Molecular developmental studies of various bilaterians have shown that the identity of the anteroposterior body axis is controlled by Hox and ParaHox genes. Detailed Hox and ParaHox gene expression data are available for conchiferan mollusks, such as gastropods (snails and slugs) and cephalopods (squids and octopuses), whereas information on the putative conchiferan sister group, Aculifera, is still scarce (but see Fritsch et al., 2015 on Hox gene expression in the polyplacophoran Acanthochiton acarunita). In contrast to gastropods and cephalopods, the Hox genes in polyplacophorans are expressed in an anteroposterior sequence similar to the condition in annelids and other bilaterians. Here, we present the expression patterns of the Hox genes Lox5, Lox4, and Lox2, together with the ParaHox gene caudal (Cdx) in the polyplacophoran A. crinita. To localize Hox and ParaHox gene transcription products, we also investigated the expression patterns of the genes FMRF and Elav, and the development of the nervous system. Similar to the other Hox genes, all three Acr-Lox genes are expressed in an anteroposterior sequence. Transcripts of Acr-Cdx are seemingly present in the forming hindgut at the posterior end. The expression patterns of both the central class Acr-Lox genes and the Acr-Cdx gene are strikingly similar to those in annelids and nemerteans. In Polyplacophora, the expression patterns of the Hox and ParaHox genes seem to be evolutionarily highly conserved, while in conchiferan mollusks these genes are co-opted into novel functions that might have led to evolutionary novelties, at least in gastropods and cephalopods.
Aronowicz and Lowe, 2006) and Ecdysozoa (e.g., Wang et al., '93; Averof and Akam, '95; Averof and Patel, '97; Orii et al., '99; Peterson et al., '99). Detailed Hox and ParaHox gene expression data within the third bilaterian superclade, Lophotrochozoa, are available to a much lesser degree such as for various annelid and two nemertean species (Nardelli-Haefliger and Shankland, '92; Nardelli-Haefliger et al., '94; Wong et al., '95; Kourakis et al., '97; Irvine and Martinbide, 2000; Kulakova et al., 2007; Fröbius et al., 2008; Bakalenko et al., 2013; Gharbaran et al., 2013; Hiebert and Maslakova, 2015a,b).

As for the majority of lophotrochozoan phyla, however, in Mollusca, the phylum with the widest spectrum of body plans, research into Hox and ParaHox gene expression is still in its infancy (Biscotti et al., 2014; Wanninger and Wollesen, 2015). A maximum of 11 Hox and three ParaHox genes were identified in gastropods (Giusti et al., 2000; Barucca et al., 2003, 2006; Hinman et al., 2003; Canapa et al., 2005; Pérez-Parallé et al., 2005; Iijima et al., 2006; Pernice et al., 2006; Biscotti et al., 2007; Samadi and Steiner, 2010a,b; Simakov et al., 2013) and, most recently, in one bivalve (Takeuchi et al., 2016), 11 Hox and two or three ParaHox genes in other bivalves (Barucca et al., 2003, 2006; Canapa et al., 2005; Pérez-Parallé et al., 2005; Iijima et al., 2006; Pernice et al., 2006; Biscotti et al., 2007; Zhang et al., 2012; De Oliveira et al., in review), nine Hox and two ParaHox genes in scaphopods (Iijima et al., 2006; Wollesen et al., 2015a), and ten Hox and three ParaHox genes in cephalopods (Callaerts et al., 2002; Lee et al., 2003; Iijima et al., 2006; Pernice et al., 2006; Biscotti et al., 2007; Wollesen et al., 2015a). In the octopod Octopus bimaculoides, eight Hox genes (the number of the ParaHox genes remains unknown) were identified (Albertin et al., 2015). Within the aculiferans, in Polyplacophora nine Hox and three ParaHox genes (Barucca et al., 2006; Iijima et al., 2006; Biscotti et al., 2007), in Solenogastres seven to eight Hox genes, and in Caudofoveata four ParaHox genes were identified, and at least one ParaHox gene in the latter taxon (Iijima et al., 2006). However, detailed data on the temporal expression of these genes are known for very few species only (Giusti et al., 2000; Hinman et al., 2003; Lee et al., 2003; Le Gouar et al., 2003; Samadi and Steiner, 2009, 2010a,b; Focareta et al., 2014; Fritsch et al., 2015).

In contrast to other bilaterians, the gastropod and cephalopod Hox and ParaHox gene expression data suggest that these genes have been co-opted into the formation of distinct organs such as the mantle, shell, radula, or the light organ of certain squids (Giusti et al., 2000; Hinman et al., 2003; Lee et al., 2003; Le Gouar et al., 2003; Samadi and Steiner, 2009, 2010a,b; Focareta et al., 2014). Recent data on the polyplacophoran Acanthochitona crinita showed that the Hox genes have preserved their hypothetical ancestral mode of expression, which is in a colinear manner along the anteroposterior axis (Fritsch et al., 2015). Herein, we describe the expression of the three missing lophotrochozoan-specific central class Hox genes Lox5, Lox4, and Lox2, together with the ParaHox gene caudal (Cdx), which is often believed to have a function in hindgut formation (Brooke et al., '98; Holland, 2001; de Rosa et al., 2005; Kulakova et al., 2008; Hui et al., 2009), in the polyplacophoran A. crinita.

**MATERIALS AND METHODS**

**Collection, Fixation, and Terminology**

Adult individuals of Acanthochitona crinita were collected in the intertidal zone along the coastline of the Biological Station Roscoff in France. Spawning was induced by water temperature variations and sun light exposure. Eggs were fertilized with a concentrated sperm solution for 30 min and reared at 21–23°C. Animals were fixed in 4% paraformaldehyde in MOPS buffer, dehydrated by a graded methanol series, and stored in 100% methanol at −20°C (see Fritsch et al., 2015). For whole-mount immunostaining, larvae were fixed in 4% paraformaldehyde for 45 min at room temperature, dehydrated, and stored in 100% methanol at 4°C.

The entirely lecithotrophic larval development was divided into three different larval stages. Early-stage trochophore larvae are equipped with an apical tuft and a prototroch, which divides the larva into an anterior episphere and a posterior hyposphere. Mid-stage trochophore larvae are slightly longer than the earliest stage, about 280 μm, and the anlagen of the ventral foot and the dorsal shell plates are discernible in the hyposphere region. Late-stage trochophore larvae are approximately 360 μm in length and seven dorsal shell plate anlagen are present in the hyposphere. At the end of larval development, larvae undergo metamorphosis and commence their benthic lifestyle. Herein, terminology and descriptive larval terms are used following Fritsch et al. (2015).

