# Noncanonical role of Hox14 revealed by its expression patterns in lamprey and shark

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***Hox* genes are arranged in uninterrupted clusters in vertebrate genomes, and the nested patterns of their expression define spatial identities in multiple embryonic tissues. The ancestral *Hox* cluster of vertebrates has long been thought to consist of, maximally, 13 *Hox* genes. However, recently, Hox14 genes were discovered in three chordate lineages, the coelacanth, cartilaginous fishes, and amphioxus, but their expression patterns have not yet been analyzed. We isolated Hox14 cDNAs from the Japanese lamprey and cloudy catshark. These genes were not expressed in the central nervous systems, somites, or fin buds/folds but were expressed in a restricted cell population surrounding the hindgut. The lack of Hox14 expression in most of the embryonic axial elements, where nested *Hox* expressions define spatial identities, suggests a decoupling of Hox14 genes' regulation from the ancestral regulatory mechanism. The relaxation of preexisting constraint for collinear expression may have permitted the secondary losses of this *Hox* member in the tetrapod and teleost lineages.**

Hox-code | secondary gene loss | posterior Hox

The collinear expression of clustered *Hox* genes and their role in specifying regional identities along the anteroposterior embryonic axis in various metazoans are two of the most important discoveries in the field of evolutionary developmental biology (1, 2). All vertebrates surveyed so far have multiple *Hox* clusters (3), in which *Hox* genes located closer to the 3′ end are expressed more anteriorly and earlier than those closer to the 5′ end. This spatially as well as temporally collinear expression pattern of *Hox* genes (Hox-code; ref. 3) is found in the CNS, pharyngeal arches, digestive tract, and limb buds of the gnathostome (jawed vertebrate) embryos (4–6).

The ancestral vertebrate *Hox* gene cluster was thought to consist of 13 paralogous groups (Hox1–13) (7). This is consistent with the absence of any more paralogous group in mammals, birds, and teleosts. However, an additional gene, designated as Hox14, was found in the amphioxus genome between the Hox13 and *even-skipped* ortholog (*Evx*) (8). Recently, Hox14 genes were also found in an equivalent genomic location in the HoxA cluster of the coelacanth and in the HoxD cluster of the horn shark and the elephant fish (9, 10). These findings suggested that the ancestral *Hox* gene cluster of gnathostomes may have consisted of 14 *Hox* genes, not 13 as previously thought. Possession of Hox14 genes on different clusters between the coelacanth and the chondrichthyans (cartilaginous fishes) indicates that their origin antedates the cluster duplications (9, 11). All of the Hox14 genes reported so far have a unique intron in the homeodomain (8, 9), whereas all of the other vertebrate *Hox* genes do not have any intron in the homeodomain. Therefore, the sharing of this intron and the genomic location (between Hox13 and *Evx*), which are usually regarded as conservative traits, suggested that all of the known Hox14 genes originated from a common ancestral gene. It is still controversial, however, whether the gnathostome Hox14 and the amphioxus Hox14 are orthologous or not (9, 11, 12), because molecular phylogeny of Hox13/14 genes suggested independent origins of Hox14 in the amphioxus and gnathostomes (Fig. 1*A*) (9). This dispute makes an impact on a controversy over the deuterostome phylogeny. A recent large-scale molecular phylogenetic study suggested that not the cephalochordates, but the urochordates, which seem to possess no Hox14 (13, 14), are the sister group of vertebrates (15). Based on this phylogeny and a single origin of Hox14, we must assume an additional loss of Hox14 gene in the urochordate lineage (Fig. 1*C*). Otherwise, the traditional phylogenetic hypothesis, which combines cephalochordates with vertebrates (16), could be supported under the assumption of a single origin of Hox14 (Fig. 1*B*). To fill the gap between existing data, additional analyses in early vertebrates, such as cyclostomes (lampreys and hagfishes), could aid in the understanding on the origin of Hox14.

