The Hox8 of the hemichordate Balanoglossus misakiensis

The *Hox8* of the hemichordate *Balanoglossus misakiensis*

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ABSTRACT

Deuterostomes comprise a monophyletic group of animals that include chordates, xenoturbellids, and the Ambulacraria, which consists of echinoderms and hemichordates. The ancestral chordate probably had 14 *Hox* genes aligned linearly along the chromosome, with the posterior six genes showing an independent duplication compared to protostomes. In contrast, ambulacrarians are characterized by a duplication of the posterior *Hox* genes, resulting in three genes known as *Hox11/13a*, *Hox11/13b*, and *Hox11/13c*. Here, we isolated 12 *Hox* genes from the hemichordate *Balanoglossus misakiensis*, and found an extra *Hox* gene that has not been reported in hemichordates. The extra *B. misakiensis* gene was suggested to be *Hox8* from paralog-characteristic residues in its hexapepetide motif and homeodomain and a comparison with *Strongylocentrotus purpuratus Hox* genes. Our data suggest that the ancestor of echinoderms and hemichordates may have had a full complement of 12 *Hox* genes.

Key words: Ambulacraria, *Balanoglossus misakiensis*, Echinoderm, Hemichordate, *Hox* gene

INTRUDUCTION

Deuterostomes consist of four phyla: Chordata, Echinodermata, Hemichordata, and Xenoturbellida (Bourlat et al. 2006). Studies of molecular phylogeny, larval morphology, and the adult heart/kidney complex suggest that echinoderms and hemichordates are sister taxa, forming a group known as the Ambulacraria (Smith et al. 2004; Swalla and Smith 2008). Since hemichordates share gill slits, an endostyle, and a post-anal tail with chordates, the deuterostome/chordate ancestor has been argued to have been a benthic wormlike creature (Brown et al. 2008).

The *Hox* complex is a duplicated set of genes that frequently occurs in a single cluster on the chromosome and controls spatial patterning mechanisms along the anteroposterior axis in bilateral animals (Carroll 1995). The ancestral chordate probably had 14 *Hox* genes aligned linearly along the chromosome, with the posterior six genes showing an independent duplication compared to protostomes (Amemiya et al. 2008; Kuraku et al. 2008). In the sea urchin *Strongylocentrotus purpuratus*, three anterior *Hox* genes have been translocated in an inverse orientation to the 5' end of the cluster, which lacks *Hox4* (Cameron et al. 2005). The loss of *Hox4*, however, appears to be a derived state in sea urchins, since Long et al. (2003) and Hara et al. (2006) isolated a *Hox* gene encoding Hox4-characteristic residues from the asteroid *Patiriella exigua* and the crinoid *Metacrinus rotundus*, respectively. In contrast, 11 and eight *Hox* genes, respectively,

have been isolated from the hemichordates *Saccoglossus kowalevskii* and *Ptychodera flava* (Aronowicz and Lowe 2006; Peterson 2004). Peterson (2004) found that ambulacrarians have four posterior *Hox* genes, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c*; *Hox9/10* is shared by deuterostomes, whereas the *Hox11/13* groups are specific to hemichordates and echinoderms.

We previously described the development of the hemichordate *Balanoglossus misakiensis* (Urata and Yamaguchi 2004). In this study, we amplified 12 *Hox* genes by PCR and RACE from *B. misakiensis*, and found an extra *Hox* gene that has not been reported in hemichordates. The extra *B. misakiensis* gene was suggested to be *Hox8* from paralog-characteristic residues in its hexapepetide motif and homeodomain and a comparison with *Strongylocentrotus purpuratus Hox* genes. Our data suggest that the ancestor of echinoderms and hemichordates may have had a full complement of 12 *Hox* genes.

MATERIALS AND METHODS

Animals

Adult *B. misakiensis* were collected at Masuho-ga-ura Beach, Ishikawa, Japan. After artificial insemination, embryos were cultured at 23-25°C as described previously (Urata and Yamaguchi, 2004).

