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Novel and Conserved Features of the Hox Cluster of *Entoprocta* (*Kamptozoa*)

Julia Merkel[1], **Andreas Wanninger**[2] **and Bernhard Lieb**[1*]

[1]*Institute of Zoology, Johannes Gutenberg University, 55099 Mainz, Germany*

[2]*Department of Integrative Zoology, University of Vienna, 1090 Vienna, Austria*

[*]**Corresponding author:** Bernhard Lieb, Institute of Zoology, Johannes Gutenberg University, 55099 Mainz, Germany, Tel: 004961313923158; E-mail: lieb@uni-mainz.de

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Abstract

Hox genes are highly conserved developmental genes involved in the patterning of the anterior-posterior axis of nearly all metazoan animals. While *Hox* genes have been characterized for many bilaterians, several cryptic taxa, often comprising microscopic specimens, have hitherto been neglected. We here present the first combined transcriptomic and genomic *Hox* gene study for Entoprocta (=Kamptozoa), a phylum of microscopic, sessile, tentacle-bearing animals with unresolved phylogenetic affinities. We identified 10 of the 11 Hox genes commonly found in other lophotrochozoans. The analyses of transcriptomic data of different developmental stages of three species (regenerating stages of the colonial species *Pedicellina cernua*, budding stages of the solitary species *Loxosomella vivipara* and embryos of the solitary species *Loxosomella murmanica*/*atkinsae*) yielded the Hox genes Labial, Hox3, Lox5, and Post2 in all species. Pb and Dfd were only found being expressed in the colonial species *P. cernua*. *Lox4* was uniquely expressed in the solitary species *L. vivipara* and *L. murmanica*/*atkinsae*. Other homeobox genes belonging to the ANTP-class genes, e.g., ParaHox and NK-like genes, were also found. Thus, in addition to newly identified Hox genes (*PceLox2-like* & *LviPost2-like*), Entoporocta show the typical lophotrochozoan *Hox* pattern besides the loss of the posterior class *Hox* gene *Post*1.

Keywords: *Hox*; Entoprocta; Lophotrochozoa; Transcriptome; Genome; Regeneration

Introduction

The identity of the antero-posterior axis of nearly all cnidarians and bilaterian animals is controlled by a group of transcription factors, the *Hox* genes, which are characterized by a highly conserved 60 amino acid polypeptide motif, the homeodomain [1-5]. Even though *Hox* genes are mainly found during early developmental processes, such as embryogenesis, larval and post-larval development [6-9]; see Wanninger [10] for detailed reviews on *Hox* gene expression and function in invertebrate animals), it could be shown that *Hox* genes also have an important role during regeneration events such as, e.g., in *Cnidaria* [11], *Annelida* [12], *Platyhelminthes* [13-18], *Echinodermata* [19] and *Vertebrata* [20-23]. So far, many *Hox* genes have been characterized among the Metazoa [6-9,24-32], but only a few among less species-rich *lophotrochozoan* phyla that mainly contain cryptic, microscopic species.

One of these little investigated phyla is *Entoprocta* (=*Kamptozoa*). Its members are microscopic, sessile, colonial or solitary, mostly marine animals. Their bodies can be subdivided into calyx, stalk and foot [33-35]. The calyx comprises the characteristic tentacle crown, which surrounds both, mouth and anus, the U-shaped gut, one pair of *protonephridia*, the reproductive organs and the cerebral ganglion. They reproduce asexually by budding or sexually, whereby two different larval types can be found: the lecithotrophic and supposedly basal creeping larval type and the more common planktotrophic trochophore-like swimming larval type [36-39]. So far, approximately 150 species are known from four families: the solitary Loxosomatidae and the colonial *Loxokalypotidae*, *Barentsiidae* and *Pedicellinidae* [36,40]. Due to environmental conditions and injuries the calyx of *Pedicellinidae* and *Barentsiidae* can die off and a new "head" forms from the remaining stalk; alternatively, parts of the stalk are rebuilt prior to calyx regeneration [41-43]. For the Loxosomatidae, so far only one species, *Loxosomella antarctica*, is known to have regeneration capabilities comparable to colonial entoprocts [42].

The phylogenetic position of *Entoprocta* is still a matter of debate. Classical morphological and some molecular studies favor a grouping of *entoprocts* with *ectoprocts* as sistergroup [37-38]. Other molecular studies comprise *entoprocts* and *cycliophorans* as a sistergroup to ectoprocts to form the monophyletic *Polyzoa* [44-45]. In contrast, the so-called Tetraneuralia-concept (also *Sinusoida* or *Lacunifera*) places *mollusks* and *entoprocts* as sistergroups, since the creeping-type larva resembles a mosaic of larval and adult molluscan characters, such as the tetraneury of the longitudinal nerve cords or the number of flask-shaped cells in the apical organ [46-51].

So far, Hox genes have not been characterized for any *entoproct* species. However, Hox genes play an important role in determining the body plan, may be used to study and analyze both, the early development in embryos and regeneration processes in adults (see above), e.g. by in situ hybridization experiments, and are also useful characters for phylogenetic studies. We therefore sequenced three transcriptomes of regeneration stages of the colonial species *Pedicellina cernua*, budding stages of the solitary *Loxosomella vivipara*, and embryonic stages of the solitary *Loxosomella murmanica*, in order to reveal the expression of Hox genes during the different developmental processes in these species. In addition, we mind the genome of *P. cernua* to identify the entire entoproct Hox gene

cluster in order not to overlook any non- or less expressed Hox genes in species that were analyzed by transcriptomic data only.

Materials and Methods

Animals and fixation

Adults of the colonial species *P. cernua* live epizooically on the *ectoproct Bugula* sp. or the ascidian *Styela* sp., which inhabit the wharfs of the island Neeltje Jans, The Netherlands. Individuals of *P. cernua* were removed from their hosts and maintained in glass dishes on a shaker in seawater at a temperature of approximately 16°C. Cultured animals were fed once a week and water was changed ~24 h after feeding. For the collection of different regeneration stages, approximately 60 animals were decapitated and collected after a period of four, six, eight, ten, twelve and fourteen days, fixed in RNAlater and stored at -18°C. For genomic analyses, animals were transferred into 100% ethanol.