To date, there is no report on expression patterns of Hox14 genes. It is already known that posterior *Hox* genes (*Abdominal-B* group; Hox9–13) are coordinately expressed in the limb buds to establish the anteroposterior regional identities (5). This is also observed in the fin buds of chondrichthyans (17), suggesting that the Hox-code in the paired appendages, and that in the CNS and somites, was established before the divergence between chondrichthyans and osteichthyans (the rest of gnathostomes). In the lamprey, which lacks paired fins, Hox9 and Hox10 genes were shown to be expressed in the mesenchyme of dorsal fin fold (18). This is thought to be a portion of ancestral Hox-code before its deployment into the patterning of paired fins. Here, we selected a lamprey and a cartilaginous fish as targets of our study to examine whether Hox14 genes play these roles as posterior *Hox* genes, and to explore an evolutionary background that permitted the losses of Hox14 in multiple vertebrate lineages.

## Results

**Identification of Hox13 and Hox14 Genes in Lamprey.** To sample ancient member of vertebrate Hox14, we conducted a cDNA survey in a posterior half of the Japanese lamprey (*Lethenteron japonicum*) embryos at stage 28 (see *Materials and Methods*) and identified a Hox14 gene, *LjHox14α*. Its deduced amino acid sequence shows a high similarity with those of gnathostome Hox14 genes (Fig. 2*A*). We also identified two additional lamprey genes, *LjHox13α* and *LjHox13β*, which are similar to gnathostome Hox13 genes (Fig. 2*A*). An additional intron in the homeodomain is found not only in the *LjHox14α* gene but also

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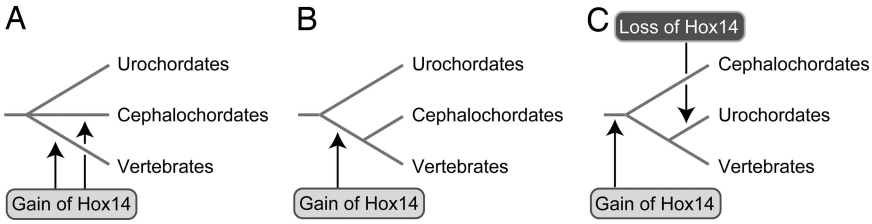


**Fig. 1.** Alternative scenarios for gains and a loss of Hox14. (*A*) Hox14 might have been gained independently in the cephalochordate and vertebrate lineages. (*B*) Under the assumption of traditional phylogenetic relationships between chordate subphyla (see ref. 15), if Hox14 of the amphioxus and vertebrates arose with a single event, the presence of Hox14 can be interpreted as a synapomorphy for the cephalochordate-vertebrate clade. (*C*) Under the phylogenetic relationships suggested by Delsuc *et al.* (15), if Hox14 of amphioxus and vertebrates are orthologous, we must assume a secondary loss of the Hox14 gene in the urochordate lineage.

in the *LjHox13β* gene (Fig. 2*A*). The amino acid stretch WFQNRR, which is conserved in all of the reported vertebrate Hox1–13 genes, has been revealed to be converted into WFQNQR in all of the vertebrate Hox14 genes reported so far, including *LjHox14α* (Fig. 2*A*). There are also other amino acid residues in the homeodomain that are conserved specifically among vertebrate Hox14 genes (Fig. 2*A*).

Our survey in genomic shotgun traces of *Petromyzon marinus* detected putative orthologs of *LjHox14α*, *LjHox13α*, and *LjHox13β*, but did not detect any other Hox14 member (see *Materials and Methods*). The same intron–exon structures were shared among *L. japonicum* genes identified in this study (*LjHox14α*, *LjHox13α*, and *LjHox13β*), and their *P. marinus* orthologs. None of assembled genomic contigs in the Ensembl *Pre!* was long enough to reveal the gene orders in flanking regions of these genes.

