

A PCR survey for posterior *Hox* genes in amphibians

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Abstract

Hox genes encode transcription factors that play a key role in specifying the body plan in metazoans and are therefore essential in explaining patterns of evolutionary diversity. As an ancient tetrapod group with diverse limb types, amphibians are important for understanding the origin and diversification of limbs in land vertebrates. We conducted a PCR survey in two species of each amphibian order to identify *Hox-9* to *Hox-13*, known to function in limb development. Fifteen distinct posterior *Hox* genes and one retro-pseudogene were identified, and the former confirm the existence of four *Hox* clusters in each amphibian order. Some genes expected to occur in all tetrapods, based on the posterior *Hox* complement of mammals, fishes and coelacanth, were not recovered from our survey, and may have been lost. *Hoxd-12* is absent in frogs and possibly other amphibians. Considering its function in autopodial development, the loss of this gene may be related to the absence of the fifth finger in frogs and salamanders.

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1. Introduction

One of the major changes in vertebrate evolution is the origin of the tetrapod limb, an adaptation to a terrestrial lifestyle. Because amphibians diverged early from other tetrapods (Ruta et al., 2003), they play a key role in understanding the genetic basis of this evolutionary transition. Among the three amphibian orders, Anura (frogs and toads) mainly have long hind limbs for saltatory locomotion, Caudata (salamanders and newts) generally have four limbs of similar size, and Gymnophiona (caecilians) are limbless. Both frogs and salamanders usually have four fingers and five toes, but several exceptions are known. For example, amphiumid and proteid salamanders, as well as brachycephalid and some microhylid frogs have a reduced number of digits, and Sirenidae lack hind limbs. Frogs additionally have evolved a large diversity of adaptive limb structures, including suctorial pads (e.g., tree frogs), large tubercles for digging (e.g., burrowing frogs), and extended

webbing for swimming (e.g., *Xenopus*) or gliding (e.g., *Rhacophorus*).

Changes in the number and regulation of *Hox* genes may have strongly influenced the diversification of metazoan body plans (Carroll, 2001). In particular, this family of DNA-binding transcription factors probably played an important role in the differentiation of amphibian limbs because, in addition to their function in anterior–posterior body patterning in all Bilateria, they are involved in morphogenesis of paired appendages (Carroll et al., 2001; Dollé et al., 1989). *Hox* genes are characterized by a 180-base-pair (bp) homeobox that is highly conserved among distantly related taxa. They are typically arranged in clusters with up to 14 genes that originated from a series of tandem duplications (Ruddle et al., 1994). Subsequent rounds of duplication in vertebrate evolution produced multiple *Hox* clusters (Bailey et al., 1997): three or four in agnathans (lamprey and hagfish; Force et al., 2002; Fried et al., 2003; Irvine et al., 2002; Stadler et al., 2004), typically four in gnathostomes (*HoxA–D*; Koh et al., 2003; Longhurst and Joss, 1999; Powers and Amemiya, 2004a), and up to eight in teleost fishes (Amores et al., 1998, 2004). At present, the origin of

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these clusters, either by whole-genome duplications or by cluster duplications, is still the subject of debate (reviewed by Wolfe, 2001). The multiple lamprey and hagfish *Hox* clusters probably originated by two independent cluster duplications in each lineage (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002; Stadler et al., 2004). Teleost fishes experienced an additional genome duplication, resulting in eight different *Hox* clusters (Málaga-Trillo and Meyer, 2001; Taylor et al., 2003). Paralogous groups (PG; i.e., genes on different clusters that originated from the same ancestral gene during the cluster duplication events) differ in numbers of genes, indicating patterns of secondary gene loss.

Patterning of the tetrapod limb is controlled by complex interactions between several genes, such as *Hox*, *Shh*, *Msx*, *Gli-3*, *Alx4*, members of the transforming growth factor- β (TGF- β) and bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) (reviewed by Capdevila and Izpisua Belmonte, 2001). A combination of *Hoxb-5*, *Hoxc-6*, and *Hoxc-8* is mainly involved in positioning the limb field (Nelson et al., 1996; Rancourt et al., 1995), while the posterior *Hox* genes (PG9–13) have been identified as important pathway genes in outgrowth of the limb bud (Dollé et al., 1989; Kondo et al., 1997; Shubin et al., 1997). Particularly, *Hoxa* and *Hoxd* genes function in formation of stylopodium (humerus/femur: *Hoxa-9*, *Hoxd-9*), zeugopodium (radius, ulna/tibia, fibula: *Hoxa-10*, *Hoxd-10*, *Hoxa-11*, *Hoxd-11*), and autopodium (hand/foot: *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *Hoxa-13*) (reviewed by Zákány and Duboule, 1999). Outside the limb bud, these genes also play a role in morphogenesis of the urogenital organs and the digestive tract (Dollé et al., 1991; Yokouchi et al., 1995).

In contrast to mammals and ray-finned fishes, amphibian *Hox* clusters have not been studied extensively, despite the limb diversity in this group. Some *Hox* genes have been characterized in frogs (*Xenopus laevis*: e.g., Lombardo and Slack, 2001 and *Silurana tropicalis*: Chiu et al., 2000) and salamanders (*Ambystoma mexicanum*: e.g., Gardiner et al., 1995; *Pleurodeles waltl*: Nicolas et al., 2003 and *Notophthalmus viridescens*: e.g., Belleville et al., 1992), but no caecilian *Hox* genes have yet been identified. In salamanders, these genes have been studied mainly for their importance in the regeneration of amputated limbs (reviewed by Nye et al., 2003).

