Rotiferan Hox genes give new insights into the evolution of metazoan bodyplans

Andreas C. Fröbius & Peter Funch

The phylum Rotifera consists of minuscule, nonsegmented animals with a unique body plan and an unresolved phylogenetic position. The presence of pharyngeal articulated jaws supports an inclusion in Gnathifera nested in the Spiralia. Comparison of Hox genes, involved in animal body plan patterning, can be used to infer phylogenetic relationships. Here, we report the expression of five Hox genes during embryogenesis of the rotifer Brachionus manjavacas and show how these genes define different functional components of the nervous system and not the usual bilaterian staggered expression along the anteroposterior axis. Sequence analysis revealed that the loxS-parapeptide, a key signature in lophotrochozoan and platyhelminthean Hox6/loxS genes, is absent and replaced by different signatures in Rotifera and Chaetognatha, and that the MedPost gene, until now unique to Chaetognatha, is also present in rotifers. Collectively, our results support an inclusion of chaetognaths in gnathiferans and Gnathifera as sister group to the remaining spiralians.
Originally discovered in *Drosophila melanogaster*[^1],[^2], *Hox* gene transcription factors have been researched extensively in a phylogenetically diverse range of animals over the last 30 years. Being present in the genomes of nearly all animals with the exception of Porifera, Ctenophora, and Placozoa, this family of highly conserved transcriptional regulators controls some of the most fundamental processes of embryonic development. During morphogenesis of all triploblastic metazoans studied, *Hox* genes exhibit expression in body regions along the antero-posterior axis of the embryo at some point. Regional identities are imprinted by either single or combined expression of different *Hox* genes, referred to as "*Hox* code"[^3]. This is clearly seen in the segmented taxa Arthropoda[^4],[^5], Annelida[^6],[^7], and Chordata[^8],[^9] where the exact identity of body segments is regulated by the "*Hox* code". Here, deviations from the appropriate expression profiles result in homeotic transformations of the body regions involved. Amazingly, even across distantly related taxa, body regions comparable to each other (anterior, median, posterior) are patterned by orthologous *Hox* genes. Thus, evolutionary changes of *Hox* gene expression may have led to evolutionary shifts accounting for the emergence of new body plans[^8]. Having arisen by tandem duplication, *Hox* genes are often physically linked in genomic clusters. A correlation of the spatial order of *Hox* gene expression along a primary body axis and sometimes even temporal order of *Hox* gene activation during embryogenesis with the order of the genes in the *Hox* cluster, referred to as colinearity[^9], has been observed in a broad range of metazoans. In some bilaterian taxa, however, the *Hox* cluster has been rearranged[^10] or even dispersed up to the relocation of all *Hox* genes to different locations of the genome[^11],[^12]. Degradation of the *Hox* cluster, however, does not necessarily result in destruction of the *Hox* code. In some cases, specification of appropriate body regions is maintained in taxa where *Hox* genes are no longer closely linked[^13],[^14].

Within Protostomia, *Hox* gene expression has been studied in a variety of ecdysozoan and spiralian taxa. However, *Hox* gene activation during embryonic development has not been studied in gnathiferae so far. Based on morphological characters, it has been proposed that the phylum Rotifera including the parasitic Acanthocephala, along with Gnathostomulida and Micrognathozoa, form the clade Gnathifera[^15],[^16]. Rotifera are microscopic, ecologically important aquatic animals comprising ca. 2200 described species. Their embryonic development and sexual dimorphic adult body plans exhibit special features. Protected within an egg shell, development starts with a unique cleavage pattern involving an exceptional type of D-quadrant cleavage[^18]–[^20]. Lacking a larval form, direct development with early determination gives rise to eutelic animals typically with a tripartite, pseudocoelomic body plan, consisting of a head with a ciliated corona, a trunk and a post-cloacial foot. Several rotiferan tissues are syncytial but musculature and nervous system are cellular[^21]. Muscles are mostly formed by few cells directly innervated by nerves formed by simple chains of single neurons[^22]. The excretory system consists of paired protonephridia with both cellular and syncytial sections[^21].

