Unexpected co-linearity of Hox gene expression in an aculiferan mollusk

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Abstract

Background: Mollusca is an extremely diverse animal phylum that includes the aculiferans (worm-like aplacophorans and eight-shelled polyplacophorans) and their sister group, the conchiferans, comprising monoplacophorans, bivalves (clams, mussels), gastropods (snails, slugs), scaphopods (tusk shells) and cephalopods (squids, octopuses). Studies on mollusks have revealed an overall number of 11 Hox genes in seven out of eight molluscan “class”-level taxa, but expression data of key developmental regulators such as homeotic genes are only available for three gastropod and two cephalopod species. These show that Hox genes are involved in the formation of specific features including shell, foot, funnel or tentacles and not in antero-posterior body plan patterning as in most other bilaterian animals. The role of Hox genes in non-conchiferan (i.e., aculiferan) mollusks remains entirely unknown.

Results: Here we present the first data on the expression of seven Hox genes in a polyplacophoran mollusk, Acanthochitona crinita. In A. crinita the Hox genes Acr-Hox1-5, Hox7 and Post2 are expressed in a co-linear pattern along the antero-posterior axis, but not in molluscan-specific features such as the shell or the foot. The expression pattern is restricted to the post-trochal region and the transcripts are present in ecto-, endo- and mesodermal cell layers. Contrary to the situation in gastropods and cephalopods, we did neither find Hox gene expression in distinct neural subsets of A. crinita, nor in its developing shell plates.

Conclusions: Our analysis and comparison with other lophotrochozoans indicate that the basal role of Hox genes is in antero-posterior axis patterning in mollusks, similar to the vast majority of bilaterian animals, and that this role has been conserved in polyplacophorans, while co-option into patterning of evolutionary novelties emerged either at the base of Conchifera or independently in gastropods and cephalopods. These morphological innovations most likely contributed to the evolutionary success of its representatives, as exemplified by, e.g., the wide ecological range and species richness of gastropods.

Keywords: Body plan, Development, Evo-devo, Evolution, Mollusca, Novelty, Patterning, Polyplacophora
Caudofoveata, as well as the shell plate-bearing Polyplacophora). Thereby, recent developmental data have shown that the aculiferans most likely originated from a polyplacophoran-like ancestor, implying that the simple vermiform morphology of the aplacophorans evolved by secondary simplification [23].

Until today, an overall number of 11 Hox genes has been identified in seven out of the eight molluscan “class”-level taxa [24–38], but expression studies on Hox genes are restricted to representatives of two, probably derived, clades of Mollusca that belong to the conchiferans, namely Gastropoda (snails and slugs) and Cephalopoda (squids and octopuses). The existing expression data on Mollusca are limited to larvae of the gastropods Haliotis asinina, H. rufescens and Gibbula varia, and to developing embryos of the cephalopods Euprymna scolopes and Sepia officinalis. These data show that the Hox genes are involved in the formation of specific morphological features such as the shell, the ganglionic nervous system, the tentacles and the funnel, and that they are - contrary to other bilaterians - expressed in a non-co-linear pattern [24, 26, 27, 33–35, 38]. Furthermore, Hox transcription products could also be identified in sensory organs (e.g., in the apical organ and the statocyst in gastropod larvae) and in the light organ of squid embryos [27, 34]. As opposed to this, in most other bilaterians, Hox genes do show co-linearity and play a crucial role in organizing the antero-posterior body axis [4, 6, 7]. The gastropod and cephalopod expression data suggest that Hox genes may have been co-optimized into the formation of novel molluscan morphological features [27, 33–35, 38].

In contrast to gastropods and cephalopods, polyplacophorans possess a number of non-conchiferan characters such as serially arranged shell muscles, dorsal shell plates, as well as a non-ganglionic visceral and pedal nervous system with iterated commissures [23, 39, 40]. In order to assess potential functions of Hox genes in non-conchiferan mollusks, we here present the first data on Hox gene expression in an aculiferan mollusk, the polyplacophoran Acanthochitona crinita (Pennant, 1777).

Results

Hox gene orthologs and phylogenetic analysis

In the assembled transcriptome of Acanthochitona crinita (Acr), orthologous Hox gene sequences were identified. The sequences of the genes Acr-Hox1-5, Acr-Hox7 and Acr-Post2 contain a conserved homeo-domain amino-acid sequence and Acr-Hox1-2, Acr-Hox4-5 and Acr-Hox7 are flanked by the Y(P)WM-motif at the N-terminal (Fig. 1). All Acr-Hox protein sequences cluster together with their respective bilaterian orthologs, as revealed by the phylogenetic analysis (Fig. 2).

Larval development of Acanthochitona crinita

Early trochophore larvae of A. crinita are less than 200 μm long (not shown) and have an apical tuft at the anterior pole. The well-developed prototroch divides the pre-trochal area (episphere) from the post-trochal region (hyposphere). Ventro-medially, posterior to the prototroch, the mouth opening (stomodaeum) is located.

Mid-trochophore larvae are oval-shaped and approximately 280 μm in length (Fig. 3a and b). The hyposphere is more elongated, and posterior to the mouth opening the developing anlage of the foot extends in the ventral region. Dorso-laterally, on both sides of the hyposphere, a longitudinal row of epidermal and very prominent spicule-containing cells are discernible. Dorso-anteriorly, a transversal row of spicule-containing cells is present in the episphere and in the posterior region of the hyposphere. In this stage the anlagen of the seven dorsal shell plates are already visible in the hyposphere.

