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Evidence from *Hox* genes that bryozoans are lophotrochozoans

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SUMMARY Bryozoans, or moss animals, are small colonial organisms that possess a suspension-feeding apparatus called a lophophore. Traditionally, this "phylum" has been grouped with brachiopods and phoronids because of the feeding structure. Available molecular and morphological data refute this notion of a monophyletic "Lophophorata." Alternative hypotheses place bryozoans either at the base of the Lophotrochozoa or basal to the Lophotrochozoa/Ecdysozoa split. Surprisingly, the only molecular data bearing on this issue are from the 18S nuclear ribosomal gene. Here we report the results of a *Hox* gene survey using degenerate polymerase chain reaction primers in a gymnolaemate bryozoan, *Bugula turrita*. Putative orthologs to both the *Post2* and the *Lox5* genes were found, suggesting that bryozoans are not a basal protostome group but closely allied to other lophotrochozoan taxa. We also found the first definitive evidence of two *Deformed/Hox4* class genes in a nonvertebrate animal.

INTRODUCTION

Bryozoans (or ectoprocts) are a clade of small colonial suspension-feeding animals that are commonly known as moss animals. The "phylum" comprises approximately 4500 described species, but the actual number of species is certainly much higher. Since the early 1880s, bryozoans have been viewed as closely related to brachiopods and phoronids and in a group commonly referred to as the Lophophorata (Hyman 1959; Willmer 1990). This grouping was based on the inferred homology of their ciliated tentacular feeding structures (Willmer 1990; Halanych 1996). As a whole, lophophorates have been of interest to evolutionary biologists because they display a mosaic of archetypal protostome and deuterostome conditions for "classical" embryological characters (Willmer 1990; Halanych 1996; Valentine 1997). Differing interpretations of developmental and morphological traits have led to the assignment of lophophorates as protostomes (e.g., Gutmann et al. 1978), deuterostomes (e.g., Zimmer 1973), intermediates between the two groups (e.g., Seiwing 1976; Salvini-Plawen 1982), or an independent radiation (Willmer 1990).

Based on 18S rDNA data, Halanych et al. (1995) hypothesized that "lophophorate" phyla were more closely related to protostomes such as annelids and mollusks than to deuterostomes; the resulting clade was termed the Lophotrochozoa. Whereas the placement of Brachiopoda and Phoronida close to annelids and mollusks has been confirmed by multiple sources of data (Erber et al. 1998; de Rosa et al. 1999; Stechmann and Schlegel 1999; Cohen 2000; de Rosa 2001; Helfenbein et al. 2001; Balavoine et al. 2002; Ruiz-Trillo et al. 2002; Helfenbien and Boore 2003), no molecular data other than 18S rDNA sequences have been used to address the placement of the Bryozoa. Moreover, analyses based on 18S rDNA, morphology, or combined data sets repeatedly fail to recover a monophyletic Lophophorata (e.g., Halanych et al. 1995; Mackey et al. 1996; Zrzavy et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001). The placement of bryozoans within these studies varies based on the characters chosen and character scoring. Zrzavy et al. (1998) coded bryozoans as possessing a lophophore and recovered the bryozoans as an outgroup to Phoronida+Brachiopoda+Deuterostomia. In a recent study, Peterson and Eernisse (2001) defined "Lophophorata" in a very unconventional way, scoring bryozoans as lophophore absent, contradicting Hyman's definition (see Halanych 1996). They found bryozoans to be closely related to spiralian protostomes such as mollusks, annelids, and entoprocts, but not monophyletic with brachiopods and phoronids. The separate origins of bryozoans (a.k.a. ectoprocts) and phoronids and brachiopods has repeatedly been put forth by Nielsen (1977, 1985, 1987, 2001; Nielsen et al. 1996; Nielsen and Riisgård 1998) based on morphological grounds (particularly ciliary characters). Despite the lack of evidence for a monophyletic Lophophorata, the clade is still often presented as a natural entity (Knoll and Carroll 1999; Brusca and Brusca 2003).