Phylogenetic approaches show that rotiferan sequences exhibit very high evolutionary rates resulting in unstable positions within calculated trees[^23]–[^25]. Likewise, Platyhelminthes and some ecdysozoans display fast evolving sequences. As a result, Rotiferan are prone to long-branch-attraction (LBA) artifacts by grouping with e.g., Platyhelminthes. In addition, taxonomic sampling within Gnathifera is sparse. This has lead to the controversial hypothesis of a clade "Platyzoa" uniting Gnathifera with Platyhelminthes and Gastrotricha within Spiralia (Supplementary Fig. 1)[^26],[^27]. Recent phylogenomic approaches focusing on resolving spiralian phylogeny, though, support paraphyly of Platyzoa and monophyly of Gnathifera placing Gnathifera basally near the Spiralia/Ecdysozoa split as sister to Lophotrochozoa and Roupheozoa (including Platyhelminthes and Gastrotricha)[^28]–[^30]. Based on the presence of specific amino-acid residues and peptide motifs within the homeodomain and its flanking regions —referred to as signatures— *Hox* genes can be assigned to various paralogous groups (PG1-15)[^31],[^32]. These paralogous groups are quite clearly defined for anterior class PG1-2, PG3 and median class PG4-5 *Hox* genes. Evolution of these *Hox* genes predates the divergence of Protostomia and Deuterostomia. Due to independently duplicated median and posterior class *Hox* genes in different bilaterian lineages later on, the exact paralogy status of the remaining median genes (PG6-8) and posterior class genes (PG9-14) is more difficult to determine given the lack of diagnostic position and overall phylogenetic signal. However, these duplication events and subsequent selection present us with *Hox* gene orthologues along with their conserved amino-acid signatures characteristic for these specific lineages in the clades existing today, allowing us to examine phylogenetic relationships based on *Hox* gene complements and the *Hox* signatures within[^33].

In this study, we isolate and examine genes of the *Hox* complement of the monogonont rotifer *Brachionus manjavacas*. Our analysis of *Hox* gene expression during embryogenesis of amictic females shows non-canonical expression patterns in the developing nervous system consistent with an original role of *Hox* genes in neurogenesis. Our sequence analyses show the presence of a new signature in the *Hox6* paralog of *B. manjavacas*, shared by chaetognath *Hox6* genes only. Furthermore, one of the rotifer *Hox* genes possesses median- and posterior-like amino-acid residues, exhibiting similarity to chaetognath *MedPost* genes. These results provide evidence for inclusion of both Rotifera and Chaetognatha in Gnathifera and also support a basal phylogenetic position of Gnathifera as sister group to the remaining Spiralia.

### Results

**Rotiferan *Hox* genes and metazoon phylogeny.** We isolated single copies of five *Hox* genes from the monogonont rotifer *Brachionus manjavacas*. Based on phylogenetic analyses of the homeodomain and diagnostic amino-acid motifs, we assigned orthology of these genes to the anterior class *Hox* gene PG2 (*Bm-Hox2*), a PG3 gene (*Bm-Hox3*) and central class genes PG4 (*Bm-Hox4*) and PG6 (*Bm-Hox6*) (Supplementary Figs. 2, 3, 4, and 5). The fifth *Hox* gene isolated from *Brachionus manjavacas* surprisingly clusters with *MedPost* genes from the chaetognaths *Flaccisagitta enflata* and Spadella cephaloptera (Fig. 1a, Supplementary Figs. 3, 4, and 5) and is strongly supported with a posterior probability of 100% in Bayesian analysis. Maximum-likelihood (ML) bootstrap support for this grouping is only 63%, this, however, is comparable to the ML support of grouping of the ecdysozoan *AbdB* genes (67%) or all *Saccoglossus kowalevskii* *Hox11-13* genes analyzed (61%) and even higher than the support for grouping of all Lox5-genes undoubtedly related (< 50%) (Supplementary Fig. 5). Mean statistical support from ML analyses for *Hox* gene orthology assignments usually is significantly lower due to the highly conserved nature of the homeodomain[^34]. While it could be argued that accelerated evolution could have led to phylogenetic artefacts as LBA, phylogenetic analyses did not reveal branch lengths for the grouping of the chaetognath and rotifer *MedPost* genes significantly larger than those observed for some posterior class *Hox* genes in general.