PCR surveys have demonstrated their value for preliminary identification of *Hox* genes in various animals (e.g., Force et al., 2002; Irvine et al., 2002; Koh et al., 2003; Longhurst and Joss, 1999; Stadler et al., 2004). Here, we survey and characterize the homeobox sequences of the posterior *Hox* genes in two previously unexamined representatives of each amphibian order.

2. Materials and methods

2.1. Cloning and sequencing

Whole genomic DNA was extracted from muscle tissue of the following species using a standard protocol

(Sambrook et al., 1989): the frogs *Rana temporaria* (Ranidae) and *Leiopelma archeyi* (Leiopelmatidae), the salamanders *Triturus helveticus* (Salamandridae) and *Siren intermedia* (Sirenidae) and the caecilians *Ichthyophis banananicus* (Ichthyophiidae) and *Rhinatrema bivittatum* (Rhinatremitidae). These species pairs were selected to represent a broad evolutionary divergence within each order, preferably by spanning the basal-most split. For example, the basal divergences of the archaeobatrachian frog *Leiopelma* and the neobatrachian frog *Rana*, as well as of the caecilians *Rhinatrema* and *Ichthyophis*, are supported by both morphological and molecular evidence (Duellman and Trueb, 1986; Lynch, 1973; Roelants and Bossuyt, 2005; San Mauro et al., 2004; Wilkinson, 1997). In addition, most phylogenetic studies have agreed on the early divergence of Sirenidae among living salamanders (San Mauro et al., 2005; Wiens et al., 2005). Molecular clock estimations have situated each of these intra-ordinal divergences in Early Mesozoic times (Roelants and Bossuyt, 2005; San Mauro et al., 2005).

Based on known sequences of vertebrate *Hox* genes, two degenerate primers, 5'-AARAARMGITGYCCNTAYAC-3' (forward) and 5'-YTTCATICKNCKRRTTYTGRAA-3' (reverse), were designed for amplification of a 118-bp fragment of the highly conserved homeobox of PG9–11. For amplification of PG12 and 13, we used the primers from Kurosawa et al. (1999): the forward primer specific for PG12 5'-GGGGATCCAARAARMGRAARCCNTA-3', the forward primer specific for PG13 5'-GGGGATCCAARAARMGRGTNCCNTA-3'; and the general reverse primer 5'-GGGTGACYCKYCKRRTTYTGRAACCA-3'. Because of doubt about the presence/absence of the whole B-cluster in caecilians, an additional PCR-survey was performed that targeted PG1–10 in *Ichthyophis*, using the following primer set (Koh et al., 2003): 5'-GARYTNGARAARGARTT-3' (forward) and 5'-CKNCKRRTTYTGRAACCA-3' (reverse). Additionally, to confirm the absence of several genes, new degenerate primers were designed specifically for amplification of the genes that were not initially retrieved with the general primers. *Hoxc-11* was targeted with the forward primer 5'-CAARAARMGNTGYCCNTAYTC-3' and the reverse primer 5'-AARTACTGYARNCGGTCYCT-3', *Hoxb-13* with the forward primer 5'-GGCGNGRYMGNAARAARMGNAT-3' and the reverse primer 5'-SCKGTTYTGAAACCA RATSGTRAT-3'.

PCR amplification was performed with FastStart Taq DNA polymerase (Roche) using the following cycling parameters: initial denaturation at 95 °C for 4 min, 44 cycles of 94 °C for 40 s, 45–57 °C for 1 min, and 72 °C for 1 min, and final extension at 72 °C for 3 min. Amplified fragments were purified by agarose gel extraction (Qiagen) and cloned into a pGEM-T Easy vector (Promega) following the manufacturer's protocol. An additional round of PCR and purification of the clones was performed to increase the quantity. The clones were sequenced with the ABI Prism BigDye Terminator

Table 1
GenBank accession numbers of the *Hox* sequences used in the analyses

	<i>Mus musculus</i>	<i>Homo sapiens</i>	<i>Gallus gallus</i>	<i>Latimeria menadoensis</i>	<i>Ambystoma mexicanum</i>	<i>Pleurodeles waltl</i>	<i>Notophthalmus viridescens</i>
<i>Hox-a9</i>	AAH55059		Q98924	AAO43021	P50209	AAN63623	
<i>Hox-b9</i>	NP_032296					AAQ92346	
<i>Hox-c9</i>	AAH50838		XP_423451	AAO43038			
<i>Hox-d9</i>	AAH19150		P24340	AAO43045			
<i>Hox-a10</i>	AAH50839			AAO43022			
<i>Hox-c10</i>	AAH53405		XP_428107	AAO43039	AAG27630		X68975
<i>Hox-d10</i>	AAH13463		P24341	AAO43046	AAC34742		X68976
<i>Hox-a11</i>	NP_034580		P31258	AAO43023			
<i>Hox-c11</i>		O43248					
<i>Hox-d11</i>	NP_032299		P24342	AAO43048			A43783
<i>Hox-c12</i>	AAM28679			AAO38042		AAN63593	
<i>Hox-d12</i>	NP_032300		P24343	AAO43047			
<i>Hox-a13</i>	AAB03322		NP_989470	AAO43024	P50210		
<i>Hox-b13</i>	AAH13639			AAO43033	AAG27629		
<i>Hox-c13</i>	NP_034594		XP_427501	AAO43040		AAN63624	
<i>Hox-d13</i>	NP_032301		P24344			AAQ92345	

v3.1 Cycle Sequencing Kit and analyzed on an automated ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

