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Expression patterns of *Hox* genes in the direct-type developing sand dollar *Peronella japonica*: insights into the evolution of echinoderms

*Hox* 遺伝子から探る棘皮動物の進化
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**General Introduction**

Bilateria is a group of multicellular animals with bilateral symmetry and three germ layers. Bilaterians are classified into the deuterostomes and protostomes according to traits such as coelomogenesis, cleavage type, and mouth formation. Deuterostomes are a monophyletic group of animals that include chordates, hemichordates, echinoderms, acoels, and xenoturbellids (Fig. I-1; Philippe et al., 2011). Echinoderms and hemichordates are sister taxa, forming a group known as the ambulacaria (Swalla and Smith, 2008). Typical members of the ambulacraria adopt indirect development with an auricularia-type larva (Nakano et al., 2003). The larva is characterized by having three parts of coeloms along the anteroposterior (AP) axis: the protocoel, and a pair of the mesocoels and metacoels (or somatocoels) (Fig. I-2; Peterson et al., 2000). Adult hemichordates inherit fundamental body plan from the larvae. In contrast, echinoderms transform from a bilateral larva into a pentameral adult.

Echinoderms include sea lilies, starfishes, brittle stars, sea cucumbers, and sea urchins. Echinoderms have a unique water vascular system, which consists of the ring canal encircling the esophagus and the radial canal running along each ray. The radial canal sends numerous small projections (podia or tube feet) to the exterior. The surface of an echinoderm is divided into two areas, the ambulacrum and interambulacrum; symmetrically spaced radiating grooves or bands are termed ambulacra, at which the podia project to the exterior, whereas the areas between the ambulacra are termed interambulacra.

In the typical sea urchin development, adult mouth will open on the left side of the larva. Thus, the mouth-to-anus axis turns at a right angle to the left, during metamorphosis.
The left mesocoel is called the hydrocoel, because it develops into the water vascular system together with overlying ectoderm, called the vestibule. The adult rudiment is a complex of tissues, which is composed of the vestibule, hydrocoel, and left somatocoel. During the rudiment formation, the left somatocoel comes to lie under the hydrocoel, while the hydrocoel encircles the future esophagus and develops five extensions, known as the primary lobes. Each lobe gives rise to a radial canal with tube feet (podia), forming a ray called the ambulacrum.

The unique body plan of echinoderms results from superposition of the radial symmetry onto bilaterality. With respect to the ancestral AP axis in adult echinoderms, two major models have been proposed (Fig. I-3). A “rays-as-axes” model is based on the fivefold symmetry of the adult nervous system; five radial nerves connected by a central ring nerve. From the analogy to the central nervous system of chordates, each echinoderm ‘arm’ represents a duplication of the bilaterian AP axis (Raff, 1996). In contrast, a “rays-as-appendages” model regards the adult oral-aboral axis as the ancestral AP axis; each ‘arm’ is considered as an outgrowth of the water vascular system (Hotchkiss, 1998; Peterson et al., 2000).

The Hox gene complex is a duplicated set of genes that encode transcriptional regulatory proteins with a highly conserved role in patterning along the AP axis in bilaterians. Hox genes often occur in a single cluster on the chromosome. The most conserved and striking feature of the Hox gene complex is its collinear pattern of expression, in which anterior-class genes are expressed in more anterior domains along the AP axis than posterior-class genes (Carroll et al., 2005). However, increasing numbers of animal genome sequences have revealed that Hox genes are not always organized in a cluster. In the
urochordates *Ciona intestinalis* and *Oikopleura dioica*, their *Hox* gene clusters are disintegrated, and several *Hox* genes are lost, although their expression patterns along the AR axis are conserved (Spagnuolo et al., 2003; Ikuta et al., 2004; Seo et al., 2004; Ikuta and Saiga, 2007).

The sea urchin *Strongylocentrotus purpuratus* (*Sp*) Genome Project demonstrated unusual gene order and organization in the sea urchin *Hox* cluster. When compared to the chordate *Hox* cluster, the sea urchin *Hox* cluster differs in three points: (1) the anterior-most three *Hox* genes (*Hox1, Hox2, and Hox3*) are translocated to the 5’ end of the cluster in an inverse orientation, (2) it lacks *Hox4*, and (3) *Hox5* and *Hox11/13b* are inverted in situ (Fig. I-4A; Cameron et al., 2006).

