

# Independent Hox-Cluster Duplications in Lampreys

CLAUDIA FRIED<sup>‡,†</sup>, SONJA J. PROHASKA<sup>‡,†</sup>,  
AND PETER F. STADLER<sup>‡,†</sup>

<sup>‡</sup>Bioinformatik, Institut für Informatik,  
Universität Leipzig, Kreuzstraße 7b, D-04103 Leipzig, Germany

<sup>†</sup>Institut für Theoretische Chemie und Molekulare Strukturbiologie Universität  
Wien,  
Währingerstraße 17, A-1090 Wien, Austria

\*Address for correspondence:

Peter F. Stadler, Bioinformatik, Institut für Informatik, Universität Leipzig,  
Kreuzstraße 7b, D-04103 Leipzig, Germany. Phone: ++49 341 149 5120; Fax:  
++49 341 149 5119; Email: [peter.stadler@bioinf.uni-leipzig.de](mailto:peter.stadler@bioinf.uni-leipzig.de).

## Abstract.

The analysis of the publicly available Hox gene sequences from the sea lamprey *Petromyzon marinus* provides evidence that the *Hox* clusters in lampreys and other vertebrate species arose from independent duplications. In particular, our analysis supports the hypothesis that the last common ancestor of agnathans and gnathostomes had only a single *Hox* cluster which was subsequently duplicated independently in the two lineages.

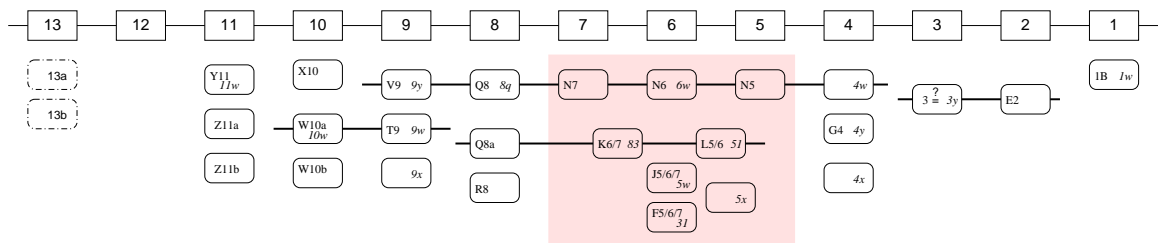
**Keywords.** Hox clusters, lamprey, phylogenetic footprints

## 1. Introduction

*Hox* genes code for homeodomain containing transcription factors which are homologous to the genes in the *Drosophila* homeotic gene clusters (McGinnis and Krumlauf, 1992; Schubert *et al.*, 1993). There is good evidence that the common ancestor of sharks, bony fish, and tetrapods, had four clusters homologous to the mammalian ones (Holland and Garcia-Fernandez, 1996; Prohaska *et al.*, 2003b). An additional duplication event in the teleost lineage increased the number of distinct clusters to at least 7, e.g. in zebrafish (Amores *et al.*, 1998; Stellwag, 1999).

The agnathan vertebrates, lampreys (*Hyperoartia*) and hagfishes (*Hyperotreti*), as the most primitive extant true vertebrates, occupy a phylogenetically intermediate position between the cephalochordates, such as amphioxus, with a single Hox cluster (Garcia-Fernández and Holland, 1994) and the gnathostomes with four or more clusters. PCR surveys (Pendleton *et al.*, 1993; Sharman and W., 1998) and recent genomic mapping data (Force *et al.*, 2002; Irvine *et al.*, 2002) indicate that lampreys have at least three and possibly four Hox clusters, Fig. 1.

Despite recent efforts, the evolutionary history of the lamprey *Hox* genes and their relationship with the quadruplicate mammalian *Hox* clusters is far from being resolved. Irvine *et al.* (2002) conclude that they have “insufficient data to determine with confidence the identities and evolutionary histories of the lamprey Hox clusters.” Amores *et al.* (1998) argue for a two-step duplication scenario, with a duplication of both ancestral agnathan clusters, possibly simultaneously by genome duplication, to produce the four cluster ancestral gnathostome arrangement. Force *et al.* (2002) report that “in general, the lamprey Hox genes do not appear to be orthologues of specific Hox genes in gnathostomes” and conclude that the most likely scenario is one genome duplication in the vertebrate ancestor producing a *HoxAB* and a *HoxCD* cluster with subsequent divergence of the agnathan and gnathostome lineages and independent subsequent duplications in each lineage. Ample evidence from other gene families (Escriva *et al.*, 2002), including *Dlx* (Neidert *et al.*, 2001) and *Otx* (Germot *et al.*, 2001) confirms at least one independent duplication in the agnathan and