Specimens of *L. vivipara* live on the alga *Amphiroa fragilissima* in 1.5 m depth in the southern reef of Heron Island, Queensland, Australia. Adults with buds were removed and relaxed in a 1:1 dilution of seawater and 7,14% $MgCl_2$ for 10 min, since they immediately glue themselves with their foot onto the glass wall of the dish. After relaxation ~100 animals were transferred into RNAlater and stored at -18°C.

Loxosomella murmanica (and *L. atkinsae*) can be found on *Phascolion strombus*. This sipunculid species resides in empty shells of the scaphopod *Antalis* sp. or the gastropod *Turritella* sp. Thus, the entoprocts were collected by dredging shells from 30 m depth at Gåsö Ränna, Gullmarsfjord closely located to the Kristineberg Marine Research Station (Sweden). Approximately 150 brooding animals were removed from their host and transferred into RNAlater and stored at -18°C.

The adult gross morphology of *L. murmanica* and *L. atkinsae* is quite similar. During sampling, a determination of the two species was only possible through their different larval types: *L. murmanica* develops via the creeping-type larva and *L. atkinsae* via the swimming-type larva. According to the amount of animals clearly identified through the larval type and the amount of species used with ambiguous determination, we assume that at least 85% were *L. murmanica*.

RNA extraction, sequencing and analyses

After storage, extraction of total-RNA of all probes (~50 to 100 individuals per probe) was performed following the instruction manual with the miRCURY RNA Isolation Kit-Tissue (Exiqon A/S, Denmark). DNase I treatment was skipped for minimizing the loss of RNA during additional washes. For the genomic analyses, DNA extraction of approximately 60 individuals of *P. cernua* was done with the NucleoSpin Tissue XS- Kit (Macherey-Nagel, Germany) following the instruction manual. Quantity and quality of the probes were determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). In preparation for sequencing, cDNA libraries were synthesized for all RNA probes and samples were sequenced paired end with an Illumina Hiseq 2000 (GENterprise Genomics Mainz, Germany). Transcriptome and genome data were analyzed with Geneious version 5.6.6 [52]. Prior to sequence analyses, a database was generated for each sample. Then, sequence search was performed against the amino acid sequence of the *Drosophila melanogaster* Hox gene Antp (Acc.-Nr. AAA70216.1; 1000 Hits, WordSize 3, Max E-value 1e-1), and the nucleotide sequences of all hits were downloaded and assembled. Hox fragments were identified through GeneBank search (National Center for Biotechnology Information). Longer gene fragments were built with the `map to reference` program of the Geneious software. Still incomplete gene fragments of *P. cernua* were elongated with the Genome Walker Universal Kit (Clontech) following the instruction manual. Gene fragments of *L. vivipara* were tried to be extended with the GeneRacer Kit L1502-01 (Invitrogen). Therefore, 4,3 µg of total RNA was used and RACE-ready cDNA was synthesized following the instruction manual. For the 5′- and 3′-RACE a nested PCR was performed with the Dream Taq PCR Master Mix (2X) (Thermo Scientific, Germany), two gene specific primers, and the GeneRacerTM 5′ (Nested) Primer and 3′ (Nested) Primer. The amplification product was gel purified and extracted with the GeneJET Gel Extraction Kit (Thermo Scientific, Germany), and cloned with the StrataClone PCR Cloning Kit (Agilent Technologies, Germany) following the manufacturer′s instructions. Plasmids of relevant clones were purified with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Germany) and sequenced (StarSEQ, Germany). All sequence data is available in Genbank: *LmuHox3* KP691958; *LmuLab* KP691959; *LmuLox4* KP691960; *LmuLox5* KP691961; *LmuPost2* KP691962; *LmuXlox* KP691963; *LviAntp* KP691964; *LviCdx* KP691965; *LviHox3* KP691966; *LviLab* KP691967; *LviLox5* KP691968; *PceCdx* KP691969; *PceDfd* KP691970; *PceEn* KP691971; *PceHox3* KP691972; *PceHox3B* KP691973; *PceLab* KP691974; *PceLox4* KP691975; *PceLox5* KP691976; *PcePb* KP691977; *PcePost2* KP691978; *PcePost2B* KP691979; *PceLox2-like* KP691980; *PceXlox* KP691981; *LviPost2* KP691982; *LviXlox* KP691983; *LviPost2-like* KP691984.

Phylogenetic analysis

A Translation Alignment, iterated with the Muscle algorithm (Geneious), was performed of 96 nucleotide sequences including the homeobox and flanking regions upstream (up to a max. 201 bp) and downstream (up to a max. of 60 bp) of the Entoprocta and six additional lophotrochozoan groups, the Ectoprocta (*Bugula turrita* Btu, *Bugula neritina* Bne), Nemertea (*Lineus sanguineus* Lsa), Brachiopoda (*Lingula anatina* Lan), Mollusca (*Euprymna scolopes* Esc, *Gibbula varia* Gva) and Annelida (*Perionyx excavatus* Pex, *Hirudo medicinalis* Hme, *Capitella teleta* Cte, *Nereis virens* Nvi, *Platynereis dumerilii* Pdu, *Chaetopterus variopedatus* Cva, *Myzostoma cirriferum* Mci). For this reason, all sequences were brought into the same translation frame. Only entoproct sequences were allowed to have incomplete homeobox sequences. The alignment was converted into Phylip format using the data converter of phylogeny.fr [53]. The ML analysis was done with raxmlGUI version 1.3 [54,55] using GTR + GAMMA model parameters with 5.000 bootstrap replications.

Expression pattern analysis

For each of the three transcriptome data bases, sequence search was performed against the amino acid sequence of the *Drosophila melanogaster Hox* gene *Antp* (AAA70216.1), and the nucleotide sequence of all hits were downloaded and assembled. In addition, only blast-hits were considered for this analysis, fitting exactly within the homeodomain. With this restriction we assumed to retrieve approximately one hit per gene expression (that would not be the case if overlaps were allowed; note: incomplete homeodomain sequences of the respective species such as *PceHox3* or *LviLox5* are excluded by this restriction). We assembled the resulting hits and determined the

relative frequency of the respective genes (see supplemental material S4 for table of absolute frequency and diagram of different expression quantity of different developmental stages).