Of particular interest, the combined analysis of Giribet et al. (2000) suggested that bryozoans are basal to other protostome taxa and not as closely allied to the other Lophotrochozoa taxa as hypothesized by Halanych et al. (1995). Between these conflicting results reported in the literature and the lack of additional molecular data, the phylogenetic position of the Bryozoa remains enigmatic. Thus, to address this problem, we explored the *Hox* gene complement of the bryozoan *Bugula turrita* (Gymnolaemata, Cheilostomata) to look for putative orthologs (sensu De Rosa et al. 1999) among the three major bilaterian clades: Ecdysozoa, Lophotrochozoa, and Deuterostomia. *Hox* genes appear to have undergone various independent duplications in the three clades, making analysis of Hox cluster composition useful for examining broad patterns of animal relationships (reviewed in Halanych and Passamaneck 2001; Balavoine et al. 2002; Halanych 2004).

MATERIALS AND METHODS

Genomic DNA

Colonies of *B. turrita* were collected from docks in Eel Pond, Woods Hole, Massachusetts, USA. Colonies were held in filtered seawater overnight to allow clearance of gut contents before extraction of genomic DNA. Colony fragments were sorted under light microscopy to avoid contamination by epibionts such as nematodes and caprellid amphipods. Genomic DNA was extracted using the DNEasy Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocols.

Homeodomain amplification and cloning

Homeodomains were amplified using the forward primer Hox1F-LELEKE (GCTCTAGARYTNGARAARGARTT) and reverse primer Hox2R-WFQNRR (CGGGATCCCKNCKRTYTY-GRAACCA). The forward primer PostF-RKKRKPY (MGIAARAARMGIAARCCNTA) and the reverse primer HoxR-WFQNRRMK (YTTCATICKICKRTTYTGRAACCA) were used to screen for posterior class genes. Polymerase chain reaction (PCR) was conducted using Taq polymerase (Promega, Madison, WI, USA) applying a "touchdown" approach and the manufacturer's recommendations. PCR conditions involved an initial denaturation (94°C, 2 min) and then 30 touchdown amplification cycles (94°C, 30 sec; 55°C [minus 0.5°C/cycle], 45 sec; 72°C, 45 sec) followed by a final extension (72°C, 5 min). PCR products were cloned using pGEM-T Vector System (Promega). Clones were purified using Qiaprep (Qiagen) miniprep kit and sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA), using Big Dye Terminator Sequencing Reaction chemistry (Applied Biosystems).

Seventy clones of PCR fragments amplified with the primers Hox1F-LELEKE and Hox2R-WFQNRR and 30 clones of PCR fragments amplified with the primers PostF-RKKRKPY and HoxR-WFQNRRMK were sequenced and compared against GenBank using a BLAST search (Altschul et al. 1997). For sequences of interest, complete homeodomains and flanking regions were obtained using the ligation-mediated PCR technique, as described by Balavoine (1996). Seminested ligation-mediated PCR was conducted using specific primers designed from homeodomain fragments identified during degenerate screens.

Orthology assignment

Gene fragments were assigned to orthology groups based on both phylogenetic analysis and the presence of diagnostic residues in the homeodomain and flanking regions. The gene sequences used in the analysis are listed in Table 1. Inferred amino acid sequences for *Hox* gene homeodomains and flanking regions were aligned by eye using MacClade 4.0 (Maddison and Maddison 2000). Bayesian likelihood analyses were conducted using MrBayes version 2.0 (Huelsenbeck and Ronquist 2001), with a JTT model of amino acid replacement (Jones et al. 1992). Minimum evolution and parsimony analyses were conducted using PAUP* version 4.0 b10 (Swofford 2002).

RESULTS

Six unique *Hox* genes were cloned and characterized from *B. turrita* (Table 1). Orthology of isolated genes to *Hox* genes in other metazoans was initially determined by comparison of inferred amino acid sequence of homeodomains. Assignment of orthology was based on identification of peptide residues that appear to be conserved among members of specific orthology groups (de Rosa et al. 1999; Balavoine et al. 2002) (Fig. 1). *Bugula turrita Hox* genes were designated with the prefix "*Btu-*."