The expression patterns of *Hox* genes have been reported in three echinoderm species. The *S. purpuratus* larva at pentagonal disc stages has a U-shaped digestive tract, which is flanked by a pair of the somatocoels on either side. In the somatocoels, five *Hox* genes ordered in the cluster, *Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b*, are expressed collinearly along the mouth-anus axis (Fig. I-4B; Arenas-Mena et al., 2000). In contrast, translocated *Hox3* is expressed in the dental sacs protruding from the left somatocoel between the primary podia in alternate positions (Arenas-Mena et al., 1998). Similarly, in the somatocoels of the sea lily *Metacrinus rotundus, Hox5, Hox7, Hox8, and Hox9/10* are expressed in their numeric order along the AP axis of the larva (Hara et al., 2006). On the other hand, in the vestibula larva of *Holopneustes purpurescens*, a direct-developing sea urchin, *Hox5* and *Hox11/13b* are expressed in the vestibule that gives rise to the adult ectoderm (Morris and Byrne, 2005). In either case, however, information on the *Hox* expression pattern is insufficient in both the developmental stages and complements.
*Peronella japonica* is a direct-type developing sand dollar, first characterized by Mortensen (1921). Its eggs are ~300 µm in diameter, making them the smallest known among direct-developing echinoids (Okazaki and Dan, 1954; Wray and Raff, 1991). The embryo develops into an abbreviated pluteus larva and then metamorphoses on day three without feeding. Furthermore, Hano et al. (2001) have isolated eleven *Hox* gene fragments from *P. japonica*. Thus, *P. japonica* is considered to be one of the most suitable echinoderm species to examine the *Hox* expression patterns. However, the processes underlying formation of coelomic compartments, particularly the origin of the hydrocoel, remain undefined.

In the present study, I have investigated the evolution of the echinoderm body plan using *P. japonica*. In part 1, the developmental processes underlying formation of coelomic compartments are reported. I show that the left coelom develops by both schizocoely and enterocoely from the archenteron tip, whereas the hydrocoel and right coelom forms by enterocoely from the archenteron. In part 2, the expression patterns of the *Hox* genes are examined by whole-mount in situ hybridization. Evolution of the echinoderm body plan is discussed based on the expression patterns. In the general discussion, I discuss the origin of the pentameral body plan on the basis of the information obtained from the studies and progressing experiments using the sand dollar *Peronella japonica*. 
Fig. I-1. Phylogenetic relationships of deuterostomes. Deuterostomes are a monophyletic group of animals that include chordates, hemichordates, echinoderms, acoels, and xenoturbellids (Philippe et al., 2011). Echinoderms and hemichordates are sister taxa, forming a group known as the ambulacaria (Swalla and Smith, 2008).
Fig. I-2. The coelomic architectures of the auricularia-type larva, and adult hemichordate and echinoderm. The larva is characterized by having three parts of coeloms along the anteroposterior axis: the protocoel (orange), and a pair of the mesocoels (blue and green) and metacoels (or somatocoels; red and purple) (modified from Fig. 2 in Peterson et al., 2000). Yellow indicates the guts. Hemichordate adults inherit fundamental body plan from the larvae. In contrast, echinoderms transform from a bilateral larva into a pentameral adult. In sea urchin development, adult mouth will open on the left side of the larva. Thus, the mouth-to-anus axis turns at a right angle to the left, during metamorphosis. The left mesocoel is called the hydrocoel, because it develops into an echinoderm-specific water vascular system together with overlying larval ectoderm, called the vestibule. The adult rudiment is a complex of tissues, which is composed of the vestibule, hydrocoel, and left somatocoel. During the rudiment formation, the left somatocoel comes to lie under the hydrocoel, while the hydrocoel encircles the future esophagus and develops five extensions, known as the primary lobes. Each lobe gives rise to a radial canal with tube feet (podia), forming a ray called ambulacrum.
Two hypotheses regarding the orientation of the ancestral bilaterian AP axis in the adult echinoderm body plan (modified from fig. 6-16b in Carroll et al., 2005). **Left**: A “rays-as-axes” model is based on the fivefold symmetry of the adult nervous system; five radial nerves connected by a central ring nerve. From the analogy to the central nervous system of chordates, each echinoderm ‘arm’ represents a duplication of the bilaterian AP axis (Raff, 1996). **Right**: A “rays-as-appendages” model regards the adult oral-aboral axis as an ancestral AP axis; each ‘arm’ is considered an outgrowth of the water vascular system (Hotchkiss, 1998; Peterson et al., 2000).

**Figure I-3**

“rays-as-axes” model

“rays-as-appendages” model
Fig. I-4.  

A: The sea urchin *Hox* cluster (Cameron et al., 2006).  Half-arrows indicate the direction of transcription.  

B: Summary of patterns of five medial/posterior *Hox* gene expression in the somatocoels of *S. purpuratus* (modified from Fig. 7 in Arenas-Mena et al., 2000).  The expression domains are depicted using the same colors than the genes, represented in the sea urchin cluster (A).  Domains of overlapping gene expression are represented as bi-colored stripes.  Each stage is shown from both left and right views to illustrate both somatocoels: left views, where pentameral rudiment can be seen.  

| a, anal | abn, abanal | abo, aboral | o, oral |
Part 1

Unusual coelom formation in the direct-type developing sand dollar *Peronella japonica*
Abstract

*Peronella japonica* is a sand dollar with a zygote that develops into an abbreviated pluteus but then metamorphoses on day three. The adult rudiment formation is unique; it uses a median position of the hydrocoel and a stomodeum-like invagination of vestibule that covers the dorsal side of the hydrocoel. However, the developmental processes underlying coelom formation remain unclear. In this study, I examined this process by reconstructing three-dimensional images from serial sections of larvae. I show that the left coelom developed by both schizocoely and enterocoely from the archenteron tip, whereas the hydrocoel and right coelom formed by enterocoely from the archenteron. This coelom formation arranged the coelomic compartments directly along the adult oral-aboral axis by skipping the initial bilateral phases. Furthermore, my data indicate *P. japonica* retains ancestral asymmetry along the left-right axis in the location of the adult rudiment.
**Introduction**

In echinoids, most species adopt one of two developmental modes. Indirect developers make numerous small eggs, which develop into larvae that require a feeding period before metamorphosis. In contrast, direct developers make few large eggs. These large eggs then develop into larvae that do not feed, reducing the time in the water column before metamorphosis (Emlet et al., 1987; Raff, 1987). Indirect development via a planktotrophic pluteus larva is thought to be the ancestral mode of echinoid development, and direct development with a non-feeding lecithorophic larva is thought to have evolved independently in several lineages (Strathmann, 1978; Raff, 1987; Emlet, 1990).