Isolation of the *B. misakiensis Hox* gene fragments

Hox genes from *B. misakiensis* were PCR-amplified using genomic DNA as the template with three degenerate primers: Hox-F1, 5’-CARYTNACNGARYTNGARAA-3’ coding for QLTELEK; Hox-F2, 5’-YTNGARYTNGARAARGARTT-3’ coding for LELEKEF; and Hox-R, 5’-TTCATNCKNCKRTTYTGRAA-3’ coding for FQNRRMK. The *BmHox11/13b* cDNA fragment was amplified by RT-PCR using total RNA from *B. misakiensis* larvae with a Hox11/13b-specific primer and Hox-R. The Hox11/13b-F primer was designed based on the N-terminal flanking region of the homeodomain, the sequence of which (5’-ACNTTYACNACNACNCC-3’) codes for TFTTTP. Total RNA was extracted using Sepasol I Super (Toyobo) from the regenerating bud of an adult or larva (Table 1). The 3’ends of the *BmHox1*, *BmHox2*, *BmHox3*, *BmHox4*, *BmHox9/10*, *BmHox11/13a*, and *BmHox11/13c* cDNAs were isolated using the 3' RACE System for Rapid Amplification of cDNA Ends (Gibco). The 3’ends of *BmHox5*, *BmHox6*, *BmHox7*, *BmHox8*, and *BmHox11/13b*, and the 5’ end of *BmHox3*, *BmHox4*, *BmHox5*, *BmHox6*, *BmHox7*, and *BmHox8* were isolated using a GeneRacer Kit (Invitrogen). The primers used were as follows: BmHox1-F, 5’-TCGACGTGTTGAAATAGCCGCCATGTT-3’; BmHox2-F, 5’-GGAGTTTAATTATAACAGATATCTCTGCAG-3’; BmHox3-F, 5’-AACAGATATCTGCAAAAATCACGCCGAGA-3’; BmHox3-R, 5’-ATGTCGTCGTATTTGTACAGTTTCT-3’; BmHox4-F,

5’-TCACTTCAATCGATACTTGACCAGGAGACG-3’; BmHox4-R,

5’-TCTTGGTTTTCGTGTTTGGAAGGTTGTGAT-3’; BmHox5-F, 5’-

TCACTTCAACCGGTACCTCACACGCA-3’; BmHox5-R,

5’-ATTCTTGGGATCCTCCATTAGCTGTGATAT-3’; BmHox6-F, 5’-

TCACTTTAACCGTTACCTGACGCGACG-3’; BmHox6-R,

5’-TGACTTCTGGTGTTCGTCGAACGGTTTTGA-3’; BmHox7-F, 5’-

AATCGAACTGTCGCACCTTCTCGGA-3’; BmHox7-R, 5’-CAGTTTCACTGTCATCTTTCTTGCT-3’;

BmHox8-F, 5’- ACGGATCGAAATCTCACAGATTGTGGGA-3’; BmHox8-R,

5’-GAAGCTAACACGTCGCCTGTCGTTC-3’; BmHox9/10-F,

5’-ATTTGACACGGGAAAGGAGAGTGGAAATAT-3’; BmHox11/13a-F,

5’-TTTGTACAATATGTACCTGACCAGGGACC-3’; BmHox11/13b-F, 5’-

GCGAACAAAGCGACGTCCGTACTC-3’; and BmHox11/13c-F,

5’-TTCCAACAAAACATGTACTTGACGCGCGA-3’. The sequences have been deposited in the DNA

Data Bank of Japan (DDBJ) under the accession numbers shown in Table 1.

RESULTS AND DISCUSSSION

We amplified 12 *Hox* gene fragments by PCR from *B. misakiensis,* and the 3' end of each cDNA as well as the 5' end of five medial *Hox* plus *Hox3* cDNAs were isolated by RACE (Table 1). Figure 1 shows alignments of the deduced homeodomain sequences and C-terminal flanking regions of *Hox1*, *Hox2*, *Hox3*, *Hox4*, *Hox5*, *Hox6*/*Hox6/8*, *Hox7*, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c* from the hemichordates *S. kowalevskii* and/or *P. flava* with six full and five partial sequences of the *B. misakiensis Hox* genes. The boxes in Figure 1 indicate paralog-characteristic residues conserved between *Drosophila* and vertebrate Hox members (Sharkey et al. 1997). From the alignment, the 11 *B. misakiensis Hox* genes were inferred to correspond to *Hox1*, *Hox2*, *Hox3*, *Hox4*, *Hox5*, *Hox6*/*Hox6/8*, *Hox7*, *Hox9/10*, *Hox11/13a*, *Hox11/13b*, and *Hox11/13c* of *S. kowalevskii* and/or *P. flava*, respectively. When compared to *P. flava*, the putative *B. misakiensis* Hox1, Hox4, Hox5, Hox6/Hox6/8, Hox9/10, Hox11/13a, Hox11/13b, and Hox11/13c were nearly identical to the *P. flava* Hox proteins, in terms of both their homeodomain sequences and their C-terminal flanking regions. Most of the sequences of the homeodomains and/or C-terminal flanking regions were conserved in the *S. kowalevskii* Hox proteins, although *S. kowalevskii* Hox11/13b sequence was fairly diverged (Fig. 1). When compared to *S. kowalevskii* Hox2, Hox3, and Hox7, a *B. misakiensis* Hox similar to *S. kowalevskii* Hox2 was identified with two Hox2-characteristic residues in

