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Xenacoelomorph-Specific Hox Peptides: Insights into the Phylogeny of Acoels, Nemertodermatids, and Xenoturbellids

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Xenacoelomorpha has recently been proposed as an animal taxon that includes acoels, nemertodermatids, and xenoturbellids. Their flattened bodies are very simple and lack discrete organs. The Acoela and Nemertodermatida (which comprise Acoelomorpha) were traditionally regarded as early-diverged extant orders of the class Turbellaria of the phylum Platyhelminthes. Recent anatomical studies and molecular phylogenetic studies demonstrate that the two groups belong to the phylum Xenacoelomorpha together with Xenoturbellida. However, debate remains in regard to whether Xenacoelomorpha is monophyletic, and whether xenacoelomorphs are sisters to all other bilaterians or have close affinity to ambulacrarians. The present study addresses the first question by examining the presence or absence of diagnostic peptide sequences shared by the three taxa. *Hox* genes have been used to investigate the phylogenetic relationships of metazoans. It has been shown that lophotrochozoans, rotifers, and chaetognaths share diagnostic peptide sequences in the C-terminal region of the Lox5 (Hox5/6/7) homeodomain proteins, which supports the clustering of these taxa. Examination of the decoded genome of the acoel *Praesagittifera naikaiensis* and reported xenacoelomorph *Hox* genes revealed that acoels share a peptide NLK(S/T)MSQ(V/I)D, which starts immediately after the homeodomain sequence of the central Hox4/5/6. In addition, we found another diagnostic peptide, KEGKL, in the C-terminal region of the anterior Hox1, which is shared by all the three groups of xenacoelomorphs, but not other bilaterians. Furthermore, two acoels, *Praesagittifera naikaiensis* and *Symsagittifera roscoffensis*, share another peptide SG(A/P)-PGM in the posterior Hox9/11/13. These results support the designation of the phylum Xenacoelomorpha, in which Acoela is a discrete group.

Key words: xenacoelomorphs, acoels, nemertodermatids, xenoturbellids, specific Hox-peptides, clustering of members

INTRODUCTION

Acoels and nemertodermatids are marine, soft-bodied, flattened acoelomates (Brusca et al., 2016) that have a unique epidermis with pulsatile activity. The mouth is located ventrally with an incomplete gut and no anus. These organisms lack discrete organs, such as a circulatory system and gonads. According to the traditional view prior to 2010, acoel flatworms and nemertodermatid flatworms (recently called acoelomorphs) were categorized as early-diverged extant orders of the class Turbellaria of the phylum Platyhelminthes, which included planarians, parasitic flukes, and tapeworms (Hyman, 1951; Brusca and Brusca, 2003). Planarians, parasitic flukes, and tapeworms are regarded as representatives of the phylum and are considered primitive bilaterians, but few studies have focused on acoels and nemertodermatids.

Xenoturbella bocki is found at depths of approximately 200 m in cold Baltic seawater and was first described only about 70 years ago (Westblad, 1949). Since then, several species of *Xenoturbella* have been recorded (Rouse et al., 2016; Nakano et al., 2017). Although morphological and anatomical similarities between *Xenoturbella* and acoelomorphs have been discussed, *X. bocki* has been considered an enigmatic animal, and its phylogenetic position has been obscure (Telford, 2008).

Molecular phylogeny is a powerful tool for inferring the phylogenetic relationships of metazoans that differ in morphology and embryology. Nucleotide substitutions in DNA likely occur neutrally, such that the substitution rate can be used to deduce the relationship without prejudicing the interpretation, which would be the case if it were based on spe-

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cific morphological traits or embryological modes and features. *X. bocki* has been extensively used for molecular phylogenetic studies. A first round of molecular phylogenetic reports discusses the phylogenetic position of *Xenoturbella* (Norén et al., 1997; Bourlat et al., 2003; Bourlat et al., 2006) in relation to their enigmatic anatomy and life cycle (Lundin, 1998; Nakano et al., 2013; Perea-Atienza et al., 2015).

There has been considerable debate about the placement of *Xenoturbella* relative to acoel and nemertodermatid flatworms. Repeated analyses of molecular phylogeny have nearly reached a consensus that acoels, nemertodermatids, and xenoturbellids form a clade called Xenacoelomorpha (Phillipe et al., 2011). However, it remains a matter of debate whether Xenacoelomorpha is monophyletic or whether these organisms are sisters to all bilaterians or to ambulacrarians (echinoderms + hemichordates) (Ruiz-Trillo et al., 1999; Telford, 2008; Hejnol et al., 2009; Mwinyi et al., 2010; Ruiz-Trillo et al., 2014; Simakov et al., 2015; Cannon et al., 2016; Dittmann et al., 2018).