*Peronella japonica* is a direct-type developing sand dollar, first characterized by Mortensen (1921). Its eggs are ~300 µm in diameter, making them the smallest known among direct-developing echinoids (Okazaki and Dan, 1954; Wray and Raff, 1991). The larva may also represent an intermediate form in the evolution from indirect to typical direct development (Raff, 1987; Amemiya and Arakawa, 1996; Yajima, 2007; Iijima et al., 2009). Indeed, the zygote forms micromeres at the 16-cell stage (Fig. 1-1A), and the descendants ingress into the blastocoel as the primary mesenchyme cells (PMCs) before hatching (Fig. 1-1B) to eventually differentiate to skeletogenic cells (Okazaki, 1975; Amemiya and Arakawa, 1996; Yajima, 2007; Iijima et al., 2009). The embryo develops into an abbreviated pluteus larva with a pair of the postoral arms (Fig. 1-1E) and then metamorphoses without feeding on day three (Fig. 1-1F; Okazaki and Dan, 1954; Okazaki, 1975). PMCs contribute exclusively to the formation of larval skeletal elements, whereas late mesenchyme cells, similar to the secondary mesenchyme
cells (SMCs) of typical indirect developers, are involved in adult skeletogenesis (Yajima, 2007; Iijima et al., 2009).

*P. japonica* exhibits unique adult rudiment formation (Mortensen, 1921; Okazaki and Dan, 1954; Okazaki, 1975). Gastrular invagination begins with the migration of late mesenchyme cells (Fig. 1-1C). Within a few hours, another invagination begins in the ectoderm in the center of the flattened oral field, eventually developing into a stomodeum-like invagination (Fig. 1-1D, G). This invagination extends along the dorsal side of the endomesoderm to the posterior end of the larva, forming the vestibule (Fig. 1-1E, H, I). The mouth does not open, and the blastopore closes, resulting in a blind sac (Fig. 1-1G). At ~24 h, the hydrocoel begins to differentiate in a nearly median position from the left or right coelom, whichever lies close to the ventral side of the larva (Okazaki and Dan, 1954; Fig. 1-1H, I). The location of the hydrocoel, together with the unusual median position of the vestibule, is strikingly different from the corresponding structures in other echinoids. However, the processes underlying formation of coelomic compartments, particularly the origin of the hydrocoel, remain undefined. After the enlargement of the hydrocoel, five lobes are pushed out and arranged in a bilaterally symmetrical fashion with regard to the midline of the larva. Metamorphosis then begins with the protrusion of rudimentary spines and tube feet from the dorsal side of the larva (Okazaki and Dan, 1954; Okazaki, 1975).

In this study, I examined the formation of coelomic compartments which are enclosed by the water vascular system or which become the main body cavities in *P. japonica*. To do this, I reconstructed three-dimensional (3D) images from serial sections of larvae. I show that the left coelom developed by both schizocoely and enterocoely from the archenteron
tip. In contrast, the hydrocoel and right coelom sequentially formed from the archenteron by enterocoely. Furthermore, I defined each ambulacrum of *P. japonica* according to Lovén’s system by raising juveniles until they had a mouth and anus.
Materials and Methods

Animals, Embryos, and Larvae

Adult *P. japonica* were collected in Matsushima beach, Noto Island, Ishikawa, Japan. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Embryos, larvae, and juveniles were cultured in plastic dishes at 24°C in Marine Art SF-1 artificial seawater (Tomita Pharmaceutical, Tokushima, Japan). To culture juveniles, the seawater was changed every other day, and a new suspension of the diatom *Chaetoceros gracilis* was added.

Reconstruction of Three-dimensional (3D) Images

For anatomical observations, embryos and larvae were fixed with 4% paraformaldehyde in artificial seawater (van’t Hoff, 1903), dehydrated in an ethanol series and acetone, embedded in Technovit 8100 (Heraeus Kulzer, Hanau, Germany), and cut serially into 2.5–3 µm thick sections on a microtome LEICA RM 2255 (Leica, Nussloch, Germany). Sections were observed with a fluorescence microscope BZ-9000 (KEYENCE, Osaka, Japan). Because *P. japonica* embryos and larvae emit autofluorescence, I recorded fluorescence images of unstained sections. To reconstruct 3D images, the fluorescence images were converted to black and white and false-colored by hand with Adobe Photoshop CS5 (Adobe systems inc., San Jose, CA). To examine the formation of coelomic compartments, the ectodermal layer, a mass of mesenchyme cells (the left coelom), and archenteron-derived epithelia were colored green, red, and yellow, respectively. In the coloring process, mesenchyme cells scattered in the blastocoel, including the blastocoelar and skeletogenic cells, were eliminated.
Three-dimensional images were reconstructed from colored section images using DeltaViewer (DeltaViewer Project, http://vivaldi.ics.nara-wu.ac.jp/~wada/Delta-Viewer/).
Results