its homeodomain, whereas another *B. misakiensis* Hox with a Hox3-related homeodomain sequence shared a Hox3-specific glycine residue in its C-terminal flanking region of the homeodomain. The other *B. misakiensis* Hox was identical to *S. kowalevskii* Hox7 in its homeodomain sequence and the C-terminal flanking four residues. Therefore, we tentatively assigned the 11 *B. misakiensis* genes to *BmHox1*, *BmHox2*, *BmHox3*, *BmHox4*, *BmHox5*, *BmHox6*, *BmHox7*, *BmHox9/10*, *BmHox11/13a*, *BmHox11/13b*, and *BmHox11/13c*, respectively, although their order on the chromosome is unknown.

We isolated an extra medial *Hox* gene fragment from *B. misakiensis* in addition to the 11 assigned *Hox* genes. Because Hox6, Hox7, and Hox8 include almost no paralog-characteristic residues in either their homeodomains or flanking regions, it is generally difficult to infer the paralogous groups to which these three *Hox* genes belong based on their sequences. Indeed, when analyzed by a neighbor-joining method (CLUSTAL W) using the homeodomain sequences plus both the N-terminal and the C-terminal flanking six residues (a total of 72 residues) of medial Hox members from chordates, echinoderms, hemichordates, and *Drosophila*, *Hox6*, *Hox7*, or *Hox8* members, including supposed *B. misakiensis Hox8*, did not form a paralog-specific clade even in deuterostomes, although *Hox4* and *Hox5* members constituted a monophyletic and a paraphyletic bilaterian clades, respectively (Fig. 2). However, in the present case, the ambulacrarian Hox proteins included residues that implied orthology. Figure 3 shows alignments of the

deduced homeodomain sequences and flanking regions encoded by *Hox6*, *Hox7*, and/or *Hox8* from the mouse *Mus musculus*, the lancelet *Branchiostoma floridae*, the sea urchin *S. purpuratus*, the sea lily *M. rotundus*, and the hemichordates *S. kowalevskii* and *P. flava* with the sequences of the *B. misakiensis Hox* genes. When compared to *S. purpuratus Hox6*, *Hox7*, and *Hox8*, which are ordered linearly on the chromosome (Cameron et al. 2005), the ambulacrarian *Hox7* and supposed *Hox8* genes, respectively, encoded several residues that were shared by paralogous ambulacrarian groups, but were not conserved in chordates, in their hexapeptide motifs and/or homeodomains (boxes in Fig. 3). Based on these ambulacrarian-characteristic residues and a comparison with the *S. purpuratus Hox* genes, we designated the extra *B. misakiensis* gene *BmHox8*, although the gene number may not reflect the gene order. Pendleton et al. (1993) reported an unidentified *S. kowalevskii* Hox sequence (accession number Q26523; homeodomain positions 21–47) that included two substitutions shared by the supposed ambulacrarian Hox8 (Fig. 3). From these observations, we propose that hemichordates have a complete set of medial *Hox* genes comparable to chordates, at least in number, together with three anterior and four posterior *Hox* genes (Fig. 4). Based on the *Hox* complements from sea lily, feather star, star fish, and sea cucumber, the common ancestor of echinoderms is parsimoniously inferred to have had a complete set of anteromedial *Hox* genes (Hara et al. 2006; Long and Byrne 2001; Long et al. 2003). Thus, the ambulacrarian ancestor may have had

a *Hox* cluster similar to one of the hemichordates in the complement. The *Saccoglossus kowalevskii* Genome Project being undertaken by Genamics GenomeSeek (http://genamics.com/) will reveal the *Hox* gene order and illuminate the ancestral *Hox* cluster of the deuterostome ancestor as well as its chordate origins.