**Molecular Phylogenetic Studies to Reveal the Origin of Vertebrate Hox14.** We inferred molecular phylogenetic trees by using the neighbor-joining, maximum-likelihood (ML), and Bayesian methods. In the ML tree, the lamprey *LjHox13α* gene is grouped with gnathostome *HoxB13* genes, whereas the lamprey *LjHox13β* gene is placed basally among the Hox13 genes of vertebrates (Fig. 2*B*). The lamprey *LjHox14α* clustered with the coelacanth and chondrichthyan Hox14 genes with high confidence (Fig. 2*B*).

To focus on the origin of vertebrate Hox14, we then constrained relationships within *HoxA13–D13* and vertebrate Hox14 (see *Materials and Methods*), and performed a maximum-likelihood analysis with seven operational taxonomic units (OTUs) (HoxA13, HoxB13, HoxC13, HoxD13, vertebrate Hox14, amphioxus Hox13, and amphioxus Hox14). In Table 1, the best 25 tree topologies, of 945 possible tree topologies, are listed with probabilities in likelihood ratio tests. Tree topologies with the vertebrate Hox14-*AmphiHox14* clustering always have lower supporting values [$\Delta \ln L = 2.84$ (standard error, 2.75) for the tree topology supporting this clustering with the highest log-likelihood; approximate unbiased (AU) test, $P < 0.28$] than those with the *AmphiHox13-AmphiHox14* clustering. The clustering of vertebrate Hox14 and amphioxus Hox14 was again rejected by an analysis including urochordate sequences (data not shown).

**Expression Analysis of Hox13/14 Genes in Lamprey and Shark.** We conducted *in situ* hybridization analyses for the three novel lamprey *Hox* genes and the cloudy catshark (*Scyliorhinus torazame*) *Hoxd14* gene [supporting information (SI) Fig. S1]. In the catshark embryos, the expression of *Hoxd14* was not detected in the CNS, somites, or