\textit{P. japonica} formed an adult anus at the anterior end of the larva

Similarly to Okazaki and Dan (1954; Fig. 1-1G), I defined the site of the vestibular opening (stomodeum-like structure in typical indirect developers) as the anterior side of the larva. I define the ventral side as the side containing the blastopore. The blastopore closes during the prism stage but remains as a pit for several hours. The vestibule, therefore, invaginates along the dorsal side of the larva along the anteroposterior (AP) axis (Fig. 1-1H, I).

Fig. 1-2 shows a \textit{P. japonica} imago three weeks after metamorphosis, which had been fed the diatom \textit{Chaetoceros gracilis}. It developed five sets of dental elements, adult six-rayed spines, and 15 tube feet (out of focus in this photo). It also retained two larval postoral rods (black and white arrowheads in Fig. 1-2), which are traces of the anterior side of the larva. In our culture condition, several percent of the imagoes (n > 100) retained the larval rods. I observed the digestive tract by diatom chlorophyll fluorescence and found that it started at the masticatory apparatus in the oral center of the imago, involuted counter-clockwise (when viewed from the aboral side) approximately three quarters around, reached the former anterior side of the larva and twisted nearly once around, and ended at the anus (red arrowhead in Fig. 1-2) between the postoral rods. This means that the AP axes of the larva and juvenile are parallel but opposite in direction. Lovén (1874) developed a numbering system for the ambulacra of echinoids based on the AP axis of Irregularia species. According to Lovén’s system, I defined each ambulacrum of \textit{P. japonica} (see below).
The left coelom developed by both schizocoely and enterocoely from the archenteron tip

To examine the formation of the coeloms in *P. japonica* larvae, I reconstructed 3D images from serial sections of larvae (2.5–3 µm thick) using DeltaViewer.

I examined gastrulae at 16 h which had developed the vestibule in the oral field (Fig. 1-3A), and found that the archenteron leaned toward the left side. I also observed a number of mesenchyme cells, similar to the SMCs of indirect developers, migrating out of the archenteron tip (Fig. 1-3B). Fig. 1-4A–D shows four of 70 horizontal serial sections (3 µm thick) along the dorsoventral (DV) axis of an early prism larva at 18 h, in which the ectodermal layer, a mass of mesenchyme cells, and archenteron-derived epithelia are colored green, red, and yellow, respectively. In the coloring process, I have eliminated the mesenchyme cells scattered in the blastocoel, including the PMCs and the blastocoelar cells. Fig. 1-4E–H indicate reconstructed 3D images of the exterior and internal endomesodermal structures viewed from the right-posterior and slightly dorsal side of the larva, respectively. Fig. 1-5 shows sixteen serial sections (from the nineteenth to thirty fourth) of 70 original horizontal serial sections along the dorsoventral axis of the early prism larva (18 h). The exterior image (Fig. 1-4E) is shown in a reduced scale compared to the internal structures (Fig. 1-4F–H). From the archenteron, a coelomic pouch, marked in yellow, elongated counter-clockwise to the anterior and then dorsal direction; the tip of coelomic pouch reached the anterodorsal side of the larva, which was adjacent to the vestibular floor (arrowheads in Fig. 1-4D, G, H). The mesenchyme, marked in red, covered the left side of the coelomic pouch (Fig. 1-4A–D, G). I should note that the boundary between the mesenchyme and enterocoelic epithelia was obscured in portions just below the tip of the coelomic pouch (blue asterisks in Fig. 1-4C, G, H; arrowheads in Fig. 1-5).
This lobe, marked in yellow, appeared to participate in the formation of the left coelom probably by enterocoely since it disappeared from the enterocoelic epithelium by the next stage, and the port ofion of the left coelomic cavity was encircled by an epithelial layer in the next stage (Fig. 1-6; arrowheads in Fig. 1-8).

Fig. 1-6 shows six of 84 horizontal serial sections (3 µm thick) of an early pluteus larva at 24 h, and Fig. 1-7 shows reconstructed 3D images of the exterior and internal endomesoderm structures, viewed from the right-posterior or left-anterior side of the larva, both with a slightly dorsal view. Color code is the same as in Fig. 1-4, except for red. Red shows the left coelom developed from the mesenchyme plus an epithelial lobe formally marked in yellow (asterisks in Fig. 1-4). Fig. 1-8 shows sixteen serial sections (from the eleventh to twenty eighth) of 84 original horizontal serial sections along the dorsoventral axis of the early pluteus larva (24 h). By this stage, the mesenchyme and lobe completely separated from the enterocoelic epithelium and started to form coelomic cavities (Fig. 1-6; Fig. 1-8). Additionally, the coelom expanded to the anterior side of the larva on either side of the enterocoelic structure. The left side of the coelom extended ventrally while the right side extended dorsally (Fig. 1-6B–F; Fig. 1-7C, G). Together with the original left side location, the C-shaped coelom dominantly covered the enterocoelic structures on the left side, with a ventral tilt of the left side (Fig. 1-7C, G).