A careful examination of the homeodomain alignment of *MedPost* genes with either central class *Hox* genes or posterior class *Hox* genes (Supplementary Fig. 3) illustrates both, similarities and differences between rotiferan and chaetognath
Fig. 1 Hox gene data places rotifers and chaetognaths in Gnathifera within Spiralia. a Phylogenetic tree depicting the relationship of MedPost genes to PG8 and posterior class Hox genes. Tree topology is from Bayesian analysis. Bayesian posterior probabilities based on 400,000 trees from 40,000,000 generations and ML support values from 1000 iterations are shown above branches. Single values represent Bayesian posterior probabilities only. Asterisks denote ML support below 50%. b Alignment of ten amino acids of the carboxy terminal region to the homeodomain of PG6 genes. Sequences highlighted with yellow contain the new signature found in rotifers and chaetognaths. Blue highlighting marks the lox5-parapeptide of lophotrochozoan genes. Neither is found in Ecdysozoa, Ambulacraria, Chordata, or Xenacoelomorpha. c Summary of representative characteristics of the Hox cluster within different metazoan taxa. Boxes in the middle depict Hox gene contingents (color coded according to the assignment of the Hox genes to the different paralogous groups) isolated from representative species. The right hand column summarizes characteristic Hox gene evolution and duplication events along with presence of special Hox signatures resulting in Hox genes characterizing the respective groups.
MedPost genes. Both groups share nine of eleven central class diagnostic amino acids: Q (position 6 of the homeodomain), LTR(R/K)RR (26–31) and E (59). Previous work on MedPost genes of Flaccisagitta enflata35 and Spadella cephaloptera36 defined diagnostic posterior class residues characteristic for chaetognath MedPost genes: K(3), A(14), R(18), Y(20) and V(21). Only two of these posterior class diagnostic residues, K(3) and Y(20) are shared between chaetognaths and rotifers, but clearly, they represent plesiomorphic characters since they are also present in posterior Hox genes of all major bilaterian clades and interestingly also in chaetognath PostA and PostB genes (Supplementary Fig. 3). The other amino-acid residues A(14), R(18), and V(21) present in chaetognaths but not in rotifers are neither found in posterior class Hox genes of Ecdysozoa nor in those of Deuterostomia, but surprisingly in Post1 genes typical of Lophotrochozoa (Supplementary Fig. 3). Amino-acid residues supposedly characteristic for a new gene class thus have to be re-evaluated once new genes sharing these characteristics have been isolated. MedPost genes in general might be defined by only some of the posterior class specific amino-acid positions while others might be specific for one of the taxa only. Hox genes belonging to the same paralogous group often exhibit diversity at some positions of the homeodomain (Supplementary Fig. 2). The presence of MedPost could also be interpreted as a possible ancestral character, but if it was lost in the Lophotrochozoa + Rouphozoan lineage chaetognaths could still be affiliated to gnathiferans.

The possible close relationship between Rotifera and Chaetognatha was further supported when the homeodomain and 3’ flanking sequences of the PG6 gene Bm-Hox6 were analyzed (Fig. 1b). Lophotrochozoans and Platyhelminthes possess some central class Hox genes containing amino-acid motifs not observed in ecdysozoan or deuterostome taxa. These genes have been named Lox5 (PG6/7), Lox2, and Lox4 (both PG8). Lox5 orthologs possess the motif “KLTGP” in the carboxy flanking region of the homeodomain. (position 64–68)31, 37, and it seems likely that this motif was present in a common ancestor of Lophotrochozoa and a newly proposed clade Rouphozoa38 consisting of Platyhelminthes and Gastrotricha. Importantly, the PG6 gene of B. manjavacas not only lacks this “Lox5-parapeptide”, it possesses a new Hox signature “KS(I/L)ND” at position 63–67 also identified in PG6 genes of the bdelloid rotifers Philodina roseola and Adineta vaga, and the chaetognath Flaccisagitta enflata (Fig. 1b). This motif is not entirely identical among rotifers and chaetognaths, however, Hox signatures exhibit some variability as known from the variant Lox5 signature of Myzostomida38 and some Platyhelminthes39 or the Ubx-A peptide of Spiralia35. Recent phylogenomic approaches support Ecdysozoa as sister group to Spiralia, and neither ecdysozoan nor deuterostome PG6 genes possess a Hox signature in the carboxy flanking region to the homeodomain. Moreover PG6 Hox genes are absent in Xenacoelomorpha40–42 sister group to Nephrozoa (Deuterostomia, Ecdysozoa, and Spiralia)43, 44. Thus both signatures, the “Lox5-parapeptide” and the “KS(I/L)ND” signature could have evolved independently after the split of Ecdysozoa and Spiralia. The alternative hypothesis that the new signature is plesiomorphic has weaker support.