**Orthology Assignment and Phylogenetic Analysis**

Local similarity searches with amino acid sequences of other organisms retrieved from NCBI against a transcriptome of A. crinita (Trinity assembled) were performed using the program Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand). The multiple amino acid sequence alignment of the herein identified Lox genes and the Cdx gene (NCBI accession numbers: Acr-Lox5, KU960944; Acr-Lox4, KU960945; Acr-Lox2, KU960946; Acr-Cdx, KU960947), the already identified Hox genes in A. crinita (see Fritsch et al., 2015), and their metazoan orthologs was performed with the program mafft v7.221 (Katoh et al., 2005), while Jalview 2 (Waterhouse et al., 2009) was used to illustrate the alignment (Fig. 1). For identification of the homodomain sequences of A. crinita, a maximum likelihood analysis using a Jones–Taylor–Thornton (Jones et al., '92) amino acid substitution model with 1,000 replicates was performed within the RAxML v7.2.6 software (Stamatakis, 2006) (Fig. 2).

**Molecular and Immunostaining Experiments**

Specific Acr-Lox gene and Acr-Cdx primers were designed with Geneious 6.1.6. PCR amplifications, cloning and ligation
| protein | homebox domain |
|--------|----------------|
| Eac-Hox | EAGQGST RFTNL TELKVE FYNKKI LTRARIE AAIAAAA | 1 4 2 3 8 | 1 4 2 3 8 |
| Gva-Hox | YAGIIGN STGSN NWFKNAFFK EKEFKNF KFTRARRT IEAIAGGNT NTVNFKF KKFKRRGQ - 1 4 2 3 8 |
| Lna-Hox | NMRITFSKTL EEFKNT LTRARIE AAGNTN EVNOKNOM IRKRMKESQ |
| Lsa-Hox | FAGOPGTN RFTNL TELKVE FYNKKI LTRARIE IAAGNTN EVNOKNOM IRKRMKETN |
| Nvi-Hox | YTPQPMIFRTNFNL TELKVE FYNKKI LTRARIE IAAGNTN EVNOKNOM IRKRMKETN |
| Sro-Hox | YTPQPMIFRTNFNL TELKVE FYNKKI LTRARIE IAAGNTN EVNOKNOM IRKRMKETN |
| Dme-Hox | SQRKKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQRKKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Tdv-Hox | EKQKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Dme-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQRKKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | PGKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Eka-Hox | AEKQGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Gpa-Hox | GQRRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Nvi-Hox | SQKRGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
| Pvu-Hox | QSGGKGK KRTAF RFTNL TELKVE FYNKKI LTRARIE AAGNTN EVNOKNOM |
A. crinita in situ hybridizations, Diagnostics GmbH, Mannheim, Germany). For whole-mount were designed with a DIG-labeling kit (#11277073910, Roche and were performed as described in Fritsch et al. (2015). Probes were designed with a DIG-labeling kit (#11277073910, Roche Diagnostics GmbH, Mannheim, Germany). For whole-mount in situ hybridizations, A. crinita larvae were decalcified, pre-treated with proteinase-K solution, and washed several times in phosphate buffer-based solutions. For reduction of probe charge, larvae were incubated in a 1% triethanolamine and 0.5% acetic anhydride solution. After preincubation overnight in 100% hybridization buffer, larvae were hybridized with a probe concentration of 0.25 ng/μL at 60°C in a water bath for 48 hr. Subsequently, larvae were washed and rinsed with a descending SSC washing buffer, then several times in a maleic acid buffer-based solution. The digoxigenin antibody conjugated to alkaline phosphatase incubation (#11093274910, Roche; 1:5,000 dilution) was carried out overnight at 4°C and for transcript visualization larvae were transferred into a color reaction buffer (7.5% polyvinyl alcohol with 2% NBT/BCIP (#11681451001, Roche) for 45–60 min. Larvae were cleared in a 1:1 benzylalcohol:benzylbenzoate solution (for further details, see Fritsch et al., 2015).

For staining of neural components, larvae were pretreated in a 4% Triton-X 100 in PBS solution. To label acetylated α-tubulin structures, a monoclonal mouse primary antibody (#T6793, Sigma-Aldrich, St. Louis, Missouri, USA; 1:250 dilutions in PBT for 48 hr) together with an Alexa680-coupled mouse secondary antibody (#A11004, Invitrogen, Carlsbad, CA, USA; dilution 1:300 in PBT for 48 hr) was used. The neurotransmitter serotonin was labeled with a polyclonal rabbit primary antibody (#S5545, Sigma; 1:250 dilutions in PBT for 48 hr) together with an Alexa633-coupled rabbit secondary antibody (#A21070, Invitrogen; dilution 1:300 in PBT for 48 hr). SYBR Green-I Nucleic Acid Gel Stain was used as nuclear counterstain (#S-7567, Thermo Fisher Scientific, Waltham, MA, USA; 1:600 dilutions in PBS for 60 min). Specimens were mounted on microscope slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and scanned with a Leica DMi6000 CFS microscope equipped with a Leica TCS SP5 II scanning system (Leica Microsystems, Wetzlar, Germany). Scans were edited with IMARIS 7.3.1 (Bitplane, Zurich, Switzerland) and figures were designed using Corel Graphic Suite X3 (Corel Corporation, Ottawa, Canada).

RESULTS

Hox and ParaHox Gene Expression in A. crinita

The Hox genes Acr-Lar5, Lar3, and Lar2 and the ParaHox gene AcrCdax are expressed in distinct domains of early-stage A. crinita trochophore larvae. All three Hox genes are expressed in the posterior region of the ventral hypophore (Figs. 3A–C, G–I, and M–O). The strongest expression pattern is that of Acr-Lar4, which is present in two prominent epidermal and subepidermal cellular strands at the posterior pole of the larva (Figs. 3G–I). The expression pattern of Acr-Cdx in early-stage trochophore larvae is restricted to a subepidermal spot near the posterior pole of the larval body (Figs. 3S–U).

In mid-stage trochophore larvae, transcripts of Acr-Lar5 are largely distributed in epidermal and subepidermal cell layers in the ventral hypophore. Expression occurs in two prominent domains in the central and posterior region of the hypophore (Figs. 3D–F and 4A–D). Transcripts are also present in individual ventrolateral cells, immediately posterior to the prototroch, and in several cells on the dorsal side of the hypophore (Fig. 3F). In the episphere, four pairs of Acr-Lar5 transcript-containing cells are present (Figs. 3D and F). Two pairs of ventrolateral cells and two pairs of dorsolateral cells are identifiable (Figs. 3D, F, and 4A–D, black and white arrowheads). In mid-stage trochophore larvae, Acr-Lar3 is expressed in the posterior hypophore in two parallel epidermal and subepidermal expression domains (Figs. 3J–L and 4E–H). Small Acr-Lar3 expression domains are present subepidermally in the dorsohypophore (Figs. 3F and H). The expression pattern of Acr-Lar2 in the posterior hypophore is less prominent than that of Acr-Lar4. Two slender subepidermal cellular domains are present ventrally, and dorsally a faint Acr-Lar2 subepidermal expression domain is discernible (Fig. 3P–R and 4I–L). The expression pattern of Acr-Cdx in mid-stage trochophore larvae is still only detectable in a single subepidermal expression domain near the posterior pole of the larval body, most likely in the developing posterior digestive system (hindgut) (Figs. 3V–X and 4M–P).

