic organizations of the *Hox* clusters during vertebrate evolution suggests that there are two major types of *Hox* cluster architectures: (1) the four-cluster type and (2) the post-3R teleost type. The former is further divided into three themes: (1A) an ancestral gnathostome type (still seen in the horn shark A, B, and D clusters); (1B) non-teleost bony fish type (as found in the coelacanth and probably in the bichir); and (1C) the tetrapod type (e.g. human). To date, there is no solid genomic data about the organization of *Hox* gene clusters in some crucial lineages, such as hagfishes, lampreys and reptiles, and marsupials. These data are needed urgently for a more complete understanding of the patterns and processes of *Hox* cluster evolution in deuterostomes.

Knowledge of expression patterns of *Hox* genes might help in the understanding of the evolutionary history, mechanisms and constraints that shaped *Hox* cluster evolution. Expression information would also aid in the identification of potentially homologous morphological structures among species that belong to phenotypically extremely diverged lineages of vertebrates.

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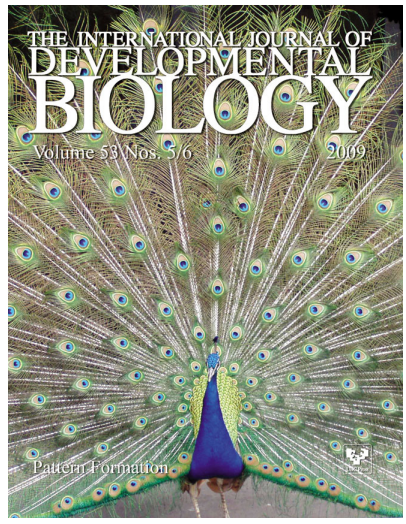

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