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Hox Genes of the Direct-type Developing Sea Urchin *Peronella japonica*

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ABSTRACT—*Peronella japonica* is a direct-type developing sea urchin that metamorphoses in 3 days without feeding, providing a good system to analyze developmental mechanisms especially at later larval stages to metamorphosis. We surveyed the *Hox* genes of *P. japonica* by PCR and RT-PCR, and isolated 13 *Hox*-type sequences. Phylogenetic analysis and database searches resulted in identification of all the *P. japonica* orthologs of the *Hox* genes that have been reported in other sea urchins, plus 3 *Hox*-type genes: the *Evx*-type, the *Xlox*-type genes, and an additional posterior *Hox* gene. This is the first report to show the *Evx* in echinoderms. The present results revealed the putative *Hox* gene cluster of *P. japonica*, which contains three anterior, four medial, and four posterior genes plus the *Evx*. The organization is essentially similar to the vertebrate *Hox* cluster except for only a single gene for *Hox4-5* types. The C-terminal amino acid sequence outside the homeodomain of *Hox4/5* suggested that loss of *Hox4* might have occurred in the echinoderm lineage.

INTRODUCTION

The *Hox* gene cluster occupies a central position in current concepts of the development and evolution of metazoan body plans. It controls spatial patterning mechanisms along the anterior/posterior axis. There exists a co-linearity between the order of the *Hox* genes in the genome and the spatial order of their domains of expression during development. This co-linearity is widely conserved among insects, vertebrates, and nematodes (McGinnis and Krumlauf, 1992; Salser and Kenyon, 1994; Garcia-Fernández and Holland, 1996). Furthermore the presence of the *Hox*-type genes has been also confirmed in nonbilaterian animals including cnidarians and sponges (Schummer *et al.*, 1992; Degnan *et al.*, 1995). Therefore the *Hox* system has been proposed to be the zootype, a fundamental mechanism shared by all metazoans (Slack *et al.*, 1993).

In contrast to the majority of deuterostomes, echinoderms have pentamerous body plans and lack cephalic structures. Echinoderms have a single *Hox* cluster (Popodi *et al.*, 1996; Mito and Endo, 1997, 2000). Martinez *et al.* (1999) have recently characterized the gene organization by physical mapping in *Strongylocentrotus purpuratus*, and shown that the echinoderm *Hox* cluster is essentially similar to those of the chordates, despite of the radically altered body plan.

For several phyla so far examined, the *Hox* genes are expressed most clearly at the phylotypic stage: a particular stage of embryonic development at which all the members of the phylum show the maximum degree of similarity (Slack *et al.*, 1993). In echinoderms, most of the *Hox* genes are not used for the early embryonic development, whereas all are expressed during the stage when the adult body plan is formed in the imaginal rudiment (Arenas-Mena *et al.*, 1998). These observations strongly suggest that metamorphic larvae/juveniles may be the phylotypic stage of echinoderms. Thus the explication of expression patterns of the echinoderm *Hox* genes in the rudiment might give us a clue to elucidate their evolutionary transformations from bilateral to penta-radial structures.

Peronella japonica is a direct-type developing sea urchin. It forms the two-armed pluteus larva but the metamorphosis completes in 3 days without feeding (Okazaki, 1975). This characteristic provides an excellent system to analyze expression patterns of the *Hox* genes both in the larva and in the rudiment. Since there is no information yet about the *P. japonica* *Hox* genes, we surveyed the *Hox* genes by genomic PCR as well as RT-PCR. In the present paper we isolated all the *P. japonica* orthologs of the *Hox* genes that have been reported in other sea urchins. In addition, three other *Hox*-type genes were identified: the *Evx*- and *Xlox*-type genes, and a further posterior *Hox* gene. This work will provide fundamental information to investigate roles of the *Hox* genes in penta-radial echinoderms.

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MATERIALS AND METHODS

Animal and gametes

The sea urchin *Peronella japonica* were collected near the Noto Marine Laboratory, Kanazawa University. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Jamarine U (JSW; Jamarine Laboratory) was used as artificial seawater throughout experiments. After insemination, fertilized eggs were washed with JSW three times and cultured at 25°C at a concentration of 50 embryos/ml in Petri dishes without agitation.