Results from phylogenetic analysis of *Hox* gene sequences are presented in Figure 2. The data set consisted of the 60 amino acids of the homeodomain for 88 taxa and is publicly available at TreeBase (www.treebase.org). Even though *Hox* gene geneology was not well resolved, *Btu-Post2* clusters with other *Post2* genes with significant support. A monophyletic grouping of *Btu-Lox5* with *Lox5* orthologs from the annelid *Nereis* and the brachiopod *Lingula* is also noteworthy.

Assignment of gene orthology was bolstered by identification of conserved peptide motifs in the regions flanking the homeodomain. Using Balavoine et al. (2002) as a guide, we highlighted conserved peptide motifs that aid with determination of gene identity in Figure 1. We identified two genes that appear to be of the *Dfd*/*Hox4* orthology class. Across the Bilateria, *Dfd*/*Hox4* orthologs possess an "LPNTK" motif C terminal to the homeodomain. This motif was found in *Btu-Dfda* (Fig. 1). At the same positions, *Btu-Dfdb* contained the motif "LSSSK," but both genes shared a motif "PEI" in the flanking region, not observed in other *Hox* genes sampled. The peptide motif "KLTG" was identified C terminal to the homeodomain of *Btu-Lox5*. This region appears to be homologous to the "KLTGP" motif in the *Lox5* gene from other lophotrochozoans.

Additional *Hox* genes (e.g., *Lox2*, *Lox4*, *Post1*) postulated to contain Lophotrochozoan amino acid motifs (de Rosa