Results

The transcriptomic analyses of the three investigated entoprocts resulted in sequences of the *Hox* genes *Labial* (*LmuLab* KP691959, *LviLab* KP691967, *PceLab* KP691974), *Hox3* (*LmuHox3* KP691958, *LviHox3* KP691966, *PceHox3* KP691972), *Lox5* (*LmuLox5* KP691961, *LviLox5* KP691968, *PceLox5* KP691976) and Post2 (*LmuPost2* KP691962, *PcePost2* KP691978, *LviPost2* KP691982) (Figure 1). The respective *Labial, Hox3* and *Post2* sequences could be clearly identified through an initial search against the NCBI database for non-redundant protein sequences (nr) using blastx and phylogenetic analyses (Figure 2). *Lox5* could be characterized by the "KLTGP"–motif, a C-terminal parapeptide flanking the homeodomain only found in Lophotrochozoa [30,56].

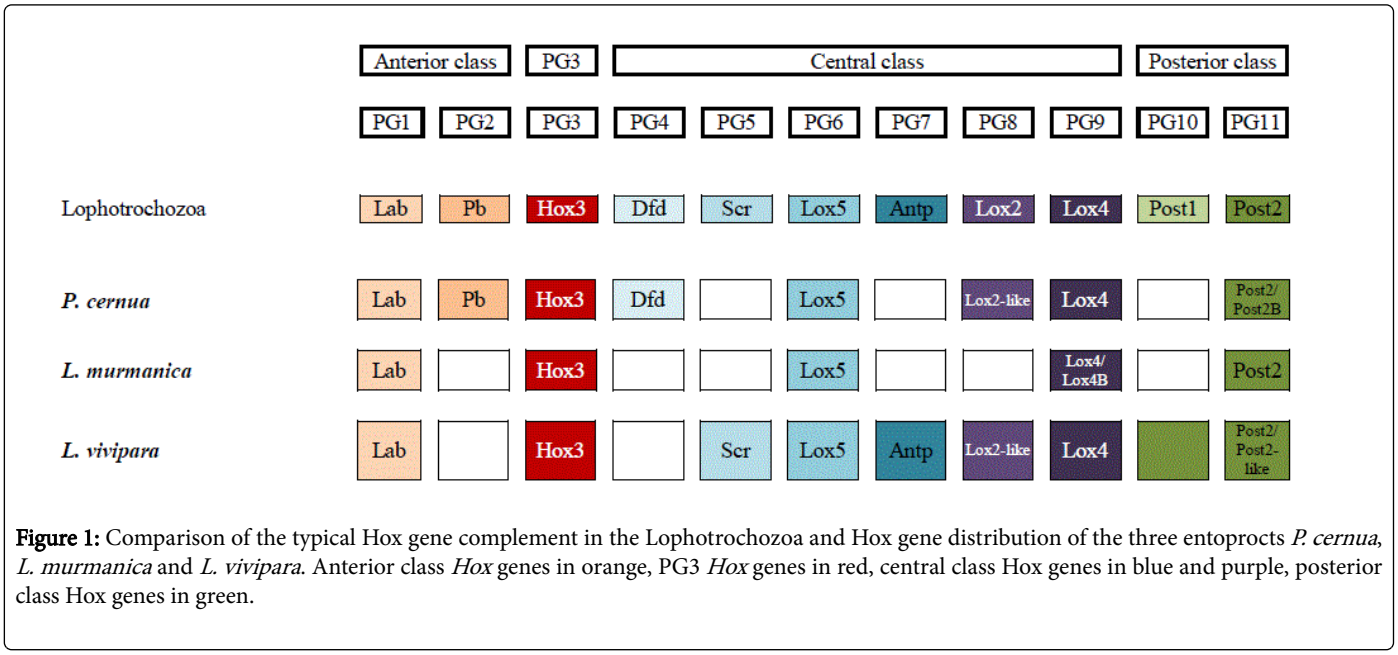

Figure 1: Comparison of the typical Hox gene complement in the Lophotrochozoa and Hox gene distribution of the three entoprocts *P. cernua*, *L. murmanica* and *L. vivipara*. Anterior class *Hox* genes in orange, PG3 *Hox* genes in red, central class Hox genes in blue and purple, posterior class Hox genes in green.

The orthologous genes of *Hox*2 and *Hox*4, *PcePb* (KP691977) and *PceDfd* (KP691970), respectively, could only be identified within the transcriptome of *P. cernua* (Figure 1) In addition, an unidentified *Hox* gene sequence, *PceLox2*-like (KP691980), was found, and the initial search against the NCBI database supports a classification as central class Hox gene. Our phylogenetic analyses weakly support a grouping with the Lox2 genes (Figure 2). The analyses of the transcriptome of *L. vivipara* only provided incomplete homeodomains of *LviLab* (KP691967), *LviHox3* (KP691966), *LviLox5* (KP691968), *LviAntp* (KP691964). *LviHox3* and *LviLox5* could be elongated performing 5´- and 3´-RACEs. A definite identification of the respective homeodomains of *LviScr* and *LviAntp* needs further investigation (Figures 2 and 3). 75 base pairs of the homeobox of an unidentified *Hox* gene sequence were sequenced by RACE. The corresponding amino acid sequence matches to 100% with the unidentified *Hox* gene sequence of *P. cernua*. The *Post2* gene of *L. vivipara*, *LviPost2*, could be unambiguously identified by GenBank analyses and also by our phylogenetic analyses (Figure 2). An additional posterior class *Hox* gene, *LviPost2-like* (KP691984), was uniquely found in *L. vivipara*. Since the homeodomains of *LviPost2* and *LviPost2-like* have only 43 identical sites (~72%), we assume that *LviPost2-like* most probably belongs to the *Post1* genes. However, *LviPost2-like* groups together with the *Post2* genes and not with *Post1* (cf. Figures 2 and 3). An additional *Lox4* cognate, *LmuLox4B*, was found in the transcriptome of *L. murmanica/atkinsae*. We could not obtain the complete homeodomain sequence of *LmuLox4B*, but of 46 detected sites, 44 amino acids were identical with Lox4 (~96%).