The hydrocoel and right coelom sequentially developed by enterocoely from the archenteron

In an early prism larva at 18 h, the tip of the coelomic pouch reached the anterodorsal
side of the larva (arrowheads in Fig. 1-4), forming the putative right coelom. During the next six hours of hydrocoel formation, the tip of the coelomic pouch elongated in the posterior direction until nearly reaching the dorsal center of the larva. At the end of elongation, the newly formed hydrocoel was inserted into the C-shaped putative left coelom (Fig. 1-6F; Fig. 1-7C, G). The putative right coelom corresponded to a turning portion of the coelomic pouch, from the anterior to the dorsal direction (Fig. 1-6D–E; Fig. 1-7D, H). This portion of the coelomic pouch became coelom-like in shape with a relatively large cavity in the pluteus larva at 28 h (Fig. 1-9B, C; Fig. 1-10D).

The hydrocoel, left somatocoel, and right somatocoel were arranged along the DV axis of the larva

Fig. 1-9 shows six of 65 horizontal serial sections (2.5 µm thick) of a pluteus larva at 28 h that were used to reconstruct the 3D images of the exterior and internal structures (Fig. 1-10). Color code is the same as in Fig. 1-6. By the pluteus larva stage, the formerly C-shaped left coelom had fused at the anterior side of the larva to encircle the hydrocoel (Fig. 1-10F, G). The hydrocoel started branching lobes to form the podium primordia. According to Lovén’s system, I defined the presumptive ambulacra I–V, although the branches of the hydrocoel in ambulacra I and V were immature compared to those in ambulacra II, III, and IV (Fig. 1-10D, H). P. japonica juveniles have a triad of podia in each ambulacrum after metamorphosis (Okazaki and Dan, 1954). At 28 h, the primordia of a triad of podia and the radial canal developed exclusively in the ambulacrum III (blue asterisks and ra, respectively, in Fig. 1-10D, H) on the future anterior side of adult sand dollars. On the other hand, the left
coelom generated projections against the vestibular floor that covered the dorsal side of the hydrocoel and left coelom (Fig. 1-10B, F). This projection was evident in presumptive interambulacra 2 and 3 (Fig. 1-10C, G). I believe that these projections are rudiments of the dental sac because their development is similar to the formation of dental sacs from the left somatocoel in indirect developers (MacBride, 1903; von Übish, 1913; Smith et al., 2008).

Fig. 1-11 shows six of 71 horizontal serial sections (2.5 µm thick) of a pluteus larva at 32 h that were used to reconstruct the 3D images of the exterior and internal structures viewed from either the dorsal side or the right-posterior (and slightly dorsal) side of the larva (Fig. 1-12). Color code is the same as in Fig. 1-6. By 32 h, the five lobes of the hydrocoel were evident, although closure of the hydrocoel crescent resulting in a closed ring canal had not yet occurred between ambulacra IV and V (arrow in Fig. 1-12D). This is consistent with what is observed in echinoids (Hotchkiss, 1995). Although the primary lobe was largely bilateral along Lovén’s axis that passes through both the ambulacrum III and the interambulacrum 5 (ra3–ds5 in Fig. 1-12C), it did not lie on the midline of the larva, but on the left side (see location of the future ring canal; arrow in Fig. 1-12D). Along with the location, the left coelom tilted leftward (see positions of dental sacs in Fig. 1-12B, F). By this stage, five dental sacs developed from the left coelom and interdigitated with five lobes of the hydrocoel (ds1–5 in Fig. 1-12C, G). This observation indicates that dental sacs in the posterior side of the larva (ds2, 3 in Fig. 1-10B, F) developed before the anterior ones, and suggests that the left coelom should actually be designated the left somatocoel, like that found in the larval anatomy of indirect developers (Smith et al., 2008).
At the same developmental time point, the right coelom, which had been largely segregated from the enteric sac (Fig. 1-11A-E), narrowed in the anterodorsal portion (arrow in Fig. 1-11D) and divided the coelom into anterodorsal and posteroventral sacs. The anterodorsal sac was connected to the hydrocoel at the base of the presumptive ambulacrum II via an epithelial duct (arrowhead in Fig. 1-11E; st in Fig. 1-12H). Although further analysis is required for definitive identification, I believe the duct is a presumptive stone canal. In indirect-developing echinoids, the stone canal that is associated with the left axocoel (ampula) and the hydroporic canal connects the ring canal to the madreporite on genital plate 2 (MacBride, 1903; Gordon, 1929; Smith et al., 2008). This presumptive stone canal suggests that the anterodorsal and posteroventral sacs derived from the right coelom may be the axocoel and right somatocoel, respectively. This classification is consistent with coelomic stacking, the echinoderm-characteristic arrangement of coeloms where the hydrocoel, left somatocoel, and right somatocoel are stacked along the oral-aboral axis of adults (David and Mooi, 1998; Peterson et al., 2000). Our data show that the hydrocoel, left somatocoel, and putative right somatocoel were arranged along the DV axis of the larva with a leftward tilt in P. japonica (Fig. 1-11, 1-12).
Discussion

Unusual coelom formation in *P. japonica*

Our observations indicate that *P. japonica* development represents an example of an extremely modified coelom formation in echinoids. The left coelom developed from mesenchyme cells that had migrated out of the archenteron tip and an epithelial lobe that had projected from the archenteron, whereas the hydrocoel and right coelom sequentially formed from the archenteron by enterocoely (Fig. 1-4, 1-5, 1-6, 1-8). Although enterocoely is the typical coelom formation strategy employed in most echinoderms, schizocoely has been described in several direct-developing species, including the spatangoid *Abatus cordatus* (Schatt and Féral, 1996), the ophiuroid *Amphipholis squamata* (Fell, 1946), and the crinoid *Oxycomanthus japonica* (Kubota, 1988).