2.2. Genome walking

For one unidentifiable *Siren Hox-13* sequence, genome walking was performed to amplify the unknown sequence adjacent to the 118-bp fragment towards both ends of exon two. The homeobox-flanking regions are less conserved and more informative and thus can facilitate identification of the paralog. Genomic DNA was digested with restriction enzymes (BsuRI, EcoRV, MssI, PvuII, and RsaI) and adaptors [up: 5'-GTAATACGACTCACTATAGGGCA CGCGTGGTTCGACGGCCCGGGCTGGT-3', down: 3'-H₂N-CCCGACCA-PO₄-5' (BD GenomeWalker)] were ligated to both ends of the fragments with T4 ligase. PCR amplification was done with gene-specific primers and adaptor primers (Siebert et al., 1995). The first PCR cycle consisted of a denaturation step at 95 °C for 10 min, seven cycles of 94 °C for 20 s and 72 °C for 3 min, followed by 32 cycles of 94 °C for 20s and 67 °C for 3 min, and a final elongation step of 67 °C for 7 min. The second PCR, with nested primers, used the product of the first amplification as a template with the same parameters, but with five and 25 cycles respectively. The adaptor-specific primers were AP1 (5'-GTAATACGACTCACTATAGGGC-3') and the nested primer AP2 (5'-ACTATAGGGCACGCGTGGT-3'). The 5' end of the *Siren Hox-13* sequence was amplified with the reverse gene-specific primers HoxGW-063 (5'-GGTTTATCACTAAGGACAAGCGGCC-3') and HoxGW-064 (5'-CGCTGGAGCACGAGTACACGGCCGGCA-3', nested). Amplification of the 3' end of this sequence used the forward gene-specific primers HoxGW-061 (5'-ATCTGAAGGCGCTGGAGCACGAGTACA-3') and HoxGW-062 (5'-TAGCACGAGTACACGGCCGGCAG GTTT-3', nested).

2.3. Sequence analyses

The obtained clones were screened for *Hox* gene fragments using a set of different BLAST searches (blastn, blastp, and tblastn) against GenBank. Derived amino acid sequences of these fragments were aligned with *Hox* genes of coelacanth, mammals, chicken and other amphibians retrieved from GenBank (Accession numbers are provided in Table 1), using ClustalX v1.81 (Thompson et al., 1994) and MacClade v4.0 (Maddison and Maddison, 2000). The database 'Xenopus tropicalis genome assembly version 3.0' (February 2005, DoE Joint Genome Institute),¹ which contains a draft version of the genomic sequence of *Silurana tropicalis*, was screened for *Hox* clusters by BLAST searches. The *Hox* genes in this draft sequence were identified by cluster alignment of *Silurana* with other vertebrates using MultiPipMaker (Schwartz et al., 2003) and by screening of the *Silurana* clusters for coding regions with GenomeScan (Yeh et al., 2001).

The newly cloned amphibian *Hox* sequences were assigned to the different PGs by phylogenetic comparison of their translated amino acid sequences with those of the other vertebrates, using maximum parsimony (MP), neighbor joining (NJ), maximum likelihood (ML) and Bayesian inference (BI). Heuristic MP searches were done with PAUP* v4.0b10 (Swofford, 2002) using tree bisection–reconnection branch swapping in 10,000 replicates. ML and Bayesian analyses used the JTT model of amino acid substitution (Jones et al., 1992), with optimized γ -shape correction for among-site rate heterogeneity (+ Γ). This model was assigned as best fitting the observed data by the different selection criteria implemented in ProtTest v1.2.6 (Abascal et al., 2005). We used PHYML v2.4.4 (Guindon and Gascuel, 2003) for the ML analyses and MrBayes v3.0b4

¹ URL: <http://genome.jgi-psf.org/Xentr3/Xentr3.home.html>

(Ronquist and Huelsenbeck, 2003) for BI. The latter was performed with four chains of 5,000,000 generations, sampled every 500 generations. Time series plots of log-likelihoods and model parameters indicated complete convergence after 1,000,000 generations, and posterior probabilities were derived from 8000 post-burnin trees. NJ analyses were performed using the JTT model of amino acid substitution with the suite of software available in the PHYLIP v3.6 package (Felsenstein, 2004). In addition to Bayesian posterior probabilities, support for the clades was evaluated by non-parametric bootstrap analyses (Felsen-

stein, 1985) under MP (10,000 replicates), NJ (1000 replicates) and ML (500 replicates).

The short size and highly conserved amino acid sequences may complicate detailed phylogenetic resolution based on conventional analyses. For further comparative studies, we identified unique combinations of amino acids for each PG, i.e., amino acid combinations that are conserved among all members of a PG but not in any other group (Sharkey et al., 1997). In addition, we indicated a unique combination of amino acids that distinguish the paralogs within a PG.