In late-stage trochophore larvae, the expression levels of all four genes gradually decrease (Figs. 5A–L). In late trochophore larvae, Acr-Lar5 transcripts are only present in some ventral subepidermal cells within the foot region (Figs. 5A–C). A faint Acr-Lar4 expression is present in late-stage trochophore larvae.
Figure 2. Phylogeny of homeodomain genes. Phylogeny of Hox genes and Cdx gene families from amino acid sequences present in the homeodomain. The best fit tree was inferred by a maximum likelihood phylogenetic analysis with the RAxML v7.2.6 software; bootstrap support values over 90% are displayed. All identified Acanthochitonacrinita Hox and ParaHox genes within the respective gene clusters are highlighted by colored arrows. For the genes of interest of our study, the Lox and Cdx genes are highlighted by colored brackets. All A.crinita Hox and ParaHox gene sequences cluster with appropriate bilaterian Hox gene orthologs. The homeotic genes distal-less and eneigned of Platynereis dumerilii and Drosophila melanogaster are used as outgroups.

in ventral epidermal and subepidermal cells of the posterior foot region (Figs. 5D–F). No Acr-Lox2 expression was found in late-stage trochophore larvae (Figs. 5G–I). In late-stage trochophore larvae Acr-Cdx is still, albeit weakly, expressed in subepidermal cells in the region of the prospective hindgut (Figs. 5J–L).

Elav and FMRF Expression in the Developing Nervous System of A. crinita
In trochophore larvae of A. crinita, a developing tetranerous nervous system is present (Figs. 6A–D). Immunostaining against 5HT (serotonin) revealed an apical organ (consisting of most probably monopolar neurons and a neuropil) and the anlage of the cerebral commissure at the anterior pole of the larva (Fig.6B). Posterior to the commissure, four longitudinal neurite bundles (two ventromedial pedal and two ventrolateral visceral nerve cords) interconnected by transversal commissures are present (Figs. 6A and B). In addition to that, by using antibodies against α-acetylated tubulin, the tubulin-containing cells of the polyplacophoran-specific larval ampullary sensory system (Haszprunar et al., 2002) was labeled in the anterior region of the episphere (Figs. 6C and D). Altogether, four ventralateral and four dorsomedial tubulin-containing cells are present.
Figure 3. Acr-Lox and Acr-Cdx gene expression pattern in early- and mid-stage trochophore larvae of Acanthochitona crinita. Apical faces up. The expression pattern of each gene is depicted in early- (upper row) and mid-stage (bottom row) trochophore larvae of A. crinita, respectively (scale 50 μm). (A–F) Acr-Lox5 is expressed in epidermal and subepidermal cells mainly in the posterior part of the ventral hyposphere. In the ventral episphere, four Acr-Lox5-positive cells are present in the same area as the FMRF-positive cells and the ampullary sensory system. Dorsally in the episphere (white arrows) and hyposphere single Acr-Lox5 transcript-containing cells are present. (G–H) The expression pattern of Acr-Lox4 in the hyposphere appears farther posterior than the expression of Acr-Lox5. In mid-stage trochophore larvae, Acr-Lox4 transcripts are mainly distributed ventrally in epidermal and subepidermal cells of the posterior hyposphere region. (M–R) Small domains of Acr-Lox2 transcripts are present ventrally in subepidermal cells at the posterior end of the hyposphere, slightly posterior to that of Acr-Lox4. (S–X) Acr-Cdx is expressed in central subepidermal cells of the prospective developing hindgut at the posterior end of the hyposphere.

ao, apical organ; ds, dorsal shell plates; f, foot; pt, prototroch.
The Elav expression pattern in trochophore larvae of A. crinita appears to colocalize with the immunostaining of the terebranular nervous system (Figs. 6E–J). Ventrally in the episphere, transcripts of Acr-Elav are present in two distinct domains, postero-laterally to the apical organ (Figs. 6E and F). The prototroch region is devoid of Acr-Elav expression (Fig. 6E). In the ventral hyposphere, two prominent putative neuroectodermal medial longitudinal and two slender, more laterally positioned longitudinal expression strands are present (Figs. 6E and H).

Transcripts of the FMRF gene in larvae of A. crinita are mainly present in the epidermal cell layers of the episphere (Figs. 6K–P). Two pairs of ventrolateral (Figs. 6K and N) and two pairs of dorsomedial cells (Figs. 6M and P) contain Acr-FMRF transcripts. These Acr-FMRF-containing cells appear to colocalize with the cells of the ampullary sensory system.

**DISCUSSION**

**Identification of Hox and ParaHox Genes in A. crinita**

The identification of a maximum of 11 Hox genes and three ParaHox genes in mollusks and annelids as their potential sister group might suggest that this was the situation in the last common ancestor of mollusks (Biscotti et al., 2014; Wanninger and Wollesen, 2015; Takeuchi et al., 2016). However, in the transcriptome of A. crinita, only ten Hox genes and one ParaHox gene were identified (present study; Fritsch et al., 2015; De Oliveira et al., in review). Although the gene Post1 was identified in almost all molluscan class-level lineages (Iijima et al., 2006), in A. crinita it was not found. Comprehensive BLAST searches and annotation investigations were performed with the program BUSCO (v1.1). BUSCO enables similarity searches between a transcriptome and a set of orthologous genes conserved in the Metazoa (Simão et al., 2015). The results showed that in the transcriptome of A. crinita about 95% conserved orthologous genes were identified, indicating that the transcriptome has a great depth and is almost complete. The definite presence or absence of Post1 and the two Parahox genes in A. crinita may only—if at all—be assessed once the genome of the species becomes available.

**Hox gene expression in putative (neuro)-ectodermal domains of polyplacophorans**

Hox and Parahox genes are key determinants for the formation of the anteroposterior body axis in the vast majority of bilaterian animals (e.g., Holland, 2001; Hughes and Kaufman, 2002; Garcia-Fernández, 2005; Fröbisch and Seaver, 2006; Aronowicz and Lowe, 2006; Kulakova et al., 2007, 2008; Hui et al., 2009; Fritsch et al., 2015). Expression studies show that these homeotic genes are also mainly expressed in the forming cells of the ectoderm, in particular the neuroectoderm (e.g., Kourakis et al., ’97; Hinman et al., 2003; Lee et al., 2003; Lowe et al., 2003; Kulakova et al., 2007, 2008; Samadi and Steiner, 2009, 2010a,b; Bakalenko et al., 2013). To localize Acr-Lox gene transcripts in ectodermal and neuroectodermal derivatives of A. crinita, the formation of the nervous system was also documented by immunostaining techniques and by analyzing the gene expression patterns of Elav and FMRF. The Elav protein is first detectable in young neurons and studies in the fruit fly Drosophila revealed that Elav is not detected in other tissue types (e.g., Robinow and White, ’91; Berger et al., 2003).

