A PCR Survey of *Xenoturbella bocki Hox* Genes

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Abstract

Xenoturbella bocki has recently been identified as one of the most basal deuterostomes, although an even more basal phylogenetic position cannot be ruled out. Here we report on a PCR survey of partial Hox homeobox sequences of *X. bocki*. Surprisingly, we did not find evidence for more than five Hox genes, one clear labial/PG1 ortholog, one posterior gene most similar to the PG9/10 genes of Ambulacraria, and three central group genes whose precise assignment to a specific paralog group remains open. We furthermore report on a re-evaluation of the available published evidence of Hox genes in other basal deuterostomes.

Key words: *Xenoturbella bocki*, Hox genes, PCR survey

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1 Introduction

Xenoturbella bocki is a small free-living marine animal, with an irregular flattened shape and a length of up to 4 cm (Israelsson, 1999). The simple body plan, the diffuse nerve net without centralization and the unusual morphology, with an epithelial epidermis and gastrodermis, but without anus and only a "statocyst" as distinct organ (Ehlers, 1991), led to highly divergent phylogenetic interpretations. All available molecular data, namely authentic nuclear SSU rDNA sequences (Bourlat *et al.*, 2003), mitochondrial protein-coding gene sequences (Israelsson and Budd, 2005; Bourlat *et al.*, 2006; Perseke *et al.*, 2007), expressed sequencing tags (ESTs) (Bourlat *et al.*, 2006) and immuno-histochemical evidence (Stach *et al.*, 2005; Israelsson, 2006) place *Xenoturbella* in a basal position among the Deuterostomia, albeit so far an even more basal position among the Bilateria cannot be ruled out with certainty (Perseke *et al.*, 2007).

Hox genes code for a subclass of homeodomain transcription factors that play a determining role in the patterning of the anterior-posterior body axis (McGinnis and Krumlauf, 1992). Due to their crucial role in early development, the analysis of Hox genes has contributed significantly to resolve important issues in metazoan phylogeny. For instance, they provide strong support for the monophyly of Bilateria (Balavoine *et al.*, 2002), they provide a major line of support (de Rosa *et al.*, 1999) for the Ecdysozoa/Lophotrochozoa hypothesis (Aguinaldo *et al.*, 1997), and they have been a major reason to classify Acoelomorpha (Ruiz-Trillo *et al.*, 2002; Jondelius *et al.*, 2002; Telford *et al.*, 2003) (including Acoela and Nemertodermatida) as basal bilaterians rather than within Platyhelmintes (Cook *et al.*, 2004; Jiménez-Guri *et al.*, 2006; Olson, 2007).

Given the likely position of *Xenoturbella* as one of the most basal deuterostomes, the analysis of its Hox gene complement is of utmost importance for our understanding of the evolution of Hox gene clusters (see e.g. Garcia-Fernández (2005); Ikuta and Saiga (2005); Lemons and McGinnis (2006); Prohaska *et al.* (2006) for recent reviews). Here we report therefore on a PCR survey of *Hox* homeobox fragments in *Xenoturbella bocki*.

2 Materials and Methods

Large specimens of *Xenoturbella bocki* were collected in Gullmarsfjorden (Bohuslän) off the Swedish West coast near Kristinebergs Marina Forskningsstation (KMF) in 2003. Since *Xenoturbella* material might be contaminated by DNA from prey, in particular from molluscs, we isolated genomic DNA from

Xenoturbella bocki in two different ways (see below) and in addition from the putative prey *Nucula nitida* and analyzed the DNA by PCR-amplification using the same primers.

