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

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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) 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. 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), Entoprocta show the typical lophotrochozoan Hox pattern besides the loss of the posterior class Hox gene Post1.

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 Loxokalyptotidae, 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 cyclophorans 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 epizoically on the *Phalacrocystis* *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% MgCl₂ 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* in species that were analyzed by transcriptomic data only.

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 mRNA 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* (Access. Nr. AA.A70216;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; PceDld KP691970; PceEn KP691971; PceHox3 KP691972; PceHox3B KP691973; PceLab KP691974; PceLox4 KP691975; PceLox5 KP691976; PcePb KP691977; PcePost2 KP691978; PcePost2B KP691979; PcePost2-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. 60 bp) of the Entoprocta and six additional lophotrochozoan groups, the Entoprocta (*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 ambiguous determination, we assume that at least 85% were *L. murmanica.*

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* (AA.A70216.1), 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].

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), hematopoietically expressed homeobox (Hmx), H6 family homeobox (Hmx1), msh homeobox (Mnx) 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 (KP691973) 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 consensus 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).
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 species, the Hox genes Ph 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 Dmeneantp (AAA70216.1) (e.g. Xlox, Gax, Mox, Nk6), belong to the group of ParaHox, EGHbox and NKL/metaHox genes. Congruent with the Hox genes, the ParaHox, EGHbox 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

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].

| ANTP class | Parahox | Extended Hox | NK-like |
|------------|---------|-------------|---------|
|            | Gax     | Cdx         | Xlox    |
| Lmu        | x       | x           | x       |
| Lvi        | x       | x           | x       |
| Pce        | x       | x           | x       |
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 need to be further assessed 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 atkinsae within the homeodomain of the three investigated entoproct species. Individual entoproct Hox genes revealed some intriguing substitutions. This gene, besides others (e.g. Hox3, Post2), is 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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