The expression domains of the genes Acr-Lar5, Lar4, and Lar2 in the ventroposterior hyposphere overlap partly with the terebranular nervous system. Within the area of the posterior developing pedal nerve cords, also the three Acr-Lox genes are expressed. In addition, in the episphere of A. crinita larvae, the Acr-Lar5-containing ventral and dorsal cells most probably colocalize with the ventral and dorsal cells of the ampullary sensory system. Thus, together with the homeotic gene Pax2/5/8/B, Acr-Lar5 also seems to play a role in the formation of the ampullary sensory system (present study; Wollesen et al., 2015b).

Transcripts of Acr-Elav in larvae of A. crinita are present within the area of the forming terebranular nervous system. In particular, in the hyposphere, all four longitudinal nerve cords overlap with the Acr-Elav expression pattern. Furthermore, transcripts of Acr-Elav in the posterior hyposphere also

---

**Figure 4.** Acr-Lox and Acr-Cdx transcript distribution pattern in mid-stage trochophore larvae of Acanthochitona crinita. Three-dimensional reconstruction and localization of the specific gene expression pattern (yellow) within mid-stage trochophore larvae of A. crinita. Morphology of the larvae is presented by autofluorescence images (cyan). From left to right, first column: longitudinal, second column: sagittal, third column: laterosagittal, and the forth column: transversal plane. (A–D) Acr-Lox5 transcription products present in ventral and dorsal subepidermal cell layers. In the ventral episphere, four single Acr-Lox5 transcript-containing cells are present (white arrows) in the same area as Acr-FMRF and ampullary sensory cells. In the dorsal episphere, also four single Acr-Lox5-positive cells (black arrows) are present. (E–H) Acr-Lox4 expressed within ventral and dorsal subepidermal cell layers in the posterior part of the hyposphere. (I–L) The expression pattern of Acr-Lar2 in ventral and dorsal subepidermal cell layers at the posterior end of the hyposphere. (S–X) Acr-Cdx transcripts are present in subepidermal cells in the region of the prospective hindgut: a, apical; aa, ab-apical; ao, apical organ; at, apical tuft; d, dorsal; ds, dorsal shell plates; f, foot; mo, mouth opening; pt, prototroch; st, stomodaeum; trb, trochoblast(s); v, ventral.
Figure 5. Acr-Lox and Acr-Cdx gene expression patterns in late trochophore larvae of Acanthochiton acrinita. In late-stage trochophore larvae, the expression level of all Acr-Lox and the Acr-Cdx gene gradually decreases (scale 50 μm). (A–C) Acr-Lox5 transcripts are only present in ventral epidermal cells within the ventral foot region. (D–F) Acr-Lox4 is weakly expressed in epidermal and subepidermal cells within the posterior ventral foot region. (G–I) Acr-Lox2 shows no expression signal in late-stage trochophore larvae. (J–L) Acr-Cdx is weakly expressed in subepidermal cells in the prospective hindgut at the posterior end of the larva. ao, apical organ; ds, dorsal shell plates; f, foot; pt, prototroch.
Figure 6. Nervous system staining and the Acr-Elav/Acr-FMRF expression patterns in mid-stage trochophore larvae of *Acanthochitona crinita*. (A–D) Immunostaining of the nervous system (serotonin and acetylated α-tubulin) in trochophore larvae. (E–J) Expression pattern of the Acr-Elav in mid-stage trochophore larvae. (K–P) Expression pattern of Acr-FMRF in mid-stage trochophore larvae. (H–J, N–O) From left to right, longitudinal, sagittal, and transversal plane. (A) Serotonin-like immunoreactive (ir) labeled tetraneural nervous system (green) and tubulin-containing cilia (white/red). (B and B’) Detailed serotonin-positive tetraneural nervous system. Anteriorly, apical organ consisting
colocalize with the medial longitudinal expression domains of the genes Acr-Lox5, Lox4, and Lox2.

Transcripts of Acr-FMRF in larvae of A. crinita are present within the ventral and dorsal ampullary sensory cells in the episphere. The matching expression pattern of Acr-FMRF and Acr-Lox5 further substantiates the assumption that the transcripts of this gene are also localized within the cells of that particular sensory structure.

Altogether, the expression patterns of the genes Acr-Lox5, Lox4, and Lox2 overlap and colocalize partly with the developing nervous system and with the expression patterns of Acr-Elav and Acr-FMRF. Thus, the herein investigated Acr-Lox genes are primarily expressed in ectodermal and neuroectodermal domains, a condition which is similar to the other Hox genes in A. crinita (see Fritsch et al., 2015). Nevertheless, as already mentioned for the formerly studied Hox genes in A. crinita, the presence of Acr-Lox gene transcription products in endo- and mesodermal cell layers cannot be excluded. Next to the tissue of the developing nervous system, transcripts from all three Lox genes are also present within the ventral region of developing muscle fibers and within the central area of the forming digestive tract (see also Fritsch et al., 2015).

Comparison of Lox Gene Expression within Mollusca

To date, Lox gene expression studies in mollusks are only available for the gastropod Gibbula varia and the cephalopod Euproymna scolopes (Lee et al., 2003; Samadi and Steiner, 2010a). Similar to the gastropod G. varia, the first transcription products of Acr-Lox5, Lox4, and Lox2 were found in early-stage trochophore larvae of A. crinita immediately after hatching. In early- and mid-stage trochophore larvae of A. crinita, all three Lox genes are expressed predominantly in the ventral ectoderm of the posterior hyposphere. Only Acr-Lox5 is additionally expressed in the episphere, namely in paired ventral and dorsal ectodermal cells. In contrast, in trochophores of G. varia, Gva-Lox5, Lox4, and Lox2 are expressed either in ectodermal cells in the episphere (Gva-Lox5 and Lox2), in the apical organ, and later in the forming cerebral ganglion (Gva-Lox5, Lox4, and Lox2), or in the ciliated cells of the prototroch and later within the velum (Gva-Lox4 and Lox2) (Samadi and Steiner, 2010a).

Overall, the expression patterns of all three Lox genes differ significantly between gastropods and polyplacophorans with only Lox5 showing a congruent expression pattern. In both G. varia and A. crinita, Lox5 is expressed in the episphere. Nevertheless, in A. crinita, Lox5 transcripts are present in four ventral and four dorsal cells, whereas Gva-Lox5 is expressed in two ventral and two dorsal cells at the base of the apical organ (Samadi and Steiner, 2010a).