**Figure 1.** *Petromyzon marinus* Hox clusters. Summarized from (Force *et al.*, 2002), Fig.1, and (Irvine *et al.*, 2002), Fig.1 and Table 1. *Hox13* genes identified in the PCR survey (Force *et al.*, 2002) but for which no cDNA or cosmid was reported in (Force *et al.*, 2002; Irvine *et al.*, 2002) are indicated by dashed boxes. The corresponding sequences are not available. Physical linkage is indicated by a line. The sequences of paralog groups 5, 6, and 7 are insufficient to resolve their mutual relationships, and are therefore excluded from further analysis.

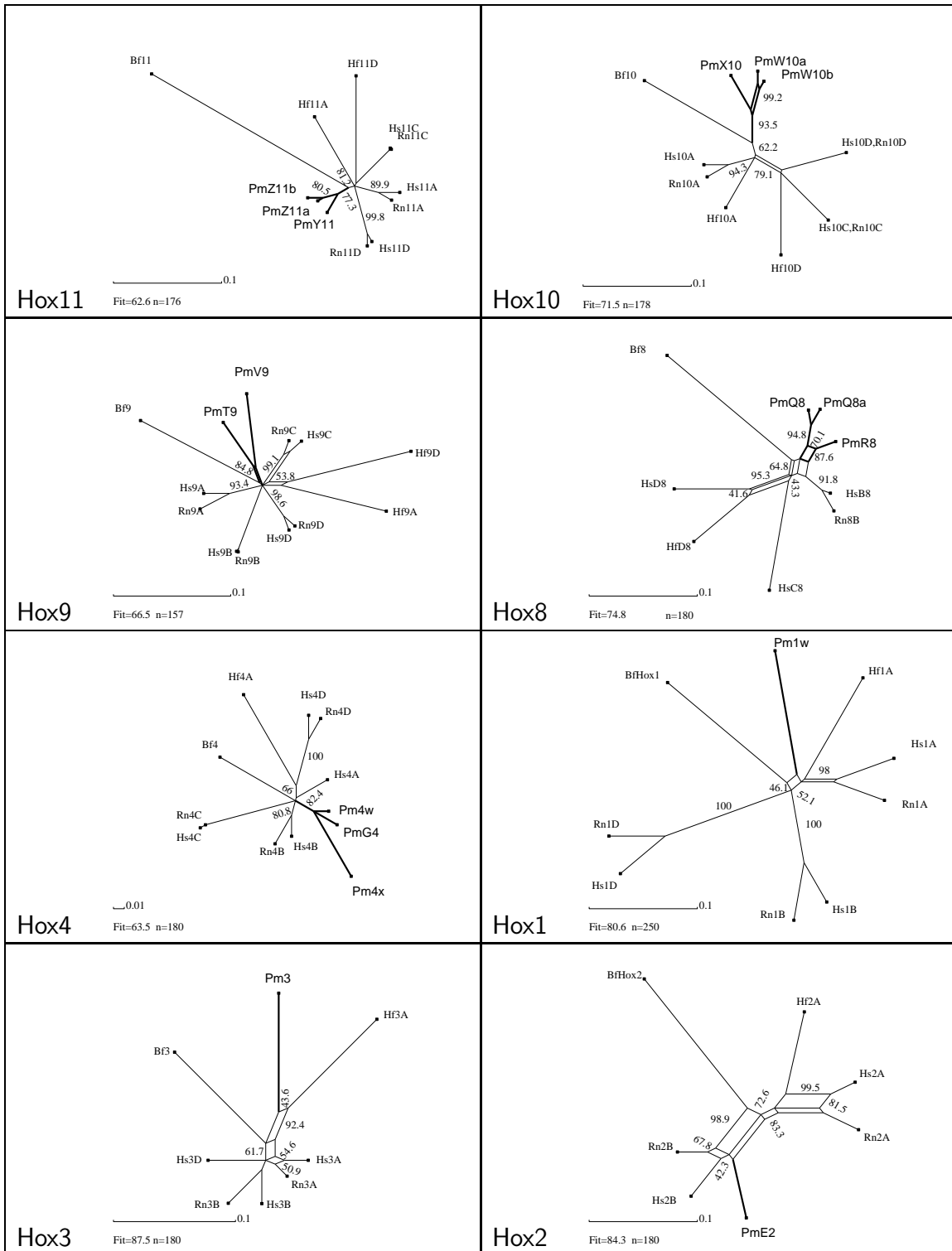
the gnathostome lineages. In this letter we report on a re-evaluation of the publicly available lamprey *Hox* sequences.