Sequence search against the amino acid sequence of the *Drosophila melanogaster Hox* gene *Antp* (AAA70216.1) with each of the three transcriptome data bases revealed the presence of additional homeobox genes belonging to the ANTP-class (Extended *Hox, ParaHox, NK-like*) homeobox genes (Table 1; for classification of homeobox genes). These are the even-skipped homeobox (*Evx*), motor neuron and pancreas homeobox (*Mnx*) and mesenchyme homeobox gene (*Mox2*), the ParaHox genes Gs homeobox (*Gsx*), caudal-type homeobox (*Cdx*; *LviCdx* KP691965, *PceCdx* KP691969) and *Xlox* (*LmuXlox* KP691963, *LviXlox* KP691983, *PceXlox* KP691981), as well as the *NK-like* genes developing brain homeobox 1 (*Dbx1*), distal-less homeobox (*Dlx*), engrailed homeobox (*En*¸ *PceEn* KP691971), hematopoietically expressed homeobox (*Hhex*), H6 family homeobox (*Hmx1*), msh homeobox (*Msx*) and *NK6* homeobox gene (Nk6; Table 1) [57].

The genome data of *P. cernua* supplemented the transcriptome data set with the *Hox8* orthologue *PceLox4* (KP691975). *PceLox4* is separated by an intron of approximately 800bp length. A cognate of *PceHox3*, *PcePost2*, *PceHox3B* (PKP691973) and *PcePost2B* (KP691979), respectively, could additionally be identified. The homeoboxes of *PceHox3* and *PceHox3B* have 137 identical sites (~76%), the homeodomains show 50 identical sites (~83%). The homeoboxes of *PcePost2* and *PcePost2B* have 137 identical sites (~76%), while the homeodomains show 54 identical sites (~90%).

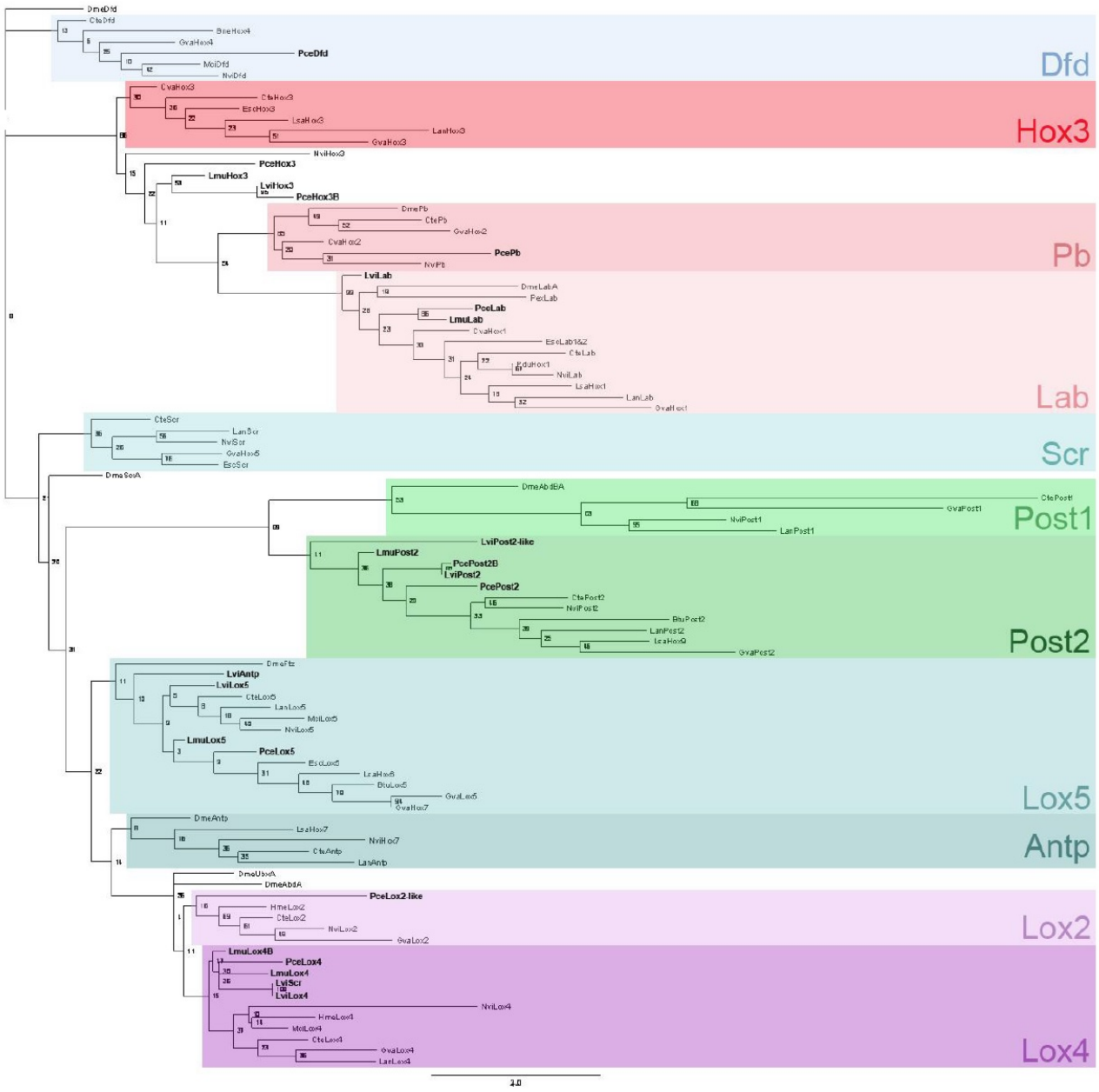

Figure 2: Maximum likelihood analysis of *Hox gene* relationships of six lophotrochozoan groups, Ectoprocta (*Bugula turrita* Btu, *Bugula neritina* Bne), Nemertea (*Lineus sanguineus* Lsa), Brachiopoda (*Lingula anatina* Lan), Mollusca (*Euprymna scolopes* Esc, *Gibbula varia* Gva) and Annelida (*Perionyx excavatus* Pex, *Hirudo medicinalis* Hme, *Capitella teleta* Cte, *Nereis virens* Nvi, *Platynereis dumerilii* Pdu, *Chaetopterus variopedatus* Cva, *Myzostoma cirriferum* Mci). Anterior class Hox genes (Lab, Pb), *Hox3*, central class *Hox* genes (*Dfd, Scr, Lox5, Antp, Lox2, Lox4*), posterior class *Hox* genes (*Post1, Post2*) (for accession numbers see supplemental material S1).