In late-stage trochophore larvae of A. crinita, the expression patterns of Acr-Lox5 and Lox4 are rather faint and restricted to ventral ectodermal cells in the hyposphere. Transcripts of Acr-Lox2 seem to be entirely absent. In contrast, in pre- and posttorsional veliger stages of G. varia, all three Lox genes are prominently expressed in the cerebral ganglion and velum (Samadi and Steiner, 2010a). In the cephalopod E. scolopes, the gene Esc-Lox4 is expressed in parts of the central nervous system, within the pedal ganglion, and Esc-Lox5 shows an expression pattern in the brachial crown (Lee et al., 2003). The expression pattern of Lox2 during cephalopod development is still unknown (Lee et al., 2003; Wanninger and Wollesen, 2015 for review).

Altogether, the Acr-Lox gene expression pattern in Polyplacophora compared with that in the gastropods and cephalopods indicates that the central Hox genes Lox5, Lox4, and Lox2 in A. crinita seem to be primarily expressed in the (neuro-)ectodermal cells or cell layers that contribute to the formation of neural tissues, but not exclusively in distinct structures of the nervous system, such as the apical organ or the cerebral commissure. Instead, in the polyplacophoran A. crinita, Lox genes are expressed in an anteroposterior colinear manner (Fig. 7). In accordance with the other polyplacophoran Hox genes, the Acr-Lox genes are also expressed in defined body regions along the anteroposterior axis (Fritsch et al., 2015). This is in contrast to the condition found in conchiferan mollusks but resembles the condition found in other bilaterians (Lewis, ’78; Scott et al., ’89; McGinnis and Krumlauf, ’92; Wang et al., ’93; Carroll, ’95; Prince et al., ’98; Orii et al., ’99; Ferrier and Holland, 2001;
Hughes and Kaufman, 2002; Lowe et al., 2003; Wray et al., 2003; Garcia-Fernández, 2005). The opposing gastropod and cephalopod Lox gene expression patterns within distinct nervous system structures (e.g., apical organ, cerebral ganglia) or in locomotion tissues/structures (e.g., trochoblasts of the prototroch, brachial crown) indicate co-option and functional plasticity of these genes at least in both conchiferan representatives.

Interestingly, throughout larval development of A. crinita, Acr-Lox5 is the only Hox gene that is expressed within the region of the episphere, however, not in cells of the apical organ. The entire lack of Acr-Hox gene transcripts in the apical organ in Polyplacophora (see Fritsch et al., 2015 and herein) contradicts the hypothesis of Marlow and colleagues (2014) that Hox genes generally play a role in the formation of the apical organ in planktonic ciliated larvae, at least for this molluscan clade.

Comparative Analysis of Hox Gene Expression in Polyplacophora and Other Lophotrochozoans

As in the polyplacophoran A. crinita, the Lox genes in polychaete annelids show a strict colinear anteroposterior expression pattern in the hyposphere of early trochophore larvae. In early trochophores of Nereis virens (now renamed as Alitta virens), the Lox5 transcript is present in a similar ventral and posterior region of the hyposphere (Kulakova et al., 2007). Nvi-Lox4 and Nvi-Lox2 are first expressed in the pygidial area of Nereis (Alitta) neotrochaete larvae and these genes do not seem to be involved in the formation of the presegmented larval body. Thereby, Nvi-Lox4 and Nvi-Lox2 are also expressed in a colinear manner (Kulakova et al., 2007). A colinear anteroposterior Lox gene expression pattern is also observed in other annelids, namely the sedentary Capitella teleta and the hirudinean Hirudo medicinalis and Helobdella triserialis (Nardelli-Haefliger and Shankland, ’92; Kourakis et al., ’97; Gharbaran et al., 2013).

Together with the remaining Hox genes (see Fritsch et al., 2015), the Lox gene transcripts appear predominantly during the patterning processes in early- and mid-stage trochophore larvae in A. crinita. As the other Hox genes in A. crinita, also the Acr-Lox genes do not appear to be restricted to the ectodermal expression domains. Instead, they also seem to be present in developing endo- and mesodermal tissues (see also Fritsch et al., 2015). A similar expression domain of Lox genes in all three germ layers is present in early larval stages, prior to the onset of segmentation, of the polychaetes Chaetopterus sp., C. teleta, Nereis (Alitta) virens, and Platynereis dumerilii. Thereby, the transcripts of the remaining Hox genes are also present in endo- and mesodermal cell layers in early trochophore larvae (Irvine and Martindale, 2000; Kulakova et al., 2007; Fröbius et al., 2008). Correspondingly, in early and presegmental embryonic stages of H. medicinalis, Helobdella robusta, and H. triserialis, Hox gene expression appears in all three germ layers (Nardelli-Haefliger and Shankland, ’92; Kourakis et al., ’97). Later, during segment formation processes in meta-trochophore larvae of Chaetopterus sp., C. teleta, Nereis (Alitta) virens, and P. dumerilii, and in late embryonic stages of H. medicinalis, H. robusta, and H. triserialis, Hox gene expression appears particularly in the germ layers of newly differentiating segments in an anteroposterior gradient.
(Nardelli-Haeffliger and Shankland, ’92; Nardelli-Haeffliger et al., ’94; Wong et al., ’95; Kouarakis et al., ’97; Irvine and Martindale, 2000; Kulakova et al., 2007; Fröbius et al., 2008; Bakalenko et al., 2013; Gharbaran et al., 2013). In contrast, in late-stage trochophores of A. crinita, all identified Hox genes (including Lox genes) are only weakly expressed or expression is entirely lacking. During this stage, the anlagen of all major serially arranged muscular and neural features are established and are subsequently further elaborated during postmetamorphic development (Friedrich et al., 2002; Voronezhskaya et al., 2002; Wanninger and Haszprunar, 2002; Scherholz et al., 2013).

Apart from the annelids, the only other detailed and comprehensive lophotrochozoan Hox gene expression data are available for the nemertean species Micrura alaskensis and Puntionemertes californiensis. Although in the pilidiophoran species M. alaskensis a clear anteroposterior Hox gene expression gradient is present only in the developing juvenile stages (but not during larval development), in the hoplonemertean species P. californiensis the genes Hox1-Hox4, Lox5, and Post2 are clearly expressed in larval and juvenile stages in a manner that suggests colinearity (Hiebert and Maslakova, 2015a,b). The pilidiophoran larva is considered an evolutionary novelty that may be patterned by genetic mechanisms other than the Hox genes (Hiebert and Maslakova, 2015a). Lox genes are similarly expressed in Nemertea, Polychaetophora, and Annelida, that is, near the posterior end of the larval or juvenile body (Hiebert and Maslakova, 2015a,b).