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FIGURE LEGENDS

Fig. 1. Alignments of the deduced homeodomain sequences and C-terminal flanking regions of *Hox* genes from the hemichordates *S. kowalevskii* (Sk) and/or *P. flava* (Pf) with six full and five partial sequences of the *B. misakiensis* (Bm) *Hox* genes. Dots indicate conserved residues between the indicated organism and *S. kowalevskii*, whereas the dash indicates a gap (inserted for Hox9/10 alignment). Asterisks denote stop codons. Boxes indicate paralog-characteristic residues conserved between *Drosophila* and the vertebrate Hox members (Sharkey et al., 1997). The accession numbers are as follows: *SkHox1*, AAP79296; *SkHox2*, ABK00018; *SkHox3*, AAP79286; *SkHox4*, AAP79297; *SkHox5*, ABK00019; *SkHox6*, ABK00020; *SkHox7*, AAP79287; *SkHox9/10*, ABK00021; *SkHox11/13a*, ABK00022; *SkHox11/13b*, ABK00023; *SkHox11/13c*, AAP79288; *PfHox1*, AAR07634; *PfHox4*, AAR07635; *PfHox5*, AAR07636; *PfHox6/8*, AAR07637; *PfHox9/10*, AAR07638; *PfHox11/13a*, AAR07639; *PfHox11/13b*, AAR07640; and *PfHox11/13c*, AAR07641.

Fig. 2. Neighbor-joining tree constructed from the homeodomain sequences plus both the N-terminal and the C-terminal flanking six residues (a total of 72 residues) of medial Hox members from chordates,

echinoderms, hemichordates, and Drosophila. Hox3 genes act as outgroups. The numbers on the branches are bootstrap values higher than 50% over 1,000 replications. Hox6, Hox7, or Hox8 members did not form a paralog-specific clade even in deuterostomes, whereas Hox4 and Hox5 members constituted a monophyletic and paraphyletic bilaterian clades, respectively. The accession numbers are as follows: *MmHoxa6*, NP_034584; *MmaHox7*, NP_034585; *MmbHox8*, NP_034591; *BfHox6*, ABX39490; *BfHox7*, ABX39491; *BfHox8*, ABX39492; *SpHox6*, GLEAN3_05171; *SpHox7*, GLEAN3_02634 and GLEAN3_05170; *SpHox8*, GLEAN3_21309; *MrHox7*, BAF43725; and *MrHox8*, BAF43726.

Fig. 3. Alignments of the homeodomain sequences and flanking regions encoded by *Hox6*, *Hox7*, and/or *Hox8* from the mouse *Mus musculus* (Mm), the lancelet *Branchiostoma floridae* (Bf), the sea urchin *S. purpuratus* (Sp), the sea lily *M. rotundus* (Mr), *S. kowalevskii* (Sk), and *P. flava* (Pf) with the sequences of the *B. misakiensis* (Bm) *Hox* genes. The sequences between the homeodomains and hexapeptide motifs have been omitted. Identical residues based on the *M. musculus* sequences are indicated by dots. Compared to *M. musculus* and *B. floridae*, the ambulacrarian Hox*7* and supposed Hox8 proteins include several paralog-characteristic residues shared by ambulacrarians, but not conserved between chordates and ambulacrarians, in their hexapeptide motifs and/or homeodomains (boxes). An unidentified *S. kowalevskii*

Hox sequence (accession number Q26523) included two ambulacrarian-Hox8-characteristic residues. The accession numbers not listed in Figs. 1 and 2 are as follows: *MmHoxb6*, NP_032295; *MmHoxc6*, NP_034595; *MmbHox7*, NP_034590; *MmcHox8*, NP_034596; *MmdHox8*, NP_032302.

Fig. 4. Deuterostome taxonomy and *Hox* phylogeny. A phylogenetic tree for three higher taxa and three hemichordate species is shown on the left. The known (solid line) or presumed *Hox* clusters for each taxon or species are shown to the right of the taxonomic tree.

Table 1. *Hox* genes isolated from *B. misakiensis*

Gene	Template for 3'RACE (5'RACE)	DDBJ accession no.
BmHox1	regenerating bud of adult	AB426592
BmHox2	regenerating bud of adult	AB426593
BmHox3	regenerating bud of adult (larva)	AB426594
BmHox4	regenerating bud of adult (larva)	AB426595
BmHox5	larva (larva)	AB426596
BmHox6	larva (larva)	AB426597
BmHox7	larva (larva)	AB426598
BmHox8	larva (larva)	AB426599
BmHox9/10	regenerating bud of adult	AB426600
BmHox11/13a	regenerating bud of adult	AB426601
BmHox11/13b	larva	AB426602
BmHox11/13c	regenerating bud of adult	AB426603