Late trochophore larvae are about 360 μm in length and the hyposphere is considerably more elongated than in previous stages (Fig. 3c and d). On the dorsal side of the hyposphere, seven differentiating shell plates are present. At the end of the entirely lecithotrophic, i.e., non-feeding larval development, late trochophore larvae settle and undergo metamorphosis, which is characterized by considerable dorso-ventral flattening of the animals as well as loss of the apical ciliary tuft and the prototroch [41].

Hox gene expression

Acanthochitona crinita-Hox1

The expression of Acr-Hox1 in early and mid-trochophore larvae is restricted to the anterior region of the hyposphere, postero-lateral to the stomodaeum. Ventrally, the expression pattern is visible in two sub-epidermal cellular spots, lateral to the median body axis (Fig. 4a–f). Dorsally, a slight transversal band of Acr-Hox1 expression is detectable within the epidermal and sub-epidermal cell layer in the anterior region of the hyposphere (Fig. 4c–f). In later stages a weak Acr-Hox1 expression pattern is discernible in the epidermal and sub-epidermal cell layer (Fig. 4g–i).

Acr-Hox2

Transcripts of Acr-Hox2 in early and mid-trochophore larvae are present in two sub-epidermal cell clusters, postero-lateral to the stomodaeum (Figs. 5a–c and 6a–c). The pattern is posteriorly more elongated than in Acr-Hox1 and each expression domain is also located lateral to the median body axis (Fig. 5a). In later stages, an indistinct, faint Acr-Hox2 expression pattern is found sub-epidermally and antero-posteriorly in the hyposphere (Fig. 7a–c).
Fig. 1 Homeodomain alignment. Sequence alignment containing Hox gene homeo-domains (Hox1-5, Hox7 and Post2) and the flanking regions. The residues are bluish colored in each column according to the percentage of identity that agrees with the consensus sequence. Residues with less than 40% of identity are not colored. Dashes represent missing data.
**Acr-Hox3**

Expression pattern of Acr-Hox3 in early and mid-trochophore larvae is found in the hyposphere, in two parallel longitudinal sub-epidermal cellular strands (Figs. 5d–f and 6d–f). The pattern is posteriorly much more elongated than that of Acr-Hox2. Each strand of expression extends laterally to the median body axis. Dorsally, a slight transversal anterior ring of Acr-Hox3 transcripts is present in the hyposphere (Figs. 5e–f and 6e–f). Acr-Hox3 in later stages is only indistinctly expressed in the sub-epidermal layers (Fig. 7d–f).

**Acr-Hox4**

In comparison to the other genes, Acr-Hox4 shows the strongest expression pattern in early and mid-trochophore larvae. Acr-Hox4 transcripts are present in two prominent epidermal and sub-epidermal expression strands in the hyposphere (Figs. 5g–i and 6g–i). Ventro-medially, both elongated strands are interconnected with a slender expression band in the anterior hyposphere region. Laterally, the expression pattern is faint. A slight transversal ring of Acr-Hox4 expression is present antero-dorsally (Figs. 5h–i and 6h–i).
6h–i). Later stages retain a less prominent expression pattern in the ventral hyposphere (Fig. 7g–i).

**Acr-Hox5**

Transcripts of *Acr-Hox5* in early and mid-trochophore larvae extend from the central to the posterior region of the hyposphere. *Acr-Hox5* is expressed sub-epidermally, in two distinct longitudinal cellular strands (Figs. 5j–l and 6j–l). Antero-medially, both strands of expression are interconnected. In later stages, *Acr-Hox5* is less strongly expressed, but still two weak, parallel, sub-epidermal cellular strands are present (Fig. 7j–l).

**Acr-Hox7**

*Acr-Hox7* expression in early and mid-trochophore larvae is present in the posterior hyposphere, sub-epidermally in a ring-like pattern (Figs. 5m–o and 6m–o). Expression is ventrally and dorsally more prominent than in the lateral cell layers. *Acr-Hox7* is expressed in later stages, indistinctly in the posterior region of the hyposphere (Fig. 7m–o).

**Acr-Post2**

Expression of *Acr-Post2* in early and mid-trochophore larvae is in the posterior pole of the hyposphere. *Acr-Post2* transcription products accumulate ventrally and dorsally in epidermal and sub-epidermal cell layers (Figs. 5p–r and 6p–r). In later stages, *Acr-Post2* is less prominently expressed, but still discernible in the posterior pole of the larva (Fig. 7p–r).

**Discussion**

**Hox gene expression in aculiferan and conchiferan mollusks**

The existing expression data of three species of gastropods (*Haliotis asinina*, *H. rufescens* and *Gibbula varia* [24, 26, 33–35]) and two cephalopod species (the squid *Euprymna scolopes* and the cuttlefish *Sepia officinalis* [27, 38]) have shown that Hox genes are predominantly involved in the formation of the ganglionated central nervous system in these conchiferan mollusks. Other Hox gene transcripts are present in sensory organs such as the apical organ or the statocyst in the gastropod trochophore larvae and in the light organ of *E. scolopes* [27, 34]. Furthermore, Hox gene expression domains include the gastropod shell and the cephalopod brachial crown and funnel.