The comparison of the Hox and Lox gene data of A. crinita with data on the well-investigated Annelida and Nemertea clearly revealed that Hox genes are expressed in a similar anteroposterior pattern during their early larval development (Nardelli-Haeffliger and Shankland, ’92; Nardelli-Haeffliger et al., ’94; Wong et al., ’95; Kouarakis et al., ’97; Irvine and Martindale, 2000; Kulakova et al., 2007; Fröbius et al., 2008; Bakalenko et al., 2013; Gharbaran et al., 2013; Hiebert and Maslakova, 2015a,b). This anteroposterior expression pattern is in stark contrast to the condition found in Gastropoda and Cephalopoda.

Comparative Aspects of Cdx Expression in Lophotrochozoa

The ParaHox gene Cdx (caudal) is often thought to pattern the posterior region of the digestive tract in bilaterian animals (Brooke et al., ’98; Holland, 2001; de Rosa et al., 2005; Fröbius and Seaver, 2006; Kulakova et al., 2008; Hui et al., 2009; Samadi and Steiner, 2010b; Altenburger et al., 2011). In addition, ParaHox gene expression studies in the gastropod O. bimaculoides (Albertin et al., 2015) have shown that Hox genes in this species are not arranged in a single cluster, which is in line with the nonanterior–posterior Hox gene expression pattern in gastropods and cephalopods. Whether this phenomenon of noncolinearity combined with distinct structural Hox gene expression domains was already present in the last common ancestor of Conchifera or is restricted to gastropods and cephalopods remains open until data on the scaphopods and bivalves become available. The Cdx expression pattern in the region of the forming hindgut of A. crinita is strikingly similar to that in chordates, ecdysozoans, and other lophotrochozoans and suggests an evolutionarily conserved function of Cdx in posterior digestive tract formation in bilaterian animals.

In A. crinita, Cdx is expressed in all trochophore stages in the posteromedian hyposphere that probably represents an ectodermal domain and forms the prospective hindgut. This expression pattern is very similar to that of the trochophores of the gastropod G. varia and Patella vulgata, the polychaete annelids Nereis (Allitta) virens and P. dumerilii, the hoplonemertean P. californiensis, as well as the brachiopod Terebratalia transversa (Le Gouar et al., 2003; Kulakova et al., 2008; Hui et al., 2009; Samadi and Steiner, 2010b; Altenburger et al., 2011; Hiebert and Maslakova, 2015b). In G. varia and P. vulgata, Gva-Cdx and Pva-Cdx are also expressed in the cells of the neuroectoderm and the mesoderm (Le Gouar et al., 2003; Samadi and Steiner, 2010b). In P. dumerilii, Pdu-Cdx is also expressed in mesodermal and potentially also in endodermal precursors (Hui et al., 2009). In A. crinita, such an expression is absent; however, the posterior Cdx expression in the trochophores of A. crinita matches the spatial expression pattern in most other protostomes (Brooke et al., ’98; Ferrier and Holland, 2001; de Rosa et al., 2005; Kulakova et al., 2008; Hui et al., 2009; Samadi and Steiner, 2010b; Altenburger et al., 2011; Hiebert and Maslakova, 2015b). Thus, the ParaHox gene Cdx seems to be involved in the formation of the posterior digestive system in the polyplacophoran A. crinita.

CONCLUSIONS

As previously shown for the Hox genes Acr-Hox1-5, Acr-Hox7, and Acr-Post2, the Acr-Lox genes are likewise expressed in a distinct anteroposterior manner in the polyplacophoran mollusk A. crinita, similar to the expression pattern in annelids and other bilaterians. This pattern differs from the expression in Gastropoda and Cephalopoda. These findings suggest that the Hox genes are involved in anteroposterior body axis patterning in Polyplacophora, similar to the proposed ancestral role of bilaterian Hox genes. The co-option of Hox genes into the formation of specific morphological features seems to be a characteristic of Conchifera, at least of gastropods and cephalopods. Recent genomic data from the octopod O. bimaculoides (Albertin et al., 2015) have shown that Hox genes in this species are not arranged in a single cluster, which is in line with the nonanterior–posterior Hox gene expression pattern in gastropods and cephalopods. Whether this phenomenon of noncolinearity combined with distinct structural Hox gene expression domains was already present in the last common ancestor of Conchifera or is restricted to gastropods and cephalopods remains open until data on the scaphopods and bivalves become available. The Cdx expression pattern in the region of the forming hindgut of A. crinita is strikingly similar to that in chordates, ecdysozoans, and other lophotrochozoans and suggests an evolutionarily conserved function of Cdx in posterior digestive tract formation in bilaterian animals.
ACKNOWLEDGMENTS

This work was supported by the German Science Foundation (Deutsche Forschungsgemeinschaft [DFG]; project FR 3392/1-1 to M. F.) and the ASSEMBLE program (grant agreement no. 227799 to M. F.). T. W. was also supported by ASSEMBLE (grant agreement no. 835 [SBR-1]) while collecting developmental stages of A. crinita. A. W. was supported by a grant of the Austrian Science Fund (FWF) on evolutionary development of putative basal mollusks (grant number P24276-B22). We gratefully acknowledge helpful comments from A. L. de Oliveira (Vienna) on phylogenetic analyses, the support by the Faculty of Life Sciences, University of Vienna, and the staff of the Biological Station Roscoff (France) for logistic support.

LITERATURE CITED

Albertin CB, Simakov O, Mitros T, Wang ZY, Pungor JR, Edsinger-Gonzales E, Brenner S, Ragsdale CW, Rokhsar DS. 2015. The octopus genome and the evolution of cephalopod neural and morphological novelties. Nature 524:220–226.

Altenburger A, Martinez P, Wanninger A. 2011. Homeobox gene expression in Brachiopoda: the role of Not and Cdx in body plan patterning, neurogenesis, and germ layer specification. Gene Expr Patterns 11:427–436.

Aronowicz J, Lowe CJ. 2006. Hox gene expression in the hemichordate Saccoglossus kowalevskii and the evolution of deuterostome nervous systems. Integr Comp Biol 46:890–901.

Averof M, Akam M. 1995. Hox genes and the diversification of insect and crustacean body plans. Nature 376:420–423.

Averof M, Patel NH. 1997. Crustacean appendage evolution associated with changes in Hox gene expression. Nature 388:682–686.

Bakalenko NI, Novikova EL, Nesterenko AY, Kulakova MA. 2013. Hox gene expression during postlarval development of the polychaete Alitta virens. Evo Devo 4:113.

Barucca M, Olmo E, Canapa A. 2003. Hox and ParaHox genes in the bivalve mollusk. Gene 317:97–102.

Barucca M, Biscotti MA, Olmo E. 2006. All the three ParaHox genes are present in Nuttallochiton mirandus (Mollusca: Polyplacophora): evolutionary considerations. J Exp Zool B Mol Dev Evol 306B:164–167.