Consistent with their absence in the publically available genome of the bdelloid rotifer Adineta vaga45, PG8 genes (Lox2/Lox4/Ubx/AbdA) and posterior Hox genes (PG9-14) were not recovered from the monogonont rotifer B. manjavacas suggesting that these genes are missing in Rotifera (Fig. 1c).
Another central class gene (PG5) has recently been recovered from the genome of Brachionus manjavacas while the presence of the second anterior class gene (PG1) as identified in A. vaga could not be confirmed (D. M. Welch, personal communication). Interestingly a single PG8 gene with similarity to ecdysozoan and lophotrochozoan PG8 genes and two posterior Hox genes sharing key residues with lophotrochozoans, ecdysozoans, and deuterostomes, have been isolated from the chaetognath F. enflata. Posterior class Hox genes are also present in the cnidianar Nemotestella vector, and have been shown to exhibit extraordinary flexibility leading to possible independent evolution in different lineages. Thus, the most parsimonious explanation is that both PG8 Hox genes and the posterior genes (PG9-14) have been lost in rotifers (Fig. 1c).

Some phylogenomic analyses found support for a group called Platyzoa consisting of Gnathifera, Gastrotricha, and Platyhelminthes, but morphological characteristics supporting this group have never been strong. All platyzoans are non-coelomate, ciliated animals with worm-like appearance and without specialized respiratory or vascular systems. Moreover, most platyzoans are microscopic, with cuticle of the chaetognath head could be homologous to the pharyngeal cuticle of the chaetognath head could be homologous to the pharynx in gnathiferans. Quite comparable to the jaws of gnathiferans, both the high chitin content of the cuticle of the chaetognath head could be homologous to the chitinous parts and membranes of the pharynx in gnathiferans. Both chaetognaths and rotifers feature a tripartite body plan consisting of head, trunk without external motile cilia and foot/tail region. In contrast to Lophotrochozoa and Gastrotricha, the anus is not located terminally but instead near the posterior border of the lateral region. Also the trunk regions of Lophotrochozoa and Gastrotricha are with motile cilia. The nervous system includes additional nerve plexi: the caudal ganglion in rotifers and the ventral ganglion in chaetognaths. Both chaetognaths and rotifers feature a tripartite body plan consisting of head, trunk without external motile cilia and foot/tail region. In contrast to Lophotrochozoa and Gastrotricha, the anus is not located terminally but instead near the posterior border of the lateral region. Also the trunk regions of Lophotrochozoa and Gastrotricha are with motile cilia. The nervous system includes additional nerve plexi: the caudal ganglion in rotifers and the ventral ganglion in chaetognaths. Both also feature lateral sensory antennae connected to the nervous system. The corona, the ciliary organ of rotifers used for feeding, is formed by two coronal nerves. The mastax ganglion of rotifers is connected to the brain via two nerves. In chaetognaths, the suboesophageal ganglion is connected to the brain in a similar fashion with two small separate ventral ganglia integrated in the nerves connecting to the brain. An additional pharyngeal ganglion has also been reported for Gnathostomulida. Embryonic development of Chaetognatha and Rotifera shares some important characteristics. Spiral cleavage, prevalent in Lophotrochozoa and Platyhelminthes, is absent in Rotifera and Chaetognatha. The latter two groups exhibit D-quadrant cleavage but do not form a 4d mesentoblast. Primordial germ cells (PGCs) of rotifers and chaetognaths are specified by preformation only, in contrast to the specification of PGCs of Platyhelminthes and Lophotrochozoa by mostly epigenesis. Unfortunately, hardly anything is known about embryonic development of Micrognathozoa and only the earliest embryonic development has been described for a gnathostomulid species once, indicating possible presence of spiral cleavage in this group. Early cleavage patterns of chaetognaths and rotifers differ from each other. In Chaetognatha, cleavage is total and equal forming a blastula. A typical invagination gastrula can be observed. This basic cleavage pattern had originally been mistaken as radial cleavage. Rotifer development involves total and unequal first cleavages. Subsequently columns of cells descending from the A-C quadrant are formed by cleavage with mitotic spindles parallel to the primary axis. The 2D blastomere is then internalized by epibolic gastrulation. These differences do not necessarily contradict the hypothesis of unison of Chaetognatha and Gnathifera. Even within the morphologically and phylogenetically well supported Gnathifera different cleavage modes can be observed. The development of the parasitic thorny-headed worms Acanthocephala is different from that reported for monogonont rotifers though data are also included in Syndermata. The cleavage modes of rotifers and chaetognaths could, therefore, be interpreted as steps in a transformation series towards spiral cleavage with chaetognaths showing a more basal pattern.