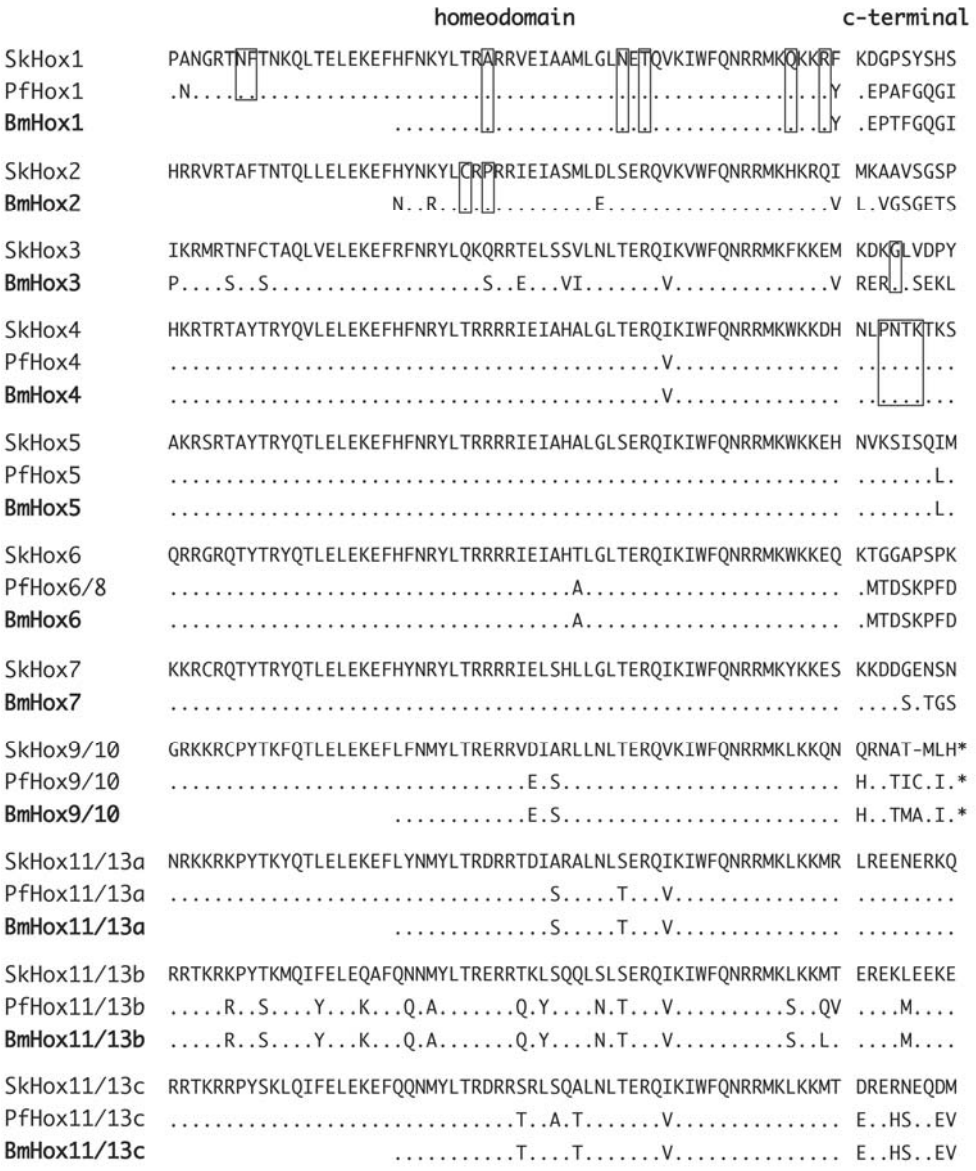
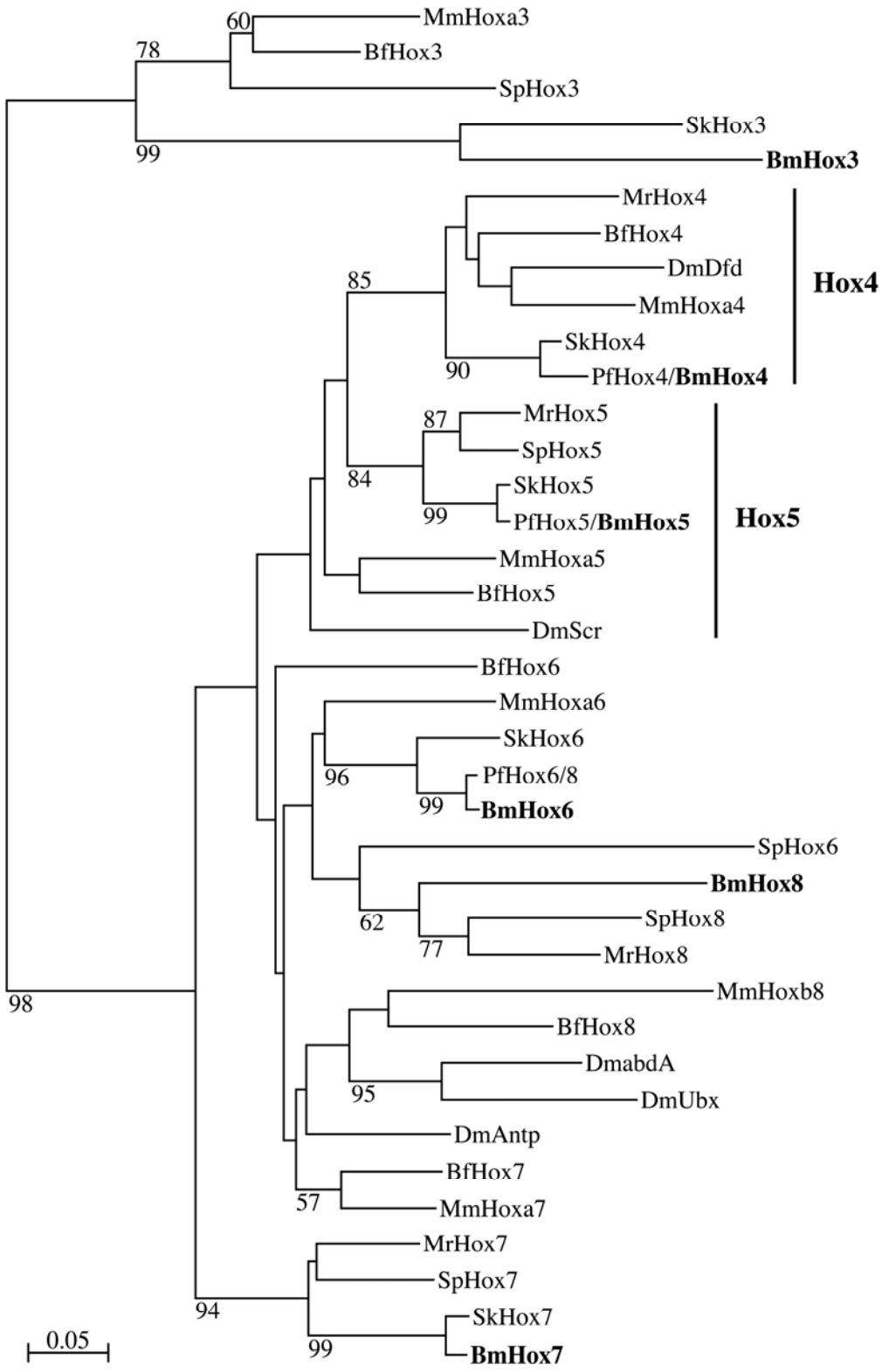