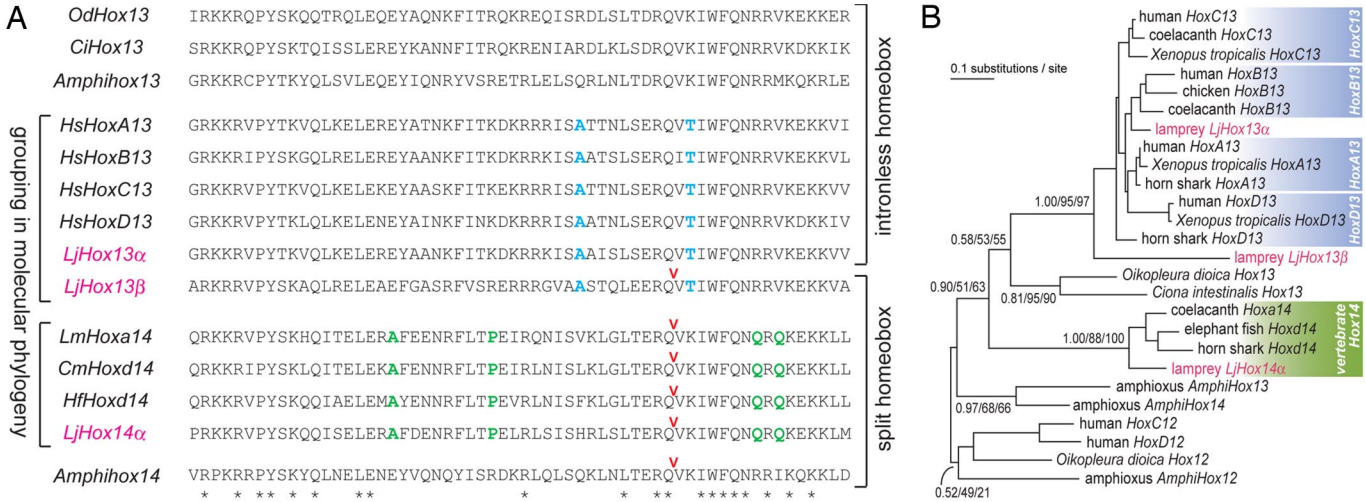


**Fig. 2.** Sequence comparison among Hox13/14 genes. (*A*) Amino acid sequences of the homeodomains of Hox13 and -14 genes. Amino acid residues that are conserved for all of these sequences are shown with asterisks below the alignment. Positions of introns are shown as ''V'' above the amino acid sequences. Amino acid residues specific to each paralogous group of *Hox* genes, based on comparison with paralogous group 1–12, are indicated with matching colors. Abbreviation of species names: *Hs*, *Homo sapiens*; *Lm*, *Latimeria menadoensis*; *Lj*, *Lethenteron japonicum*; *Hf*, *Heterodontus francisci*; *Cm*, *Callorhinchus milii*; *AmphiHox*, amphioxus *Hox*. (*B*) A molecular phylogeny of chordate Hox13 and Hox14 genes. This tree was inferred with the ML method using amino acid sequences (58 aa) using Hox12 genes as an outgroup. The JTT+I+$G_4$ model was assumed ($\alpha = 0.37$). Support for critical nodes are shown in the order, posterior probabilities of the Bayesian analysis, support values of ML analysis using quartet-puzzling, and bootstrap probabilities of the neighbor-joining tree inference with 1,000 iterations (17). Note that the clustering of vertebrate Hox13 genes and tunicate Hox13 genes is not strongly supported.

**Table 1. Results of maximum-likelihood analysis for inferring the phylogenetic relationships between Hox13/14 genes**

| Rank | Tree topology | ΔlnL | SE | ΔlnL/SE | P | | |
|---|---|---|---|---|---|---|---|
| | | | | | AU | KH | SH |
| 1 | (A13,(C13,(B13,D13)),(v14,(Amphi13,Amphi14))) | ML | — | — | 0.97 | 0.62 | 0.99 |
| 2 | (C13,(A13,(B13,D13)),(v14,(Amphi13,Amphi14))) | ML | — | — | 0.75 | 0.29 | 0.98 |
| 3 | ((v14,(Amphi13,Amphi14)),(D13,B13),(A13,C13)) | ML | — | — | 0.76 | 0.38 | 0.98 |
| 4 | ((v14,(Amphi13,Amphi14)),(D13,C13),(B13,A13)) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 5 | (C13,(D13,(A13,B13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 6 | (D13,(C13,(B13,A13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 7 | (B13,(A13,(C13,D13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 8 | (C13,(B13,(A13,D13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 9 | (D13,(A13,(C13,B13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 10 | (B13,(C13,(A13,D13)),(v14,(Amphi13,Amphi14))) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 11 | ((v14,(Amphi13,Amphi14)),(D13,A13),(B13,C13)) | 1.43 | 2.12 | 0.68 | 0.30 | 0.24 | 0.83 |
| 12 | (A13,(B13,(C13,D13)),(v14,(Amphi13,Amphi14))) | 1.37 | 2.02 | 0.68 | 0.31 | 0.23 | 0.84 |
| 13 | (A13,(D13,(C13,B13)),(v14,(Amphi13,Amphi14))) | 1.37 | 2.02 | 0.68 | 0.31 | 0.23 | 0.84 |
| 14 | (B13,(D13,(C13,A13)),(v14,(Amphi13,Amphi14))) | 0.13 | 0.14 | 0.91 | 0.96 | 0.19 | 0.96 |
| 15 | (D13,(B13,(C13,A13)),(v14,(Amphi13,Amphi14))) | 0.13 | 0.14 | 0.91 | 0.96 | 0.19 | 0.96 |
| 16 | (A13,(C13,(B13,D13)),(Amphi14,(Amphi13,v14))) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 17 | (A13,(C13,(B13,D13)),(Amphi13,(v14,Amphi14))) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 18 | (C13,(A13,(B13,D13)),(Amphi13,(v14,Amphi14))) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 19 | (C13,(A13,(B13,D13)),(Amphi14,(Amphi13,v14))) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 20 | ((Amphi14,(v14,Amphi13)),(D13,B13),(C13,A13)) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 21 | ((Amphi13,(v14,Amphi14)),(D13,B13),(A13,C13)) | 2.84 | 2.75 | 1.03 | 0.28 | 0.16 | 0.63 |
| 22 | (B13,(D13,(C13,A13)),(Amphi13,(v14,Amphi14))) | 2.85 | 2.74 | 1.04 | 0.30 | 0.15 | 0.62 |
| 23 | (D13,(B13,(C13,A13)),(Amphi13,(v14,Amphi14))) | 2.85 | 2.74 | 1.04 | 0.30 | 0.15 | 0.62 |
| 24 | (D13,(B13,(C13,A13)),(Amphi14,(Amphi13,v14))) | 2.85 | 2.74 | 1.04 | 0.30 | 0.15 | 0.62 |
| 25 | (B13,(D13,(C13,A13)),(Amphi14,(Amphi13,v14))) | 2.85 | 2.74 | 1.04 | 0.30 | 0.15 | 0.62 |