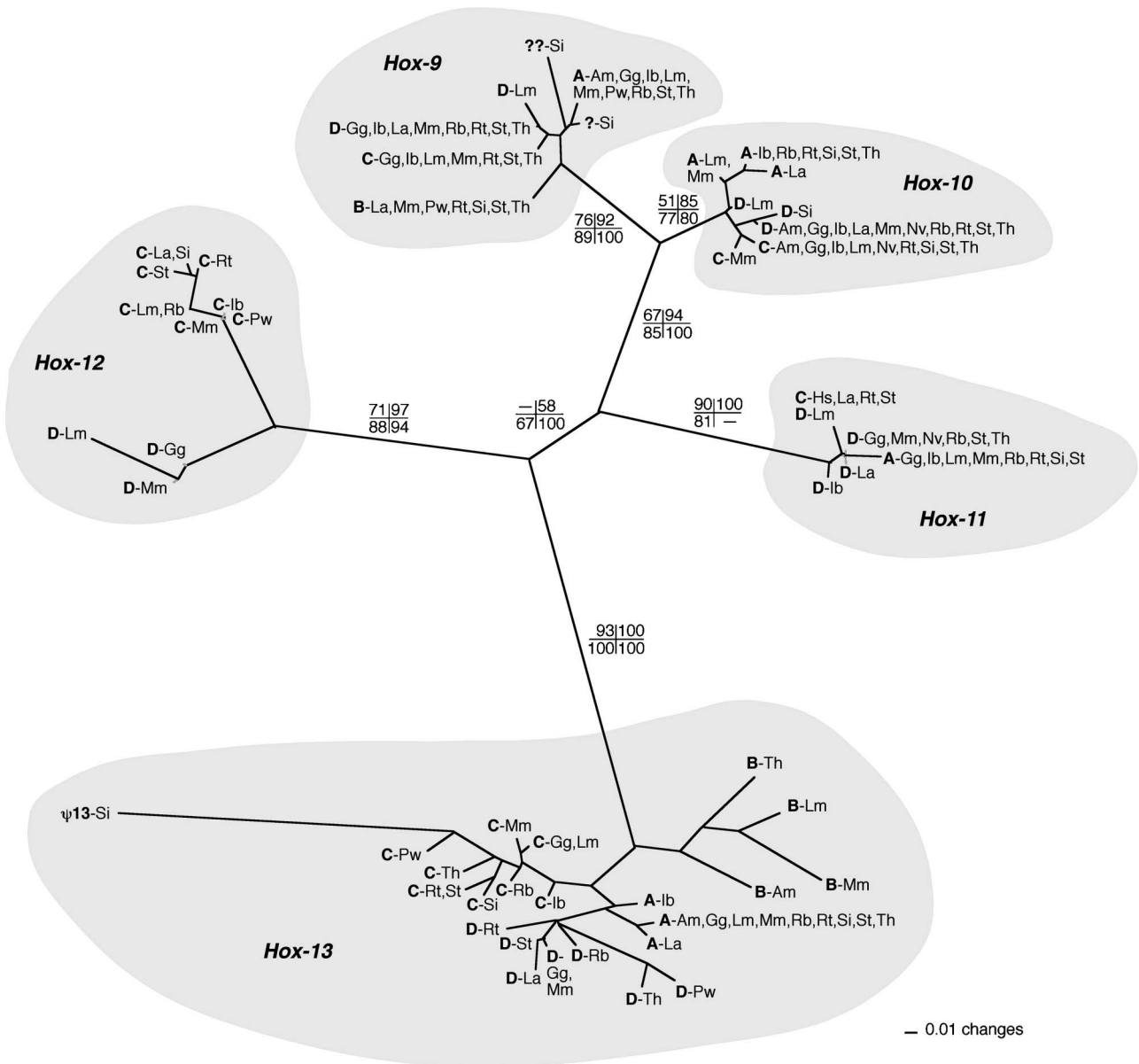


Fig. 1. Neighbor joining tree for the amphibian *Hox* protein sequences of this study combined with *Hox* sequences of mouse, chicken, coelacanth, some other amphibians from GenBank, and of *Silurana tropicalis* from the *Xenopus tropicalis* genomic database. Abbreviations of the species are as follows: Am, *Ambystoma mexicanum*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Ib, *Ichthyophis bannanicus*; La, *Leiopelma archeyi*; Lm, *Latimeria menadoensis*; Mm, *Mus musculus*; Nv, *Notophthalmus viridescens*; Pw, *Pleurodeles waltl*; Rb, *Rhinatrema bivittatum*; Rt, *Rana temporaria*; Si, *Siren intermedia*; St, *Silurana tropicalis*; Th, *Triturus helveticus*. Numbers on the branches represent bootstrap support for MP (upper left), NJ (upper right), ML (lower left), and Bayesian posterior probabilities (lower right).

3. Results

3.1. Amplification of homeobox sequences

In total, 391 clones containing a fragment of the posterior *Hox* genes of the six amphibian species were sequenced:

308 clones were obtained from amplification with the primer set for PG9–11, 23 with the PG12 primer set, 58 with the PG13 primer set and two were amplified with primers specific for *Hoxc-11*. Additionally, we sequenced 23 clones of PG1–10 of *Ichthyophis*. Occasionally, two sequences were found differing in only one nucleotide without affect-