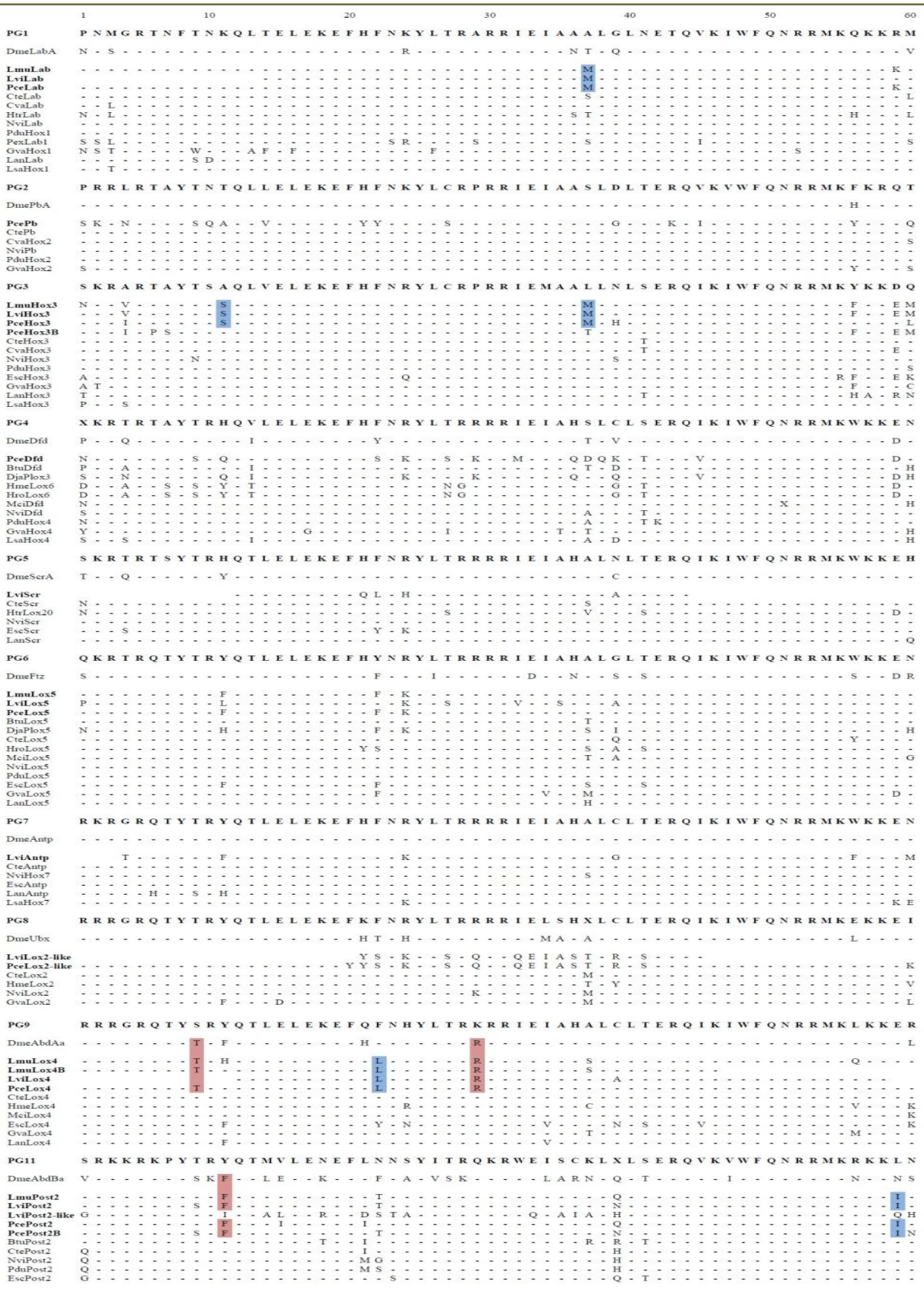

Figure 3: Muscle alignment of *Hox* gene homeodomains of *Drosophila melanogaster* and different lophotrochozoan species: Ectoprocta (*Bugula turrita* Btu), Platyhelminthes (*Dugesia japonica* Dja), Nemertea (*Lineus sanguineus* Lsa), Brachiopoda (*Lingula anatina* Lan), Mollusca (*Euprymna scolopes* Esc, *Gibbula varia* Gva) and Annelida (*Perionyx excavatus* Pex, *Hirudo medicinalis* Hme, *Helobdella triserialis* Htr, *Capitella teleta* Cte, *Nereis virens* Nvi, *Platynereis dumerilii* Pdu, *Chaetopterus variopedatus* Cva, *Myzostoma cirriferum* Mci). Hyphens mark the identity with the consenus sequence of each paralogous group. Amino acids which have been exclusively found in Entoprocta are highlighted in light blue. Similarities among *Drosophila melanogaster* and Entoprocta marked in red (for accession numbers see supplemental material S2).

	ANTP class												
	ParaHox			Extended Hox			NK-like						
	Gsx	Cdx	Xlox	Evx	Mnx	Mox2	Dbx1	Dlx	En	Hhex	Hmx1	Msx	Nk6
Lmu	x		x		x			x					
Lvi		x	x		x		x		x	x	x		
Pce		x	x	x	x	x		x	x		x	x	x

Table 1: Homeobox genes (*Hox* genes excluded) found in the transcriptome of *Loxosomella murmanica/atkinsae* (Lmu), *L. vivipara* (Lvi) and *Pedicellina cernua* (Pce). All genes belong the ANTP class homeobox genes comprising the extended *Hox*, the *ParaHox*, and the *NK-like* homeobox genes. Classification of homeobox genes after Holland et al. [57].

Discussion

The Hox gene cluster of Entoprocta

Hitherto, nothing was known about *Hox* genes in Entoprocta. Here we present the first *Hox* gene sequences for this phylum. For our analyses, we generated and investigated both, transcriptome data and genomic sequences to avoid any possibility not to obtain the complete set of entoproct *Hox* genes due to any transcriptional or sequencing bias. In addition, we discuss possible differences in the expression pattern of regeneration, budding and embryonic stages. To this end, we collected up to 150 individuals of three entoproct species and analyzed the corresponding transcriptomes in regenerating, budding and embryonic stages.