2.1 PCR analysis of DNA from dissected Xenoturbella

During the first preparation we were careful to sample only tissue away from the gastro-intestinal tract. The mitochondrial genome reported by Perseke *et al.* (2007) was obtained from the the same isolate, so that we were sure to have sampled *Xenoturbella bocki* only. Total genomic DNA was extracted using the NucleoSpin Tissue Kit (Machery & Nagel) respecting the manufacturer's protocol. Following the suggestions of Mito and Endo (1997) and Hano *et al.* (2001), we tested two different forward primers,
HoxF1 (5'-`CARYTNCANGARYTNGARAA`-3') and
HoxF2 (5'-`YTNGARYTNGARAARGARTT`-3'),
in combination with two reverse primers,
HoxR1 (5'-`TTCATNCKNCKRTTYTGRAA`-3') and
HoxR2 (5'-`CKRTTYTGRAACCADATYTT`-3') for
amplification of HOX genes in *Xenoturbella*. However, after trying different PCR conditions, positive PCR results for *Xenoturbella* were only obtained combining *HoxF2* (`LELEKEF`) primer with the reverse primers *HoxR1* (`FQNRRMK`) and *HoxR2* (`KIWFQNR`). The Hano *et al.* (2001) primer set used in this work was a *more* degenerate version of the Hox primers proposed by Mito and Endo (1997) in a PCR survey of a sea star and later used successfully in a hemichordate by Peterson (2004).

In addition we performed a PCR with more specific forward primers for Hox3,
FHox3 (5'-`AAYMRNTAYYTNCARAARCA`-3') and Hox2/3,
FHox2/3 (5'-`AAYMRNTAYYTNTGYMRNCC`-3'),
see Peterson (2004). However, no additional Hox genes were amplified. Also, amplification with the engrailed-specific *EN-F* primer (5'-`GAYGARAARMGNCCNMG`-3'), (Hano *et al.*, 2001) did not yield a positive result.

PCR amplifications were performed under the following conditions: $1\times$ reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of each primer and 2 U *FastStart* Taq DNA Polymerase (Roche) in a total volume of 50 μl. The PCR was performed in an Eppendorf Mastercycler starting with $94°C$ for 4 min to activate the previously inactive FastStart Taq DNA Polymerase and to denature the DNA template. This initial step was followed by 34 cycles at 94°C for 30s, 50°C for 30s, 72°C for 45s and one final step at 72°C for 10min. PCR products were checked on 1.5% agarose gel and extracted using Invisorb Spin DNA Extraction Kit (Invitek) following the manufacturer's protocol. Cloning of PCR fragments was carried out using the pGEM-T Vector

system (Promega) and plasmids were transferred into chemically competent *E. coli* JM109 cells. The whole PCR and cloning procedure was performed for two independent PCR reactions for each set of primers.

Altogether, 94 positive clones containing 75nt homeobox fragments were sequenced using the ABI PRISM BigDye v. 3.1 Cycle Sequencing Kit and M13 primers and analysed with an ABI Prism 3100 automated sequencer, following the manufacturer's protocol.

A 5' RACE experiment was performed according to Matz *et al.* (2003) for the *Xb-HoxM2* gene using the primer `CCGACGCCGTGTCAGGTACT` and cDNA generously provided by Drs. Leonid L. Moroz and Andreas Heyland, The Whitney Laboratory, University of Florida.

2.2   Assessment of genomic DNA contamination

In order to assess the most prevalent contamination of *Xenoturbella* DNA by prey DNA, we also investigated DNA isolated from the whole animal by PCR amplification. PCR-experiments were more successful when a standard protocol of Phenol:Chloroform:Isoamyl DNA-extraction was used compared to the Qiagen DNA Extraction kit routinely used on other animals. Successfully products were cloned using TOPO TA Cloning (Invitrogen). Subsequently, clones were picked, transformed bacteria were grown and inserts either sequenced by KMF's routinely used commercial sequencer or in the laboratory of Dr. Albert Poustka (MPI, Berlin, Germany). In total, we have produced 22 putative sequences of *Xenoturbella bocki* DNA from an undissected specimen, and three sequences from the putative prey species *Nucula nitida*.

2.3   Computational Analysis

Homeobox sequences were aligned using `clustalw` (Thompson *et al.*, 1994) to identify identical sequences. All distinct nucleic acid sequences were translated in all six reading frames and compared to a collection of deuterostome homeobox sequences, resulting in five distinct homeobox amino acid sequences for the "dissected" DNA isolation and nine for the whole animal DNA (Tab. 1). A `blast` (Altschul *et al.*, 1997) search of the nucleotide sequences against GenBank confirmed that none of the PCR clones was a contamination by human or other known DNA.