## 2. Materials and Methods

The available lamprey *Hox* sequences are compiled (together with their accession numbers) in Table 1 in the Appendix. Only short sequences of the homeobox region are available in almost all cases. In contrast to the previous studies we use the nucleic acid sequences rather than the sequences of the *Hox* proteins because of the weak phylogenetic signals in the short and highly conserved amino acid sequences. The sequence from the PCR survey of *Lampetra planeri* (Sharman and W., 1998) are much shorter (82nt) than the *Petromyzon marinus* sequence reported by Pendleton *et al.* (1993) (180nt) and Irvine *et al.* (2002) (240nt). In almost all cases it was possible to identify the homology between the *Lampetra planeri* sequences and their *Petromyzon marinus* counterparts, see Table 1. We therefore use the data from Irvine *et al.* (2002) where possible.

Canonical split decomposition (Bandelt and Dress, 1992), as implemented in the `splitstree` package (version 3.1) by Huson (1998), is used for the reconstruction of the phylogeny. The split-based methods are particularly suitable for our purposes because they are known to be very conservative in that they tend to produce multifurcations rather than poorly supported edges (Semple and Steel, 2003). For comparison we compute exact maximum parsimony trees using the program `dnapenny` which is part of the `phylip` package (Felsenstein, 1989). We use a variety of *Hox* genes from mammals (*Homo sapiens* and *Rattus norvegicus*), shark (*Heterodontus francisci*), coelacanth (*Latimeria menadoensis*), and amphioxus (*Branchiostoma floridae*) for phylogeny reconstruction. All sequences were downloaded from genbank. Alignments were constructed using `dialign` (Morgenstern, 1999). Since split-based methods tend to lose resolution with increasing number of taxa we use different combinations of lamprey and sequences from other taxa instead of using all sequences together.

An independent line of evidence is derived from the analysis of conserved non-coding DNA. The 30kb PAC clone Pm18 containing the HoxW10a region of *Petromyzon marinus* was sequenced by Irvine *et al.* (2002), accession number AF464190. Here we use the `tracker` program (Prohaska *et al.*, 2003a) to search for phylogenetic footprints in the non-coding parts of this sequence by comparing it with the corresponding regions of the publicly available sequences of human, fugu (*Takifugu rubripes*, sequences obtained from the JGI database<sup>1</sup>, release 3.0), and shark *Hox* clusters. In the case of the *HoxB* clusters, which lack *Hox-10*, *Hox-11* and *Hox-12* gene, we use the complete inter-genic region from *Hox-13* to *Hox-9* for the `tracker` run. The output is then restricted to the region between the first and the last footprint that the lamprey sequence shares with another cluster to account for the fact that Pm18 does not span the entire range to the neighboring genes.



**Figure 2.** Buneman graphs of the homeobox sequences for paralog groups 1, 2, 3, 4, 8, 9, 10, and 11. We show here the comparison with Human, rat, shark, and amphioxus sequences. Using Teleost fish or coelacanth sequences instead of mammalian data yield qualitatively the same results (data not shown).

### 3. Results

Only the paralog groups 1, 2, 3, 4, 8, 9, 10, and 11 could be used for our purposes because (i) only a single short *Hox-13* sequence from *Lampetra planeri* was found in the databanks, (ii) there does not seem to be a *Hox-12* gene at all in lampreys, and (iii) the available sequences are too short and too conserved to distinguish unambiguously between members of the paralog groups 5, 6, and 7, see also (Force *et al.*, 2002; Irvine *et al.*, 2002).