homeodomain
c-terminal
SkHox1 PANGRTNFTNKQLTELEKEFHFNKYLTRARRVEIAAMLGLNETQVKIWFQNRRMKQKKRF KDGPSYSHS
PfHox1 .N.....Y .EPAFGQGI
BmHox1 .....Y .EPTFGQGI
SkHox2 HRRVRTAFTNTQLLELEKEFHYNKYLCRPRRIEIASMLDLSERQVKVWFQNRRMKHKRQI MKAAVSGSP
BmHox2 N..R.....E.....V L.VGSGETS
SkHox3 IKRMRTNFCTAQLVELEKEFRFNRYLQKQRRTELSSVLNLTERQIKVWFQNRRMKFKKEM KDKGLVDPY
BmHox3 P....S..S.....S..E...VI.....V.....V RER..SEKL
SkHox4 HKRTRTAYTRYQVLELEKEFHFNRYLTRRRRIEIAHALGLTERQIKIWFQNRRMKWKKDH NLPNTKTKS
PfHox4 .....V..... .....
BmHox4 .....V..... .....
SkHox5 AKRSRTAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALGLSERQIKIWFQNRRMKWKKEH NVKSISQIM
PfHox5 ....L.
BmHox5 ....L.
SkHox6 QRRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHTLGLTERQIKIWFQNRRMKWKKEQ KTGGAPSPK
PfHox6/8 .....A..... .MTDSKPFD
BmHox6 .....A..... .MTDSKPFD
SkHox7 KKRCRQTYTRYQTLELEKEFHYNRYLTRRRRIELSHLLGLTERQIKIWFQNRRMKYKKES KKDDGENSN
BmHox7 ....S.TGS
SkHox9/10 GRKKRCPYTKFQTLELEKEFLFNMYLTRERRVDIARLLNLTERQVKIWFQNRRMKLKKQN QRNAT-MLH*
PfHox9/10 .....E.S..... H..TIC.I.*
BmHox9/10 .....E.S..... H..TMA.I.*
SkHox11/13a NRKKRKPYTKYQTLELEKEFLYNMYLTRDRRTDIARALNLSERQIKIWFQNRRMKLKKMR LREENERKQ
PfHox11/13a .....S.....T...V..... .....
BmHox11/13a .....S.....T...V..... .....
SkHox11/13b RRTKRKPYTKMQIFELEQAFQNNMYLTRERRTKLSQQLSLSERQIKIWFQNRRMKLKKMT EREKLEEKE
PfHox11/13b .....R..S....Y...K...Q.A.....Q.Y....N.T...V.....S..QV ....M....
BmHox11/13b .....R..S....Y...K...Q.A.....Q.Y....N.T...V.....S..L. ....M....
SkHox11/13c RRTKRRPYSKLQIFELEKEFQQNMYLTRDRRSRLSQALNLTERQIKIWFQNRRMKLKKMT DRERNEQDM
PfHox11/13c .....T..A.T.....V..... E..HS..EV
BmHox11/13c .....T....T.....V..... E..HS..EV