A preliminary analysis of the 75nt long homeobox fragments was performed using `blast` and the NCBI tree-reconstruction service that uses neighbor joining and minimum evolution operating at an alignment of all `blastp` hits. NJ trees

are shown in the electronic supplement (`http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/07-012/`).

A more detailed analysis was performed by manual inspection of alignments and the distance matrices. We applied neighbor joining and maximum parsimony analyses of a dataset including Hox gene sequences (apart from *X. bocki*) from Echinodermata, Hemichordata, Urochordata and Mollusca (see appendix for a complete list) using both nucleic acid and amino acid sequences.

In order to provide an up-to-date picture of the evolution of Hox genes in basal deuterostomes we re-evaluated the published data of earlier studies. The sequence data underlying some of the following references are not available in GenBank. In this case they were extracted from the published paper. Again, a complete list is provided in the appendix. For the Hox gene complements of cephalochordates we refer to Ferrier *et al.* (2000), for the ancestral state of the vertebrate Hox cluster prior to the genome duplications see Powers and Amemiya (2004).

3   Results

By the genomic PCR approach using degenerated primers, we found 75nt (25aa) homeobox fragments with 5 distinct nucleotide and amino acid sequences within the *Xenoturbella* DNA isolation in which care has been taken to minimize contamination by prey DNA, and another 9 distinct nucleotide and amino acid sequences within the whole animal isolation.

A detailed sequence comparison identifies homeobox fragments of four Hox genes for the first DNA preparartion. Since *Xb-HoxM1a* and *Xb-HoxM1b* differ only by a single nucleotide, they are interpreted as allelic variants. We found one member of the PG1/lab group (Xb-Hox1), one posterior gene (Xb-HoxP), which is most similar to the PG9/10 genes of Ambulacraria and the PG10 and PG9 genes of chordates and three middle group genes (Xb-HoxM1a/b and M2), which cannot be assigned to individual paralog groups with any degree of certainty (Fig. 1).

The detailed analysis of sequences of the whole animal isolate showed that five of the nine distinct sequences represented homeodomain genes of molluscs (see Supplement Material). Among the remaining, three sequences correspond to the *X. bocki* Hox genes described above. One additional middle group gene was found, which does not seem to belong to the putative prey species. We tentatively identify it as *Xb-HoxM3*. Due to the short sequence, the exact paralog group cannot be determined.

A 5'RACE of *Xb-HoxM2* resulted in a fragment of 856 nt. The analysis of this fragment did not improve a assignment of the sequence to individual paralog groups.

In summary, using seven different primer combinations and two distinct DNA isolates, we were able to identify five Hox genes of *Xenoturbella bocki* (counting the two allelic variants of the*Xb-HoxM1* as a single gene). If *Xenoturbella bocki* contains more than these five Hox genes, we suspect that they will most likely be highly derived.

We did not observe a particularly striking sequence similarity with basal bilaterians, in particular acoel flatworms (Cook *et al.*, 2004) or Nemertodermatida (Jiménez-Guri *et al.*, 2006) that would suggest that *Xenoturbella* has to be a more basal bilaterian rather than a basal deuterostome with a reduced Hox gene complement.

4   Discussion

The PCR survey of the Hox genes of *Xenoturbella bocki* detected not more than five Hox genes, one representative of PG1, a posterior gene, and three middlegroup genes, one of which was only found in a preparation of the whole animal and could be a contamination by prey DNA, although it does not seem to belong to a mollusc or annelid as is the case with the other detected prey contamination sequences.