Most molecular phylogenetic studies are based on comparisons of quantitative molecular changes. Comparative studies of qualitative molecular traits may provide additional or more reliable support for those derived from quantitative analyses. An interesting example is encountered in a diagnostic peptide motif known as the “spiralian peptide” (Bayascas et al., 1998) or the “Lox5 peptide” (de Rosa et al., 1999). This motif is composed of KLTGP pentapeptides and is present immediately carboxyterminal to the homeodomains (the functionally important centers of these molecules) that are encoded by the central *Hox* genes. These genes include *Lox5* and *Hox6* in lophotrochozoans, but not in ecdysozoans or deuterostomes (see Fig. 2).

This motif is also shared by Lox5 in *Dicyema japonicus* (Kobayashi et al., 1999), which supports the inclusion of dicyemids in the lophotrochozoan group (Lu et al., 2017). Recently, Fröbius and Funch (2017) reported the presence of another peptide motif, KSLND, in Lox5 (Hox6/7) of rotifers (see Fig. 2). Because this motif is shared with chaetognaths, the authors suggested a close relationship between rotifers and chaetognaths, which are sometimes called Gnathifera. A recent molecular phylogenetic analysis of chaetognaths also supports this rotifer/chaetognath clustering (Marlétaz et al., 2019).

Brauchle et al. (2018) recently surveyed all 11 classes of homeobox-containing genes in xenacoelomorphs and showed the presence of anterior, central, and posterior Hox genes, which are each in the ATNP-type homeodomain family. However, no studies have examined the presence of diagnostic peptides in xenacoelomorphs thus far. Recently, we decoded the genome of the acoel, *Praesagittifera naikaiensis* (Arimoto et al., 2019), which is a marine acoel worm (Yamasu, 1982) that is easily found along the seashores of the Seto Inland Sea and may be widely used for studies of acoel biology (Hikosaka-Katayama and Hikosaka, 2015). In the present study, we examined the presence or absence of spiralian peptide-like sequences in the Hox genes of acoels, nemertodermatids, and xenoturbellids.

MATERIALS AND METHODS

Genomic and transcriptomic sequence reads of the acoel,

Table 1. Homeobox genes in xenacoelomorphs.

OTU names	Organisms	References	Sequence IDs	Paralogous groups
PnHox1	*Praesagittifera naikaiensis*	This study	g27202.t1	PG1-3
SrHox1	*Symsagittifera roscoffensis*	Moreno et al. 2009	ADM48793.1	PG1-3
HmHox1	*Hofstenia miamia*	Brauchle et al. 2018	DN54918_c0_g1_i1	PG1-3
IpHox1	*Isodiametra pulchra*	Brauchle et al. 2018	DN75362_c0_g1_i3	PG1-3
NwHox1	*Nemertoderma westbladi*	Brauchle et al. 2018	Locus_35873.0_Transcript_1	PG1-3
XbHox1	*Xenoturbella bocki*	Brauchle et al. 2018	DN2325_c0_g1_i1	PG1-3
PnHox4/5/6	*Praesagittifera naikaiensis*	This study	g17009.t1	PG4-8
SrHox4/5/6	*Symsagittifera roscoffensis*	Brauchle et al. 2018	DN118344_c1_g1_i1	PG4-8
HmHox4/5/6	*Hofstenia miamia*	Brauchle et al. 2018	DN27605_c0_g1_i1	PG4-8
IpHox4/5/6	*Isodiametra pulchra*	Brauchle et al. 2018	DN17434_c0_g1_i1	PG4-8
XbHox4/5/6-1	*Xenoturbella bocki*	Brauchle et al. 2018	DN76496_c0_g1_i2	PG4-8
XbHox4/5/6-2	*Xenoturbella bocki*	Brauchle et al. 2018	DN84275_c0_g2_i1	PG4-8
XbHox4/5/6-3	*Xenoturbella bocki*	Brauchle et al. 2018	DN124767_c0_g1_i1	PG4-8
PnHox9/11/13	*Praesagittifera naikaiensis*	This study	g54685.t1	PG9-13
SrHox9/11/13	*Symsagittifera roscoffensis*	Brauchle et al. 2018	DN123370_c1_g1_i1	PG9-13
HmHox9/11/13	*Hofstenia miamia*	Brauchle et al. 2018	DN3061_c0_g2_i1	PG9-13
IpHox9/11/13	*Isodiametra pulchra*	Brauchle et al. 2018	DN66164_c4_g4_i4	PG9-13
NwHox9/11/13-1	*Nemertoderma westbladi*	Brauchle et al. 2018	Locus_10725.0_Transcript_1	PG9-13
NwHox9/11/13-2	*Nemertoderma westbladi*	Brauchle et al. 2018	Locus_54694.0_Transcript_1	PG9-13
XbHox9/11/13	*Xenoturbella bocki*	Brauchle et al. 2018	DN3733_c0_g1_i1	PG9-13
HmEvx	*Hofstenia miamia*	Brauchle et al. 2018	DN58284_c0_g1_i1	Evx
SrEvx	*Symsagittifera roscoffensis*	Brauchle et al. 2018	DN124972_c0_g3_i4	Evx
IpEvx	*Isodiametra pulchra*	Brauchle et al. 2018	DN12279_c0_g2_i1	Evx