Table 1. Homeobox gene sequences used in phylogenetic analyses

Gene Name	Species	Accession Number
Btu_pb	*Bugula turrita*	AY497421
Btu_Hox3	*Bugula turrita*	AY497422
Btu_DfdA	*Bugula turrita*	AY497423
Btu_DfdB	*Bugula turrita*	AY497424
Btu_Lox5	*Bugula turrita*	AY497425
Btu_Post2	*Bugula turrita*	AY497426
Alo_Ftz	*Archegozetes longisetosus*	AAF63162
Bfl_Hox1	*Branchiostoma floridae*	BAA78620
Bfl_Hox10	*Branchiostoma floridae*	CAA84522
Bfl_Hox11	*Branchiostoma floridae*	AAF81909
Bfl_Hox12	*Branchiostoma floridae*	AAF81903
Bfl_Hox13	*Branchiostoma floridae*	AAF81904
Bfl_Hox14	*Branchiostoma floridae*	AAF81905
Bfl_Hox2	*Branchiostoma floridae*	BAA78621
Bfl_Hox4	*Branchiostoma floridae*	BAA78622
Bfl_Hox5	*Branchiostoma floridae*	CAA84517
Bfl_Hox6	*Branchiostoma floridae*	CAA84518
Bfl_Hox7	*Branchiostoma floridae*	CAA84519
Bfl_Hox8	*Branchiostoma floridae*	CAA84520
Bfl_Hox9	*Branchiostoma floridae*	CAA84521
Dja_Abd_Ba	*Dugesia japonica*	BAB41079
Dja_Abd_Bb	*Dugesia japonica*	BAB41078
Dja_Plox2	*Dugesia japonica*	BAA77402
Dja_Plox3	*Dugesia japonica*	BAA77403
Dja_Plox4	*Dugesia japonica*	BAA77404
Dja_Plox5	*Dugesia japonica*	BAA77405
Dja_Plox6	*Dugesia japonica*	BAA77406
Dme_Abd_A	*Drosophila melanogaster*	NP_476693
Dme_Abd_B	*Drosophila melanogaster*	NP_650577
Dme_Antp	*Drosophila melanogaster*	AAA70216
Dme_Dfd	*Drosophila melanogaster*	NP_477201
Dme_Dll	*Drosophila melanogaster*	A44168
Dme_ems	*Drosophila melanogaster*	S22708
Dme_eve	*Drosophila melanogaster*	P06602
Dme_lab	*Drosophila melanogaster*	CAA31495
Dme_otx	*Drosophila melanogaster*	A35912
Dme_pb	*Drosophila melanogaster*	NP_476669
Dme_Scr	*Drosophila melanogaster*	NP_524248
Dme_Ubx	*Drosophila melanogaster*	NP_536752
Esc_Antp	*Euprymna scolopes*	AAL25809
Esc_Hox3	*Euprymna scolopes*	AAL25806
Esc_Lox4	*Euprymna scolopes*	AAL25810
Esc_Lox5	*Euprymna scolopes*	AAL25808
Esc_Post1	*Euprymna scolopes*	AAL25811
Esc_Post2	*Euprymna scolopes*	AAL25812
Esc_Scr	*Euprymna scolopes*	AAL25807
Fca_Ftz	*Folsomia candida*	AAK51915
Gti_HoxA	*Girardia tigrina*	CAA64694
Gti_HoxC	*Girardia tigrina*	CAA64696
Gti_HoxD	*Girardia tigrina*	CAA64697
Gti_HoxF	*Girardia tigrina*	CAA64692
Lan_Antp	*Lingula anatina*	AAD45590
Lan_Hox3	*Lingula anatina*	AAD45588
Lan_lab	*Lingula anatina*	AAD45587
Lan_Lox2	*Lingula anatina*	AAD45592
Lan_Lox4	*Lingula anatina*	AAD45593
Lan_Lox5	*Lingula anatina*	AAD45591
Lan_Post1	*Lingula anatina*	AAD45594
Lan_Post2	*Lingula anatina*	AAD45595
Lan_Scr	*Lingula anatina*	AAD45589
Lsa_NK	*Lineus sanguineus*	CAA76300
Lsa_Hox1	*Lineus sanguineus*	CAA76295
Lsa_Hox3	*Lineus sanguineus*	CAA76296
Lsa_Hox6	*Lineus sanguineus*	CAA76297
Lsa_Hox7	*Lineus sanguineus*	CAA76298
Lsa_Hox9	*Lineus sanguineus*	CAA76299
Mta_Ftz	*Milnesium tardigradum*	AAF63163
Pdu_otx	*Platynereis dumerilii*	CAC19028
Nvi_Dfd	*Nereis virens*	AF151666
Nvi_Hox3	*Nereis virens*	AF151665
Nvi_lab	*Nereis virens*	AF151663
Nvi_Lox2	*Nereis virens*	AF151668
Nvi_Lox4	*Nereis virens*	AF151669
Nvi_Lox5	*Nereis virens*	AF151671
Nvi_pb	*Nereis virens*	AF151664
Nvi_Post1	*Nereis virens*	AF151672
Nvi_Post2	*Nereis virens*	AF151673
Nvi_Scr	*Nereis virens*	AF151667
Pca_AbdB	*Priapulus caudatus*	AF144893
Pca_Dfd	*Priapulus caudatus*	AF144887
Pca_HB1	*Priapulus caudatus*	AF144888
Pca_Hox3	*Priapulus caudatus*	AF144886
Pca_lab	*Priapulus caudatus*	AF144884
Pca_pb	*Priapulus caudatus*	AF144885
Pca_HB2	*Priapulus caudatus*	AF144889
Pca_HB3	*Priapulus caudatus*	AF144890
Pca_HB4	*Priapulus caudatus*	AF144892
Pca_Ubx	*Priapulus caudatus*	AF144891

et al. 1999; Balavoine et al. 2002) were not observed in our screen of *B. turrita*. We have no evidence to suggest that these genes are not present, and their absence here may simply reflect the bias inherent in this type of PCR screening process.