Accordingly, we could identify and assign 10 orthologues of the 11 *Hox* genes known for Lophotrochozoa to Entoprocta. In addition, we detected a so far unidentified *Hox* gene, *Lox2-like*, present in two entoproct species, as well as an unknown posterior class *Hox* gene. The latter unknown posterior *Hox* gene was solely expressed in budding stages. Thus, this novel Hox gene might be involved in clonal reproduction by budding.

Different patterns of Hox gene expression during different developmental processes in Entoprocta

While several *Hox* genes (*Lab*, *Hox3*, *Lox5*, *Post2*) were expressed in all three species, the *Hox* genes *Pb* and *Dfd* could only be found in the transcriptome data of the regenerating stages of *P. cernua* (cf. Figures 1 and 2). The expression of the *PceHox3* cognate *PceHox3B* during regeneration is questionable, since an assembly of the *PceHox3B* sequence with the transcriptome data yielded no result. *L. vivipara* shows an additional posterior class Hox gene, *LviPost2-like*, which could not be characterized further, as well as one additional central class *Hox* gene, most probably representing an orthologue of *Hox7*. Labial is quite equally expressed in all three developmental stages. As previously mentioned, Pb (~5%) and *Dfd* (~26%) are only expressed in regeneration stages of *P. cernua*. While the budding stages of *L. vivipara* show the highest expression of *Hox3* (~15%) and *Post2-like* (~21%), *Lox4* is significantly high expressed (~56%) in embryonic stages of *L. murmanica/ atkinsae*. In regeneration stages, the expression of *Post2* (~26%) is higher than in the budding stages (~3%) and embryos (~14%). Other genes, which have been assembled to *DmeAntp* (AAA70216.1) (*e.g. Xlox, Gsx, Msx, Nk6*), belong to the group of *ParaHox, EHGbox* and *NKL/metaHox* genes. Congruent with the Hox genes, the *ParaHox, EHGbox* and *NKL/metaHox* genes belong to the homeobox-containing genes and probably arose by gene duplication events early in metazoan evolution [58-60].

The reason for this individual gene expression pattern might have its origin in the variable expression during the different developmental processes: *Pb*, *Dfd*, and *Post2* seem to play a central role during regeneration events, *Hox3* and *Post2-like* are highly expressed in budding stages and more than 50% of the expressed Hox genes in embryos belong to *Lox4*. In any case, only *in situ* hybridization experiments of numerous developmental stages will show the sites of expression of *Hox* genes involved in regeneration, embryogenesis or budding, or the persistent expression of individual *Hox* genes in adult tissues.

Species-specific sequence variation in the homeodomain and cognates

The homeodomain is a 60 amino acid long peptide motif of *Hox* genes, highly conserved among nearly all metazoans [5]. In all three of the investigated entoproct species, the homeodomain sequence of respective *Hox* genes shows modifications, similarly but also uniquely found within the Lophotrochozoa. At position 37, *labial* shows a methionine (M) instead of an alanine (A) in all entoprocts Besides some exceptions coming from some annelids, this alanine is present in all other lophotrochozoan species (blue marks, Figure 3).

The sequence of *Hox3/3B* also unravels two amino acids uniquely found in Entoprocta. At position 11, a serine (S) is present instead of an alanine (A), and at position 37, a highly conserved leucine (L) is replaced by a methionine (M) or a threonine (T), respectively (see also labial; blue marks, Figure 3).

The Lox4 sequences of Lophotrochozoa and of *D. melanogaster* usually possess an aromatic tyrosine (Y) or phenylalanine (F) at position 22. In Entoprocta, this aromatic residue is replaced by a nonpolar leucine (L). Within the same sequence, at the positions 9 and 29, respectively, a serine (S) is exchanged by a threonine (T), and a Lysine (K) is replaced by an arginine (R). At the positions 11 and 59, respectively, within the *Post2* sequences of the investigated entoprocts, a tyrosine (Y) is 'replaced' by an phenylalanine (F), and a leucine (L) is 'replaced' by an isoleucine (I) (blue and red marks, Figure 3). Remarkably, exchanges in *Lox4* at positions 9 and 29 and exchanges in *Post2* (position 11) are not common for Lophotrochozoa, but instead are typical for *D. melanogaster* (Ecdysozoa). But, due to the similar chemico-physiological characteristics of the latter mentioned exchanges (Y→F, S→T, K→R), these exchanges most probably may not affect any functionality instead of just representing isofunctional

exchanges maintaining the same conserved function of *Lox4* and *Post2* even in distantly related lineages such as Entoprocta and Arthropoda.

More strikingly, however, the exchanges observed within labial (A/S/T→M) or Pb (A→S, L→M/T) might affect the functional characters of these Hox genes. While more studies are needed to further assess functional issues, these unique features represent an apomorphy of Entoprocta, which might also be useful for further phylogenetic inferences [61-66].

Conclusions

We analyzed the transcriptomes of three entoproct species, one colonial and two solitary forms. In total, we detected 11 different *Hox* gene sequences and we also identified other homeobox-genes, which belong to the ANTP class homebox genes (Extended *Hox*, *ParaHox*, and *NK-like*). A definite assignment of a *Lox2* orthologue was not possible. Instead, we found a closely related *Lox2-like* gene. A *Post1* orthologue could not be found, even by screening the genomic data of *P. cernua*. Thus, we assume that *Post1* was lost before, during or after the evolutionary emergence of Entoprocta. Nevertheless, the presence of typical lophotrochozoan Hox genes such as *Lox5* and *Post2* further corroborates Entoprocta as being a member of the Lophotrochozoa.

While *Labial*, *Hox3*, *Lox5*, and *Post2* were present in the transcriptomes of all investigated species, we only found *Pb* and *Dfd* in the transcriptome of the colonial species *P. cernua* and *Lox4* in the transcriptomes of both solitary species, *L. vivipara* and *L. murmanica/atkinsae*. Our findings clearly reflect the specificity and accuracy of the controlled expression pattern and recruitment of different *Hox* genes for different processes also in Entoprocta.