The coelom formation of *P. japonica* is unique in two regards. The first is that *P. japonica* uses two means of coelom formation: both schizocoely and enterocoely for the left somatocoel, and enterocoely for formation of the rest of coelomic compartments, including the hydrocoel, stone canal, axocoel, and right somatocoel. The second unique feature is that *P. japonica* uses sequential coelom formation from the archenteron tip. In indirect developers, the left and right coelomic pouches pinch off from the respective sides of the archenteron near the end of gastrulation. Then, during the eight-arm pluteus stage, each coelom divides into three compartments, the axocoel, hydrocoel, and somatocoel, along either side of the esophagus and stomach (Smith et al., 2008). Even in the direct developers *Asthenosoma iijimai* and *Heliocidaris erythrogramma*, the left and right coeloms form independently from the
archenteron tip, and then the left coelom divides into the hydrocoel and left somatocoel (Amemiya and Emlet, 1992; Ferkowicz and Raff, 2001). Thus, unlike both the indirect and the direct developers, the *P. japonica* archenteron tip sequentially generates coelomic compartments: first, mesenchyme cells plus an epithelial lobe that give rise to the future left somatocoel, and then the future hydrocoel, axocoel, and right somatocoel by enterocoely. By skipping the initial bilateral phases of the coelom formation, sequential coelom formation results in direct arrangement of the coelomic compartments along the adult oral-aboral axis in both stacking order and connection via the stone canal. Additionally, *P. japonica* probably skips formation of the hydroporic canal and hydropore. In indirect developers, these structures develop from the left coelom prior to its differentiation into the left axocoel, hydrocoel, and somatocoel (Smith et al., 2008). However, I did not observe these types of tubular structures or an opening in either the serial sections or the 3D images of *P. japonica* larvae from the early prism (18 h) to pluteus larva (32 h) stages. In fact, the dorsal and ventrolateral sides of the endomesoderm were covered with the vestibular floor consisting of stratified epithelia and a gap-less larval ectodermal layer with a blastopore, respectively.

*P. japonica* does not form larval mouth (stomodeum), but instead develops a vestibule in the region (Okazaki and Dan, 1954; Kitazawa and Amemiya, 1997). In addition to the direct arrangement of coelomic compartments along the adult oral-aboral axis, this precocious formation of the vestibule may contribute to the rapid adult rudiment formation in *P. japonica*.

*P. japonica* retains traits of indirect-developing sand dollars
Unlike in Echinacea species (so-called regular urchins; Smith, 1984), the bilateral symmetry of adult skeletal elements in *Echinarchnious parma*, an indirect-developing sand dollar, is marked along Lovén’s axis rather than von Übisch’s axis (Gordon, 1929). Furthermore, Gordon (1929) showed *E. parma*-characteristic features; the ambulacrum III develops more rapidly than the others, and the skeletal plates in interambulacra 2 and 3 are larger and more numerous than those in the three posterior areas of adults. In *P. japonica*, I observed bilateral symmetry of the primary lobe along Lovén’s axis (Fig. 1-10, 1-12) and precocious formation of both the triad of podia in ambulacrum III and the dental sacs in interambulacra 2 and 3 (Fig. 1-10, 1-12). Thus, *P. japonica* appears to conserve traits of indirect-developing sand dollars, except for adult rudiment location.

Kitazawa et al. (2004) discovered two asymmetric traits along the left-right axis in *P. japonica* larvae: shifts toward the left dorsal side of both the vestibular opening and the ciliary band. Together with subsequent enlargement of the vestibular opening and dorsal expansion of the oral field, Kitazawa et al. (2004) observed part of the adult rudiment, such as the podia, through the vestibular opening by scanning electron microscopy. This external observation is consistent with our internal observations that there is a leftward tilt of the left somatocoel encircling the hydrocoel and a concomitant leftward shift of the primary lobe (Fig. 1-10, 1-12). These observations indicate that *P. japonica* retains ancestral asymmetry along the left-right axis and furthermore that the location of the adult rudiment in *P. japonica* is not as exceptional as previously thought.
Figure 1-1

Fig. 1-1. Development of *P. japonica* (A–F) and schematic drawings of larvae (G–I; modified from fig. 1 in Okazaki and Dan, 1954).  