Abbreviations: A13, HoxA13; B13, HoxB13; C13, HoxC13; D13, HoxD13; v14, vertebrate Hox14; Amphi13, AmphiHox13; Amphi14, AmphiHox14; Δ lnL, difference of log-likelihood in the ML analysis deviated from the ML tree. SE, standard error of log-likelihood; AU, approximate unbiased test; KH, Kishino–Hasegawa test; SH, Shimodaira–Hasegawa test. Similar results were obtained even in the analysis containing Hox13 genes of tunicates and also Hox9–12 genes (as outgroups).

the fin buds, but was only detected in a small cell population ventral to the hindgut at the late pharyngula stage (Fig. 3 *A–C* and Fig. S2). In the lamprey, *LjHox14α* transcripts were detected in a small cell population surrounding the hindgut (Fig. 3 *D* and *G*), which was later expanded into a larger cell population (Fig. 3 *J* and *M*). Expression of *LjHox13α* and *LjHox13β* was also detected in a similar region surrounding the hindgut (Fig. 3 *H*, *I*, *N*, and *O*), with additional signals in the tailbud for *LjHox13α* (Fig. 3*N*), and in the cloaca for *LjHox13β* as well (Fig. 3 *I* and *O*). None of these three lamprey genes was expressed in the mesenchyme of the dorsal fin fold (Fig. 3 *D–O*). Notably, our analysis at embryonic stages did not identify signals of Hox14 expression in the CNS, somites, and fin buds/folds, where the Hox-code is expected to function (Fig. 3 *B–D*, *G*, *J*, *M*).

## Discussion

Previously, two cDNA fragments of possible Hox13 genes have been reported for two lamprey species (19, 20), but their extremely short sequences did not permit a rigorous phylogenetic analysis that would have categorized them as Hox13 genes. In addition, no Hox12 genes had been reported for lampreys so far (18–23). Therefore, we report here phylogenetic evidence for Hox12 or more posterior *Hox* genes in lampreys. Molecular phylogenetic trees based on the alignable portion of the *Hox* genes (the homeodomain of 60 aa in length) do not provide sufficient resolution that would permit an unambiguous assignment of particular *Hox* genes to clusters (A–C or D), as reported for many of other lamprey *Hox* genes (18–23). We were able, however, to identify these newly isolated genes as members of the vertebrate Hox13 and Hox14 paralogous groups (Fig. 2*B*).

The presence of additional intron in the homeodomain in the newly identified *LjHox13β* points out that this feature is not an unambiguously specific hallmark of Hox14 genes. Moreover, our phylogenetic analysis suggested that the amphioxus and vertebrate Hox14 genes are not orthologous, but have arisen independently through tandem gene duplications of Hox13 genes (Fig. 1*A*). This result, based on a short alignment, should be treated with caution. It is not necessarily likely, however, that the result of our phylogenetics analysis, which already uses maximal length of conserved homeodomain and also includes major chordate taxa (Fig. 2*B*), would be challenged with higher confidence in the future.