Fig. 1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BfHox3
SpHox3
SkHox3
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MrHox4
BfHox4
DmDfd
MmHoxa4
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PfHox4/BmHox4
Hox4
MrHox5
SpHox5
SkHox5
PfHox5/BmHox5
Hox5
MmHoxa5
BfHox5
DmScr
BfHox6
MmHoxa6
SkHox6
PfHox6/8
BmHox6
SpHox6
BmHox8
SpHox8
MrHox8
MmHoxb8
BfHox8
DmabdA
DmUbx
DmAntp
BfHox7
MmHoxa7
MrHox7
SpHox7
SkHox7
BmHox7
60
78
99
85
90
87
84
99
96
99
62
77
98
95
57
94
99
0.05

Fig. 2

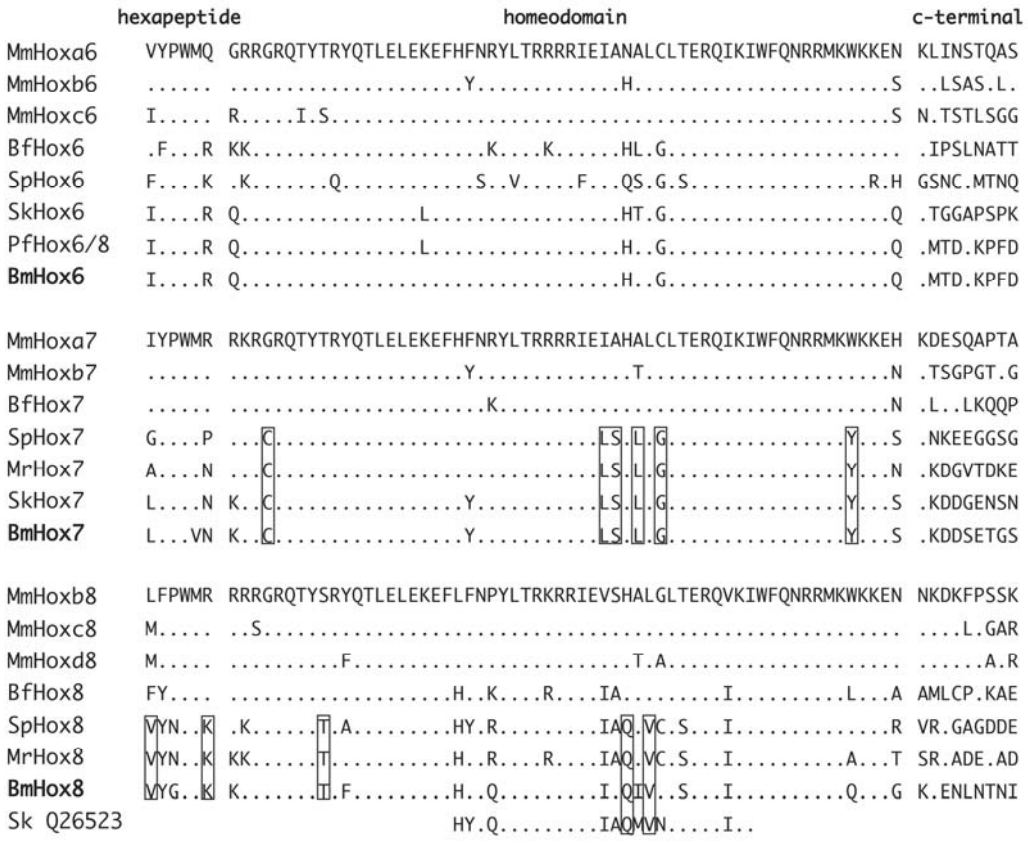
hexapeptide
homeodomain
c-terminal
MmHoxa6 VYPWMQ GRRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQNRRMKWKKEN KLINSTQAS
MmHoxb6 ....Y.....H.....S ..LSAS.L.
MmHoxc6 I..... R.....I.S.....S N.TSTLSGG
BfHox6 .F...R KK.....K....K.....HL.G..... .IPSLNATT
SpHox6 F....K .K.....Q.....S..V.....F...QS.G.S.....R.H GSNC.MTNQ
SkHox6 I....R Q.....L.....HT.G.....Q .TGGAPSPK
PfHox6/8 I....R Q.....L.....H..G.....Q .MTD.KPFD
BmHox6 I....R Q.....H..G.....Q .MTD.KPFD
MmHoxa7 IYPWMR RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEH KDESQAPTA
MmHoxb7 ....Y.....T.....N .TSGPGT.G
BfHox7 ....K.....N .L..LKQQP
SpHox7 G....P ...C.....LS.L.G.....Y...S .NKEEGGSG
MrHox7 A....N ...C.....LS.L.G.....Y...N .KDGVTDKE
SkHox7 L....N K..C.....Y.....LS.L.G.....Y...S .KDDGENSN
BmHox7 L...VN K..C.....Y.....LS.L.G.....Y...S .KDDSETGS
MmHoxb8 LFPWMR RRRGRQTYSRYQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQNRRMKWKKEN NKDKFPSSK
MmHoxc8 M..... ..S..... ....L.GAR
MmHoxd8 M..... .....F.....T.A..... .....A.R
BfHox8 FY..... .....H..K....R....IA.....I.....L...A AMLCP.KAE
SpHox8 VYN..K .K.....T.A.....HY.R.....IAQ.VC.S...I.....R VR.GAGDDE
MrHox8 VYN..K KK.....T.....H..R....R....IAQ.VC.S...I.....A...T SR.ADE.AD
BmHox8 VYG..K K.....T.F.....H..Q.....I.QIV..S...I.....Q...G K.ENLNTNI
Sk Q26523 HY.Q.....IAQMVN.....I..