Praesagittifera naikaiensis, have been deposited in the sequence read archive of the DNA Data Bank of Japan (DDBJ) under accession No. PRJDB7329 (Arimoto et al., 2019). All data are also available from the *GigaScience* GigaDB repository (http://dx.doi.org/10.5524/100564) and at http://marinegenomics.oist.jp/p_naikaiensis/viewer/info?project_id=71. Gene models have been annotated using BLAST searches (Camacho et al., 2009) against the NCBI RefSeq protein database release 88, and the results have been integrated into the database (Arimoto et al., 2019).

Among the 53 predicted homeobox genes in the database,

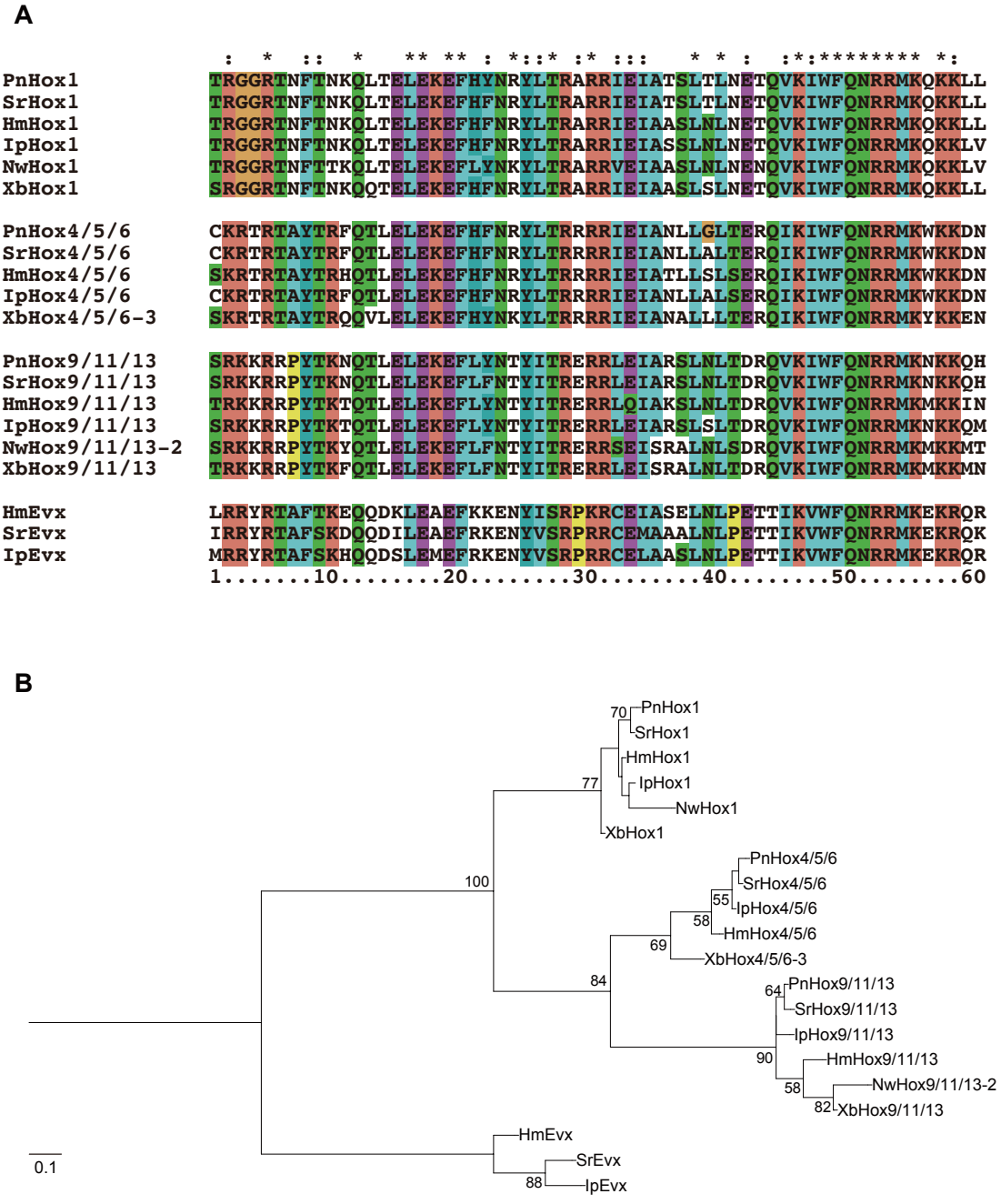

Fig. 1. Hox genes in acoels, a nemertodermatid, and *Xenoturbella*. **(A)** Alignments of amino acid sequences of the homeodomain encoded by anterior *Hox1*-related genes (top), central *Hox4/5/6*-related genes (upper middle), and posterior *Hox9/11/13*-related genes (lower middle). An alignment of the acoel *Evx* homeodomain is shown as a control (bottom). Sequence identity and similarity among homeodomains are indicated by * and :, respectively. Conserved amino acid residues are colored according to the properties of their side chains using the Clustal X color scheme (http://www.jalview.org/help/html/colorSchemes/clustal.html). Abbreviations of species names: (acoels) Pn, *Praesagittifera naikaiensis;* Sr, *Symsagittifera roscoffensis*; Hm, *Hofstenia miamia*; Is, *Isodiametra pulchra*; (a nemertodermatid) Nw, *Nemertoderma westbladi*; and (a xenoturbellid) Xb, *Xenoturbella bocki*. **(B)** Molecular phylogeny showing relationships among homeobox genes in xenacoelomorphs. A maximum-likelihood tree was constructed using the sequences of residues 1–60. Acoel Evx was used as an outgroup. Numbers at nodes show bootstrap support (1000 iterations) for the clustering. Clustering of anterior *Hox-1*-related, central *Hox4/5/6*-related, and posterior *Hox9/11/13*-related genes is evident.