Previously, we reported expression of Hox9–11 genes in the lamprey tailbud (18). The tailbud-associated expression of *LjHox13α* was more restricted posteriorly, indicating the sharing of upstream regulation by some of Hox9–13 genes (18) (Fig. 3*N*). Expression patterns of Hox13/14 genes in these early vertebrates also provide an insight into how the posterior *Hox* genes have established their roles in multiple embryonic tissues (Fig. 4). First, in the lamprey, the Hox13 genes, and the Hox14 gene, were not expressed in the medial fin fold (Fig. 3 *H*, *I*, *N*, *O*). This indicate that the deployment of posterior *Hox* genes in the fin-patterning developmental program had not been completed yet at the divergence between the cyclostome and gnathostome lineages, or that the lamprey might possess other Hox13 genes that would be expressed in the fin fold. More importantly, in the lamprey and catshark, Hox14 genes were not expressed in the embryonic CNS nor somites, but in the hindgut-surrounding cells (Fig. 3 *C*, *G*, *M*). It has been already reported that *Hox* genes are expressed in the gut, and their nested expression patterns has been interpreted as "endodermal" Hox-code (6). Especially, in mouse and chicken, Hox9–13 genes are expressed at around

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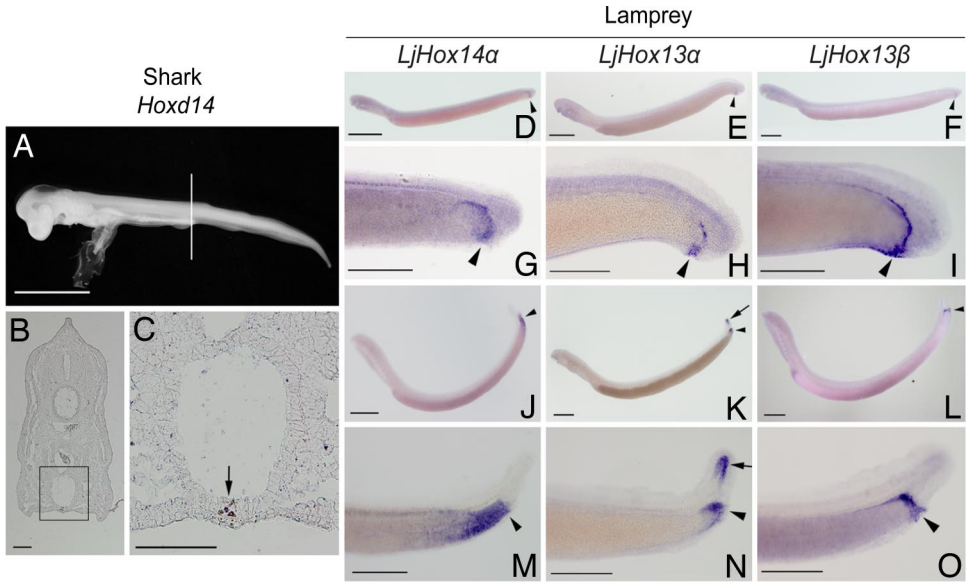


**Fig. 3.** Expression patterns of Hox13 and Hox14 genes in shark and lamprey. (*A*) An embryo of *S. torazame* at stage 27. (*B*) A transverse section stained with a *Hoxd14* riboprobe with *in situ* hybridization at the level of a bar in *A*. (*C*) A magnified view of a region shown in *B*. Cells expressing *Hoxd14* are indicated with an arrow. (*D–F*) Whole-mount lamprey (*L. japonicum*) embryos at stage 26 stained with *LjHox14α*, *LjHox13α*, and *LjHox13β* riboprobes, respectively. (*G–I*) Magnification of the posterior regions of *D–F*, respectively. (*J–L*) Whole-mount *L. japonicum* embryos at stage 28 stained with *LjHox14α*, *LjHox13α*, and *LjHox13β* riboprobes, respectively. (*M–O*) Magnification of the posterior regions of *J–L*, respectively. Arrowheads indicate the location of the cloaca. Arrows indicate the expression signals in the tail bud. (Scale bars: *A*, 5 mm; *B* and *C*, 0.1 mm; *D–F* and *J–L*, 0.5 mm; *G–I* and *M–O*, 0.25 mm.)

