A PCR survey of Hox genes in the myzostomid *Myzostoma cirriferum*

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Abstract Using degenerate primers, we were able to identify seven Hox genes for the myzostomid *Myzostoma cirriferum*. The recovered fragments belong to anterior class (*Mci_lab*, *Mci_pb*), central class (*Mci_Dfd*, *Mci_Lox5*, *Mci_Antp*, *Mci_Lox4*), and posterior class (*Mci_Post2*) paralog groups. Orthology assignment was verified by phylogenetic analyses and presence of diagnostic regions in the homeodomain as well as flanking regions. The presence of *Lox5*, *Lox4*, and *Post2* supports the inclusion of Myzostomida within Lophotrochozoa. We found signature residues within flanking regions of *Lox5*, which are also found in annelids, but not in Platyhelminthes. As such the available Hox genes data of myzostomids support an annelid relationship.

Keywords Myzostomida · Annelida · Hox genes · Platyhelminthes · Phylogeny

Introduction

Myzostomida comprise a group of marine worms which are either ectocommensals or parasites of echinoderms. The phylogenetic position of this taxon is still controversially discussed. Whereas the presence of many morphological characters is congruent with an annelid affinity (e.g., parapodia-like structures, chitinous chaetae, a ladder-like nervous system, trochophora larva, serial nephridia), some recent molecular analyses point to a platyzoan (Platyhelminthes and relatives) origin of these organisms (Eeckhaut et al. 2000; Dunn et al. 2008). However, a recent analysis using mitochondrial gene order and sequence data strongly supports an annelid affinity of Myzostomida (Bleidorn et al. 2007). Nevertheless, myzostomids show a unique mode of development and the relationship of segment formation in myzostomids (Jägersten 1940) to segment formation, as described for various annelids remains unclear.

The genetical basis of animal development has been a key issue in recent evolutionary investigations to homologize structures across animal phyla (e.g., Tessmar-Raible et al. 2007). *Hox* genes have been found to be important regulators involved in embryonic development, e.g., in the patterning of the anterior–posterior axis or segmentation (Lemons and McGinnis 2006). These genes comprise a gene family of which eight to 14 different paralog groups (PGs) are found in most bilaterians and due to whole genome duplication more copies of each gene are described for vertebrate PGs (e.g., de Rosa et al. 1999). Kulakova et al. (2007) reported *Hox* genes belonging to 11 PGs for the annelid *Nereis virens*. The information on flatworm *Hox* genes is scarce, but a scattering of surveys suggests that the complement might be as in other lophotrochozoans (Olson 2008). Typically, these genes are clustered and respect the colinearity rule, as it has been recently shown for the
capitellid polychaete *Capitella* (Fröbius et al. 2008). This means that PGs are expressed along the body axis in the same order as they are arranged on the *Hox* cluster (Carroll 1995). However, examples for disrupted *Hox* cluster organization have been described and one case includes a parasitic flatworm (Pierce et al. 2005).

For further exploration of phylogenetic relationships, we conducted a *Hox* gene survey in the myzostomid *Myzostoma cirriferum*. Here, we report the first *Hox* gene sequences for Myzostomida and discuss the phylogenetic significance of these data. Analyses of *Hox* genes will be a prerequisite for future developmental studies (e.g., comparing the expression of *Hox* genes in the course of segmentation between Myzostomida and Annelida) of this enigmatic lophotrochozoan taxon.

**Material and methods**

Individuals of *M. cirriferum* were collected from its host *Antedon bifida* (Echinodermata, Crinoidea) in Morgat (Britany, France). DNA extraction was performed using the Qiagen DNeasy™ Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

We used two different primer sets for amplifying homoeodomains. The first primer pair (pair 1) *Hox*1F-LELEKE (GCTCTAGARYTNGARAAARGARTT) and *Hox*1R-WFQNRR (CGGGGATCCCKNCKRTYTYGRAACCA) (Passamanecck and Halanych 2004) was used to screen (mainly) for anterior and medial class *Hox* genes, whereas a second primer set (pair 2) published by Lee et al. (2003) was used to screen for multiple classes of homoeodomains. PCR amplifications were carried out in a total volume of 37.5 μl containing 1 mM Tris–HCL, pH 9.0, 5 mM KCl, 0.15 mM MgCl2, 0.05 mM of each dNTP, 0.13 μM of both forward and reverse primers, and 0.75 U Taq polymerase (QBiogene, Heidelberg, Germany). PCR profile for all reactions was as follows: 94°C for 1 min as initial denaturation; 30 cycles with 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min; final extension at 70°C for 10 min. All PCR products were purified using the NucleoSpin Extract II Kit (Macherey & Nagel, Düren, Germany). Purified PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen).