		1	10	20	30	40
<i>Hoxa-9</i>	coelacanth	TKHQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	TERQVKIW
	amphibians	-KHQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	TERQVKIW
<i>Hoxb-9</i>	mouse	TKHQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	TERQVKIW
	amphibians	-KYQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	SERQVKIW
<i>Hoxc-9</i>	mouse	TKYQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	SERQVKIW
	coelacanth	TKYQ	TLELEKEFLFNMYL	TRDRRYEVAR	VNL	TERQVKIW
<i>Hoxd-9</i>	amphibians	-KYQ	TLELEKEFLFNMYL	TRDRRYEVAR	VNL	TERQVKIW
	mouse	TKYQ	TLELEKEFLFNMYL	TRDRRYEVAR	VNL	TERQVKIW
<i>Hoxd-9</i>	coelacanth	TKYQ	TLELEKEFLFNMYL	TRDRRYEVAR	L	DLTERQVKIW
	amphibians	-KYQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	TERQVKIW
<i>?-Si</i>	mouse	TKYQ	TLELEKEFLFNMYL	TRDRRYEVAR	LNL	TERQVKIW
	<i>Siren</i>	-KHQ	TLELEKEFLFNMYL	TRDRRYEVAR	VNL	TERQVKIW
<i>??-Si</i>	<i>Siren</i>	-KHQ	TLELEKEFLFNMYL	TRDRRYEVAR	L	LALTERQVKIW
	<i>Siren</i>	-KHQ	TLELEKEFLFNMYL	TRDRRYEVAR	L	LALTERQVKIW
<i>Hoxa-10</i>	coelacanth	TKHQ	TLELEKEFLFNMYL	TRRRRLEISRS	VHL	DRQVKIW
	amphibians	-KHQ	TLELEKEFLFNMYL	TRRRRLEISRS	VHLS	DRQVKIW
<i>Hoxc-10</i>	<i>Leiopelma</i>	-KHQ	TLELEKEFLFNMYL	TRRRRLEISRC	VHLS	DRQVKIW
	mouse	TKHQ	TLELEKEFLFNMYL	TRRRRLEISRS	VHL	DRQVKIW
<i>Hoxc-10</i>	coelacanth	TKHQ	TLELEKEFLFNMYL	TRRRRLEISKS	INL	DRQVKIW
	amphibians	-KHQ	TLELEKEFLFNMYL	TRRRRLEISKS	INL	DRQVKIW
<i>Hoxd-10</i>	mouse	TKHQ	TLELEKEFLFNMYL	TRRRRLEISK	TINL	DRQVKIW
	coelacanth	TKHQ	TLELEKEFLFNMYL	TRRRRLEISRS	VNL	DRQVKIW
<i>Hoxd-10</i>	amphibians	-KHQ	TLELEKEFLFNMYL	TRRRRLEISKS	VNL	DRQVKIW
	<i>Siren</i>	-KHQ	TLELEKEFLFNMYL	TRRRRLEISKS	VNL	DRQVKIW
<i>Hoxa-11</i>	mouse	TKYQ	I	RELE	REFFFSVY	INKEKRLQLSRMLNLTDRQVKIW
	coelacanth	-KYQ	I	RELE	REFFFSVY	INKEKRLQLSRMLNLTDRQVKIW
<i>Hoxc-11</i>	amphibians	TKYQ	I	RELE	REFFFSVY	INKEKRLQLSRMLNLTDRQVKIW
	human	-KFQ	I	RELE	REFFFN	VYINKEKRLQLSRMLNLTDRQVKIW
<i>Hoxd-11</i>	coelacanth	TKYQ	I	RELE	REFFFN	VYINKEKRLQLSRMLNLTDRQVKIW
	amphibians	-KYQ	I	RELE	REFFFN	VYINKEKRLQLSRMLNLTDRQVKIW
<i>Hoxd-11</i>	<i>Ichthyophis</i>	TKYQ	I	RELE	REFFFN	VYINKEKRLQLSRMLNLTDRQVKIW
	mouse	-KYQ	I	RELE	REFFFN	VYINKEKRLQLSRMLNLTDRQVKIW
<i>Hoxc-12</i>	<i>Rhinatrema</i>	SKLQ	LAE	EGEF	MVNEFITR	QRRRELSDRNLNSDQOVKI-
	<i>Ichthyophis</i>	SKLQ	LAE	EGEF	LVNEFITR	QRRRELSDRNLNSDQOVKI-
<i>Hoxc-12</i>	<i>Siren</i>	SKLQ	LAE	EGEF	MVNEFITR	QRRRELSDRNLNSDQOVKI-
	<i>Rana</i>	SKFQ	LAE	EGEF	MVNEFITR	QRRRELSDRNLNSDQOVKI-
<i>Hoxd-12</i>	<i>Leiopelma</i>	SKLQ	LAE	EGEF	MVNEFITR	QRRRELSDRNLNSDQOVKI-
	mouse	SKLQ	LAE	EGEF	LVNEFITR	QRRRELSDRNLNSDQOVKI-
<i>Hoxd-12</i>	coelacanth	TKQO	L	AHLE	NEFLINEFITN	RQKRELSDRNLNSDQOVKI-
	mouse	TKQO	L	AHLE	NEFLINEFITN	RQKRELSDRNLNSDQOVKI-
<i>Hoxa-13</i>	coelacanth	TKVQL	KELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
	amphibians	TKVQL	KELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxb-13</i>	<i>Ichthyophis</i>	TKIQL	KELE	REYASN	KFITKDKRRRISAT	TNLSERQVTIW
	<i>Leiopelma</i>	TKVQL	KELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxc-13</i>	mouse	TKVQL	KELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
	coelacanth	TKVQL	KELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	<i>Triturus</i>	SKSQL	RELE	REYATN	KFITKDKRRRISAT	TNLSERQVTIW
	mouse	SKGQL	RELE	REYAA	NKFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxc-13</i>	coelacanth	TKIQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
	<i>Rhinatrema</i>	TKLQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	<i>Ichthyophis</i>	TKLQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
	<i>Siren</i>	TKIQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	<i>Triturus</i>	TKVQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
	mouse	TKIQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	coelacanth	TKVQL	KELE	REYAA	SKFITKDKRRRISAT	TNLSERQVTIW
	<i>Rhinatrema</i>	TKLQL	KELE	TEYAI	NKFINKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	<i>Triturus</i>	TKLQL	KELE	TEYAI	NKFINKDKRRRISAT	TNLSERQVTIW
	<i>Leiopelma</i>	TKLQL	KELE	TEYAI	NKFINKDKRRRISAT	TNLSERQVTIW
<i>Hoxd-13</i>	mouse	TKLQL	KELE	NEYAG	NKFINKDKRRRISAT	TNLSERQVTIW
	<i>Siren</i>	TKLQL	KELE	NEYAG	NKFINKDKRRRISAT	TNLSERQVTIW
<i>ψHox-13</i>	<i>Siren</i>	SKAQ	T	KALE	YEYTAG	RFITKDKQRRIAAGSGLSERQVTIW