cecal/cloacal regions or more posterior regions of the hindgut (24, 25). Recent observations in chondrichthyans underline that the hindgut-associated expression of posterior *Hox* genes was gained before the divergence between the chondrichthyans and the osteichthyans (26). In the sea urchin embryo, one of the orthologs of posterior *Hox* genes (*Sphox11/13b*) is expressed in the hindgut (27), suggesting that the gut-associated expression of posterior *Hox* genes could have already been established at the root of deuterostomes. Therefore, it is reasonable to assume that the hindgut-associated expression of Hox14 genes is directly derived from Hox13, its ancestral copy, and that this expression domain has become restricted into a small cell population secondarily in the chondrichthyan lineage.

We conclude that the paralogous group Hox14, previously found in coelacanth and cartilaginous fishes, had been already established through the tandem duplication before the era of the last common ancestor of all extant vertebrates, including cyclostomes. The lack of Hox14 expression in the embryonic CNS, somites, nor the fin folds/buds suggests that they might have been decoupled from the ancestral Hox-code functioning in these embryonic structures, whereas their hindgut-associated expression, which is probably derived from Hox13 genes, has been maintained (Fig. 4). Clearly, the absence of Hox14 genes in genomes of tetrapods and teleosts indicates their secondarily losses in these lineages (Fig. 4). These secondary gene losses might have been permissible because of the less functional constraint, as is manifested by the decoupling of Hox14 genes partially from the canonical Hox-code.

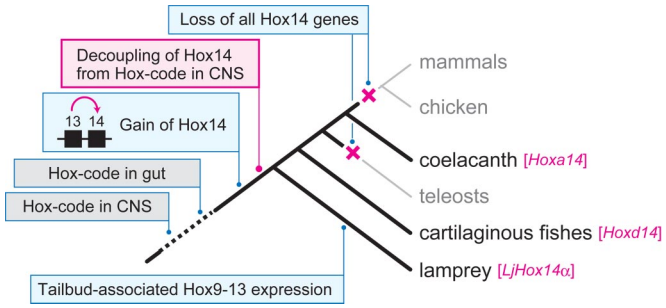


**Fig. 4.** A hypothesized evolutionary scenario of repertoires and roles of Hox14. Possession of Hox14 genes is indicated with the gene names beside the names of animal groups. The timings of possible losses of all Hox14 members are indicated with an "X."

## Materials and Methods

**Animals.** Embryonic stagings of *S. torazame* and *L. japonicum* were based on Ballard *et al.* (28) and Tahara (29), respectively. Total RNA of *S. torazame* was extracted from a whole embryo at stage 12. Total RNA of *L. japonicum* was extracted from a posterior half of an embryo at stage 28.

**cDNA Cloning and Sequencing.** Total RNAs were reverse transcribed into cDNA by using Thermo-X reverse transcriptase (Invitrogen). These cDNAs were used as templates for PCR amplification with the GC-rich PCR System (Roche). The sense degenerate primers 5′-CC GAR MGN CAR GTN AAR ATH TGG TT-3′ and 5′-GG ATC TGG TTY CAR AAY CAR MGN CA-3′ were designed based on amino acid residues in the homeobox shared by vertebrate Hox14 genes, namely, TERQVKIWF and KIWFQNQRQ, respectively. These primers were used for primary and nested amplification of the stretch spanning from the end of the homeodomain to the 3′-end using adaptor primers, as in 3′-RACE (30): *LjHox13α* and *LjHox13β* were obtained in the primary amplification, whereas *LjHox14α* were obtained in the nested one. PCR was conducted as described in ref. 31. Lengths of PCR products were confirmed with 2100 Bioanalyzer (Agilent). Amplified cDNA fragments were purified by using MinElute (Qiagen) and cloned into a pCRII-TOPO vector (Invitrogen). More than three independent clones per gene were sequenced by using a 3130 Genetic Analyzer (Applied Biosystems). Upstream nucleotide sequences of isolated lamprey cDNAs were amplified and sequenced by 5′-RACE. The partial catshark cDNA (Fig. S1) was isolated by the nested PCR amplification as described above. Nucleotide and deduced amino acid sequences determined in this study were deposited in the GenBank with accession nos. AB293596–AB293599. Presence and position of introns were analyzed with genomic PCR by using gene-specific primers.