DISCUSSION

The presence of *Post2* and *Lox5* orthologs in the *Hox* gene complement of *B. turrita* supports the placement of bryozoans with other lophotrochozoans and not as a basal lineage of protostomes. The identification of *Btu-Post2* was supported by phylogenetic analysis and the presence of diagnostic amino acid residues. The monophyly of *Post2* genes, including *Btu-Post2*, was recovered under all phylogenetic reconstruction criteria employed. Following Telford's (2000a) guideline for using paralogous genes as outgroups to root analyses, *Post2* is supported as having a derived condition relative to other posterior class *Hox* genes. Thus, the *Post2* sequence appears

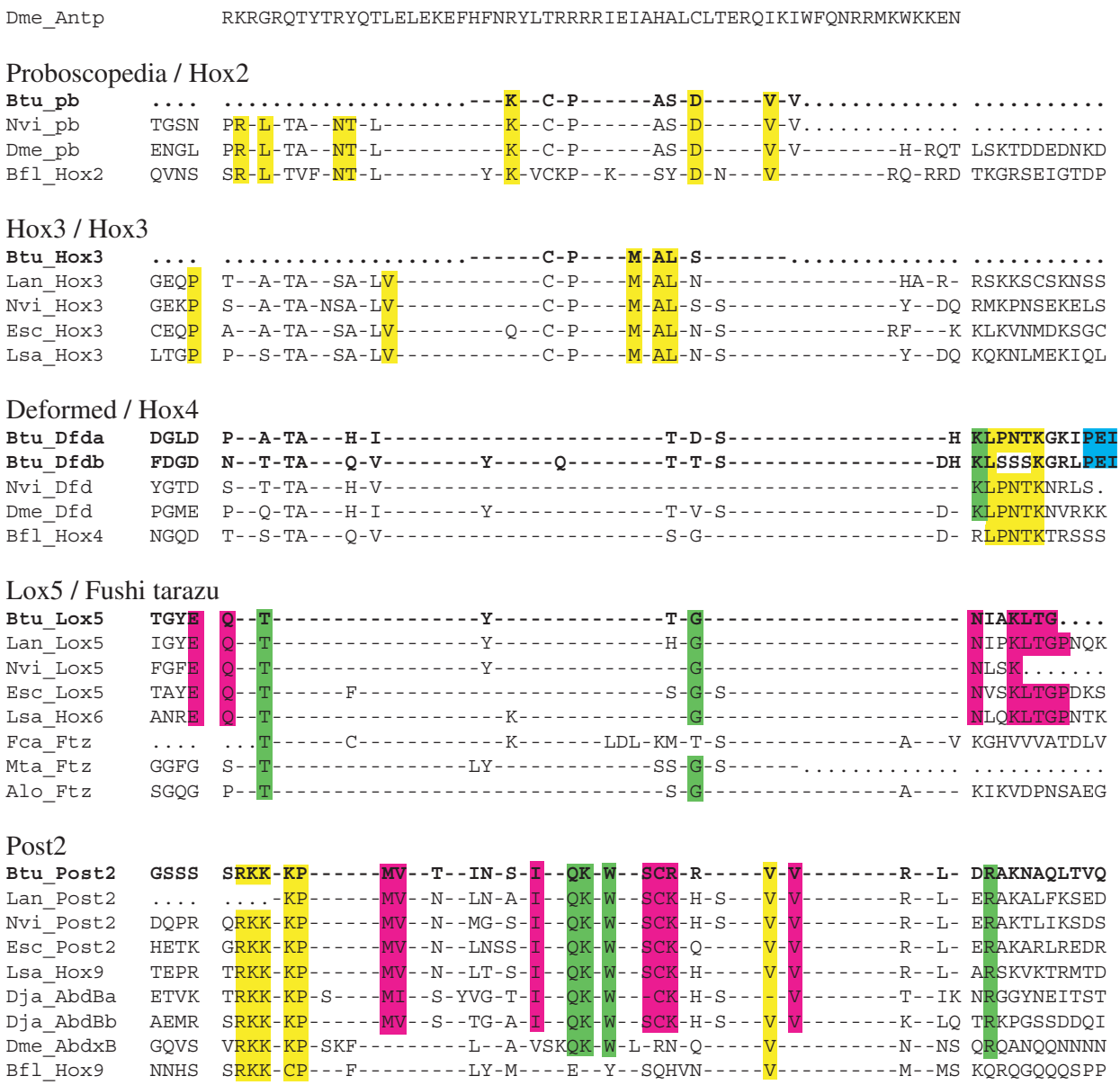