Berger C, Renner S, Lüer K, Technau GM. 2007. The commonly used marker ELAV is transiently expressed in neuroblasts and glial cells in the Drosophila embryonic CNS. Dev Dyn 236:3562–3568.

Biscotti MA, Canapa A, Olmo E. 2007. Hox genes in the Antarctic polyplacophoran Nuttallochiton mirandus. J Exp Zool B Mol Dev Evol 308B:507–513.

Biscotti MA, Canapa A, Forconi M, Barucca M. 2014. Hox and ParaHox genes: a review on molluscs. Genesis 52:935–945.

Brooke NM, Garcia–Fernández J, Holland PW. 1998. The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. Nature 392:920–922.

Callaerts P, Lee PN, Hartmann B, Farfan C, Choy DWY, Ikek K, Fishbach K-F, Gehring WJ, de Couet HG. 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.

Canapa A, Biscotti MA, Olmo E, Barucca M. 2005. Isolation of Hox and ParaHox genes in the bivalve Pecten maximus. Gene 348:83–88.

Carroll SB. 1995. Homoeotic genes and the evolution of arthropods and chordates. Nature 376:479–485.

Choo SW, Russell S. 2011. Genomic approaches to understanding Hox gene function. In: Friedmann T, Dunlap JC, Goodwin SF, editors. Advances in genetics, Vol. 76, 1st ed. Philadelphia, PA, USA: Elsevier Inc. p 55–91.

De Oliveira AL, Wollesen T, Kristof A, Scherholz M, Redl E, Todt C, Bleidorn C, Wanninger A. Comparative transcriptomics enlarges the toolkit of known developmental genes in mollusks. BMC Genomics (in review).

De Rosa R, Prud’homme B, Balavoine G. 2005. Caudal and evolution skipped in the annelid Platyneirois dumerilii and the ancestry of posterior growth. Evol Dev 7:574–587.

Friedrich S, Wanninger A, Brückner M, Haszprunar G. 2002. Neurogenesis in the mossy chiton, Mopalia muscosa (Gould) (Polyplacophora): evidence against molluscan metamerism. J Morphol 253:109–117.

Fritsch M, Wollesen T, de Oliveira A, Wanninger A, Fritsch M. 2015. Unexpected co-linearity of Hox gene expression in an aculiferan mollusk. BMC Evol Biol 15:1–17.

Fröbisch AC, Seaver EC. 2006. ParaHox gene expression in the polychaete annelid Capitella sp. I. Dev Genes Evol 216:81–88.

Fröbisch AC, Matus DQ, Seaver EC. 2008. Genomic organization and expression demonstrate spatial and temporal Hox gene colinearity in the lophotrochozoan Capitella sp. I. PLoS One 3:e4004.

Garcia-Fernández J. 2005. Hox, ParaHox, ProtoHox: facts and guesses. Hereditas (Edinb) 94:145–152.

Gehring WJ, Affolter M, Burglin T. 1994. Homeodomain proteins. Annu Rev Biochem 63:487–526.

Gharbaran R, Alvarado S, Aisenberg GO. 2013. Regional and segmental differences in the embryonic expression of a putative leech Hox gene, Lox2, by central neurons immunoreactive to FMRFamide-like neuropeptides. Invertebr Neurosci 14:51–58.

Giusti AF, Hinman VF, Degnan SM, Degnan BM, Morse DE. 2000. Expression of a Scr/Hox5 gene in the larval central nervous system of the gastropod Haliotis. Evo Devo 1:45–54.

Gonzales E, Brenner S, Ragsdale CW, Rokhsar DS. 2015. Patterns of cephalopod Hox gene expression demonstrate spatial and temporal Hox gene colinearity in the lophotrochozoan Sepia officinalis. PLoS One 9:e109627.

Haszprunar G, Friedrich S, Wanninger A, Ruthensteiner B. 2002. Fine structure and immunocytochemistry of a new chemosensory
system in the chiton larva (Mollusca: Polyplacophora). J Morphol 251:210–218.

Hiebert LS, Maslakova SA. 2015a. Hox genes pattern the anterior-posterior axis of the juvenile but not the larva in a maximally indirect developing invertebrate, *Micrura alaskensis* (Nemertea). BMC Biol 13:23.

Hiebert LS, Maslakova SA. 2015b. Expression of Hox, Cdx, and Six3/6 genes in the hoplonemertean *Pantinonemertes californiensis* offers insight into the evolution of maximally indirect development in the phylum Nemertea. Evo Devo 6:26.

Hinman VF, O’Brien EK, Richards GS, Degnan BM. 2003. Expression of anterior Hox genes during larval development of the gastropod *Halocynthia asinina*. Evol Dev 5:508–521.

Holland PWH. 2001. Beyond the Hox: how widespread is homeobox gene clustering? J Anat 199:13–23.

Hueber SD, Lohmann I. 2008. Shaping segments: Hox gene function in the genomic age. BioEssays 30:965–979.

Hughes CL, Kaufman TC. 2002. Hox genes and the evolution of the arthropod body plan. Evol Dev 499:459–499.

Hui JHL, Raible F, Korchagina N, Dray N, Samain S, Magdelenat G, Jubin C, Segurens B, Balavoine G, Arendt D, Ferrier DEK. 2009. Features of the ancestral bilaterian inferred from *Platynereis dumerilii* ParaHox genes. BMC Biol 7:43.

Iijima M, Akiba N, Sarashina I, Kuratani S, Endo K. 2006. Evolution of Hox genes in molluscs: a comparison among seven morphologically diverse classes. J Molluscan Stud 72:259–266.

Irvine SQ, Martindale MQ. 2000. Expression patterns of anterior Hox genes in the polychaete *Chaetopterus*. Eur J Dev Biol 27:333–351.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282.

Kato H, Kuma K, Toh H, Miyata T. 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518.

Kourakis MJ, Master VA, Lohkorst DK, Nardelli-Haefliger D, Wedeen CJ, Martindale MQ, Shankland M. 1997. Conserved anterior boundaries of Hox gene expression in the central nervous system of the leech *Helobdella*. Dev Biol 190:284–300.

Kulakova M, Bakalenko N, Novikova E, Cook CE, Eliseeva E, Steinmetz PRH, Kostyuchenko RP, Dondua A, Arendt D, Akam M, Andreeva T. 2007. Hox gene expression in larval development of the polychaetes *Nereis virens* and *Platynereis dumerilii* (Annelida, Lophotrochozoa). Dev Genes Evol 217:39–54.

Kulakova MA, Cook CE, Andreeva TF. 2008. ParaHox gene expression in larval and postlarval development of the polychaete *Nereis virens* (Annelida, Lophotrochozoa). BMC Dev Biol 8:61.

Lee PN, Calaerts P, de Couet HG, Martindale MQ. 2003. Cephalopod Hox genes and the origin of morphological novelties. Nature 424:1061–1065.