In the polyplacophoran *Acanthochitona crinita*, none of the seven investigated Hox gene orthologs (*Acr-Hox1*–5, *Hox7* and *Post2*) show a comparable structural expression pattern during development. By contrast, the Hox genes are expressed in epidermal and sub-epidermal cell layers, in a co-linear manner along the antero-posterior axis in the hyposphere of early and mid-trochophore larvae of *A. crinita* (Figs. 4, 5, 6, 7 and 8).

In early and mid-stage *Acanthochitona crinita* trochophores, *Acr-Hox1* transcripts are found in two ventro-lateral cell clusters, next to the stomodaemum, and dorsally in a slender transversal band. In contrast, in the gastropod *Gibbula varia*, the Hox1 ortholog (*Gva-Hox1*, Fig. 9c) is expressed in the larval dorso-posterior shell anlage [33]. *Acr-Hox2* transcripts are present in two ventral, short and parallel longitudinal strands, postero-lateral to the stomodaemum, and *Acr-Hox3* expression is
Fig. 4 (See legend on next page.)
also present in two distinct ventral parallel longitudinal strands that extend farther in posterior direction than the Acr-Hox2 expression domain. The Acr-Hox4 ortholog is highly expressed in two domains which are antero-medially interconnected by a slender band. Gva-Hox2-4 and Has-Hox2-4 (Haliotis asinina-Hox2-4) transcripts are distributed in the ventral ectoderm, posterolaterally around the foot anlage [26, 33]. Acr-Hox5 is expressed in two ventral longitudinal strands in the posterior region of the hyposphere, whereas in gastropod trochophore larvae only a faint endodermal Gva-Hox5 expression adjacent to the foot rudiment was found [24, 26, 33, 34]. Acr-Hox7 is expressed in a ring-like pattern in the posterior hyposphere; this pattern differs strongly from that observed in the trochophores of the gastropod Gibbula varia, where Gva-Hox7 is exclusively expressed in the trochoblasts [34]. Acr-Post2 expression is present in the posterior pole of the hyposphere, while in Gibbula, a certain number of cells that surround the posterior foot anlage dorsally express Gva-Post2 [34].

In late Acanthochitonina crinita trochophore larvae, the expression pattern is faint at best or entirely absent (summarized in Fig. 8), while a distinct distribution of orthologous Hox gene transcripts is present in pre- and post-torsional veliger stages of the gastropods Gibbula varia and Haliotis asinina. Thereby, Gva-Hox1, Gva-Post2 and Has-Hox1 are expressed in the mantle and especially in the shell field [26, 33]. Gva- and Has-Hox2-4 transcripts are present in the operculum and in the foot anlage [26, 33]. However, the main expression domains of Gva-Hox2-5 and Has-Hox2-5, as well as that of Haliotis rufescens-Hox5 (Hru-Hox5) and Gva-Hox7 are in the cerebral, branchial, esophageal, pedal and pleural ganglia [24, 26, 33, 34]. Orthologs of Hox1, 3, 5 and 7 in the embryos of the cephalopod Euprymna scolopes (Esc-Hox1, 3, 5 and 7) are expressed in the developing cerebral, palliovisceral and pedal ganglia, and Esc-Hox1, 3 and Post2 in the stellar ganglion [27]. Esc-Hox1, 3, 5, 7 and Post2 are also expressed in cephalopod-specific structures such as the brachial crown and the funnel [27]. As in E. scolopes, in embryos of the European cuttlefish Sepia officinalis the ortholog of Sof-Hox3 is also expressed in distinct nervous system and locomotory structures. Sof-Hox3 transcripts are present in the sub-esophageal masses, brachial, buccal and stellar ganglia and in parts of the funnel [38].

In comparison to the Hox gene expression data on gastropods and cephalopods, the polyplacophoran A. crinita Hox gene transcripts are not restricted to particular subsets of the developing nervous system (although we do not rule out the presence of Hox transcripts during neurogenesis). This may be due to the medullary cord-like character of the polyplacophoran nervous system which, in contrast to the gastropods and cephalopods, lacks distinct ganglia [41–43]. Data on polyplacophoran neurogenesis have shown that in early and mid-trochophore larvae of Ischnochiton hakodadensis and Mopalia muscosa the primary pedal neurons (derivatives of the ectoderm) develop and extend posteriorly [42, 43]. Interestingly, in the post-trochal region in early and mid-stage A. crinita trochophore larvae, the transcripts of the Acr-Hox1-5 genes are present in two parallel antero-posterior medial expression domains, which overlap with the region of the developing neurons of the future paired pedal nerve cords. In contrast, in the lateral post-trochal areas of the developing visceral nerve cords, no overlapping Acr-Hox gene expression pattern is present. However, in A. crinita, the overall expression pattern is not restricted to, e.g., the developing pedal nerve cords. Instead, the Acr-Hox gene transcripts are also found in more lateral and more central sub-epidermal tissues which clearly do not contribute to neural tissues (Figs. 4–6).