Fig. 1. Alignment of *Hox* gene homeodomains and flanking regions. Dashes represent identity with the *Drosophila melanogaster Antp* homeodomain sequence shown at the top of the alignment. Peptides diagnostic of Hox paralogs across bilaterians are highlighted in yellow. Diagnostic peptides of protostomes (i.e., lophotrochozoans and ecdysozoans), but not in deuterostomes, are highlighted in green. Diagnostic peptides found only in lophotrochozoans are highlighted in red. The "PIE" motif shared between *Btu-DfdA* and *Btu-DfdB* are highlighted in blue. Btu, *Bugula turrita*; Esc, *Euprymna scolopes*; Lan, *Lingula anatina*; Lsa, *Lineus sanguineus*; Nvi, *Nereis virens*; Alo, *Archegozetes longisetosus*; Fca, *Folsomia candida*; Mta, *Milnesium tardigradum*; Dme, *Drosophila melanogaster*; Bfl, *Branchiostoma floridae*; Dja, *Dugesia japonica*.

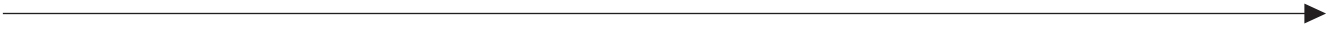
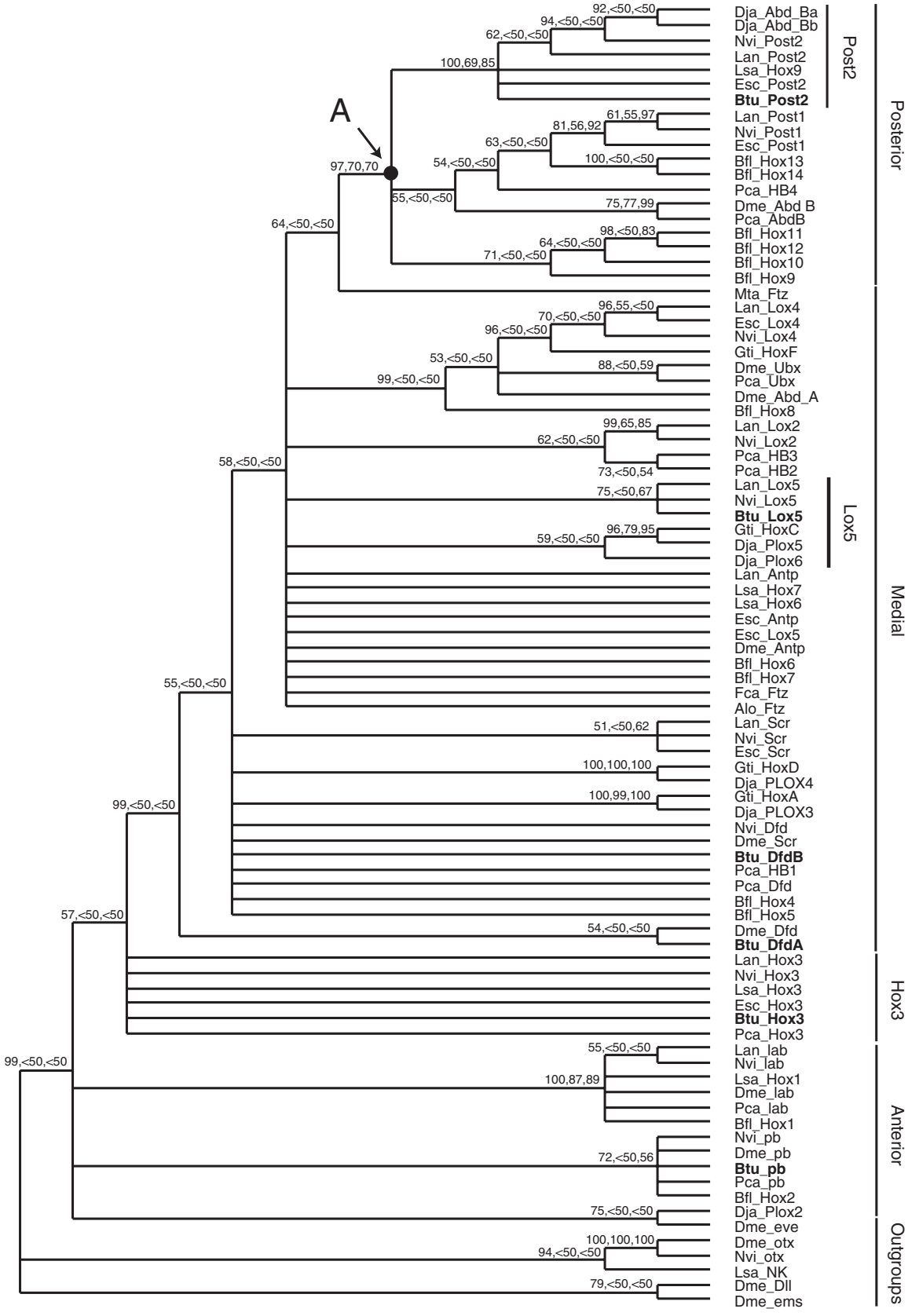