These findings are consistent with the results of phylogenetic analyses based on EST data, mitochondrial genomes and tropomyosin where Chaetognatha is sister group to Lophotrochozoa or sister group to Protostomia. Moreover, a phylogenomic study based on EST sequences of 197 genes from 66 metazoan species including both Rotifera and Chaetognatha but unfortunately lacking other gnathiferan groups supports this grouping, albeit weakly. Newer phylogenetic studies that took LBA artefacts into account placed Gnathifera as sister to Lophotrochozoa and Rouphoza but excluded chaetognaths from the analysis. Thus, both Rotifera and Chaetognatha were placed at the same position in different phylogenomic studies, indicating a possible close relationship of this taxa. Intriguingly, the newest phylogenomic study including Gnathostomulida, Rotifera, and Chaetognatha shows strong support for a clade including Gnathifera and Chaetognatha as sister to all lophotrochozoans after Bayesian analysis (posterior probability = 1.0) and mediocre support for a clade formed by Gnathifera and Chaetognatha alone (pp = 0.69).

Non-canonical expression of rotifer Hox genes. The most fascinating and highly conserved feature of Hox genes is the correlation of spatial expression along the anteroposterior axis with the structure of the genomic Hox cluster. This spatial collinearity results in the formation of nested Hox expression domains by shifting anterior borders of expression in the developing nervous system and other tissues. Unique combinations of Hox genes activated within a body region specify that region’s identity (Hox-code). Here, we report Hox gene expression patterns in Rotifera. During embryogenesis of the monogonont rotifer Brachionus manjavacas all five isolated Hox genes are expressed in parts of the nervous system and display unique expression patterns unrelated to anteroposterior axis formation (Figs. 2 and 3a).

The anterior class Hox gene Brachionus manjavacas Hox2 (Bm-Hox2) is expressed in cells forming the main ventrolateral nerves connecting the brain to the caudal ganglion, a secondary nerve centre at the base of the foot. Initially upregulated in a pair of cells on the ventral side of the embryo near the anterior pole at the beginning of morphogenesis, these Bm-Hox2-positive cells move laterally and undergo cell divisions in an anterior-to-posterior fashion resulting in paired nerve cords consisting of...
three interconnected neurons each (Fig. 2 and Supplementary Fig. 6a). Interestingly, anteroposterior patterning of the main longitudinal nerves by nested expression of several Hox genes does not occur. Faint expression of Bm-Hox2 is also detectable in the mastax plexus after hatching (Fig. 2 and Supplementary Fig. 6d, e), consistent with the observation of anterior Hox gene expression in structures associated with the stomatogastric nervous system in other taxa. Expression of Bm-Hox4 in the rotifer brain or in the coronal nerves (Supplementary Fig. 6b, c) has not been detected.

Strikingly, most Hox orthologues (Bm-Hox3, Bm-Hox4, Bm-Hox6, and Bm-MedPost) are expressed in a neurogenic region of the embryo near the posterior pole giving rise to morphological innovations: e.g., the caudal ganglion and foot primordium (Fig. 2). The central class gene Bm-Hox6 is expressed in the developing disc-shaped caudal ganglion originating from a single expression domain shifting inwards from the posterior pole of the embryo. Protruding laterally and ventrally the caudal ganglion resembles a clover leaf. Bm-Hox3 also participates in patterning of this secondary nerve centre. Bilateral symmetrical domains expressing Bm-Hox3 at the base of the forming foot fuse during morphogenesis and are integrated into the caudal ganglion. This ganglion serves as the control hub for the posterior part of the trunk providing neuromuscular control of the pedal muscles and innervation of the foot. Bm-Hox3 is also involved in patterning of the pedal nerves. An expression domain in the distal part of the foot primordium gives rise to six cells with neural morphology in the trunk region connecting to the caudal ganglion. The position of the labeled pericaryon is consistent with the six horns of the pedal muscles in Brachionus. Remarkably, Bm-Hox6 was recruited to pattern an additional functional domain of the nervous system, forming an asymmetrical nerve loop on the dorsal left side of the animal connecting the single germovitellaria to the caudal ganglion (Supplementary Fig. 6a). This observation indicates neuronal control of the germovitellaria by or via the caudal ganglion.