A: Embryo at the 16-cell stage (2.5 h, lateral view). Micromeres are formed at the vegetal pole.  
B: Blastula before hatching (9 h, lateral view). The primary mesenchyme cells ingress into the blastocoel.  
C: Gastrula (16 h, lateral view). Mesenchyme cells migrated out of the archenteron tip.  
D: Late gastrula (18 h, oral view). Stomodeum-like invagination in the oral field is vestibule.  
E: Early pluteus larva (24 h, dorsal view). Vestibule extends on the dorsal side of the larva.  
F: Juvenile after metamorphosis (4 days, aboral view). 

G: Prism larva (longitudinal section).  
H: Pluteus larva (longitudinal section).  
I: Pluteus larva (transverse section).  

A, anterior; P, posterior; D, dorsal; V, ventral; bp, blastopore; co, coelom; ec, enterocoelic sac; es, enteric sac; hy, hydrocoel; vc, vestibular cavity; ve, vestibule. Scale bar = 100 µm.
Fig. 1-2. Imago three weeks after metamorphosis fed with diatoms (aboral view). A: Bright field image. B: Fluorescence image. Green and red arrowheads indicate adult dental elements and anus, respectively. White and black arrowheads show remnant larval postoral rods, traces of the former anterior side of the larva. The digestive tract, fluorescing from chlorophyll, starts from the masticatory apparatus, involutes twice, and ends at the anus between the postoral rods. This indicates that the anterioposterior axes of larvae and adults are parallel, but opposite in direction. Scale bar = 100 µm.
Fig. 1-3. Transverse sections of a gastrula at 16 h along the oral-aboral axis.  
B: Medial section.  Archenteron leans toward the left side of the embryo.  
A number of mesenchyme cells have migrated out of the archenteron tip.  
Scale bar = 100 µm.
**Figure 1-4**

**Fig. 1-4.** Horizontal sections along the dorsoventral axis (A–D) and reconstructed three-dimensional images (E–H) of an early prism larva (18 h). (A–D) Four of 70 serial sections (3 µm thick) of the larva from the ventral bottom to dorsal top. The ectodermal layer, a mass of mesenchyme cells, and archenteron-derived epithelia are colored green, red, and yellow, respectively. Mesenchyme cells scattered in the blastocoel are eliminated. **A:** Ninth section from the ventral bottom (9/70). **B:** Fifteenth section (15/70). **C:** Twenty-fifth section (25/70). **D:** Thirty-fifth section (35/70). (E–H) Three-dimensional images reconstructed from serial sections using DeltaViewer, viewed from the right-posterior, slightly dorsal side of the larva. **E:** Exterior image with axial coordinates (A, anterior; P, posterior; D, dorsal; V, ventral; L, left; R, right). The image is shown in a reduced scale compared to those of internal structures (F–H). **F:** Image of a mesenchymal mass. **G:** Image of whole endomesoderm. **H:** Image of archenteron-derived structures. Arrowheads (in D, G, and H) indicate the tip of the coelomic pouch adjacent to the vestibular floor. Asterisks (in C, G, and H) show portions where the boundary between the mesenchyme and enterocoelic epithelia is obscure. ar, archenteron; rc, right coelom. Scale bar = 100 µm.
Fig. 1-5. Original horizontal sections along the dorsoventral axis of an early prism larva (18 h). (A–P) Sixteen serial sections (from the nineteenth to thirty fourth) of 70 serial sections (3 µm thick) of the larva from the ventral bottom to dorsal top. Arrowheads show an epithelial lobe projected posteriorly from the coelomic pouch. Scale bar = 25 µm.
Figure 1-6

Fig. 1-6.  Horizontal sections along the dorsoventral axis of an early pluteus larva (24 h).  (A–F) Six of 84 serial sections (3 µm thick) of the larva from the ventral bottom to dorsal top. The ectodermal layer, the left coelom developed from the mesenchyme plus an epithelial lobe, and archenteron-derived epithelia are colored green, red, and yellow, respectively.  A: Sixth section from the ventral bottom (6/84).  B: Ninth section (9/84).  C: Thirteenth section (13/84).  D: Twenty-third section (23/84).  E: Twenty-eighth section (28/84).  F: Thirty-second section (32/84).  ar, archenteron; hy, hydrocoel; lc, left coelom; rc, right coelom; vf, vestibular floor.  Scale bar = 100 µm.
Figure 1-7