The comparison of mammalian, shark, lamprey, and amphioxus sequences for a given paralog group presents a striking pattern. We find that the lamprey sequences cluster together outside the gnathostome *Hox* sequences for paralog groups 11, 10, 9, 8, and 4 according to the split decomposition analysis, Fig. 2. Paralog group 1 is at least consistent with this picture. The single paralog group 3 sequence shows affinity with the shark *HoxA* sequence but is well separated from the mammalian *HoxA-3* genes in the split data. The *PmE2* sequence, which is physically linked to *Pm3*, is more similar to the mammalian *HoxB-2* genes. Replacing the rat sequences by coelacanth sequences from the work of Koh *et al.* (2003) yields very similar results (data not shown).

The same picture is obtained from maximally parsimonious trees, see Table 2 in the Appendix, for groups 11, 10, 9, and 8. In contrast to the split decomposition method, the best trees for both paralog group 3 and 2 place the lamprey and amphioxus sequences together and as outgroup to the gnathostome clusters. Furthermore, the single *Hox-13* sequence of *Lampetra planeri* reported by Sharman and W. (1998) branches outside the other vertebrate genes. Paralog group 1 yields one tree that shows the 1w sequence outside the mammalian cluster and two alternative trees placing 1w with mammalian A clusters. In paralog group 4 the lamprey sequences also lie outside the mammalian clusters but form two separate branches. In no case do we find a clear assignment of the lamprey clusters to either a single or a pair of mammalian and/or fish clusters.

At present the genomic context of only a single lamprey Hox gene, *Hox-W10a* from *Petromyzon marinus*, has been published. Irvine *et al.* (2002) report footprint clusters shared with both *HoxA* and *HoxC* clusters. The footprint cliques detected by the **tracker** program in a comparison with Fugu, Shark, Human, and Ciona *Hox* clusters are summarized in Table 3 in the Appendix. Non-colinear cliques have been removed because they are most likely not homologous (Prohaska *et al.*, 2003a). There is no clear evidence that the non-coding part of the Pm18 sequence is more closely related to either a particular single gnathostome cluster or pair of clusters. The total length of available footprints is unfortunately insufficient for an independent reconstruction of the phylogeny. The most significant footprint cliques are those shared with the *HoxA* and *HoxC* clusters, in particular, and an element designated **pp** that is most likely the proximal promotor of the *Hox-10* genes and also appears in the *HoxD* clusters. The elements **A1**, **A2**, **C1**, and **C3** are described already in the work of Irvine *et al.* (2002). Both **A1**, and **A2** were also detected in comparisons of *HoxA* clusters only by Chiu *et al.* (2002). It is interesting to note that both **A2** and the **C1**, **C2** motifs

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<sup>1</sup><http://genome.jgi-psf.org/fugu6/fugu6.home.html>

also have their counterparts in the Human *HoxB* cluster, even though it lacks the *HoxB-10* gene.

#### 4. Discussion

The re-evaluation of the available lamprey hox genes strongly supports an independent origin of the three (or four) lamprey *Hox* clusters and suggest that the common ancestor of agnathans and gnathostomes had only a single *Hox* cluster. This is consistent with the *Dlx* gene phylogeny described by Neidert *et al.* (2001). These authors proposed that a tandem duplication of an ancestral *Dlx* gene predated the divergence of lampreys from gnathostomes, which was then followed by independent chromosomal or genome duplications and gene loss in each lineage. Our evaluation of the *Hox* clusters supports this hypothesis. Similar patterns have been reported for other developmentally important gene families. The neural crest marker *AP-2*, for which no duplicates have been found in lampreys, also fails to group with any one gnathostome AP-2 isoform (Meulemans and Bronner-Fraser, 2002). Consistent with an independent duplication history it is impossible to assign any one of the lamprey (and hagfish) *Otx* sequences to one of the three classes identified in gnathostomes (Germot *et al.*, 2001).

The phylogenetic signal in the *Hox* clusters is not as strong as one would like so that a definitive result will have to await more complete sequencing. This will in particular allow the unambiguous identification of the genes of paralog group 5, 6, and 7, and their use as additional phylogenetic information. At present, at least, the publicly available sequence information does not contain evidence for a Hox-cluster duplication preceding our common ancestor with the lampreys.

**Acknowledgments.** Funding for this research is gratefully acknowledged: DFG Bioinformatics Initiative BIZ-6/1-2 to SJP, CF, and PFS.