Developmental data on the muscular system have shown that in early and mid-trochophore larvae of Mopalia muscosa and Leptochiton asellus several muscle fibers start to develop in the post-trochal region (the future dorso-ventral muscles [23, 41]). The Hox gene transcripts in early and mid-stage A. crinita trochophore larvae are expressed in a region comparable to that where these (mesoderm-derived) muscles form. However, the Acr-Hox gene expression pattern is not restricted to individual muscular structures.

Henry and colleagues [44] labeled derivatives of the endoderm in the polyplacophoran species Chaetopleura apiculata via injected fluorescent markers in cell lineage experiments. Thereby, it was found that in early and mid-trochophore larvae of C. apiculata, third-quarter macromeres give rise to the developing body wall and
Fig. 5 Acr-Hox2-5, Hox7 and Post2 expression in mid-trochophore larvae of Acanthochitona crinita. Anterior faces up. Left and middle column: stereomicrographs. Right column: light micrograph.  

a–c Acr-Hox2 is expressed postero-laterally, next to the stomodeum, in two ventral, short and parallel longitudinal strands.  
d–f Transcripts of Acr-Hox3 are present in two distinct ventral parallel longitudinal strands.  
g–i Acr-Hox4 is expressed prominently in the hyposphere in two distinct domains which are antero-medially interconnected.  
j–l The expression pattern of Acr-Hox5 is present in two ventral longitudinal strands in the posterior region.  
m–o Acr-Hox7 transcripts are present in a ring-like arrangement in the posterior hyposphere.  
p–r The expression pattern of Acr-Post2 is present at the posterior pole of the larva. Scale: 50 μm. ao apical organ, f foot, pt prototroch
Fig. 6 Distribution pattern of the Acr-Hox2-5, Hox7 and Post2 transcription products in mid-trochophore larvae of Acanthochitona crinita. Anterior faces up. Autofluorescence (grey) and specific transcription product distribution (yellow). Left column: single sagittal section. Middle and right column: ventral and ventro-lateral whole mount clipping plane projection. Transcripts of Acr-Hox2 are present sub-epidermally. Acr-Hox3 is ventrally expressed in sub-epidermal cell layers. Acr-Hox4 transcripts are present in the epidermal and sub-epidermal cell layers. Transcripts of Acr-Hox5 are present sub-epidermally. The posterior ring of Acr-Hox7 transcription products is present in sub-epidermal cell layers. Acr-Post2 transcription products accumulate in epidermal and sub-epidermal cell layers. ao apical organ, mo mouth opening, pt prototroch, rs radula sac, st stomatodaeum, tb troctoblast(s)
the (endoderm-derived) gut in the post-trochal region. In early and mid-stage \textit{A. crinita} trochophore larvae, the transcripts of anterior Hox genes (such as \textit{Acr-Hox1-4}) are present post-trochally around the forming stomodeaum and adjacent to the central area of the forming digestive tract (as part of the endoderm).

Indeed, in the polyplacophoran \textit{Acanthochitona crinita}, the Hox gene expression patterns are not restricted to distinct morphological structures (e.g., ganglia, shell plates, etc.). However, comparable developmental polyplacophoran data and also the Hox gene expression patterns indicate, that the \textit{Acr-Hox} gene transcripts are seemingly present in tissues derived from all three germ layers (ectoderm, endoderm and mesoderm). These expression patterns differ significantly from the Hox gene expression pattern in gastropods and cephalopods (see above), indicating that Hox genes are involved in different, taxon-specific developmental processes in gastropods and cephalopods, but not in polyplacophorans. In contrast, the polyplacophoran transcripts \textit{Acr-Hox1-5}, \textit{Hox7} and \textit{Acr-Post2} are distributed in a specific spatial antero-posterior pattern. Anterior Hox genes (e.g., \textit{Acr-Hox1-2}) are only expressed in the anterior hyposphere, next to the stomodeaum, and posterior Hox gene transcripts (such as \textit{Acr-Post2}) are only present in the posterior hyposphere. This antero-posterior mode of expression resembles that of the co-linear Hox gene expression in the vast majority of bilaterian animals, including Acoelomorpha, Annelida, Ecdysozoa and Deuterostomia (e.g., [14, 16, 17, 26, 34, 45–54]) and thus appears to have been conserved in polyplacophoran mollusks.

In this study, the expression of seven out of the putatively 11 molluscan Hox genes was investigated in the polyplacophoran \textit{Acanthochitona crinita}. Preliminary data on the yet missing genes \textit{Acr-Lox5}, \textit{Lox4} and \textit{Lox2} (\textit{Acr-Post1} could not be identified) are in line with the antero-posterior Hox gene expression pattern described herein.
Antero-posterior Hox gene expression in Polyplacophora and Annelida