Fig. 2. Alignment of the partial amino acid sequences of the homeodomain of PG9–13 of the six amphibian species with the homologous regions of coelacanth and mouse. The fragments correspond to amino acids 9–48 of the homeodomain. Only one amphibian sequence is shown when the sequences of frogs, salamanders and caecilians were identical. Amino acids that are diagnostic for the paralogous group are represented by black letters highlighted in light grey; white letters highlighted in darker grey indicate amino acids characteristic for each paralog. Deviating diagnostic amino acids are highlighted in black.

ing the amino acid sequence. When each of these sequences was present in more than one clone, they were considered allelic variants. Conversely, non-synonymous substitutions of one or two nucleotides in a single clone, but not in all the others of the same gene, were regarded as PCR or sequencing artifacts and were excluded from further analyses. This analysis revealed 71 different haplotypes. All sequences we used are available in GenBank under Accession Nos. DQ158007–DQ158072.

3.2. Identification of obtained gene fragments

The BLAST searches revealed the close similarity of 70 amphibian sequences with 20 distinct *Hox* genes, and of a single sequence with a related homeobox gene, *Gbx*. Phylogenetic analyses under MP (1679 equally parsimonious trees with tree length = 141), NJ, ML ($-\ln L = 792.43$) and in a Bayesian framework produced similar topologies, recognizing the five PGs as distinct clades, supported by moderate to high bootstrap values and posterior probabilities (Fig. 1).

The phylogenetic analyses distinguish most paralogs, but as expected, close relationships are generally not well resolved. For each posterior PG, we identified a set of amino acids that is diagnostic when present in that particular combination (Fig. 2). The amino acid sequences of PG9–11 are highly conserved, and genes on different clusters usually differ from each other by unique combinations of only two to three amino acids, which can be considered characteristic. However, occasional deviation occurs, as seen for two unidentified *Hox-9* *Siren* clones (indicated as “?-Si” and “?-Si” in the *Hox-9* ensemble in Figs. 1 and 2). Although these two genes share amino acid similarities with *Hoxa-9* and *Hoxd-9* sequences (Fig. 2), they could also represent paralogs of additional clusters. Indeed, because karyological data suggest that all members of Sirenidae are potential tetraploids (Duellman and Trueb, 1986), we cannot exclude the possibility that an ancient polyploidization event produced more than four *Hox* clusters in these salamanders.

Hox-13 genes are more variable, and diagnostic residues could not be indicated for every paralog. Our phylogenetic analyses show two PG13 fragments of *Siren* among *Hoxc* paralogs (Fig. 1). Because one of them ($\psi 13$ -Si in Figs. 1 and 2) has a highly derived amino acid sequence, we performed a genomic walk in both directions to acquire the complete sequence of its exon two. Translation of this extended sequence and subsequent alignment with amino acid sequences of other organisms revealed a stop codon upstream of the homeobox, as well as loss of the intron between exons one and two. This DNA sequence may be a retro-pseudogene ($\psi Hox-13$), arisen by retrotransposition of a *Hox-13* gene transcript.

3.3. ‘Missing’ genes

Two genes, *Hoxb-10* and *Hoxa-14*, occur in the coelacanth, but not in mammals; *Hoxb-10* also exists in the

zebrafish. The presence or absence of these genes in amphibians could provide an answer to the question whether they were lost before or after the Amphibia–Amniota divergence. Since we did not find them in the six amphibian species, and screening of the *Silurana* genome also did not expose these genes, they were probably lost before this split.

The gene *Hoxd-12* was present in all vertebrate genomes examined so far (Powers and Amemiya, 2004b), but we did not detect this gene in our survey. Screening of the genomic sequence of the *Silurana* database also did not reveal this gene. However, the sequencing of the *Silurana* genome is still unfinished, and the scaffold encompassing the *HoxD*-cluster contains a gap of approximately 1.1 kb, about 1.3 kb downstream of *Hoxd-13*. We therefore examined the intergenic regions in the posterior section of the D-cluster. In mouse, human, chicken and zebrafish, two conserved regulatory elements have been identified between *Hoxd-13* and *Hoxd-12*, and between *Hoxd-12* and *Hoxd-11* (RXI and RX, respectively), and the former is involved in *Hoxd-12* expression during posterior trunk development (Beckers et al., 1996; Héroult et al., 1998). Hence, if *Hoxd-12* were present, this RXI sequence is expected to occur in its vicinity. When aligning the *Hoxd-13* to *Hoxd-11* fragment of the *Silurana* sequence with the homologous region of human, mouse, chicken and zebrafish, the RX element, mainly regulating *Hoxd-11*, was present, but we were unable to locate the RXI element. Provided that the distance covered by RXI and *Hoxd-12*, approximating 2.3 kb in other vertebrates (Héroult et al., 1998), is conserved in *Silurana*, both *Hoxd-12* and RXI can hardly be located in the 1.1-kb gap of the genomic sequence of *Silurana* (Fig. 3). Altogether, these data strongly suggest that *Hoxd-12* is lost in this frog, as noted by Hoegg and Meyer (2005). Considering the absence of this gene in our survey, we hypothesize that *Hoxd-12* is lost in all amphibians.