Using the amplified homoeodomains as a starting point, we performed genome walking to amplify adjacent flanking regions towards both ends. For this purpose, genomic DNA was digested with restriction enzymes (EcoR V, Dra I, Pvu II, Ssp I) and adaptors supplied by the Genome Walker Kit (Clonetch) were ligated to both ends of the fragments. Two rounds of PCR amplifications were performed with adaptor-specific and gene-specific primer (gene-specific primers are available on request). The first cycle was set as follows: 94°C for 1 min as initial denaturation; seven cycles with 94°C for 25 s and 72°C for 3 min, followed by 32 cycles of 94°C for 25 s and 67°C for 3 min; final extension at 70°C for 7 min. The second PCR, with nested primers, used the product of the first amplification as a template with the same parameters. All PCR products were purified using the NucleoSpin Extract II Kit (Macherey & Nagel, Düren, Germany). Purified PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen).

After a screening using T3/T7 colony PCRs, selected products were sequenced on an AB 3100 mult-capillary automatic sequencer (Applied Biosystems, Foster City, CA, USA). Cycle sequencing reactions were performed with the T7 primer using the BigDye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). All sequences have been submitted to NCBI GenBank under accession numbers FJ640069 to FJ640075.

The obtained clones were screened for *Hox* genes using BLAST searches against NCBI GenBank. Orthology assignment of gene fragments is based on both phylogenetic analysis and inspection of diagnostic residues in the homoeodomain and flanking regions. We compiled a *Hox* gene alignment including representatives of Annelida (*Capitella sp. I, Cca; Chaetopterus variopedatus, Cva; Ctenodrilus serratus, Cse; Helobdella robusta, Hro; Nereis virens, Nvi; Perionyx excavatus, Pex; Platynereis dumerilii, Pdu) and Platyhelminthes (*Echinococcus granulosus, Eg; Dugesia japonica, Dja; Girardia tigrina, Gti; Hymenolepis microstoma, Hmi; Schistosoma mansoni, Sma), as well as Mollusca (*Euprymna scolopes, Esc; Nautilus macromphalus, Nma), Nemertea (*Lineus sanguineus, Lsa), Bryozoa (*Bugula turrita, Btu), Brachiopoda (* Lingula anatina, Lan), Arthropoda (*Drosophila melanogaster, Dme), and Deuterostomia (*Metacrinus rotundus, Mro) (see Fig. 1 for accession numbers). Additional to available data from NCBI GenBank, we screened the *Capitella* sp. I (http://genome.jgi-psf.org/Capca1/Capca1.home.html) and *Helobdella robusta* (http://genome.jgi-psf.org/Helr1/) draft genomes for *Hox* genes using the *Hox* gene complement published for *Nereis virens* (Kulakova et al. 2007) as BLAST query. For phylogenetic analysis, only the homeodomain (no flanking regions) was included. Sequences were easily aligned by eye. ML analysis of the dataset was conducted by using RAxML version 7.0.3 (Stamatakis 2006), using GTR + WAG + I model of amino acid evolution. Support values were estimated by 1,000 bootstrap replicates. Maximum parsimony analysis with tree-bisection-reconnection (TBR) branch swapping, random
sequence addition, and holding of trees limited to 10,000 was conducted using PAUP*, version 4.0b10 (Swofford 2001). Clade support was assessed with 1,000 nonparametric bootstrap replicates.

Additionally, alignments including flanking regions of a selected set of taxa were constructed for each of the recovered myzostomid Hox fragments.