In *P. cernua* and *L. vivipara*, we additionally found a yet unidentified *Hox* gene. We termed this gene *PceLox2-like*, because our phylogenetic analyses unraveled its closest relationship to *Lox2*. Accordingly, *PceLox2-like* most probably represents a novel central class *Hox* gene that to date is unique to Entoprocta. In addition to *PceLox2-like*, we also detected a so far unknown posterior class *Hox* gene, which is highly expressed in budding stages of *L. vivipara*. We therefore assume that this gene, besides others (*e.g. Hox3*), most probably plays a major role during the budding processes and thus should be investigated more intensely in the near future. The detailed comparisons of the individual entoproct Hox genes revealed some intriguing substitutions within the homeodomain of the three investigated entoproct species that are unique among the Lophotrochozoa. Whether this might have been a driving force for Entoprocta splitting off from its lophotrochozoan sister group or whether this constitutes a later event that occurred after the establishment of the phylum remains a matter of further studies.

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References

1. Scott MP, Tamkun JW, Hartzell GW (1989) The structure and function of the homeodomain. Biochim Biophys Acta 989: 25-48.
2. McGinnis W, Garber RL, Wirz J, Kuroiwa A, Gehring WJ (1984) A homologous protein-coding sequence in Drosophila homeotic genes and its conservation in other metazoans. Cell 37: 403-408.
3. Murtha MT, Leckman JF, Ruddle FH (1991) Detection of homeobox genes in development and evolution. Proc Natl Acad Sci USA 88: 10711-10715.
4. McGinnis W, Krumlauf R (1992) Homeobox genes and axial patterning. Cell 68: 283-302.
5. Lemons D, McGinnis W (2006) Genomic evolution of Hox gene clusters. Science 313: 1918-1922.
6. Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, et al. (2007) Hox gene expression in larval development of the polychaetes Nereis virens and Platynereis dumerilii (Annelida, Lophotrochozoa). Dev Genes Evol 217: 39-54.
7. Korchagina NM, Bakalenko NI, Kulakova MA (2010) Hox-Cluster and evolution of morphogenesis. Russ J Dev Biol 41: 302-311.
8. Samadi L, Steiner G (2010) Expression of Hox genes during the larval development of the snail, Gibbula varia (L.)-further evidence of non-colinearity in molluscs. Dev Genes Evol 220: 161-172.
9. Bakalenko NI, Novikova EL, Nesterenko AY, Kulakova MA (2013) Hox gene expression during postlarval development of the polychaete Alitta virens. Evodevo 4: 13.
10. Wanninger A (2015) Evolutionary Developmental Biology of Invertebrates. 1-6.
11. Schummer M, Scheurlen I, Schaller C, Galliot B (1992) HOM/HOX homeobox genes are present in hydra (Chlorohydra viridissima) and are differentially expressed during regeneration. Embo J 11: 1815-1823.
12. Novikova EL, Bakalenko NI, Nesterenko AY, Kulakova MA (2013) Expression of Hox genes during regeneration of nereid polychaete Alitta (Nereis) virens (Annelida, Lophotrochozoa). EvoDevo 4: 14.
13. Bayascas JR, Castillo E, Muñoz-Màrmol AM, Baguñà J, Saló E (1988) Synchronous and early activation of planarian Hox genes and the re-specification of body axes during regeneration in Dugesia (G.) tigrina (Turbellaria; Tricladida). Hydrobiologia 383: 125-130.
14. Bayascas JR, Castillo E, Muñoz-Màrmol A, Saló E (1996) Hox genes disobey colinearity and do not distinguish head from tail during planarian regeneration. Int J Dev Biol 1: 173S-174S.
15. Bayascas JR, Castillo E, Saló E (1998) Platyhelminthes have a Hox code differentially activated during regeneration, with genes closely related to those of spiralian protostomes. Dev Genes Evol 208: 467-473.
16. Orii H, Kato K, Umesono Y, Sakurai T, Agata K, et al. (1999) The planarian HOM/HOX homeobox genes (Plox) expressed along the anteroposterior axis. Dev Biol 210: 456-468.
17. Nogi T, Watanabe K (2001) Position-specific and non-colinear expression of the planarian posterior (Abdominal-B-like) gene. Dev Growth Differ 43: 177-184.
18. Saló E, Tauler J, Jimenez E, Bayascas JR, Gonzales-Linares J, et al. (2001) Hox and ParaHox genes in flatworms: characterization and expression. Am Zool 41: 652-663.
19. Thorndyke MC, Chen WC, Beesley PW, Patruno M (2001) Molecular approach to echinoderm regeneration. Microsc Res Tech 55: 474-485.
20. Simon HG, Tabin CJ (1993) Analysis of Hox-4.5 and Hox-3.6 expression during limb regeneration: differential regulation of paralogous Hox genes suggest different roles for members of different Hox clusters. Development 117: 1397-1407.