We detected the gene *Hoxb-13* in salamanders, but despite additional amplification using gene-specific primers, it was not encountered in frogs and caecilians. The genomic database of *Silurana* did not reveal whether *Hoxb-13* is lost in frogs, since the B-cluster contains a 5-kb gap at the location where this gene is expected. In light of both the

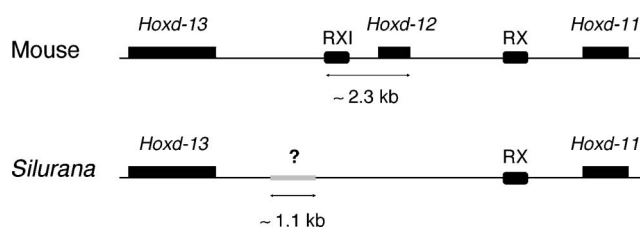


Fig. 3. Comparison of the posterior region of the *HoxD*-cluster in mouse (top) and *Silurana* (bottom). Square blocks represent *Hox* genes; rounded blocks denote the position of the RX and RXI conserved regulatory sequences. A 1.1-kb gap in the *Silurana* genome draft is indicated in grey (accompanied by a “?”). Given the conserved distance between RXI and *Hoxd-12* in most vertebrates (Héroult et al., 1998), it is unlikely that this gap encompasses both features in *Silurana*.

Batrachia-hypothesis (a frog–salamander sister relationship: San Mauro et al., 2004; Zardoya and Meyer, 2001) and the Procera-hypothesis (a salamander–caecilian sister relationship: Feller and Hedges, 1998), the absence of *Hoxb-13* requires two independent losses of this gene to explain this distribution.

Apart from *Hoxd-12* and *Hoxb-13*, one other gene, *Hoxb-9*, was not retrieved in caecilians. To check whether

this absence was caused by loss of the entire cluster, we performed an additional survey of PG1–10 in *Ichthyophis* to find at least one *Hoxb* paralog. Fast identification of the *Hoxb-3* gene excluded the possibility that the B-cluster as a whole is lost in caecilians.

Hoxc-11 could not be amplified with the general primer set because of one amino acid difference in the primer-binding sequence. The use of degenerate primers specifically