Nucleic acid extraction

Genomic DNA was extracted from sperm of *P. japonica*. Dry sperm was suspended in 20 vol of calcium-magnesium-free sea water, and to the suspension 10 vol of DNA extraction buffer (0.15 M NaCl/10 mM Tris-HCl, pH 8.0/10 mM EDTA/0.1% SDS) containing 100 µg/ml Proteinase K was added. The mixture was incubated at 55°C for 2 hr then 37°C for 16 hr, and followed by extraction with phenol and then phenol/chloroform. DNA was precipitated by adding 2 vol of ethanol to the mixture, and dissolved in TE. Total RNA was extracted from eggs or larvae with LiCl/urea method of Auffray and Rougeon (1980). Poly (A)⁺ RNA was isolated with Oligotex-dT30 (Roche) from total RNA.

Synthesis of cDNA

Complementary DNA was synthesized using 3' RACE System (Gibco BRL) from a mixture of poly(A)⁺ RNAs that were extracted from all the larval stages at 6 hr-intervals from the unfertilized egg to the metamorphosis (0–72 hr after fertilization).

PCR, RT-PCR, and 3' RACE

Four degenerated primers, F0, F, R1, and R2, were used to amplify *Hox* fragments of *P. japonica*. F0 and F, forward primers, corresponded to partially overlapping amino acid sequences, QLTELEK and LELEKEF, in the first helix of the homeodomain of the *Hox* genes, respectively. On the other hand, R1 and R2, reverse primers, located to the sequences, FQNRRMK and KIWFQNR, in the third helix, respectively. Sequences of the primers were as follows. F0; 5'-CARYTNACNGARYTNGARAA-3', F; 5'-YTNGARYTNGARAARGARTT-3', R1; 5'-TTCATNCKNCKRTTYTGAA-3', R2; 5'-CKRTTYTGRAACCADATYTT-3'. To amplify the *engrailed* gene fragment, we designed another primer, EN-F (5'-GAYGARAARMGNCNMG-3'), which located at N-terminal of the homeodomain, and used with R2 primer. We amplified the 3' end of Pj3 cDNA using 3' RACE System (Gibco BRL) and Pj3-specific primer (5'-CGATATCTCACCCGACG-3').

For each 25 µl reaction, 100 ng of genomic DNA or cDNA synthesized from 10 ng of poly (A)⁺ RNA was used as a template. Reaction mixtures contained 10 µM of each primer, 200 µM of deoxynucleotides, 1 unit of Taq DNA polymerase (Toyobo), and 1 unit of anti-Taq antibody (Toyobo) in a buffer supplied by the manufacturer. PCR cycles for genomic PCR and RT-PCR were as follows: 2 min at 94°C, 33 cycles (30 sec at 94°C, 2 min at 50°C, 30 sec at 72°C), 20 min at 72°C. In the 3' RACE, we elongated the extension time from 30 sec to 2 min. After PCR, the reaction mixture was applied to an agarose gel, and electrophoresed in TAE buffer. Gels were stained with ethidium bromide (0.5 µg/ml) to detect PCR products.

Sequencing PCR products

PCR products from excised agarose gel bands were purified using MERmaid Kit (Bio 101) or Prep-A-Gene DNA Purification Systems (BioRad), and ligated into pT7/T-vector (Novagen). Plasmid DNAs were purified using Plasmid Miniprep Kit (BioRad) and subjected to sequencing reactions (Amersham). Sequences were determined with an automated sequencer LI-COR 4200.

Sequence analysis

Derived amino acid sequences of *P. japonica Hox*-type genes were compared to those of *Tripneustes gratilla* (Dolecki *et al.*, 1986; Dolecki *et al.*, 1988; Wang *et al.*, 1990), *Paracentrotus lividus* (Di Bernardo *et al.*, 1994), *Lytechinus variegatus* (Ruddle *et al.*, 1994), *Heliocidaris erythrogramma* (Popodi *et al.* 1996), *Hemicentrotus pulcherrimus* (Ishii *et al.*, 1999), *Strongylocentrotus purpuratus* (Martinez *et al.*, 1999), *Asterina minor* (Mito and Endo, 1997), *Oxycomanthus (Comanthus) japonicus*, and *Stegophiura sladeni* (Mito and Endo, 2000). Multiple alignments and neighbor-joining analyses were performed using CLUSTAL W (Thompson *et al.*, 1994).