three genes were identified as ANTP-type homeodomain proteins and used in this study (Table 1). Homeodomain amino-acid sequences of xenacoelomorphs were retrieved from the NCBI data bank (Table 1). Data sources for other metazoans are shown in Supplementary Table 1. Amino-acid alignments were generated using MAFFT version 7.305 (Katoh et al., 2002), and a molecular phylogenetic tree was constructed using the maximum likelihood method and IQ-TREE version 1.6.10 (Nguyen et al., 2015). All homeodomain sequences were compared, and the LG+G4 substitution model was selected as the optimal one for inferring a phylogenetic tree. The nodes of the tree were evaluated using 1000 bootstrap replications. Spiralian-peptide-like sequences were identified by manually aligning amino-acid sequences at positions 50–60 of the homeodomain and its carboxyl-terminal flanking region. The identities and similarities of amino-acid sequences were checked by visual inspection.

RESULTS

Praesagittifera naikaiensis contains three Hox genes

Among the 53 predicted homeobox genes in the *P. naikaiensis* genome database, three genes were identified as ANTP-type homeodomain proteins (Table 1; Fig. 1A). The three genes correspond to an anterior *Hox1-like* gene (tentatively called *PnHox1*), a central *Hox4/5/6-like* gene (*PnHox4/5/6*), and a posterior *Hox9/11/13-like* gene (*PnHox9/11/13*) (Table 1; Fig. 1A). The presence of one representative for the anterior Hox-cluster gene (tentatively called *Hox1*), the central one (tentatively called *Hox4/5/6*), and the posterior one (tentatively called *Hox9/11/13*) has been reported in the acoels *Symsagittifera roscoffensis* (Moreno et al., 2009), *Hofstenia miamia*, and *Isodiametra pulchra* (Cook et al., 2004) (Table 1).