**Molecular Phylogenetic Analysis.** Amino acid sequences of posterior *Hox* genes were retrieved from the public databases. An optimal multiple alignment of these amino acid sequences and those obtained in this study was constructed

by using the alignment editor XCED, in which the MAFFT program is implemented (32), with manual inspection. The molecular phylogenetic trees were inferred by using the regions that were unambiguously aligned with no gaps. The neighbor-joining tree reconstruction (33) was processed by using XCED.

To calculate the support values for the molecular phylogenetic tree shown in Fig. 2*B*, we used MrBayes 3.1 (34) and Tree-Puzzle [quartet-puzzling mode (35)]. To investigate the phylogenetic relationship between *AmphiHox14* and vertebrate Hox14 (Table 1), the ML tree was inferred by using Tree-Puzzle (35), assuming JTT+I+$\Gamma_4$ model (shape parameter $\alpha = 0.47$) (36). In this ML analysis, we performed an exhaustive search of the ML tree in the ''user-defined trees'' mode of all 945 possible topologies consisting of seven OTUs, namely, *HoxA13*, *HoxB13*, *HoxC13*, *HoxD13*, vertebrate Hox14, *AmphiHox13*, and *AmphiHox14*. Phylogenetic relationships within each taxon were constrained as follows: [(human, *Xenopus*), horn shark] for *HoxA13*; [(human, chicken), coelacanth] for *HoxB13*; [(human, *Xenopus*), coelacanth] for *HoxC13*; [(human, *Xenopus*), horn shark] for *HoxD13*; ([*LmHoxa14* (*CmHoxd14*, *HfHoxd14*)], *LjHox14a*) for vertebrate Hox14. *LjHox13*$\alpha$ and *LjHox13*$\beta$ were excluded from this analysis, because precise phylogenetic positions of these genes are unknown. Likelihood ratio tests were performed by using CONSEL (37).

**Survey of Hox13/14 Genes in *P. marinus* Genomic Sequences.** In addition to performing RT-PCR, we surveyed Hox13/14 genes in the sea lamprey (*P. marinus*), whose whole genome sequencing project is now underway (Washington University Genome Sequencing Center; http://genome.wustl.edu/genome.cgi?GENOME=Petromyzon%20marinus), using the Ensembl *Pre!* Interface (http://pre.ensembl.org/Petromyzon_marinus/index.html) and shotgun genomic sequences downloaded from the Ensembl Trace server (ftp://ftp.ensembl.org/pub/traces/petromyzon_marinus/fasta/).

***In Situ* Hybridization.** Whole-mount *in situ* hybridization was performed as described in ref. 38. *In situ* hybridization with sections was also performed following the previous description (39). In detection of expression signals of catshark *Hoxd14*, we performed whole-mount staining by using *S. torazame* embryos at stages 12–27. For each of *LjHox14*$\alpha$, *LjHox13*$\alpha$, and *LjHox13*$\beta$, riboprobes were prepared by using cDNA templates containing two different stretches (results are shown only for *in situ* hybridization using more downstream riboprobes): nucleotide position 1–312 and position 296 to the end for *LjHox14*$\alpha$ (AB293599); position 1–550 and position 757–1,221 for *LjHox13a* (AB293597); position 1–966 and position 855 to the end for *LjHox13*$\beta$ (AB293598). For catshark *Hoxd14* (AB293596), a whole sequenced stretch was used as a template for riboprobe synthesis. Negative control experiments were done by using sense-strand riboprobes, in which we detected no equivalent signals to those detected in this study (data not available).

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