RESULTS AND DISCUSSION

PCR-amplification of *Hox* genes

In order to survey the *Hox* genes of *P. japonica*, we PCR-amplified the *Hox*-type sequences using genomic DNA and cDNA as templates. Complementary DNA was synthesized from poly (A)⁺ RNA that was prepared from all the larval stages from the unfertilized egg to the metamorphosis. We first used two degenerated primers, F and R1, corresponding to the first and the third helices of the homeodomain, which are highly conserved among the *Hox* genes (Bürglin, 1994). As the result, 11 *Hox*-type sequences of 85 nucleotides long were identified, and named Pj1–Pj11 in order of the determination. For their sequence determination, at least two clones of identical sequence were independently obtained to exclude the possibility of mutations introduced by PCR. Still a variation was observed on Pj7 sequence, where T was replaced by C at the nucleotide 53. It is reported that the DNA of *S. purpuratus* displays 4–5% intraspecific sequence polymorphism (Britten *et al.*, 1993). Since the substitution was synonymous in the amino acid sequence, we interpreted that the variation was due to a polymorphic allele.

We next used other primer sets, F/R2 for genomic PCR and F0/R1 for RT-PCR, and use of the latter set resulted in identification of two more *Hox*-type sequences, Pj12 and Pj13. Fig. 1 shows the sequences of Pj1–Pj13 (homeobox positions 60–144), the derived amino acid sequences (homeodomain positions 21–48), and also the number of clones obtained by genomic PCR and RT-PCR.

Phylogenetic analysis of *Hox* genes

So far eight *Hox* genes as well as other types of the homeobox genes have been isolated from several sea urchin species, and they have been termed *Hbox1-12* in order of the isolation. Recently Martinez *et al.* (1999) showed the organization of the *Hox* gene cluster in the *S. purpuratus* genome, and renamed the *Hox* genes as *SpHox 1-11/13b* on the basis of both their order in the cluster and their paralogous affinities with the vertebrate *Hox* genes. In order to infer paralog (cognate) groups to which the *P. japonica Hox*-type genes belong, we compared sequences of Pj1–Pj13 with those of the *Hox* genes of *S. purpuratus* and other sea urchins. Fig. 2 shows a neighbor-joining tree based on 25 amino acids of the homeodomains from *P. japonica* and 6 other species of sea urchins. This tree showed the corresponding relationship as follows. Pj3, Pj6, and Pj10 were identical with *Hox4/5*, *Hox6*,