Fig. 2. Phylogenetic reconstruction of *Hox* gene relationships. Tree is from Bayesian likelihood analysis using MrBayes: half compatibility consensus from 1,000,000 replicates, burn-in of 200,000 replicates. Percent support values above branches are from Bayesian likelihood, parsimony bootstrap, and minimum evolution bootstrap, respectively. Parsimony bootstrap values are from 1000 replicate resampling analyses, each of which used 100 heuristic replicates with tree-bisection-reconnection. Minimum evolution values are from 1000 replicate resampling analyses, each with 100 heuristic replicates. Node "A" represents the last common ancestor of posterior class genes in lophotrochozoans, ecdysozoans, and deuterostomes.


A
92,<50,<50
94,<50,<50
62,<50,<50
100,69,85
61,55,97
81,56,92
63,<50,<50
100,<50,<50
54,<50,<50
97,70,70
55,<50,<50
75,77,99
64,<50,<50
98,<50,83
64,<50,<50
71,<50,<50
96,55,<50
70,<50,<50
96,<50,<50
53,<50,<50
88,<50,59
99,<50,<50
99,65,85
62,<50,<50
58,<50,<50
73,<50,54
75,<50,67
96,79,95
59,<50,<50
55,<50,<50
51,<50,62
100,100,100
100,99,100
99,<50,<50
57,<50,<50
54,<50,<50
55,<50,<50
99,<50,<50
100,87,89
72,<50,56
75,<50,<50
100,100,100
94,<50,<50
79,<50,<50
Dja_Abd_Ba
Dja_Abd_Bb
Nvi_Post2
Lan_Post2
Lsa_Hox9
Esc_Post2
Btu_Post2
Lan_Post1
Nvi_Post1
Esc_Post1
Bfl_Hox13
Bfl_Hox14
Pca_HB4
Dme_Abd B
Pca_AbdB
Bfl_Hox11
Bfl_Hox12
Bfl_Hox10
Bfl_Hox9
Mta_Ftz
Lan_Lox4
Esc_Lox4
Nvi_Lox4
Gti_HoxF
Dme_Ubx
Pca_Ubx
Dme_Abd_A
Bfl_Hox8
Lan_Lox2
Nvi_Lox2
Pca_HB3
Pca_HB2
Lan_Lox5
Nvi_Lox5
Btu_Lox5
Gti_HoxC
Dja_Plox5
Dja_Plox6
Lan_Antp
Lsa_Hox7
Lsa_Hox6
Esc_Antp
Esc_Lox5
Dme_Antp
Bfl_Hox6
Bfl_Hox7
Fca_Ftz
Alo_Ftz
Lan_Scr
Nvi_Scr
Esc_Scr
Gti_HoxD
Dja_PLOX4
Gti_HoxA
Dja_PLOX3
Nvi_Dfd
Dme_Scr
Btu_DfdB
Pca_HB1
Pca_Dfd
Bfl_Hox4
Bfl_Hox5
Dme_Dfd
Btu_DfdA
Lan_Hox3
Nvi_Hox3
Lsa_Hox3
Esc_Hox3
Btu_Hox3
Pca_Hox3
Lan_lab
Nvi_lab
Lsa_Hox1
Dme_lab
Pca_lab
Bfl_Hox1
Nvi_pb
Dme_pb
Btu_pb
Pca_pb
Bfl_Hox2
Dja_Plox2
Dme_eve
Dme_otx
Nvi_otx
Lsa_NK
Dme_Dll
Dme_ems
Post2
Lox5
Posterior
Medial
Hox3
Anterior
Outgroups