The rotiferan foot is a remarkable structure enabling transient attachment to surfaces via a glue-like secretion. Expression of Bm-Hox4 during foot formation marks cells in the proximal and
central parts of the foot, giving rise to the caudal nerves and their connection to the caudal ganglion. Cells clustered at the tip of the foot have long been regarded to be simple gland cells. However, is strongly expressed in 12–14 cells connected to the caudal nerves and both Bm-Hox4 and Bm-MedPost exhibit overlapping expression in one or two cells at the base of the "toes". In deuterostomes, posterior class Hox genes play key roles in patterning of the postanal tail. In rotifers, posterior class genes are missing, leaving the key role of modeling of the nervous system of the postanal foot to central and MedPost class Hox genes. Judging from the innervation of this body region with FMRFamide- and serotonin (5HT)-positive nerve cells (Supplementary Fig. 6f, g) and expression of several Hox genes in the foot, we conclude that secretion of the "glue" is under neuronal control and the cells in the foot might represent an additional nerve plexus.

As most rotifer tissues are syncytial, and nerves consist of only a few interconnected neurons, code-like Hox expression is somewhat limited. This and newly evolved neuronal structures might have led to Hox gene regulation being adapted to modulate functional subsets of the rotifer nervous system (Fig. 3a). That the evolution of new and individual regulatory elements allowed the uncoupling of Hox expression from the constraints of collinearity is supported by the dispersed Hox cluster structure reported in the bdelloid rotifer Adineta vaga. However, the genomic structure of a monogonont rotifer Hox cluster has not yet been published. Surprisingly, Hox gene expression in Brachionus manjavacas does not seem to violate spatial colinearity completely as the patterns observed exhibit shifting anterior borders of expression to some extent. Temporal colinearity has been reported for some taxa, a correlation of the temporal order of Hox gene activation during embryogenesis with the order of

![Diagram of Hox gene expression in the nervous system of Brachionus manjavacas.](image)

![Comparison of rotiferan and chaetognath body plans with respect to the structure of the nervous system. Both groups have a dorsal brain and additional nerve plexi: mastax nerves and ganglia in rotifers and vestibular, and esophageal ganglia in chaetognaths as well as a caudal ganglion in rotifers and a ventral nerve centre in chaetognaths with the latter possibly incorporating functional subsets, these are still separated from the caudal ganglion in rotifers, e.g., innervation of sensory lateral antennae](image)
Hox genes in a cluster. The rather rapid morphogenesis in rotifers complicates determination of the order of Hox gene activation in this case. In B. manjavacas, transcription of the anterior Hox gene Bm-Hox2 and Bm-Hox3 is indeed upregulated earlier (stage 1) than transcription of the other Hox genes (stage 2). The central class Hox genes analyzed are likely to be activated more or less simultaneously, but are all involved in patterning of the caudal ganglion and the nerves of the foot. Differing onsets of transcription may, therefore, be based on the order of morphological processes these genes are involved in rather than correlation with Hox cluster structure.

**Discussion**

Though not typically used for reconstructing phylogenies, analyses of Hox genes for diagnostic residues and conserved motifs give important phylogenetic clues. Patterning of animal body plans during ontogenesis is linked to the Hox gene complement in a very unique way. Major evolutionary changes of body plans have been accompanied and most probably have been made possible by changes of the Hox cluster structure.6,8-9 Hox gene duplications enabled imprinting of additional positional information and thus evolution of additional body regions along the antero-posterior axes of animals. Reflected by possession of conserved amino-acid residues encoded in Hox genes these evolutionary changes and gene duplications seem to have happened independently in different clades. Hox genes play key roles in axial patterning and segment identity in many taxa. Our findings, however, suggest a different original role of Hox genes in metazoan evolution. In the diploblast metazoan Nematostella vectensis, Hox genes are predominantly expressed asymmetrically on one side of the body column of the polyp exhibiting slightly staggered epithelial expression patterns with large overlap.14 Nematostella vectensis Hox genes are predominantly expressed in the endodermal layer. Neural expression has not been reported. Within triploblast metazoans, recent phylogenomic studies revealed a sister group relationship of Xenacoelomorpha (consisting of Acoela, Nemertodermatida, and Xenoturbella) and Nephrozoa (Deuterostomia and Protostomia)13, 44. Analyses of Hox gene expression in the acoel Convolutriloba longissuara indicate possible participation of acoel Hox genes in axial patterning of the nervous system due to subepidermal localization and coexpression with neural markers.20 Consistent with morphological characteristics, phylogenomic analyses place Gnathifera near the base of Spiralia.28-30 Rotifers possess a miniature but rather complex nervous system. Here Hox genes have been recruited to pattern the nervous system in a non-canonical way. Amazingly, rotiferan Hox genes show expression domains specifying functional subsets, with a strong bias in patterning of the caudal ganglion and the postanal foot as morphological novelties rather than exhibiting canonical Hox code expression along the anteroposterior axis. Unfortunately, Hox expression analysis has been reported for only a single chaetognath Hox gene;61 however, expression of Spadella Hox4 in the ventral ganglion seems to be comparable to Bm-Hox4 expression in the caudal ganglion. These results might suggest an original role of Hox genes in neurogenesis. Consequently, their function in bauplan development would have been co-opted for other tissues during evolution.