A

Pj1 CCTTTACAAC ATGTACCTCA CGCGTGATCG GCGCTCGCAT ATCGCCCGCG CTCTCAGTCT CACGGAGCGC CAGGTCAAGA TATGG
 Pj2 CCATTTCAAC CGCTACCTGA CGCGGAAGCG ACGCATCGAG ATCGCACAAAG CTGTTTGCTT GACAGAACGA CAGATCAAGA TCTGG
 Pj3 CCATTTCAAC CGATATCTCA CCCGACGTCG ACGGATCGAG ATCGCCCACG CTCTCGGACT CACGGAGAGG CAGATCAAAA TTTGG
 Pj4 CCGACTCAAT AAATACCTCT GTCGACCCCG TCGGATCGAG ATCGCCGACT TCCTGGAGCT GTCAGAAAAGA CAGGTCAAAA TATGG
 Pj5 TCAGTCCAAC ATGTACCTGA CTCGAGATCG CCGGACCAAG ATGTCACAGG CCCTCGACCT CACGGAGCGA CAGGTCAAAA TCTGG
 Pj6 TCACTTCAGT CGTTACGTGA CGCGAAGGAG ACGCTTCGAG ATCGCACAGA GTCTCGGTCT CAGCGAACGT CAGATCAAGA TCTGG
 Pj7 CCTCTTCAAC ATGTACTTGA CCCGAGATCG ACGCCTCGAC ATCGCACGGA TGTTGAGCCT GACCGAGCGC CAAGTCAAGA TCTGG
 Pj8 TGCACGAGAG AACTATGTGT CCCGTCCGAA GAGGTGTGAG CTTGTACCCG CGCTCAATCT GCCTGAAACT ACTATTAAGG TTTGG
 Pj9 CCACTTCAAC AAATACATCT CACGACCTCG ACGCATCGAA CTCGCAGCCA TGCTGAATCT CACCGAGCGT CACATCAAAA TTTGG
 Pj10 CCACTTCAAC CGCTACCTAA CGCGACGACG ACGGATCGAA CTCAGCCACC TCCTCGGCTT GACAGAACGA CAGATCAAGA TCTGG
 Pj11 CAATTTCAAC CGGTATTTGT GCCGACCTCG GAGGGTTCGAA ATGGCTAAAT CACTGAGCCT AACCGAAAAGA CAAATAAAAA TATGG
 Pj12 TCATTTCAAC AAATATCTGA CGAGAGCAAG GAGAATAGAG ATTGCCGCCA TGTTGGGACT CAATGAAACG CAGGTCAAGA TCTGG
 Pj13 CCAAGAAAAC ATGTACCTGA CGAGGGACCG AAGGAATAGG ATTTCCGAGG CATTGAACCT TAGTGAGAGA CAGGTAAAAA TCTGG

B

Pj1 LYNMY LTRDR RSHIA RALSL TERQV KIW
 Pj2 HFNRY LTRKR RIEIA QAVCL TERQI KIW
 Pj3 HFNRY LTRRR RIEIA HALGL TERQI KIW
 Pj4 RLNKY LCRPR RIEIA DFLEL SERQV KIW
 Pj5 QSNMY LTRDR RTKMF TGPRL TERQV KIW
 Pj6 HFSRY VTRRR RFEIA QSLGL SERQI KIW
 Pj7 LFNMY LTRDR RLDIA RMLSL TERQV KIW
 Pj8 ARENY VSRPK RCELA TALNL PETTI KIW
 Pj9 HFNKY ISRPR RIEIA AMLNL TERHI KIW
 Pj10 HFNRY LTRRR RIEIA HLLGL TERQI KIW
 Pj11 NFNRY LCRPR RVEMA KSLSL TERQI KIW
 Pj12 HFNKY LTRAR RIEIA AMLGL NETQV KIW
 Pj13 QENMY LTRDR RNRIS EALNL SERQV KIW

C

	genomic PCR	RT-PCR
Pj1	13 clones	2 clones
Pj2	4	3
Pj3	6	1
Pj4	1	1
Pj5	1	2
Pj6	9	3
Pj7	11	21
Pj8	0	5
Pj9	1	4
Pj10	0	2
Pj11	0	4
Pj12	0	3
Pj13	0	6

Fig. 1. (A) *P. japonica* Hox-type sequences at positions 60–144 of the homeobox, (B) the derived amino acid sequences at positions 21–48 of the homeodomain, and (C) the number of clones obtained by genomic PCR and RT-PCR.

and *Hox7* of *S. purpuratus*, respectively. Pj1, Pj2, Pj4, Pj5, Pj7, Pj10, Pj11, and Pj12 were strongly suggested to be orthologous to *Hox11/13a*, *Hox8*, *Hox2*, *Hox11/13b*, *Hox6*, *Hox9/10*, *Hox7*, *Hox3*, and *Hox1*, respectively, with high percentages of support (82–99%). Pj8, Pj9, and Pj13, however, had no counterparts in other sea urchin *Hox* genes reported so far. As is shown in Fig. 2, our PCR survey of the *P. japonica* *Hox* genes succeeded in identification of all the orthologs of sea urchin *Hox* genes. Pj8, 9, and 13 isolated in this study were considered to be novel *Hox*-type genes in sea urchins.