A nemertodermatid, *Nemertoderma westbladi*, contains one anterior, one central, and two posterior *Hox* genes (Jimenez-Guri et al., 2006; Brauchle et al., 2018) (Table 1). Since the reported nemertodermatid central *Hox4/5/6* ortholog was partial (Jimenez-Guri et al., 2006), we could not include it in further analyses. In addition, *NwHox9/11/13-2* was only used in this analysis because *NwHox9/11/13-2* shows more similarity to others than *NwHox9/11/13-1*. On the other hand, *X. bocki* contains five copies, one anterior, three central (*XbHox4/5/6-1*, *-2*, and *-3*), and one posterior gene (Brauchle et al., 2018) (Table 1).

Molecular phylogeny was carried out for xenacoelomorph Hox genes using 60 amino-acid residues of the homeodomain. Tree topologies with high bootstrap support were not obtained in the first several trials using a dataset that included all three *XbHox4/5/6s* of *X. bocki* due to a long-branch attraction, which was mainly caused by *XbHox4/5/6-1*. We selected *XbHox4/5/6-3* since this homeodomain sequence exhibited greater similarity to those of other acoelomorphs (Fig. 1A). In addition, we found that using *Evx* (another member of the ANTP family) as an outgroup resulted in the most reliable tree topology (Fig. 1B). The tree demonstrated clustering of xenacoelomorph members *Hox1*, *Hox4/5/6* and *Hox9/11/13*, which had 77, 69, and 90% bootstrap support, respectively.

The tree topology differed among the three *Hox* genes but generally displayed grouping of acoel homeodomains, especially as seen in *Hox4/5/6*, as well as a close relationship between *P. naikaiensis* and *S. roscoffensis* in all three clusters. The topology also indicates that *Xenoturbella* homeodomains diverged first in the *Hox1* and *Hox4/5/6* clusters (Fig. 1B). These results paved the way for the subsequent examination of the presence of spiralian Hox-peptide-like sequences in xenacoelomorph *Hox* genes.