Comparing our results with those of Annelida, a taxon probably closely related to Mollusca (e.g., [19, 36, 55–57]), important similarities in the spatial Hox gene expression patterns along the antero-posterior axis become obvious (Fig. 9a–b). Early trochophore larvae of the annelids *Nereis virens* and *Platynereis dumerilii* show a strikingly similar co-linear Hox gene expression pattern to that of *Acanthochitona* in the ventral hyposphere. In *A. crinita*, the Acr-Hox1 expression is located in the anterior hyposphere; orthologs of *N. virens*-Hox1 (*Nvi-Hox1*) and *P. dumerilii*-Hox1 (*Pdu-Hox1*) are similarly expressed in the anterior hyposphere, the future first parapodial segment [17]. The Acr-Hox2 expression postero-lateral to the stomodaeeum is similar to the stomodeal *Pdu*-Hox2 expression [17]. The two lateral expression strands of Acr-Hox3 resemble the *Nvi*-Hox3 and *Pdu*-Hox3 expression strands [17]. Acr-Hox4 transcription products appear along the ventral and lateral hyposphere of the polyplacophoran, similar to the ventral and lateral expression of *Nvi*-Hox4 and *Pdu*-Hox4 in the developing second and third parapodial segments and the site of Acr-Hox5 expression is comparable to that of *Nvi*-Hox5 and *Pdu*-Hox5 expression in the developing second and third parapodial segment [17]. Acr-Hox7 transcripts are distributed posteriorly in a dorso-ventral ring-like pattern; Nvi-Hox7 and Pdu-Hox7 are also expressed posteriorly, namely in the developing third parapodial and pygidial segment [17]. Acr-Post2 expression is restricted to the posterior pole, similar to the pygidial expression of *Nvi*-Post2 and *Pdu*-Post2 [17]. In addition to the expression data in *N. virens* and *P. dumerilii*, a relatively similar AP expression pattern is also known from other early annelid developmental
stages (e.g., Chaetopterus [16], Helobdella robusta and H. triseriata [46], Capitella sp. [51], Platynereis [53]). Overall, the relative spatial antero-posterior Hox gene expression pattern in early developmental stages of Nereis virens and Platynereis dumerilii, as well as other annelids, is comparable to that of the polyplacophoran Acanthochitona crinita. In particular, the spatial antero-posterior Hox gene expression pattern in early polyplacophoran trochophores is also found in the ecto-, endo- and mesodermal cell layers and is thus not restricted to specific organs. Annelid Hox gene expression in the nervous system appears for the first time in meta-trochophore larvae [16, 17, 46, 51].

Conclusion
The relatively similar co-linear Hox gene expression signature in Polyplacophora, Annelida and other bilaterians demonstrates that the ancestral role of Hox genes in Mollusca was also in patterning of the antero-posterior body axis. After the aculiferan-conchiferan split, the Hox genes were secondarily recruited into novel functions in gastropods and cephalopods (either individually or at the base of Conchifera), but data on bivalves, scaphopods and monoplacophorans are needed to further substantiate this assumption. Overall, the plasticity of Hox gene functions in mollusks may well explain the high morphological variability exhibited among the various molluscan sub-lineages and may thus have been an important driving force for the evolutionary success of gastropods and cephalopods.

Methods
Rearing and larval development of Acanthochitona crinita
Adult A. crinita were collected at the Biological Station Roscoff, Brittany, France. Spawning was induced by alternating hot (30–40 °C) and cold (3–6 °C) shocks as well as sunlight exposure. After spawning, one to two drops of concentrated sperm solution was used to fertilize the eggs in 150–200 ml seawater for 30 min at 21–23 °C. After spawning and fertilization, developmental stages were reared in filtered seawater. First cleavage stages appeared 3–5 h after fertilization. First trochophore larvae hatched nine to 10 h after fertilization and post-metamorphic animals appeared three to 4 days after fertilization.

Fixation and RNA extraction
Developmental stages were fixed in 4 % paraformaldehyde in MOPS buffer (0.5 M Mops, 10 mM MgSO4, 5 mM EGTA, 2.5 M NaCl) for 45 min at room temperature, stepped into 100 % methanol and stored in 100 % methanol at −20 °C. To extract RNA with the RNasy mini Kit (#74104, Qiagen), a pool of representative developmental stages was used. RNA was sequenced by Illumina technology and reads were assembled into contigs by Trinity [58].

Orthology assignment and phylogenetic analysis of Hox genes
The assembled sequences from the Acanthochitona crinita transcriptome were used in local similarity searches using the program blastn [59] against known and well-curated Hox sequences retrieved from GenBank non-redundant protein database. The top 10 BLAST hits of each similarity search were individually and manually analyzed. The Hox domain-containing sequences were assigned according to the presence of diagnostic residues/motif in the homeodomain as well as in the flanking regions and through Bayesian molecular phylogenetic analysis (Fig. 1). The program Jalview 2 [60] was used to illustrate the multiple sequence alignment of the Hox genes. All sequences of species used in the phylogenetic analysis and corresponding accession numbers are listed in Table 1. The Bayesian analyses were carried out with MrBayes v3.2.2 software [61] with Jones-Taylor-Thornton model of amino-acid substitution [62], gamma-distributed rates, 25,000,000 generations and sampling frequency of 1000.

Primer design and probe synthesis
Acr-Hox gene-specific primer design was performed with the sequence assembling software Geneious 6.1.6 (Biomatters Limited) and primers were purchased from Life Technologies Company (Thermo Fischer Scientific). First strand cDNA was synthesized by reverse transcription of RNA pooled from representative developmental stages covering the entire larval and early post-metamorphic development (cDNA synthesis kit, #04379012001, Roche Diagnostics). Hox gene sequences were amplified with the gene-specific primers via standard PCR. PCR products were cloned by insertion into pGEM-T easy vectors (#A1360, Promega) and plasmid minipreps were purified with Qia-miniprep kit (#27106, Qiagen). Antisense and sense probes from linearized plasmid sequences were synthesized with a DIG-labeling kit (#11277073910, Roche Diagnostics).