Fig. 3

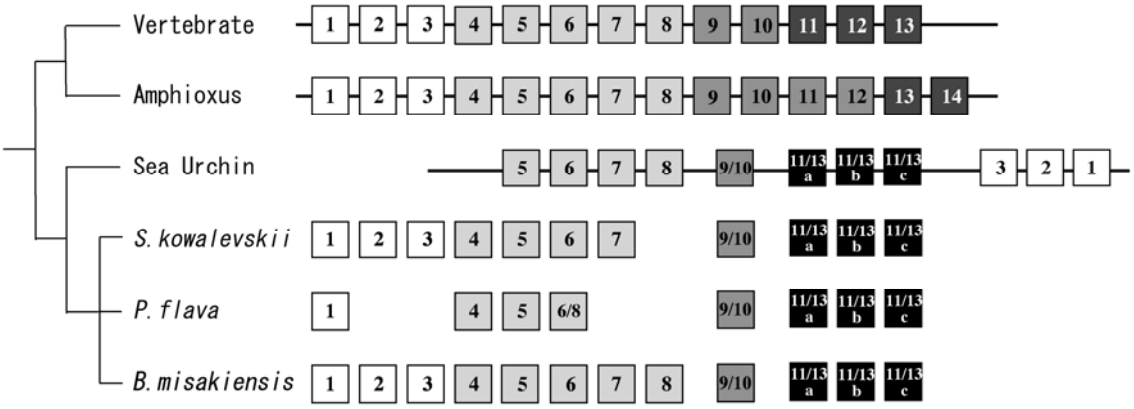
Vertebrate
1 2 3 4 5 6 7 8 9 10 11 12 13
Amphioxus
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Sea Urchin
5 6 7 8 9/10 11/13 a 11/13 b 11/13 c 3 2 1
S. kowalevskii
1 2 3 4 5 6 7 9/10 11/13 a 11/13 b 11/13 c
P. flava
1 4 5 6/8 9/10 11/13 a 11/13 b 11/13 c
B. misakiensis
1 2 3 4 5 6 7 8 9/10 11/13 a 11/13 b 11/13 c

Fig. 4