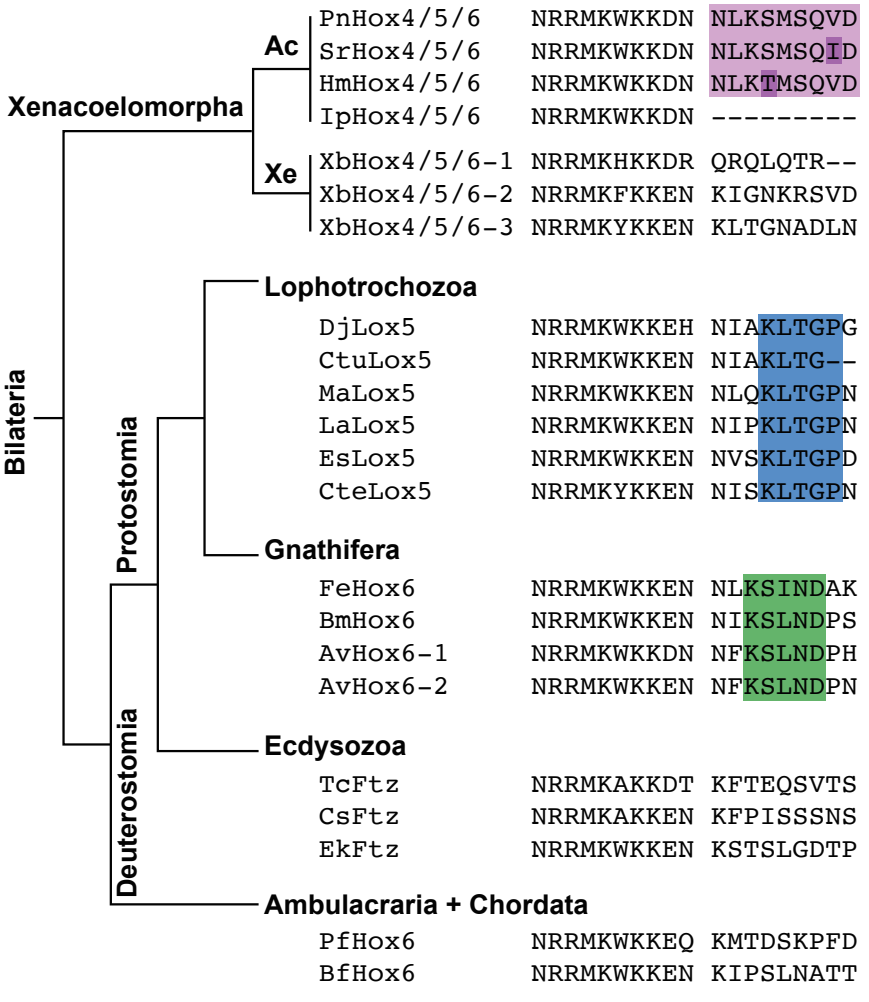

Fig. 2. Alignments of amino acid sequences at positions 50–60 of the homeodomain and the adjacent carboxyl flanking region of central *Hox4/5/6* genes. Shared peptide sequences are boxed by magenta in acoels, blue in lophotrochozoans, and green in gnathiferans. No comparable sequence of nemertodermatids has been reported yet. Ac, acoels; Xe, *Xenoturbella bocki*. For abbreviations of metazoan species, see Supplementary Table 1. A broad relationship of bilaterians is drawn based on Simakov et al. (2015) and Giribet (2016).

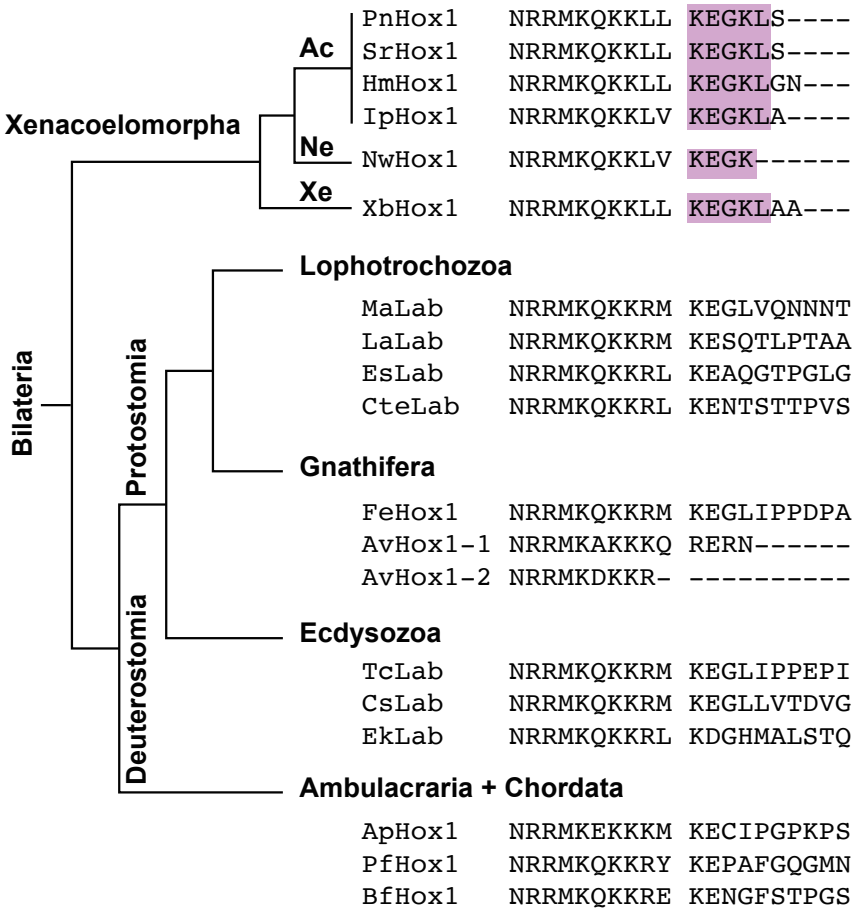

Fig. 3. Alignments of amino acid sequences at positions 50–60 of the homeodomain and following the carboxyl-flanking region of anterior *Hox1* genes. Peptide sequences shared by all three clades of Xenacoelomorph are boxed in magenta. Ac, acoel; Ne, nemertodermatid; Xe, *Xenoturbella bocki*. For abbreviations of metazoan species, see Supplementary Table S1.

The Hox4/5/6-specific peptide is shared by acoels, but not xenoturbellids

Figure 2 shows the alignment of the deduced amino-acid sequences at residues 50–60 of the homeodomain and the carboxyl terminal-flanking region of Hox4/5/6-related proteins. As reported previously, lophotrochozoans shared the peptide KLTG(P/S) (Bayascas et al., 1998; De Rosa et al., 1999; Kobayashi et al., 1999), while gnathiferans (rotifers and chaetognaths) have KSIND (Fröbius and Funch, 2017). We found that the three acoels, *S. roscoffensis*, *H. miamia*, and *P. naikaiensis*, all contained the nonapeptide NLK(S/T)MSQ(V/I)D starting immediately after position 60 of the homeodomain sequence for asparagine. Seven amino acids were identical, and two were similar.