Results and discussion

We screened 192 clones in total, of which 144 and 48 were identified as products amplified by primer pair 1 and pair 2, respectively. All fragments that were subsequently annotated as anterior or central class Hox genes were amplified with pair 1 (primers from Passamaneck and Halanych 2004), whereas the posterior class Hox gene fragment was amplified with pair 2 (primers from Lee et al. 2003). In total, our PCR survey using degenerated primers recovered seven Hox fragments and we were able to sequence flanking regions for four of them following the genome walking approach. Orthology assignment using phylogenetic analysis (ML-tree shown in Fig. 1) and alignments including flanking regions (Fig. 2) revealed that the sequences belong to Hox paralog groups (PG) including Hox1 (Mci_lab—M. cirriferum Hox genes were designated with the prefix Mci), Hox2 (Mci_pb), Hox4 (Mci_Dfd), Lox5 (Mci_Lox5), Hox7 (Mci_Antp), Lox4 (Mci_Lox4), and Post2 (Mci_Post2). The strict consensus of 10,000 equally parsimonious trees of the MP-analysis revealed a tree that is less resolved, but largely congruent with the ML-tree regarding assignment of myzostomid Hox gene fragments (see electronic supplement S1)

The presence of Lox5, Lox4, and Post2 orthologs supports the placement of Myzostomida within Lophotrochozoa. The Lox5 ortholog shows the typical C-parapeptide, except that it bears a “KLTGP” instead of “KLTGP” as usually found in most lophotrochozoans (de Rosa et al. 1999; Balavoine et al. 2002). Moreover, the flanking region upstream of the homeodomain shows a motif similar to that of annelids included in the present study (Fig. 2, “FGFE” motif). Flanking regions obtained for Mci_Lox4 strongly support its assignment to the Lox4 PG. Mci_lab is a member of the PG 1 (Hox1-PG) and typical PG 1 signature residues can be identified in its homeodomain (Fig. 2). Mci_pb was identified as a PG 2 (Hox2-PG) member. Interestingly, our phylogenetic reconstruction (Fig. 1) recovered unambiguously only one platyhelminth sequence within this group, the tapeworm Echinococcus granulosus. However, BLAST searches revealed a vertebrate similarity for this last sequence, which might be due to a contamination by its host’s DNA, as E. granulosus parasitizes dogs. Moreover, additional BLAST searches within the available Schistosoma mansoni genome data (http://www.sanger.ac.uk/Projects/S_mansoni/) have not recovered any sequence that groups within the PG 2 cluster. The sequences that Olson (2008) tentatively assigned to a combined PG 2 + PG 3 clade cluster within the PG 3 clade in our analysis or outside both PGs. In summary, the existence of PG 2 genes has still to be proven for Platyhelminthes and in fact might even have been lost in this lineage. A myzostomid PG 4 member (Mci_Dfd) has been found. A “LPNTK” motif in the downstream flanking region (Fig. 2) is present in the myzostomid, in the annelids, in one of the bryozoan sequences, and in the arthropod and deuterostome representatives, but not in any of the available flatworm sequences. A parsimonious explanation for this pattern is that annelids and myzostomids retained the plesiomorphic condition, which must have been already available in the last common bilaterian ancestor, whereas flatworms show a derived condition. We obtained a short fragment assigned to Mci_Antp belonging to the PG 7, but we were not able to amplify its flanking regions and the homeodomain itself appears to be conserved across the included taxa (Fig. 2).

The phylogenetic position of myzostomids is still under discussion and most analyses either recover an annelid or platyzoan relationship (Bleidorn et al. 2007; Dunn et al. 2008). Hox genes have been repeatedly used for phylogenetic inference (e.g. de Rosa et al. 1999), but the “right” way to analyze these data has been disputed (Telford 2000). One method is to search for so called signatures (a progression of diagnostic residues) in alignments of ortholog genes (e.g. de Rosa et al. 1999). As more sequences are included in this approach, the more diluted the picture becomes and it would be straightforward to combine single sets of ortholog sequences into a supermatrix for subsequent phylogenetic analyses. However, the ambiguous orthology of some Hox gene fragments, especially those of many of the flatworms, weakens this approach. We thus limit our analysis to the former method. Myzostomids share signatures with annelids (e.g., Lox5, see details above) and a member of the PG 2 (Mci_pb) has been identified, which might have been lost in the platyhelminth lineage. The present findings can be interpreted as a (weak) support of the annelid affinity hypothesis and is hence in concordance with morphology and mitochondrial data (Bleidorn et al. 2007). In addition, these first available Hox genes sequences for myzostomids form a starting point for future studies on their ontogeny, relative to their putative phylogenetic relationships.
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