Compared to the Hox gene complement of the Protostome-Deuterostome Ancestor (PDA) this would correspond to a loss of two of the seven ancestral Hox genes: PG2/pb and PG3/zen. In comparison to the evolution of the Hox gene clusters in other deuterostome lineages (Fig. 2) this amount of gene loss is not unusual in particularly in the light of the simple morphology of *Xenoturbella*. The urochordate *Oikopleura dioica*, for example has secondarily lost the PG3 gene as well as all but one middle group genes.

An alternative explanation for the small number of Hox genes would be a more basal phylogenetic position of *Xenoturbella*. Basal bilaterians such as Acoela (Cook *et al.*, 2004) and Nemertodermatida (Jiménez-Guri *et al.*, 2006) have 3 or 4 Hox genes: one posterior gene, one anterior gene, and one or two central group Hox genes. The *Xenoturbella* homeodomain fragments, however, do not show convincing similarities to these sequences. In particular, the *Xb-HoxP* sequence clearly clusters with ambulacrarian PG9/10 and chordate PG10 and PG9 sequences, to the exclusion of protostome and basal bilaterians. Similarly, *Xb-Hox1* can be unambiguously distinguished from PG2 sequences.
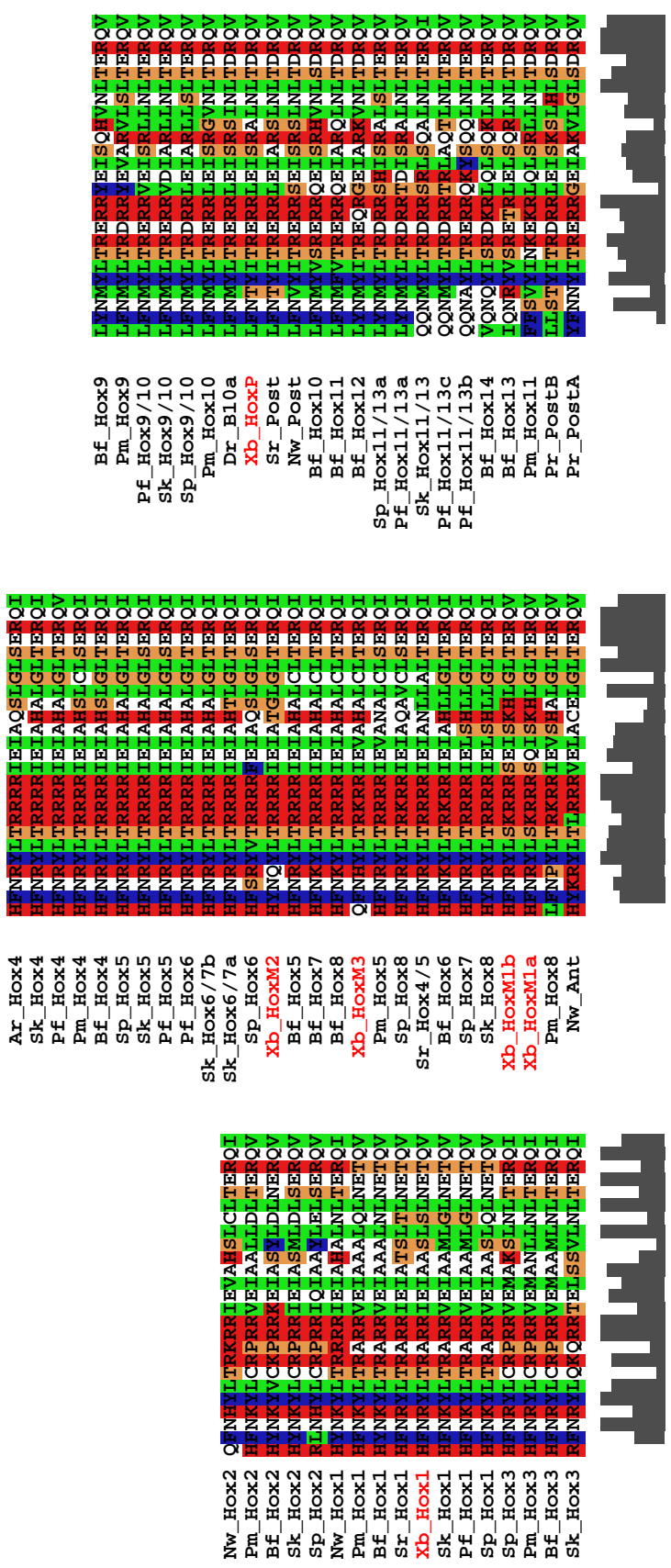
6