Lewis EB. 1978. A gene complex controlling segmentation in *Drosophila*. Nature 276:565–570.

Lowe CJ, Wu M, Salic A, Evans L, Lander E, Stange-Thomann N, Gruber CE, Gerhart J, Kirschner M. 2003. Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. Cell 113:853–865.

Marlow H, Tosches MA, Tomer R, Steinmetz PR, Lauri A, Larsson T, Arendt D. 2014. Larval body patterning and apical organs are conserved in animal evolution. BMC Biol 12:7.

McGinnis W, Krumlauf R. 1992. Homeobox genes and axial patterning. Cell 68:283–302.

Nardelli-Haefliger D, Shankland M. 1992. Lox2, a putative leech segment identity gene, is expressed in the same segmental domain in different stem cell lineages. Development 116:697–710.

Nardelli-Haefliger D, Bruce AEE, Shankland M. 1994. An axial domain of HOM/Hox gene expression is formed by morphogenetic alignment of independently specified cell lineages in the leech *Helobdella*. Development 120:1839–1849.

Orii H, Kato K, Umesono Y, Sakurai T, Agata K, Watanabe K. 1999. The planarian HOM/HOX homeobox genes (*Plox*) expressed along the anteroposterior axis. Dev Biol 210:456–468.

Pérez-Paralé LM, Carpintero P, Pazos AJ, Abad M, Sánchez JL. 2005. The HOX gene cluster in the bivalve mollusc *Mytilus galloprovincialis*. Biochem Genet 43:417–424.

Pernice M, Deutsch JS, Andouche A, Boucher-Rodoni R, Bonnaud L. 2006. Unexpected variation of Hox genes’ homeodomains in cephalopods. Mol Phylogenet Evol 40:872–879.

Peterson MD, Rogers BT, Popadíč A, Kaufman TC. 1999. The embryonic expression pattern of *labial*, posterior homeotic complex genes and the *teashirt* homologue in an apterygote insect. Dev Genes Evol 209:77–90.

Prince VE, Price AL, Ho RK. 1998. Hox gene expression reveals regionalization along the anteroposterior axis of the zebrafish notochord. Dev Genes Evol 208:517–522.

Robinow S, White K. 1991. Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. J Neurobiol 22:443–461.

Samadi L, Steiner G. 2009. Involvement of Hox genes in shell morphogenesis in the encapsulated development of a top shell gastropod (*Gibbula varia* L.). Dev Genes Evol 219:523–530.

Samadi L, Steiner G. 2010a. Conservation of ParaHox genes’ function in patterning of the digestive tract of the marine gastropod *Gibbula varia*. BMC Dev Biol 10:1–15.

Samadi L, Steiner G. 2010b. 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.

Scherholz M, Redl E, Wollesen T, Todt C, Wanninger A. 2013. Aplacophoran mollusks evolved from ancestors with polyplacophoran-like features. Curr Biol 23:2130–2134.

Scott MP, Tamkun JW, Hartzell GW, Ill. 1989. The structure and function of the homeodomain. Biochem Biophys Acta 989:25–48.

Simakov O, Marleiz F, Cho S-J, Edsinger-Gonzales E, Havlak P, Hellingsten U, Kuo D-H, Larsson T, Lv J, Arendt D, Savage R, Osoegawa K,
de Jong P, Grimwood J, Chapman JA, Shapiro H, Aerts A, Otillar RP, Terry AY, Boore JL, Grigoriev IV, Lindberg DR, Seaver EC, Weisblat DA, Putnam NH, Rohksar DS. 2013. Insights into bilaterian evolution from three spiralian genomes. Nature 493:526–531.

Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31:3210–3212.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

Takeuchi T, Koyanagi R, Gyoja F, Kanda M, Hisata K, Fujie M, Goto H, Yamasaki S, Nagai K, Morino Y, Miyamoto H, Endo K, Endo H, Nagasawa H, Kinoshita S, Asakawa S, Watabe S, Satoh N, Kawashima T. 2016. Bivalve-specific gene expansion in the pearl oyster genome: implications of adaptation to a sessile lifestyle. Zoological Lett 2:3.

Voronezhskaya EE, Tyurin SA, Nezlin LP. 2002. Neuronal development in larval chiton Ischnochiton hakodadensis (Mollusca: Polyplacophora). J Comp Neurol 38:25–38.

Wang BB, Müller-Immergluck MM, Austin J, Robinson NT, Chisholm A, Kenyon C. 1993. A homeotic gene cluster patterns the antero-posterior body axis of C. elegans. Cell 74:29–42.

Wanninger A, Haszprunar G. 2002. Chiton myogenesis: perspectives for the development and evolution of larval and adult muscle systems in molluscs. J Morphol 251:103–113.

Wanninger A, Wollesen T. 2015. Mollusca. In: Wanninger A, editor. Evolutionary developmental biology of invertebrates 2: Lophotrochozoa (Spiralia). Wien: Springer Verlag. p 103–153.

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25:189–191.

Wollesen T, Rodríguez Monje SV, McDougall C, Degnan BM, Wanninger A. 2015a. The ParaHox gene Gsx patterns the apical organ and central nervous system but not the foregut in scaphopod and cephalopod mollusks. EvoDevo 6:41.

Wollesen T, Rodríguez Monje SV, Todt C, Degnan BM, Wanninger A. 2015b. Ancestral role of Pax2/5/8 in molluscan brain and multimodal sensory system development. BMC Evol Biol 15:231.

Wong VY, Aisenberg GO, Gan WB, Macagno ER. 1995. The leech homeobox gene Lox4 may determine segmental differentiation of identified neurons. J Neurosci 15:1377–1419.

Zhang G, Fang X, Guo X, Li L, Luo R, Xu F, Yang P, Zhang L, Wang X, Qi H, Xiong Z, Que H, Xie Y, Holland PWH, Paps J, Zhu Y, Wu F, Chen Y, Wang J, Peng C, Meng J, Yang L, Liu J, Wen B, Zhang N, Huang Z, Zhu Q, Feng Y, Mount A, Hedgecock D, Xu Z, Liu Y, Domazet-Loso T, Xu Y, Jiang X, Li J, Fan D, Wang W, Fu W, Wang T, Wang B, Zhang J, Peng Z, Li Y, Li N, Wang J, Chen M, He Y, Tan F, Song X, Zheng Q, Huang R, Yang H, Du X, Chen L, Yang M, Gaffney PM, Wang S, Luo L, She Z, Ming Y, Huang W, Zhang S, Huang B, Zhang Y, Qu T, Ni P, Miao G, Wang J, Wang Q, Steinberg CEW, Wang H, Li N, Qian L, Zhang G, Li Y, Yang H, Liu X, Wang J, Yin Y, Wang J. 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490:49–54.