Although there were no available data from the acoel *I. pulchra*, it is highly likely that acoels share a Hox4/5/6-specific peptide, which provides support for a single, discrete acoel taxon. On the other hand, we failed to find this peptide in *X. bocki*, and no data are available for nemertodermatids (Fig. 2). Furthermore, this diagnostic peptide was not found in ecdysozoans, spiralians, lophotrochozoans, or deuterostomes (Fig. 2).

A Hox1-specific peptide shared by xenacoelomorphs

Because xenacoelomorphs contain three Hox-cluster genes, we also compared the deduced amino acid sequences of the carboxyl terminal-flanking region for Hox1-related and Hox9/11/13-related proteins. As a result, we found the peptide KEGKL in Hox1 starting immediately after the 60th homeodomain residue (L/V, Fig. 3). This peptide was shared by acoels, a nemertodermatid, and a xenoturbellid. None of the other examined metazoans shared this motif (Fig. 3), although the first two amino acids (KE) are commonly seen in other metazoans (Fig. 3). This may indicate that the pentapeptide is specific to xenacoelomorphs, which supports the monophyly of the phylum Xenacoelomorpha.

A Hox9/11/13-specific peptide shared by two acoels

We also found that the two acoels *S. roscoffensis* and *P. naikaiensis* conserved an SG(A/G)PGM motif from the carboxyterminal flanking region for Hox9/11/13-related proteins (Fig. 4). This motif was not shared by any other acoels, a nemertodermatid, a xenoturbellid, or other bilaterians, which suggests a close relationship between the two acoels.

DISCUSSION

We have reported on the presence of specific Hox-peptide sequences in xenacoelomorphs: (a) a Hox4/5/6-specific peptide sequence shared by acoels but not by *Xenoturbella*, although the status of nemertodermatids remains unclear; (b) a peptide sequence in Hox1 shared by all three xenacoelomorph members; and (c) a peptide sequence in Hox9/11/13 shared by the two acoels, *S. roscoffensis* and *P. naikaiensis*. Together with previous reports on clustering of the three xenacoelomorphs, the present results suggest that (1) Xenacoelomorpha is a discrete taxon or phylum that unites acoels, nemertodermatids, and xenoturbellids; (2) Acoela is a discrete clade in that phylum; and (3) the acoels *S. roscoffensis* and *P. naikaiensis* are closely related in comparison with other acoel species. The third notion is also supported by molecular phylogeny results based on a comparison of the whole mitochondrial genome sequences (Arimoto et al., 2019).

One possible argument against the present analysis is whether so few amino acids genuinely constitute a diagnostic peptide motif. Nevertheless, we identified this motif as constituting at least five consecutive identical amino acids that correspond to those in lophotrochozoan-specific Lox5 peptide (De Rosa et al., 1999) and rotifer/chaetognath Hox6 peptide (Fröbius and Funch, 2017). In particular, the acoel-