Fig. 1. Alignments of anterior (left), central (middle), and posterior (right) homeodomain fragments of *Xenoturbella bocki* with corresponding sequences from the cephalochordate *Branchiostoma floridae* (BF), the chordate *Petromyzon marinus* (Pm) and *Danio rerio* (Dr), the hemichordates *Saccoglossus kowalevskii* (Sk) and *Ptychodera flava* (Pf), the echinoderm *Strongylocentrotus purpuratus* (Sp) as well as the acoel *Symsagittifera roscoffensis* and the nemertodermatid *Nemertoderma westbladi*. The Hox4 gene of the sea star *Asterias rubens* is included since sea urchins have lost Hox4. The color scheme of the alignments is the default of clustalw, which highlights similarity in amino acid properties. Conservation curves are shown below the alignments.
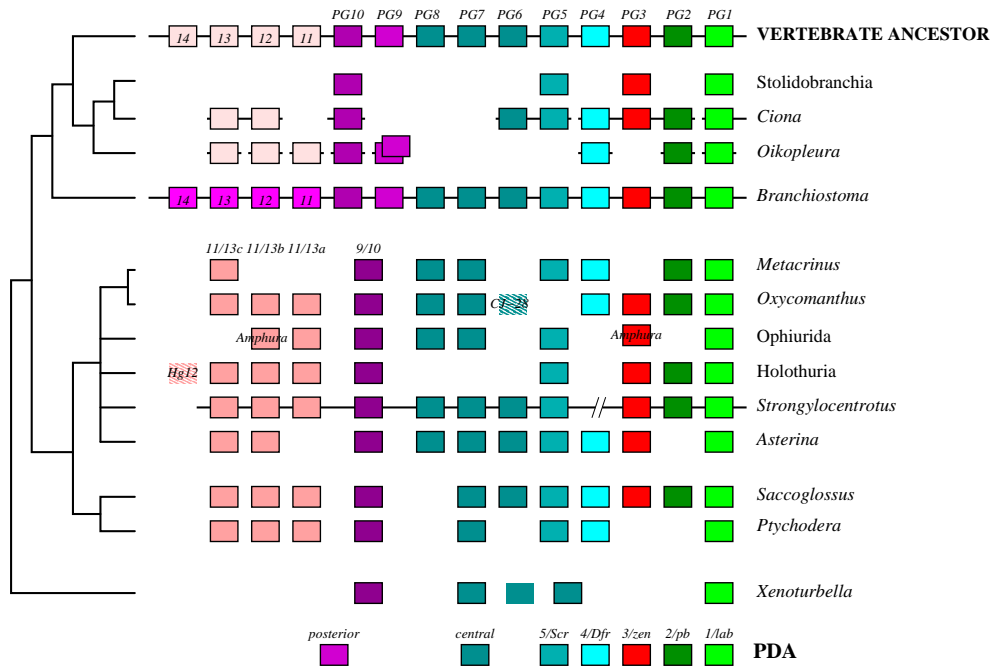
7

Fig. 2. Evolution of Hox gene clusters in deuterostomes.

Data and references underlying this figure are described in the Appendix. The Protostome-Deuterostome ancestor (PDA) is taken from Garcia-Fernández (2005), the phylogenetic tree follows the Olfactores hypothesis of Delsuc *et al.* (2006), which is consistent with the apparent orthology of the vertebrate and urochordate PG11, PG12, and PG13 genes. In this picture, the posterior genes in Olfactores, Cephalochordata, and Ambulacraria would have arisen by independent series of tandem duplications. A few published homeobox fragments (shaded) can be assigned only tentatively to a particular paralog group. For instance, the sea cucumber *Holothuria glaberrima* has an additional posterior gene beyond the usual complement of echinoderms and hemichordates.