Database searches revealed that Pj8 and Pj9 were 93% and 100% identical in the amino acid sequence with the mouse *Evx-1/2* and the Amphioxus *Xlox*, respectively. The *Evx-1/2* are homologs of the *Drosophila even-skipped* gene that controls segmentation along the anterior/posterior axis. They are not only similar to the *Hox* genes in their homeodomain sequences, but each linked to the 5' end of *Hox* gene clusters (Bürglin, 1994). A cnidarian *Evx*-like gene also locates adjacent to a *Hox*-like gene (Miller and Miles, 1993). Therefore the sequence similarity between the *Evx* and *Hox* genes reflects tandem duplication. This explains the reason the primers designed for the *Hox* genes amplified not only the *Hox* genes but also the *Evx*-type gene (Pj8) in the present study. Similarly, it is also the case with isolation of the *Xlox*-type gene

(Pj9). The *Xlox* was first isolated as a *Hox*-type gene that does not map to the *Hox* cluster, and was later shown to be a member of the *ParaHox* gene cluster, a duplicated sister of the *Hox* gene cluster (Brooke *et al.*, 1998). This is the first report to show the *Evx*-type gene in the echinoderm, while *Xlox*-type sequences have been isolated in the PCR survey of the *Hox* genes of other classes of echinoderms (Mito and Endo, 1997, 2000).

In vertebrates, five paralogous groups of posterior genes, PG9–13, have been identified. Phylogenetic analysis of the homeodomain sequences suggests that two ancestral posterior genes, one for PG9–10 and another for PG11–13, had already existed before divergence of protostomes and deuterostomes (Zhang and Nei, 1996). Tandem duplication of the ancestral genes is thought to have occurred in the deuterostome lineage to increase posterior genes in number. Amphioxus has at least four posterior genes, two of which are orthologous to the vertebrate PG9–10, but the rest two genes are uncertain in the relationship to the vertebrate paralog groups (Garcia-Fernández and Holland, 1996). In sea urchins, three posterior genes, *Hox9/10*, *Hox11/13a*, and *Hox11/13b*, have been isolated. Although *Hox9/10* is very similar to the chordate PG9–10 genes, there is no specific orthologous relationship between *Hox11/13* and the chordate PG11–13 genes