Whole mount in situ hybridization
Acanthochitona crinita trochophore larvae were de-calcified in ME (90 % MeOH and 50 mM EGTA) for 10 min and in PPE (4 % PFA, 1 × PBS, 50 mM EGTA) for 45 min and subsequently washed in PBT (1 × PBS, 0.1 % Tween 20). Larvae were incubated in a 60 μg/ml proteinase-K solution at 37 °C for 10 min and then washed in 0.2 % glycine in PBT. To reduce charged probe binding, larvae were put in 1 % triethanolamine
| Species name/Abbreviation | Phylum | Gene Name | Accession Number |
|---------------------------|--------|-----------|------------------|
| *Acanthochitona crinita/Acr* | Mollusca | *Acr_Hox1* | KR190463 |
|                           |        | *Acr_Hox2* | KR190464 |
|                           |        | *Acr_Hox3* | KR190465 |
|                           |        | *Acr_Hox4* | KR190466 |
|                           |        | *Acr_Hox5* | KR190467 |
|                           |        | *Acr_Hox7* | KR190468 |
|                           |        | *Acr_Post2* | KR190469 |
| *Branchiostoma floridae/Bfl* | Chordata | *Bfl_Hox1* | BAA78620 |
|                           |        | *Bfl_Hox2* | BAA78621 |
|                           |        | *Bfl_Hox4* | BAA78622 |
|                           |        | *Bfl_Hox5* | ABX39489 |
|                           |        | *Bfl_Hox7* | ABX39491 |
| *Bugula turrita/Btu* | Bryozoa (Ectoprocta) | *Btu_Hox2* | AAS77225 |
|                           |        | *Btu_Hox3* | AAS77226 |
|                           |        | *Btu_Hox4a* | AAS77227 |
|                           |        | *Btu_Hox4b* | AAS77228 |
|                           |        | *Btu_Post2* | AAS77230 |
| *Caenorhabditis elegans/Cel* | Nematoda | *Cel_Hox1* | CAA34929 |
| *Ciona intestinalis/Cin* | Chordata | *Cin_Hox1* | NP_001122333 |
|                           |        | *Cin_Hox2* | CAD59668 |
|                           |        | *Cin_Hox4* | NP_001027781 |
|                           |        | *Cin_Hox5* | NP_001027665 |
| *Drosophila melanogaster/Dme* | Arthropoda | *Dme_Hox1* | CABS7787 |
|                           |        | *Dme_Hox2* | CAA45271 |
|                           |        | *Dme_Hox3* | P09089 |
|                           |        | *Dme_Hox4* | P07548 |
|                           |        | *Dme_Hox5* | NP_524248 |
|                           |        | *Dme_Hox7* | CAA27417 |
|                           |        | *Dme_Dll* | NP_726486 |
| *Euprymna scolopes/Esc* | Mollusca | *Esc_Hox1* | AAL25804 |
|                           |        | *Esc_Hox3* | AAR16188 |
|                           |        | *Esc_Hox5* | AAR16189 |
|                           |        | *Esc_Hox7* | AAL25809 |
|                           |        | *Esc_Post2* | AAL25812 |
| *Flaccisagitta enflata/Fen* | Chaetognatha | *Fen_Hox1* | ABS18809 |
|                           |        | *Fen_Hox3* | ABS18810 |
|                           |        | *Fen_Hox4* | ABS18811 |
|                           |        | *Fen_Hox5* | ABS18812 |
| *Gibbula varia/Gva* | Mollusca | *Gva_Hox1* | ACX84671 |
|                           |        | *Gva_Hox2* | ADJ18233 |
|                           |        | *Gva_Hox3* | ADJ18232 |
|                           |        | *Gva_Hox4* | ACX84672 |
|                           |        | *Gva_Hox5* | ADJ18234 |
|                           |        | *Gva_Hox7* | ADJ18235 |
|                           |        | *Gva_Post2* | ACX84674 |
| Species, genes and GenBank accession numbers used in the phylogenetic analysis (Continued) |
|-------------------------------------------------------------------------------------------------