21. Gardiner DM, Bryant SV (1996) Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. Int J Dev Biol 40: 797-805.
22. Christen B, Beck CW, Lombardo A, Slack JMW (2003) Regeneration-specific expression of three posterior Hox genes. Dev Dynamics 226: 349-355.
23. Thummel R, Ju M, Sarras Jr MP, Godwin AR (2007) Both Hoxc13 orthologs are functionally important for zebrafish tail fin regeneration. Dev Genes Evol 217: 413-420.
24. Martinez P, Lee JC, Davidson EH (1997) Complete sequence of SpHox8 and its linkage in the single Hox gene cluster of Strongylocentrotus purpuratus. J Mol Evol 44: 371-377.
25. Amores A, Force A, Yan YL, Joly L, Amemiya C, et al. (1998) Zebrafish hox clusters and vertebrate genome evolution. Science 282: 1711-1714.
26. Kmita-Cunisse M, Loosli F, Bièrne J, Gehring WJ (1998) Homeobox genes in the ribbonworm Lineus sanguineus: evolutionary implications. Proc Natl Acad Sci USA 95: 3030-3035.
27. De Rosa R, Grenier JK, Andreeva T, Cook CE, Adoutte A, et al. (1999) Hox genes in brachiopods and priapulids and protostome evolution. Nature 399: 772-776.
28. Irvine SQ, Martindale MQ (2000) Expression patterns of anterior Hox genes in the polychaete Chaetopterus: correlation with morphological boundaries. Developmental Biology 217: 333-351.
29. Callaerts P, Lee PN, Hartmann B, Farfan C, Choy DWY, et al. (2002) Hox genes in the sepiolid squid Euprymna scolopes: implications for the evolution of complex body plans. Proc Natl Acad Sci USA 99: 2088-2093.
30. Iijima M, Akiba N, Sarashina I, Kuratani S, Endo K (2005) Evolution of Hox genes in molluscs: a comparison among seven morphologically diverse classes. J Molluscan Studies 72: 259-266.
31. Biscotti MA, Canapa A, Olmo E, Barucca M (2007) Hox genes in the antarctic polyplacophoran Nutallochiton mirandus. J Exp Zool 308B: 507-513.
32. Bleidorn C, Lanterbecq D, Eeckhaut I, Tiedemann R (2009) A PCR survey of Hox genes in the myzostomid Myzostoma cirriferum. Dev Genes Evol 219: 211-216.
33. Brusca RC, Brusca GJ (2003) Invertebrates. Sinauer Associates, Sunderland Mass.
34. Westheide W, Rieger R (2013) Spezielle Zoologie, Teil 1: Einzeller und Wirbellose Tiere. Stuttgart: Gustav Fischer Verlag.
35. Wanninger A (2015) Entoprocta. In: A. Wanninger, Evolutionary Developmental Biology of Invertebrates 2: Lophotrochozoa (Spiralia). Springer.
36. Emschermann P (1995) Kamptozoa. In Süßwasserfauna von Mitteleuropa (Schwoerbel J & Zwick P, eds.) Gustav Fischer, Stuttgart 113-141.
37. Nielsen C (1971) Entoproct life-cycles and the entoproct/ ectoproct relationship. Ophelia 9: 209-341.
38. Nielsen C (2001) Animal evolution: Interrelationships of the living phyla. Oxford Univ Press, Oxford.
39. Merkel J, Wollesen T, Lieb B, Wanninger A (2012) Spiral cleavage and early embryology of a loxosomatid entoproct and the usefulness of spiralian apical cross patterns for phylogenetic inferences. BMC Dev Biol 12: 11.
40. Nielsen C (2010) Some aspects of spiralian development. Acta Zool 91: 20-28.
41. Emschermann P (1969) Ein Kreislauforgan bei Kamptozoen. Z Zellforsch: 97: 576-607.
42. Emschermann P (1993) On antarctic Entoprocta: nematocyst-like organs in a loxosomatid, adaptive developmental strategies, host specifity, and bipolar occurence of species. Biol Bull 184: 153-185.
43. Mukai H, Makioka T (1978) Studies on the regeneration of an entoproct, Barentsia discreta. J Exp Zool 205: 261-276.
44. Cavalier-Smith T (1998) A revised six-kingdom system of life. Biol Rev Camb Philos Soc 73: 203-266.
45. Hejnol A, Obst M, Stamatakis A, Ott M, Rouse GW, et al. (2009) Assessing the root of bilaterian animals with scalable phylogenomic methods. Proc R Soc Lond B 276: 4261-4270.
46. Ax P (1999) Das System der Metazoa, 2nd ed Gustav Fischer, Stuttgart.
47. Haszprunar G (2000) Is the Aplacophora monophyletic? A cladistic point of view. Amer Malac Bull 15: 115-130.
48. Wanninger A, Fuchs J, Haszprunar G (2007) The anatomy of the serotonergic nervous system of an entoproct creeping-type larva and its phylogenetic implications. Invertebr Biol 126: 268-278.
49. Haszprunar G, Wanninger A (2008) On the fine structure of the creeping larva of Loxosomella murmanica: additional evidence for a clade of Kamptozoa (Entoprocta) and Mollusca. Acta Zool 89: 137-148.
50. Wanninger A (2008) Comparative lophotrochozoan neurogenesis and larval neuroanatomy: recent advances from previously neglected taxa. Acta Biol Hung 59: 127-136.
51. Wanninger A (2009) Shaping the things to come: ontogeny of lophotrochozoan neuromuscular systems and the Tetraneuralia concept. Biol Bull 216: 293-306.
52. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, et al. (2012) Geneious v5.6. Available from http://www.geneious.com.
53. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, et al. (2008) Phylogeny fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36: W465-469.
54. Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688-2690.
55. Silvestro D, Michalak I (2012) raxmlGUI: a graphical front-end for RAxML. Org Divers Evol 12: 335-337.
56. Balavoine G, de Rosa R, Adoutte A (2002) Hox clusters and bilaterian phylogeny. Mol Phyl Evol 24: 366-373.
57. Holland PW, Booth HA, Bruford EA (2007) Classification and nomenclature of all human homeobox genes. BMC Biol 5: 47.
58. Brooke NM, Garcia-Fernandez J, Holland PW (1998) The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. Nature 392: 920-922.
59. Pollard SL, Holland PW (2000) Evidence for 14 homeobox gene clusters in human genome ancestry. Curr Biol 10: 1059-1062.
60. Ferrier DE, Holland PW (2001) Sipunculan ParaHox genes. Evol Dev 3: 263-270.
61. Heffer A, Xiang J, Pick L (2013) Variation and constraint in Hox gene evolution. Proc Natl Acad Sci USA 110: 2211-2221.
62. Mann RS, Chan S-K (1996) Extra specificity from extradenticle: the partnership between Hox and PBX/EXD homeodomain proteins. Trends Genet 12: 258-262.
63. Mavilio F, Simeone A, Giampaolo A, Faiella A, Zappavigna V, et al. (1986) Differential and stage-related expression in embryonic tissues of a new human homeobox gene. Nature 324: 664-668.
64. Taylor WR (1986) The classification of amino acid conservation. J Theor Biol 119: 205-218.
65. Zhang Z, Holmer LE, Skovsted CB, Brock GA, Budd GE, et al. (2013) A sclerite-bearing stem group entoproct from the early Cambrian and its implications. Scientific Reports 3: 1066.
66. Zhao JJ, Lazzarini RA, Pick L (1996) Functional dissection of the mouse Hox-a5 gene. EMBO J 15: 1313-1322.