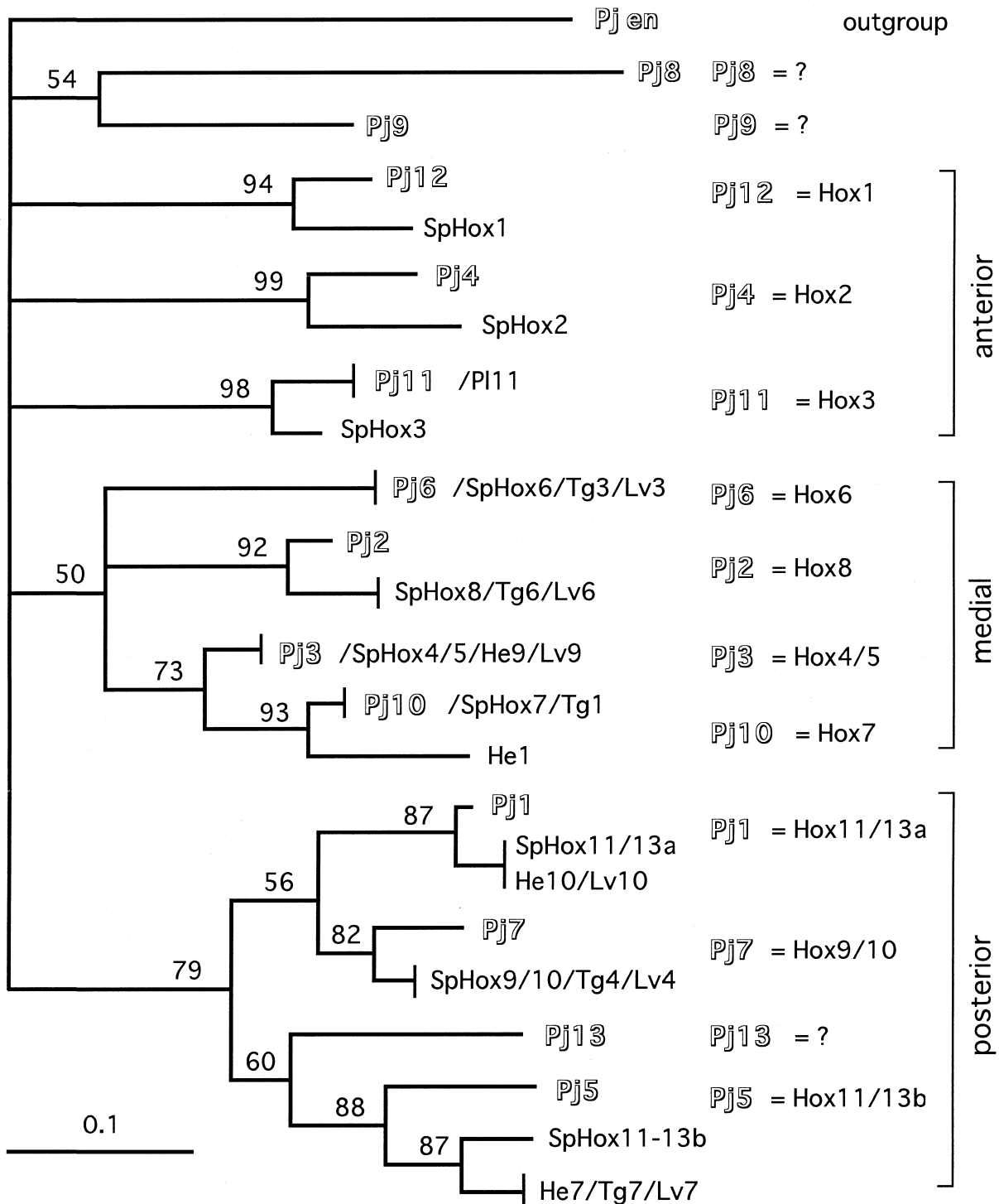
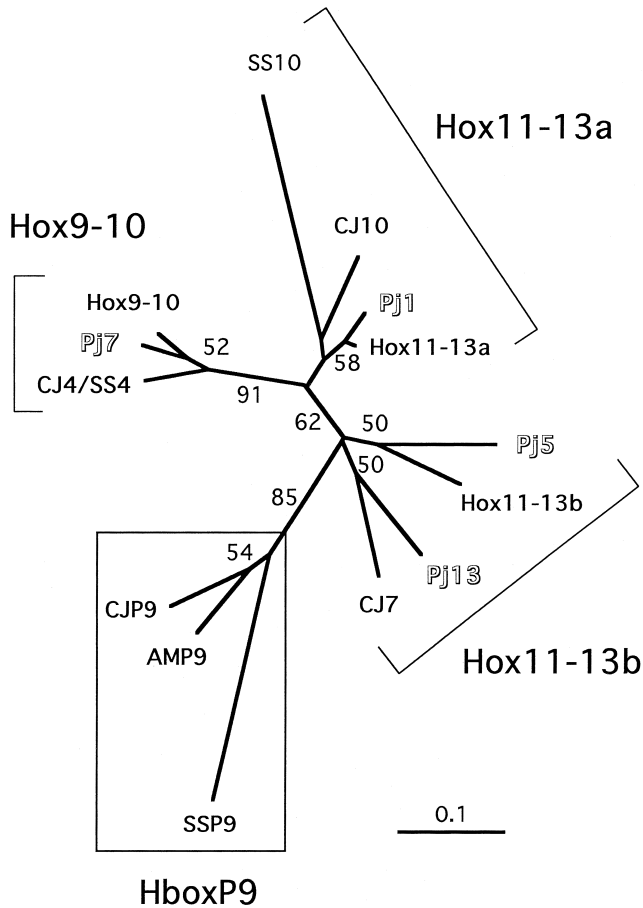


Fig. 2. Neighbor-joining tree based on 25 amino acids of the homeodomains from *P. japonica* (Pj), *S. purpuratus* (Sp), *H. erythrogramma* (He), *T. gratilla* (Tg), *L. variegatus* (Lv), *P. lividus* (Pl), and *H. pulcherrimus* (Hp). The tree is rooted with corresponding sequence of *P. japonica* engrailed (QQSNYLTEQRRRTLAKELTLESQI). Percentage of support (50% or higher) in 1000 bootstrap searches is shown on the branches. *SpHox* is a new name of sea urchin *Hox* genes based on its order in the cluster and its affinities with vertebrate *Hox* genes. Numerals of Pj are in order of the determination in the present study, while those of other sea urchins except Sp indicate *Hbox* numbers (previous names of sea urchin *Hox* genes).