| **Haliotis asinina/Has** | Mollusca | Has_Hox3 | AAK17185 |
|                        |          | Has_Hox4 | AAK11240 |
|                        |          | Has_Hox5 | AAF78248 |
| **Lingula anatina/Lan** | Brachiopoda | Lan_Hox1 | AAD45587 |
|                        |          | Lan_Hox3 | AAD45588 |
|                        |          | Lan_Hox5 | AAD45589 |
|                        |          | Lan_Hox7 | AAD45590 |
|                        |          | Lan_Post2 | AAD45595 |
| **Lithobius atkinsoni/Lat** | Arthropoda | Lat_Hox1 | AAL36907 |
|                        |          | Lat_Hox2 | AAL36908 |
|                        |          | Lat_Hox3 | AAL36906 |
|                        |          | Lat_Hox4 | AAL36902 |
|                        |          | Lat_Hox5 | AAL36909 |
|                        |          | Lat_Hox7 | AAL36901 |
| **Lineus sanguineus/Lsa** | Nemertea | Lsa_Hox1 | CAA76295 |
|                        |          | Lsa_Hox3 | CAA76296 |
| **Mus musculus/Mmu** | Chordata | Mmu_Hox1 | NP_034579 |
|                        |          | Mmu_Hox2 | NP_034581 |
|                        |          | Mmu_Hox3 | NP_034582 |
|                        |          | Mmu_Hox4 | NP_032291 |
|                        |          | Mmu_Hox5 | NP_034583 |
|                        |          | Mmu_Hox7 | NP_034585 |
| **Nymphon gracile/Ngr** | Arthropoda | Ngr_Hox1 | ABD45723 |
|                        |          | Ngr_Hox2 | ABD45725 |
|                        |          | Ngr_Hox5 | ABD45729 |
|                        |          | Ngr_Hox7 | ABD45732 |
| **Nereis virens/Nvi** | Annelida | Nvi_Hox1 | AAD46166 |
|                        |          | Nvi_Hox2 | AAD46167 |
|                        |          | Nvi_Hox4 | AAD46169 |
|                        |          | Nvi_Hox5 | AAD46170 |
|                        |          | Nvi_Hox7 | ABD04657 |
|                        |          | Nvi_Post2 | AAD46176 |
| **Platynereis dumerilii/Pdu** | Annelida | Pdu_Hox3 | ABD04656 |
|                        |          | Pdu_Hox4 | ABD04658 |
|                        |          | Pdu_Hox5 | ABD04660 |
|                        |          | Pdu_Post2 | ABD04651 |
|                        |          | Pdu_Dlx1 | CAJ38799 |
| **Sacculina carcini/Sca** | Arthropoda | Sca_Hox1 | ABB46347 |
|                        |          | Sca_Hox2 | AAD00340 |
|                        |          | Sca_Hox4 | AAD00345 |
|                        |          | Sca_Hox5 | AAMS0457 |
and 0.5 % acetic anhydride solution, washed in PBT and post-fixed for 45 min in 4 % PFA. Afterwards, larvae were stepped into 100 % hybridization buffer and pre-incubated at 60 °C in a water bath overnight. Larvae were hybridized with antisense probes which are complementary to the transcript sequence or with sense probes as negative controls (0.25–0.7 ng/μl probe concentration) at 60 °C for 48 h. After hybridization, larvae were washed several times in 4 × Wash (25 % formamide, 4 × SSC, 0.1 % Tween 20), 2 × Wash (with 2 × SSC) and 1 × Wash (with 1 × SSC). Afterwards, specimens were stepped into and washed several times in 1 × SSC, then several times in MAB (100 mM maleic acid, 150 mM NaCl, 0.02 % Tween 20). Larvae were washed in 2 % blocking solution (#11096176001, Roche) in MAB for 2–3 h and incubated in 1:5000 DIG antibody solution (#11093274910, Roche); the reaction was stopped with PBT washing steps and then larvae were post-fixed in 4 % PFA at 4 °C overnight. Larvae were cleared in 1:1 benzylalcohol:benzylbenzoate mixture and mounted on glass slides.