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## Appendix (or Supplement)

**Table 1.** Lamprey Hox sequences used in this study.

Hox	<i>Petromyzon marinus</i>					<i>Lampetra planeri</i>	
	genomic clones			PCR surveys			
	Irvine	Force	Acc. No.	Pendleton	Acc. No.	Sharman	Acc. No.
13						Lp13A	AF044814
11	Y11	11w	AF410923	11.1			
	Z11a		AF410924	11.8			
	Z11b		AF410925				
				11.6			
10	X10	Hx13(9)	AF410922	10x	L14900	Lp10B	AF044812
	W10a	10w	AF410920	10w	L14895		
	W10b		AF410921				
9	V9	9y	AF410919	9v	L14889	Lp9A	AF044809
				9s	L14911		
	T9	9w	AF410918	9t	L14894	Lp9B	AF044810
		9x		9u	L14910	Lp9C	AF044811
8	R8		AF035588	8r		Lp8A	AF044807
	Q8		AF035591				
	Q8a		AF035589	8q	L14901	Lp8B	AF044808
4	G4	4y	AF410911	4g	L14912		
		4w	AF434666	4n	L14896	? Lp4-7B	AF044803
		4x	AY056469	4l	L14891	? Lp4-7A	AF044802
				(4h	L14909)	? Lp4-7E	AF044806
3	3	3y	AF410909			Lp3A	AF044801
2	E2		AF410908	2e		Lp2A	AF044800
1	1B	1w	AF434665	1b	L14902	Lp1B	AF044798
				1a	L14893	Lp1A	AF044797
				1c	L14908	Lp1C	AF044799
				(1d	L14904)		

The sequence shown in parentheses are not included because we could not confirm their assignment to a paralog group based on their nucleic acid sequence.

**Table 2.** Maximum parsimony trees of the homeobox sequences obtained with the program **dnapenny** from the **phylip** package Felsenstein (1989). *Petromyzon marinus*, **Pm**, sequences are indicated in bold. For *Hox-13* we used the short *Lampetra planeri*, **Lp** sequence. Grey boxes indicated that all available lamprey paralogs form a subtree, dark gray boxes are used when all lamprey and the amphioxus sequence are separated from the vertebrate hox clusters. Horizontal lines indicate the two exons of the *Hox-10* in the *HoxA*, *HoxB*, and *HoxD*, as well as the *Ciona Hox* cluster Dehal *et al.* (2002).

Hox	Maximum parsimony tree
13	( <b>Lp13</b> ,((( <i>Bf13</i> ,( <b>RnC13</b> ,HsC13)),(( <b>RnD13</b> ,HsD13),(( <b>RnA13</b> ,HsA13),(HfD13,HfA13))),(HsB13,RnB13))) ( <b>Lp13</b> ,((( <i>Bf13</i> ,( <b>RnC13</b> ,HsC13)),(( <b>RnD13</b> ,HsD13),HfD13,(( <b>RnA13</b> ,HsA13),HfA13))),(HsB13,RnB13)))
11	( ( <i>Bf11</i> ,( <b>PmY11</b> ,( <b>PmZ11a</b> , <b>PmZ11b</b> ))) ,(((Hf11D,(Hs11C,Rn11C)),(( <b>Rn11A</b> ,Hs11A),Hf11A)),(Rn11D,Hs11D)))
10	( ( <i>Bf10</i> ,( <b>PmX10</b> ,( <b>PmW10b</b> , <b>PmW10a</b> ))) ,((Rn10A,Hs10A),(Hf10A,(( <b>Rn10C</b> ,Hs10C),((Hs10D,Rn10D),Hf10D))))))
9	( ( <i>BfHox9</i> ,( <b>HoxT9</b> , <b>HoxV9</b> )) ,(((Hs9D,Rn9D),((( <b>Rn9A</b> ,Hs9A),Hf9A),(Hs9B,Rn9B)),((Hs9C,Rn9C),Hf9D)))) ( ( <i>BfHox9</i> ,( <b>HoxT9</b> , <b>HoxV9</b> )) ,((((Hs9D,Rn9D),((Hs9C,Rn9C),Hf9D)),(Hf9A,(Hs9B,Rn9B))),(Rn9A,Hs9A))))
8	( ( <i>Bf8</i> ,( <b>PmQ8</b> ,( <b>PmR8</b> , <b>PmQ8a</b> ))) ,((HsC8,(HsB8,Rn8B)),(HfD8,HsD8)))
4	(( <i>Bf4</i> , <b>PmG4</b> ),((( <b>Pm4x</b> , <b>Pm4w</b> ),(Hs4A,(Hf4A,(Rn4D,Hs4D))))),(Hs4B,Rn4B),(Hs4C,Rn4C)) (( <i>Bf4</i> , <b>PmG4</b> ),(( <b>Pm4x</b> , <b>Pm4w</b> ),((Hs4B,Rn4B),(Hs4A,(Hf4A,(Rn4D,Hs4D))))),(Hs4C,Rn4C))
3	( ( <i>Bf3</i> , <b>Pm3</b> ) ,((Hf3A,(Hs3D,((Hs3A,Rn3A),(Hs3B,Rn3B))))))
2	( ( <i>Bf2</i> , <b>PmE2</b> ) ,(((Hf2A,(Rn2A,Hs2A)),(Rn2B,Hs2B)))) ( ( <i>Bf2</i> , <b>PmE2</b> ) ,((((Hf2A,(Rn2A,Hs2A)),Hs2B),Rn2B))))
1	( ( <i>Bf1</i> , <b>Pm1w</b> ) ,((((Hs1D,Rn1D),Hf1A),(Rn1A,Hs1A)),(Hs1B,Rn1B))) ( <i>Bf1</i> ,(((Hs1D,Rn1D),Hf1A),((Hs1B,Rn1B),((Rn1A,Hs1A), <b>Pm1w</b> )))) ( <i>Bf1</i> ,((((Hs1D,Rn1D),(Hs1B,Rn1B)),(Hf1A,((Rn1A,Hs1A), <b>Pm1w</b> ))))))