(Martinez *et al.*, 1999). In the present study, four posterior genes were identified from *P. japonica*: three orthologs of the known posterior genes and a novel one (Pj13). Pj13 was sug-

gested the most similar to the posterior *Hox* genes by database searches as well as phylogenetic analysis (Fig. 2). Recently Mito and Endo (2000) have reported that at least four



cognate groups are recognizable among the echinoderm posterior genes, and designated this new group *HboxP9*. Figure 3 shows a neighbor-joining tree of the echinoderm posterior genes including *HboxP9*. It suggested, however, that Pj13 might be rather related to *Hox11-13b* than to *HboxP9* since the *C. japonicus Hbox7* is considered to be an ortholog of *S. purpuratus Hox11-13b* (Mito and Endo, 2000). These observations imply that some of these posterior gene duplications may have occurred independently in different lineage within a phylum or even a class.

Putative Hox gene cluster

Based on the estimation of orthology to the known *Hox* genes mainly from *S. purpuratus*, the present results revealed the putative *Hox* gene cluster of *P. japonica* (Fig. 4). The putative cluster contains three anterior, four medial, and four posterior *Hox* genes plus the *Evx*, although their physical linkage remains to be elucidated. They represent all the paralog groups of vertebrate *Hox* clusters except for a single gene of *Hox4-5* types. The feature that the cluster lacks either *Hox4* or *Hox5* has been also suggested in other classes of echino-

Fig. 3. Neighbor-joining tree of the echinoderm posterior *Hox* genes. Percentage of support (50% or higher) in 1000 bootstrap searches is shown on the branches. Pj13, a novel sea urchin posterior gene, may be rather related to *Hox11-13b* than to *HboxP9*, the fourth posterior group identified in the sea star *A. minor* (AM), the feather star *C. japonicus* (CJ), and the brittle star *S. sladeni* (SS). Numerals of AM, CJ, and SS indicate *Hbox* numbers (see Fig. 2).