Morphological or molecular ambiguities often lead to difficulties in phylogenetic placement of taxa. Several morphological traits strongly support Gnathifera. The support of a grouping of Rotifera and Chaetognatha based solely on either morphological traits (Fig. 3b) or developmental features may be weak; the combined analyses of morphology, development, and Hox sequences, however, provide an informative basis for this relationship. In addition, the newest and most comprehensive phylogenomic studies show some albeit moderate support of a close relationship between Gnathifera and Chaetognatha consistent with our results.29 Though Hox gene information from Gnathostomulida and Micrognathozoa is currently not available, we expect these groups to show Hox characteristics consistent with this study. Exhibiting ambiguous characteristics indicating a possible close relationship to either Platychelminthes or Gnathifera, placement of Gastrotricha has always been problematic. Phylogenomic approaches support both, a relationship with Platychelminthes in Roupouzoa or a close relationship to Rotifera. Based on this study, we suggest an inclusion of chaetognaths in gnathiferans and Gnathifera as sister group to the remaining spiralian. The rather unusual expression of Hox genes in Brachionus manjavacas is additional evidence of this proposed phylogeny.
fragments were cloned into pGEM-Teasy vector (Promega) and sequenced at Macrogen Inc (South Korea) or StarSeq (Germany).

Riboprobe synthesis. Digoxigenin-labeled riboprobes were generated by in vitro transcription using MEGAScript High Yield SP6 or T7 transcription kits (Ambion) with PCR products of suitable clones flanked by SP6- or T7 RNA polymerase promoter sites as templates.

Orthology assignment and phylogenetic analyses. Assignment to paralog groups (PG) was based on the phylogenetic analyses as well as the presence or absence of diagnostic amino-acid residues or motifs in homoeodomain or flanking region of Hox genes, commonly regarded as apomorphies for specific PGs and even specific taxonomic groups. For phylogenetic analyses sequences including the homeodomain and 12 amino acids of the carboxy flanking region next to the homeodomain were aligned using MacVector 8.0. Genome accession numbers of Adneta vaga Hox genes and GenBank accession numbers of all other Hox gene sequences used in phylogenetic analyses are given in Supplementary Table 2. The most suitable amino-acid substitution model LG + I = 1 was determined by ProtTest 3.4.2. Bayesian phylogenetic analyses were conducted with MrBayes V8.1.7 on XSEDE via the CIPRES science gateway V3.3 with two independent runs sampled every 100 generations and four chains each. A summary tree file was drawn using Figtree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and CorelDraw 12. Nexus alignments are available upon request.

Data availability. Additional data associated with this study are available in the Supplementary Information of this publication. Assembled sequences for all Hox genes isolated from Brachionus manjavacas have been deposited with GenBank under accession numbers KT989538 (Bm-Hox2), KT989539 (Bm-Hox3), KT989540 (Bm-Hox4), KT989541 (Bm-Hox5), KT989542 (Bm-MedPost). The amino-acid sequence of Bm-Hox5 is available in the Fishegare Repository under the identifier 10.6084/m1.fishgare.4616125.

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Author contributions

P.F. initially conceived the project. A.C.F. oversaw the project and performed acquisition, analysis, and interpretation of the data including cloning of the Hox genes, staging of morphogenesis of Brachionus, analysis of gene expressing patterns and phylogenetic analyses. A.C.F. wrote the first draft of the paper and generated the figures. P.F. made substantial contributions to revisions of the draft of the article. Both authors discussed the results and commented on the manuscript.

Additional information

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