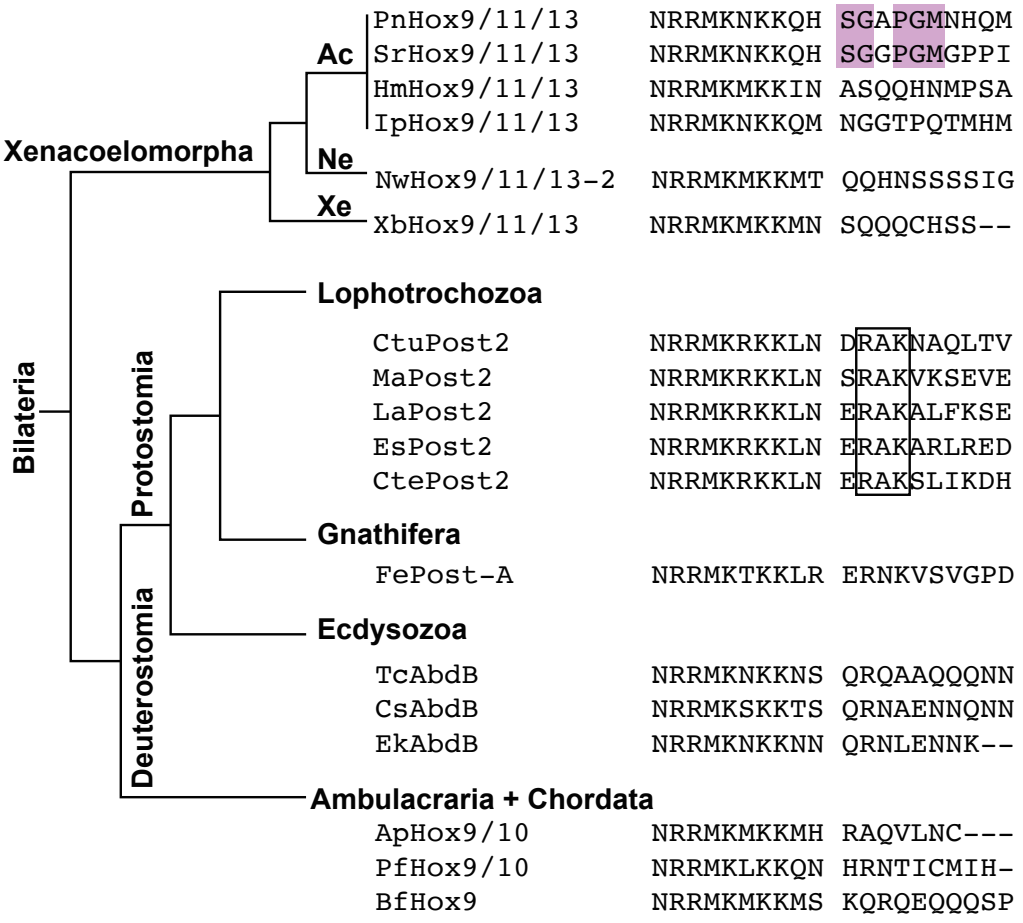

Fig. 4. Alignments of amino acid sequences at positions 50–60 of the homeodomain and adjacent carboxyl-flanking region of posterior *Hox9/11/13* genes. A shared peptide sequence was found in both the acoels *Praesagittifera naikaiensis* and *S. roscoffensis*. Three peptides shared by lophotrochozoans are boxed. Ac, acoels; Ne, nemertodermatid; Xe, *X. bocki.* For abbreviations of metazoan species, see Supplementary Table S1.

specific Hox4/5/6 peptide consists of seven identical amino acids (Fig. 2), while the xenacoelomorph-specific Hox1 peptide consists of five (Fig. 3), as does the Hox9/11/13 peptide shared by *P. naikaiensis* and *S. roscoffensis* (Fig. 4). The homeodomain is the functionally important center of these proteins, so it is expected for the amino acids adjoining both the N- and C-terminal regions to manifest sequence similarity among phylogenetically closely related species. For example, lophotrochozoans share an -RAK- sequence at the C-terminus of Post2 (Fig. 4). The presence of such peptides also supports the clustering of lophotrochozoan members (Fig. 4).

The present results provide further qualitative support of the clustering of Acoela, Nemertodermatida, and Xenoturbellida into the phylum Xenacoelomorpha. Molecular phylogenetic studies (e.g., Phillipe et al., 2011; Simakov et al., 2015) indicate that Acoela and Nemertodermatida comprise a discrete sister clade to Xenoturbellida. However, due to a lack of nemertodermatid Hox4/5/6-peptide data, the present study could not provide a support for the sister relationship between Acoela and Nemertodermatida.

On the other hand, the present results shed little light on the question of whether Xenacoelomorpha is a sister to all bilaterians or to deuterostomes. We carefully compared amino acid sequences of the C-terminal of the homeodomains and the adjacent carboxyl-flanking region between xenacoelomorphs and ambulacrarians (deuterostomes) or protostomes, but we did not find any clues about their relationship. In other words, specific Hox peptides are likely conserved among some clades of metazoans like xenacoelomorphs, lophotrochozoans, or rotifers/chaetognaths. Because Hox peptides of xenacoelomorphs were not shared with deuterostomes (Figs. 2–4), it is impossible to deduce whether there is a close relationship between Xenacoelomorpha and Ambulacraria. This interesting issue should be investigated in future studies.

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COMPETING INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUSIONS

UT and NS designed the experiments. UT, AA, and KT conducted the analyses. UT, AA, KT, and NS wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online (URL: https://bioone.org/journals/supplementalcontent/10.2108/zs190058/10.2108.zsj.36.395.s1.pdf)

Supplementary Table S1. Homeobox genes in bilaterians.

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