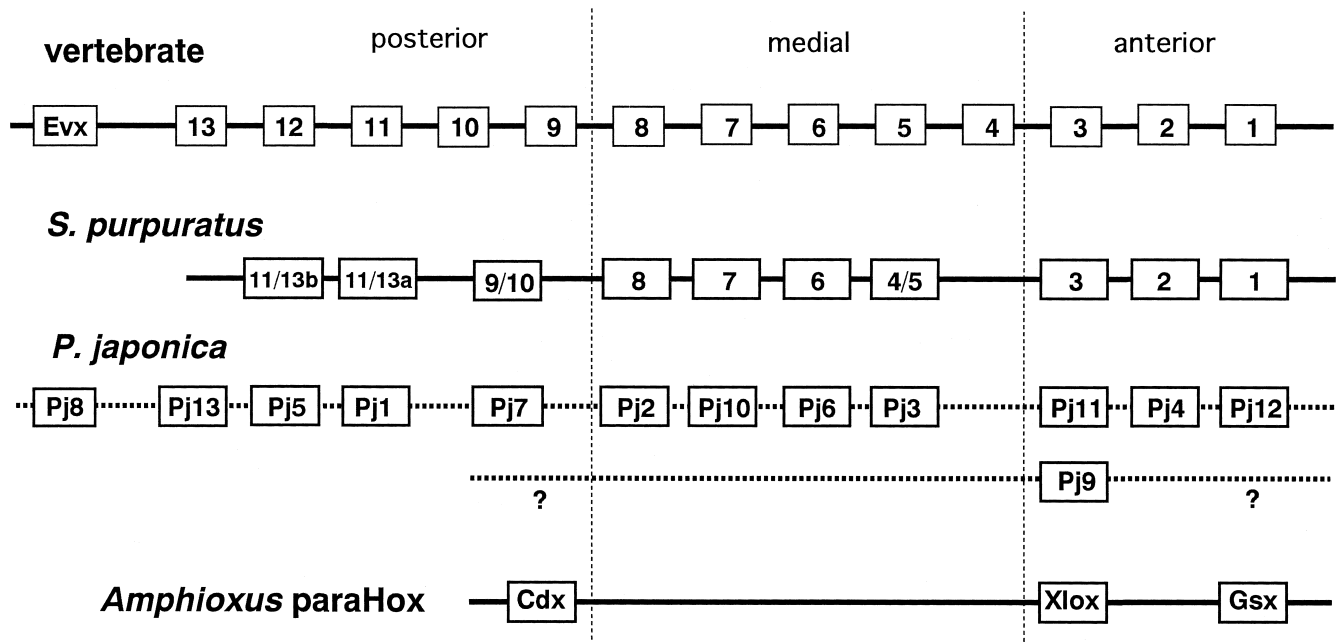


Fig. 4. Organization of the vertebrate *Hox* cluster, *S. purpuratus Hox* cluster, Amphioxus *ParaHox* cluster, and the putative *Hox* cluster of *P. japonica*. Each box indicates a *Hox* gene, and the number in the box shows the vertebrate paralog group. The vertebrate cluster is based on Bürglin (1994), and the *S. purpuratus* cluster is on Martinez *et al.* (1999). The Amphioxus *ParaHox* cluster is by Brooke *et al.* (1998).

Homeodomain	C-terminal									
	1	2	3	4	5	6	7	8	9	
Hox4		L	P	N	T	K			R	S
Hox5		K	K							
Pj3		N	V	K	S	I	S	Q	L	I

Fig. 5. Conserved and paralog characteristic residues of Hox4 and Hox5 in the C-terminal side of the homeodomain, and the corresponding amino acid sequence of Pj3. Amino acids represented in bold are conserved by all vertebrate members of the paralog group, while those in white are conserved by *Drosophila* as well as vertebrates (Sharkey *et al.* 1997). Note that Pj3 (Hox4/5) sequence includes one of the Hox5-characteristic lysine residues, but does not match the Hox4-characteristic residues at all.

derms including sea stars, feather stars, and brittle stars (Mito and Endo, 1997, 2000). On the other hand hemichordates contain both *Hox4* and *Hox5* (Pendleton *et al.*, 1993). Since the hemichordate is suggested to be a sister group of the echinoderm (Wada and Satoh, 1994), loss of either *Hox4* or *Hox5* may be characteristic of the echinoderm *Hox* cluster, a synapomorphy of echinoderms.

Paralog groups of the vertebrate *Hox* clusters can be correlated with some of the *Hox* genes of Amphioxus, sea urchins, and even *Drosophila*. This fact indicates that the paralogous relationships have still remained distinctive over hundreds million of years. Sharkey *et al.* (1997) have identified characteristic residues that define the different paralog groups of the *Hox* genes. Although Hox4 and Hox5 are almost identical in their homeodomain sequences, both conserve several paralog-characteristic residues outside the homeodomain. In order to infer a paralog group to which Pj3 (*Hox4/5*) belongs, we isolated the 3' end of Pj3 cDNA by 3' RACE and compared the derived amino acid sequence with those of Hox4 and Hox5. Fig. 5 shows the conserved paralog-characteristic residues in the C-terminal amino acid sequences outside the homeodomain of Hox4 and Hox5, and the corresponding sequence of Pj3. Four continuous residues of 'PNTK' are characteristic to Hox4, which are conserved in *Drosophila* as well as all vertebrate members. On the other hand, Hox5 conserves two characteristic lysine residues among vertebrates. Pj3 sequence included one of the Hox5-characteristic lysine residues, but it did not match widely conserved Hox4-characteristic residues at all. We inferred from the sequence that Pj3 may be *Hox4* rather than *Hox5*.

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