Stained larvae were documented with a Nikon Eclipse E800 microscope and a Nikon Fi2-U3 camera. Additionally, larvae were scanned with a Leica DMI6000 CFS confocal laser scanning microscope equipped with a Leica TCS SP5 II scanning system. Specific NBT/BCIP stainings were detected with 633 nm laser wavelength reflection scanning [63]. Confocal stacks were edited using IMARIS 7.3.1 (Bitplane AG) and figure plates were assembled using Coral Graphic Suite X3 (Corel Corporation).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MF collected, reared and fixed the material together with TW, conducted cloning experiments, probe synthesis, in situ hybridization and microscopic documentation, wrote the initial drafts of the paper and prepared all figure plates. TW isolated RNA, prepared cDNA-libraries, initiated the transcriptome assembly and analysis, and contributed to data interpretation. AO and MF conducted the phylogenetic analysis. AW designed the study, supervised research and contributed to data interpretation and writing of the manuscript. All authors discussed the results, commented on and approved the final version of the manuscript.

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References
1. Lewis EB. A gene complex controlling segmentation in Drosophila. Nature. 1978;276:565–70.
2. Scott MP, Tamkun JW, Hartzell III GW. The structure and function of the homeodomain. Biochem Biophys Acta. 1989;989:25–48.
3. McGinnis W, Krumlauf R. Homeobox genes and axial patterning. Cell. 1992;68:283–302.
4. Caroll SB. Homeotic genes and the evolution of arthropods and chordates. Nature. 1995;376:479–85.
5. Ferrier DE, Holland PW. Ancient origin of the Hox gene cluster. Nat Rev Genet. 2001;2:33–8.
6. Derelle F, Holland PW. Ancient origin of the Hox gene cluster. Nat Rev Genet. 2001;2:33–8.
7. Garcia-Fernández J. Hox, ParaHox, ProtoHox: facts and guesses. Heredity (Edinb). 2005;94:145–52.
8. Wang BB, Müller-Immergluck MM, Austin J, Robinson NT, Chisholm A, Kényon J. A homeotic gene cluster patterns the anteroposterior body axis of C. elegans. Cell. 1993;74:29–42.
9. Ori H, Kato K, Umesono Y, Sakurai T, Agata K, Watanabe K. The planarian Hom/Hox/homeobox genes (Plox) expressed along the anteroposterior axis. Dev Biol. 1999;210:456–68.
10. Averof M, Akam M. Hox genes and the diversification of insect and crustacean body plans. Nature. 1995;376:420–3.
11. Averof M, Patel NH. Crustacean appendage evolution associated with changes in Hox gene expression. Nature. 1997;386:62–6.
12. Prince VE, Price A L, Ho RK. Hox gene expression reveals regionalization along the anteroposterior axis of the zebra fish notochord. Dev Genes Evol. 1998;208:517–22.
13. Peterson MD, Rogers BT, Popadic a, Kaufman TC. The embryonic expression pattern of labial, posterior homeotic complex genes and the headshield homologue in an annelid insect. Dev Genes Evol. 1999;209:77–90.
14. Hughes CL, Kaufman TC. Hox genes and the evolution of the arthropod body plan. Evol Dev. 2002;4:99:459–99.
15. Lowe CJ, Wu M, Salic A, Evans L, Lander E, Stange-Thomann N, et al. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell. 2003;113:853–65.
16. Irvine SQ, Martindale MQ. Expression patterns of anterior Hox genes in the polychaete Chaetopterus: correlation with morphological boundaries. Dev Biol. 2000;221:333–51.
17. Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, Steinmetz PRH, et al. Hox gene expression in larval development of the polychaetes Nereis virens and Platynereis dumerilii (Annelida, Lophotrochozoa). Dev Genes Evol. 2007;217:39–54.
18. Bakalenko N, Novikova E, Nesterenko AY, Kulakova M A. Hox gene expression during post larval development of the polychaete Alitta virens. EvoDevo. 2013;4:13.
19. Kocot KM, Cannon JT, Tödt C, Citaarella MR, Kohn AB, Meyer A, et al. Phylemogenics reveals deep molluscan relationships. Nature. 2011;477:452–65.
20. Smith SA, Wilson NG, Goetz FE, Feehery C, Andrade SCS, Rouse GW, et al. Resolving the evolutionary relationships of molluscs with polyplacophoran-like features. Nature. 2011;480:646–47.
21. Sutton MD, Briggs DEG, Swetter DJ, Swetter DJ, Sigwart JD. A Silianium armourade placophoran and implications for molluscan phylogeny. Nature. 2012;490:94–7.
22. Osca D, Irismi I, Tödt C, Grande C, Zardoya R. The complete mitochondrial genome of Scutopus ventrolineatus (Mollusca: Chaetodermomorpha) supports the Aculifera hypothesis. BMC Evol Biol. 2014;14:197.
23. Scherholz M, Redl E, Wollesen T, Tödt C, Wanninger A. Aplacophoromolluscs evolved from ancestors with polyplacophoran-like features. Curri Biol. 2013;23:2130–4.
24. Giusti AF, Himman VF, Degnan SM, Degnan BM, Morse DE. Expression of a Scl/Hox gene in the larval central nervous system of the gastropod Halocline, a non-segmental spiralian lophotrochozoan. Dev Evol. 2000;2:294–302.
25. Callaerts P, Lee PN, Hartmann B, Farhan C, Choy DWW, Ike K, et al. Hox genes in the cephalopod squid Euprymna scolopes: implications for the evolution of complex body plans. Proc Natl Acad Sci U S A. 2002;99:2088–93.
26. Himman VF, O’Brien ER, Richards GS, Degnan BM. Expression of anterior Hox genes during larval development of the gastropod Halocline asinina. EvoDevo. 2003;5:508–21.
27. Lee PN, Callaerts P, de Couet HG, Martindale MQ. Cephalopod Hox genes and the origin of morphological novelties. Nature. 2003;424:1061–5.
28. Canapa A, Biscotti MA, Olmo E, Barucca M. Isolation of Hox and ParaHox genes in the bivalve Pecten maximus. Gene. 2005;348:83–8.
29. Pérez-Parañé ML, Carpintero P, Pazos AJ, Abad M, Sánchez JL. The Hox gene cluster in the bivalve mussel Mytilus galloprovincialis. Biochem Genet. 2005;43:147–79.
30. Iijima M, Akiha N, Sakaiha S, Kuratani S, Endo K. Evolution of Hox genes in molluscs: a comparison among seven morphologically diverse classes. J Mol Evol. 2006;62:259–66.
31. Perrine M, Deutsch JS, Andouche A, Bouchet-Rodoni R, Bonnal A. Unexpected variation of Hox genes’ homeodomain in cephalopods. Mol Phylogenet Evol. 2006;40:872–9.
32. Biscotti MA, Canapa A, Olmo E. Hox genes in the Antarctic polycaspochopus Nucula(nuculanidae). J Exp Zool. 2007;308:507–13.
33. Samadi L, Steiner G. Involvement of Hox genes in shell morphogenesis in the encapsulated development of a top shell gastropod (Gibbula varia L.). Dev Genes Evol. 2009;219:523–30.
34. Samadi L, Steiner G. Expression of Hox genes during the larval development of the snail, Gibbula varia (L.) – further evidence of non-colinearity in molluscs. Dev Genes Evol. 2010;220:161–72.
60. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009;25:189–1191.
61. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61:539–42.
62. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 1992;8:275–82.
63. Jékely G, Arendt D. Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy. Biotechniques. 2007;42:531–5.