Our data on the Hox genes of *Xenoturbella bocki* are thus at least consistent with a basal position within the Deuterostomia. Based on the available information, it is impossible to assign the three middle group genes to specific paralog groups with any certainty. Tentatively, *Xb-HoxM1* and *Xb-HoxM3* appear to belong the PG6/7/8 group, while the *Xb-HoxM2* gene could belong to PG5 or PG4. This suggests that *Xenoturbella* represents an early stage in the evolution of the deuterostome Hox cluster, in which the middle group genes have not yet been completely expanded to the situation common to Chordata and Ambulacraria.

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Acknowledgments

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Appendix

The sources of used sequence data:

Echinodermata: Sea Cucumber *Holothuria glaberrima* (Mendéz, 2000) (PCR); Sea Stars *Asterina minor* (Mito and Endo, 1997) (PCR) and *Patiriella exigua* (Long *et al.*, 2000) (PCR), Hox4 gene (Long *et al.*, 2003); Crinoid *Oxycomanthus japonicus* (Mito and Endo, 2000) (PCR) *Metacrinus rotundus* (Hara *et al.*, 2006); Ophiurid *Stegophiura sladeni* (Mito and Endo, 2000) (PCR), *Amphiura filiformis* (PCR, P. Martinez *et al*, Genbank submissions). For echinoids we used the completely sequenced Hox cluster (Cameron *et al.*, 2006) as references.
Hemichordata: *Ptychodera flava* (Peterson, 2004); *Saccoglossus kowalevskii* (Lowe *et al.*, 2003; Aronowicz and Lowe, 2006).
Urochordata: For *Ciona intestinalis* (Ikuta *et al.*, 2004; Spagnuolo *et al.*, 2003) and *Oikopleura dioica* (Seo *et al.*, 2004) information was available from genome sequencing, reviewed in (Ikuta and Saiga, 2005). For Stolidobranchia most sequence data from PCR surveys are available only as direct GenBank submissions: Styela (Ge *et al.*, 1994), *Polyandrocarpa misakiensis* (Fujiwara, S. *et al.* Genbank submissions), *Herdmania momus* (Kennett, C.V.D, Genbank submissions).

Table 1
Summary of PCR Experiments

		PCR Primers		
Homeobox	accession no.	F2/R1	F2/R2	total
Xb-Hox1	*AM697644*	4	14	18
Xb-HoxM1a	*AM697647*	18	3	21
Xb-HoxM1b	*AM697648*	17	5	22
Xb-HoxM2	*AM697646*	6	7	13
Xb-HoxM3	*AM886320*	different preparation		
Xb-HoxP	*AM697645*	6	14	20

Table 2
Number of pairwise differences between the 5 bona fide *Xenoturbella Hox* sequences including the variant of the XbHoxM1 fragment. Lower left: nucleotide diffences, upper right: amino acid differences.

Homeodomain	XbHox1	XbHoxM1a	XbHoxM1b	XbHoxM2	XbHoxM3	XbHoxP
XbHox1	*	11	10	9	9	12
XbHoxM1a	29	*	1	10	12	12
XbHoxM1b	28	1	*	9	11	11
XbHoxM2	24	28	27	*	7	12
XbHoxM3	29	33	32	30	*	10
XbHoxP	32	37	36	31	30	*

Table 3
Summary of PCR Experiments: clones per run

Homeobox	Xb-Hox1	Xb-HoxM1a	Xb-HoxM1a	Xb-HoxM2	Xb-HoxP	clones per run
12.12.06	-	6	6	1	-	**13**
19.12.06	3	1	1	-	3	**8**
20.12.06	5	3	3	6	9	**26**
10.01.07	3	3	4	1	2	**13**
12.01.07	-	1	4	-	1	**6**
18.01.07	4	2	2	2	3	**13**
19.01.07	3	5	2	3	2	**15**
overall	**18**	**21**	**22**	**13**	**20**	94