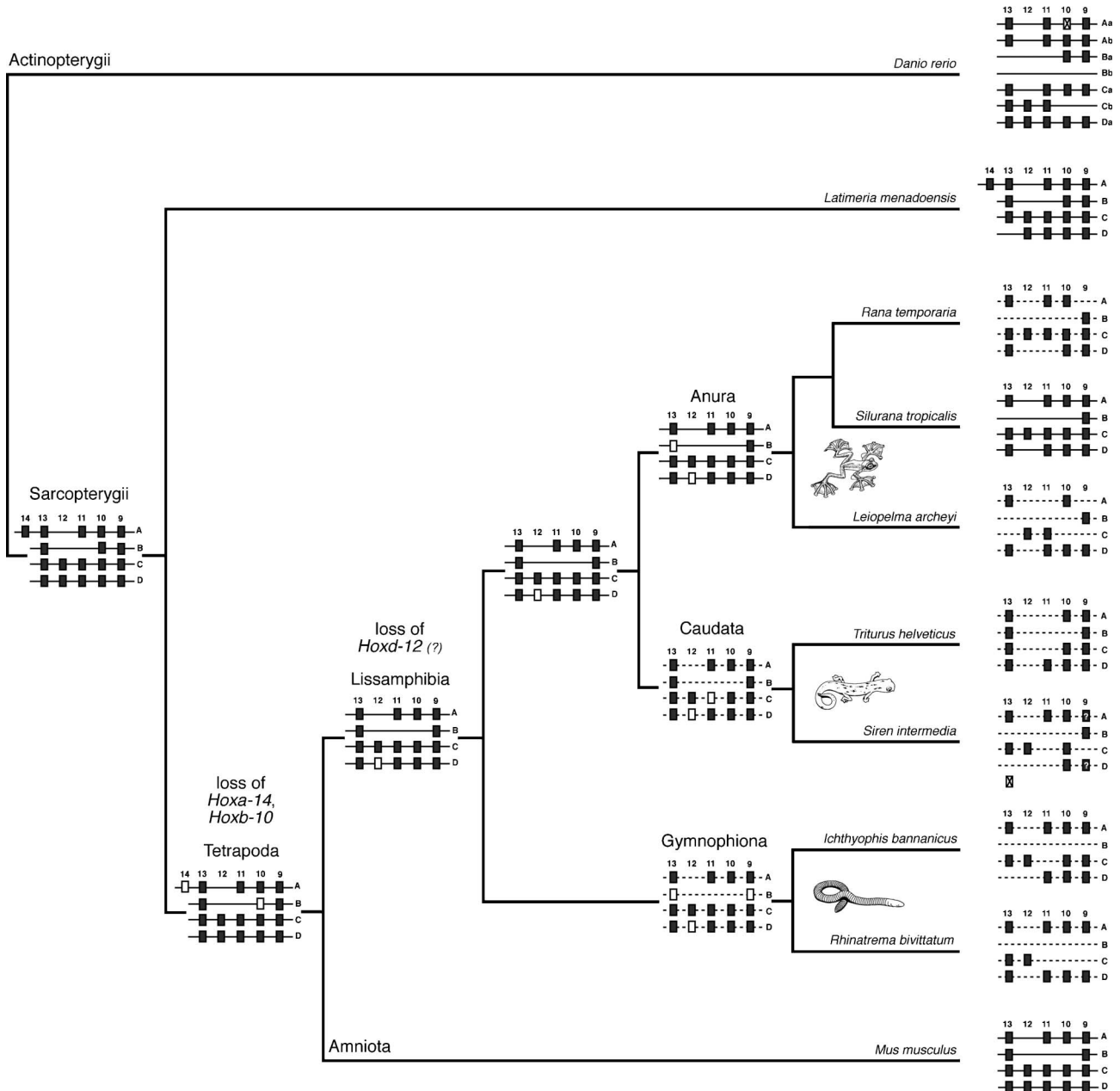


Fig. 4. Dollo parsimony reconstruction of the ancestral posterior *Hox* gene clusters in vertebrates. Frogs and salamanders are presented as sister clades, according to the most commonly accepted Batrachia hypothesis (San Mauro et al., 2004; Zardoya and Meyer, 2001). The clusters are arranged from 5' to 3', with the genes symbolized by boxes. We have no evidence for the linkage of the genes in our survey and thus the clusters are represented by dashed lines. The black boxes in the clusters on the right represent genes observed either from our survey or from literature (*D. rerio*, *L. menadoensis*, and *M. musculus*). In case of ambiguous identification, genes are indicated with a question mark, and crossed boxes denote pseudogenes. The white boxes in the hypothetical ancestral clusters represent genes that are possibly lost. Considering the fact that *Hoxd-12* was never found, we hypothesize that this gene was lost in the amphibian ancestor, rather than in the frog–salamander ancestor or the frog ancestor, but these possibilities cannot be ruled out. Our data were combined with the *Silurana Hox* genes from the genomic database and with data from Powers and Amemiya (2004b) to infer the ancestral *Hox* gene complement with the program MacClade.

designed for this gene was not successful in salamanders, either because of sequence deviation or gene loss, although we were able to amplify *Hoxc-11* with these primers in frogs and some caecilians (data not shown).

4. Discussion

This study provides a first comprehensive insight in amphibian posterior *Hox* cluster architecture. PCR surveys of *Hox* genes have shown that a bias of the primers is a phenomenon often encountered and therefore, the actual number of existing genes is underestimated (Misof and Wagner, 1996; Stadler et al., 2004). In zebrafish, for example, 26 different *Hox* genes were initially identified, suggesting no more than four clusters (Misof et al., 1996), while sequencing of PAC libraries produced 48 genes on seven clusters (Amores et al., 1998). Medaka has at least 33 *Hox* genes (Naruse et al., 2004), whereas the initial PCR survey generated 27 genes (Kurosawa et al., 1999). In coelacanth, 33 *Hox* genes were identified in a PCR survey (Koh et al., 2003), but characterization of the clusters produced 42 genes, including an unexpected 14th PG (Powers and Amemiya, 2004a,b). Also, different sets of lamprey *Hox* genes were identified in different studies, both by PCR and by screening of genomic libraries, suggesting that none of the surveys is complete and that some *Hox* genes were missed (Force et al., 2002; Irvine et al., 2002; Pendleton et al., 1993). To circumvent this problem, we included more species, for which the primer preference may be different. Furthermore, when possible, we selected two species that span the basal-most split in each amphibian order to cover a broad evolutionary range, a strategy that maximizes the probability of finding all genes.

The expected amphibian *Hox* gene complement can be inferred from the *Hox* genes present in mammals, coelacanth, fishes and *Silurana*. Using the sequences of the 64 different posterior *Hox* genes identified in this survey, as well as previously published data and the genomic sequence of *Silurana tropicalis*, we reconstructed the ancestral posterior *Hox* cluster content of amphibians using Dollo parsimony and mapped losses of posterior *Hox* genes in vertebrate evolution (Fig. 4). In contrast to ray-finned fishes, amphibians have four *Hox* clusters, like all other tetrapods examined. Since *Hoxb-10* and *Hoxa-14* are present in the coelacanth, but not in mammals or in *Silurana*, they were likely lost in the ancestor of tetrapods.

The loss of *Hoxd-12* may be reflected in amphibian morphology. Inactivation experiments in mice have shown that in absence of *Hoxd-12*, several autopodial bones are reduced in length. Especially digits II and V are significantly shorter, as a result of reduction of the metacarpals and some phalanges (Davis and Capecchi, 1996). Amphibians have experienced evolutionary reductions in digit number, resulting in four fingers, instead of the general pentadactyl hand and foot of tetrapods (Hinchliffe, 2002). Digit V is not formed in frogs and salamanders, but it is still

visible as an *Anlage*. This suggests a developmental constraint on reductions in digit number that is weaker in amphibians than in amniotes, as suggested by the higher variation in the number of phalanges, carpals and tarsal bones in amphibians (Galis et al., 2001). We propose that the absence of *Hoxd-12* in frogs may be related to the apparently weaker constraint on digit reduction.

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