to have originated subsequent to the divergence of lophotrochozoans and ecdysozoans and therefore represents a synapomorphy for the Lophotrochozoa. If bryozoans were basal protostomes, we would have expected *Btu-Post2* to be basal to, or part of a polytomy with, the last common ancestor of posterior class genes in other bilaterians (Fig. 2, node A).

Lox5 was first identified in the leech, *Helobdella robusta* (Kourakis et al. 1997) and subsequently in a brachiopod, a polychaete, and a nemertean, prompting the suggestion that *Lox5* represents a synapomorphy for the Lophotrochozoa (de Rosa et al. 1999). This assertion was based on the similarity in homeodomain sequence and the presence of the conserved "KLTGP" motif in the C terminal flanking region. Telford (2000b) suggested, based on similarities in homeodomain sequences, that *Lox5* is orthologous to *fushi tarazu* (*ftz*) in arthopods and possibly also to *Hox6* in deuterostomes. Although *Lox5* may be orthologous to *ftz*, the "KLTGP" motif has only been identified in the *Lox5* genes of lophotrochozoans. If this motif was present in the taxon basal to the Lophotrochozoan/Ecdysozoan split, at least one evolutionary loss (after the initial gain in the common ancestor) must be posited to explain its absence in Ecdysozoans. It is therefore more parsimonious to assume that bryozoans possess the *Lox5* "KLTGP" motif due to a single acquisition in lophotrochozoans after the divergence from Ecdysozoa.

Our Hox survey also produced two putative copies of the *Dfd*/*Hox4* class of genes. The phylogenetic analysis offers little resolution in this region of the gene tree. However, the presence of the "PEI" flanking motif might suggest that these two gene copies shared a recent common history. To our knowledge, this is the first case where two copies of a *Dfd*/*Hox4* type gene have been reported from the same species, suggesting the *Btu-Dfd* copies are the product of a duplication event that occurred after bryozoans formed an independent lineage. Kourakis and Martindale (2001) reported two copies of a *Dfd* type gene from the leech *Helobdella triserialis*. However, only one copy, *Lox18*, was isolated from *H. triserialis*. The other *Dfd* class gene, *Lox6*, was isolated from *H. robusta* (Kourakis et al. 1997) and *Hirudo medicinalis* (Wong and Macagno 1998) and only inferred to be in *H. triserialis*. The Lox18 sequence was considerably different from Lox6, and thus the authors concluded they must be paralogs rather than Lox18 being a derived Lox6 ortholog. Because *Lox18* and *Lox6* have not been reported from the same species, the conclusion of two *Dfd* type genes in leeches is subject to debate. In the case of multiple copies in *Bugula*, we did not find strong evidence that bryozoans have more than one Hox cluster (assuming that *Hox* genes are linked as in other animals) and suspect our findings indicate duplication within the cluster. Verification of this hypothesis will require sampling of other taxa and more complete information from the bryozoan genome.

Interpretation of the phylogenetic position of Bryozoans has relied solely on morphology and 18S rDNA data. As pointed out by Jenner (2001), the noncritical recycling of morphological data to assess relationships between recognized animal phyla is problematic. Also, based on these sources of data, conflicting results have been presented. The *Hox* gene data in hand support the hypothesis that bryozoans are members of the lophotrochozoan clade (Halanych et al. 1995) and not basal protostomes as suggested by Giribet et al. (2000). This result has also been supported by novel nuclear ribosome large subunit data (unpublished data). Although the ribosomal data suggest a basal position of bryozoans in the Lophotrochozoa, more data are needed to confidently determine their placement within the clade.

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