**Table 3.** Summary of co-linear footprint cliques produced by the **tracker** program in the range of the *Petromyzon marinus* Pm18 sequence. Hs *Homo Sapiens*, Hf *Heterodontus fransisci*, Tr *Takifugu rubripes*, Ci *Ciona intestinalis*. Numbers in parentheses are non-colinear with the footprints in this species. The last column marks previously described footprints. **pp** is the proximal promotor of the *Hox-10* gene, numbers in sans serif font are cliques listed in Prohaska *et al.* (2003a) for a comparison of *HoxA* clusters. PFC, “phylogenetic footprint cluster”, names from Chiu *et al.* (2002) are given in normal text font.

#	Pm	HsA	HsC	HsD	HfA	HfD	TrAa	TrAb	TrD	TrCa	Ci	TrBa	HsB	Remark
69	1150 85	8425 50	9641 88	7528 161	11071 223	8404 162	4984 94	3360 139	8112 126					<b>pp</b> , 42
124									9229 58	7258 69				
70	1535 48									10137 42				
71			11172 36										6867 36	
73	2977 50		12879 49										39418 55	<b>C1</b>
77	9139 35		12879 70										39418 47	<b>C2</b>
74	5107 52												39793 52	
75	5671 44					10460 44								
76	6509 30												39919 30	
96		9475 37										6722 37		
106			13364 73	8657 76										
80						11114 37						15608 37		
125				10744 52					10133 50					
83						12657 27							59050 27	
88						13213 26						19306 26		
90	10538 33					15207 33								
93	15694 23										22400 23			
94	17246 19												60196 19	
95	17946 43										22578 43			
98					12558 29								63422 29	
99		12127 39			14376 43									<b>10-9a</b> , 43
100		12248 24			14522 24									<b>10-9a</b> , 44
101	21911 64	12292 187			14566 189		7525 108							<b>A1</b> , <b>10-9a</b> , 45
109					15213 42								60500 42	
105			13293 36										78949 36	
102	23635 27											22252 27		
103	25443 21												80312 21	
104	26904 26											25684 26		
81										19399 39			82476 39	
108			15006 169							19761 170				
110	27436 105	13224 116	16328 105		15518 116		7896 99			20562 106		(18814) (83)	94203 98	<b>A2</b> , <b>10-9b</b> , 46
85							8219 11	6226 10						
86							8438 82	6389 78						
92											(11940) (51)	26975 26	98721 29	<b>10-9c</b> , 48
122		14160 49			16310 77		8304 99	6287 73						
126		15372 59		10890 63										