Fig. 1-7. Three-dimensional images of an early pluteus larva (24 h) reconstructed from serial sections. (A–D) Images viewed from the right-posterior and slightly dorsal side of the larva. (E–H) Images viewed from left-anterior and slightly dorsal side. Color code is the same as in Fig. 1-6. The left coelom, marked in red, expands to the anterior side of the larva on either side of the enterocoelic structures. The tip of the coelomic pouch elongates to the dorsal center of the larva to form the hydrocoel. Together with the original left side location, the C-shaped left coelom dominantly covers the enterocoelic structures on the left side. ar, archenteron; hy, hydrocoel; lc, left coelom; rc, right coelom.
Fig. 1-8. Original horizontal sections along the dorsoventral axis of an early pluteus larva (24 h). (A–P) Sixteen serial sections (from the eleventh to twenty eighth) of 84 serial sections (3 µm thick) of the larva from the ventral bottom to dorsal top. Arrowheads show a part of the left coelom that developed probably by enterocoely from an epithelial lobe projected from the coelomic pouch at 18 h, whereas asterisks indicate coelomic cavities that formed probably by schizocoely in the mesenchyme. Scale bar = 25 µm.
Fig. 1-9. Horizontal sections along the dorsoventral axis of a pluteus larva (28 h). (A–F) Six of 65 serial sections (2.5 µm thick) of the larva from the ventral bottom to dorsal top. Color code is the same as in Fig. 1-6. A: Eleventh section from the ventral bottom (11/65). B: Nineteenth section (19/65). C: Twenty-fifth section (25/65). D: Twenty-eighth section (28/65). E: Thirty-fifth section (35/65). F: Fortieth section (40/65). hy, hydrocoel; lc, left coelom; rc, right coelom; vf, vestibular floor. Scale bar = 100 µm.
Fig. 1-10. Three-dimensional images of a pluteus larva (28 h) reconstructed from serial sections. (A–D) Images viewed from the right-posterior, slightly dorsal side of the larva. (E–H) Images viewed from left-anterior on the slightly dorsal side. Color code is the same as in Fig. 1-6. The formerly C-shaped left coelom has fused at the anterior side of the larva to encircle the hydrocoel, whereas the hydrocoel starts branching lobes in ambulacra II–IV. A triad of podia (blue asterisks) and radial canal develop exclusively in ambulacrum III. I–V, ambulacra I–V; ds2, dental sac in interambulacrum 2; ds3, dental sac in interambulacrum 3; en, enteric sac; ra, radial canal; rc, right coelom.
Figure 1-11

Fig. 1-11. Horizontal sections along the dorsoventral axis of a pluteus larva (32 h). (A–F) Six of 71 serial sections (2.5 µm thick) of the larva from the ventral bottom to dorsal top. Color code is the same as in Fig. 1-6. A: Eighth section from the ventral bottom (8/71). B: Twelfth section (12/71). C: Sixteenth section (16/71). D: Twenty-third section (23/71). E: Thirtieth section (30/71). F: Thirty-third section (33/71). The right coelom, which has been largely segregated from the enteric sac (A–E), is narrowed in the anterodorsal portion (arrow in D) to divide it into the axocoel and right somatocoel. The axocoel is connected to the hydrocoel via a narrow duct (arrowhead in E). ac, axocoel; en, enteric sac; hy, hydrocoel; lsc, left somatocoel; rsc, right somatocoel; vf, vestibular floor. Scale bar = 100 µm.
**Figure 1-12**

(A–D) Images viewed from the dorsal side of the larva. (E–H) Images viewed from the right-posterior and slightly dorsal side. Color code is the same as in Fig. 1-6. The five lobes of the hydrocoel are evident, although closure of the hydrocoel crescent to form the ring canal has not yet occurred between ambulacra IV and V (arrow in D). A podium primordia develops in ambulacrum II (arrowheads in D and H) along with a triad of podia in ambulacrum III (blue asterisks). Although the primary lobe is largely bilateral along Lovén’s axis, it lies on the left side of the larva (C, D), together with a leftward tilt of left coelom (B, F). The left somatocoel has projected five dental sacs, which are interdigitated with five lobes (C, G). The axocoel is connected to the hydrocoel at the base of ambulacrum II via the stone canal. I–V, ambulacra I–V; ac, axocoel; ds1–5, dental sacs in interambulacra 1–5; en, enteric sac; ra2, 3, radial canal in ambulaclum II, III; rsc, right somatocoel; st, stone canal.
Part 2

Expression patterns of *Hox* genes in the direct-type developing sand dollar *Peronella japonica*
Abstract

Echinoderm adults exhibit a remarkable pentaradial body plan, although echinoderms belong to the bilateria and have bilateral larvae. The *Strongylocentrotus purpuratus* Genome Project revealed that the sea urchins have a *Hox* cluster with unusual gene order and organization (Cameron et al., 2006). Although *Hox* expressions in echinoderm larvae have been examined (Arenas-Mena et al., 1998, 2000; Morris and Byrne, 2005; Hara et al., 2006), current data are fragmental in both developmental stages and complements. I comprehensively analyzed expression patterns of ten out of eleven *Hox* genes in the sand dollar *Peronella japonica* by whole-mount in situ hybridization. Like in *S. purpuratus* larvae, five medial/posterior *Hox* genes, *Hox7, Hox8, Hox9/10, Hox11/13a*, and *Hox11/13b*, were expressed in a C-shaped left somatocoel clockwise in their numeric order, when viewed from the adult oral side. This expression pattern conserved regardless of developmental mode suggests that the ancestral AP axis resides in the somatocoels of echinoderms. In the body cavity, adult digestive tract is suspended by the mesenteies, mesodermal membranes formed by epithelial fusion between the left and right somatocoels, and the juvenile digestive tract largely turns clockwise in the body cavity. Given that the somatocoelar *Hox* expression controls the turn and development of the digestive tract, the collinear/numeric-order *Hox* expression in the somatocoel supports a “rays-as-appendages” model, although the oral-aboral (mouth-anus) axis is twisted. In contrast, *Hox1, Hox3, Hox5*, and *Hox11/13b* were expressed pentamerally in the vestibule that generate the adult ectoderm, in either the ambulacral or interambulacral regions. This expression pattern makes it difficult to regard rays of echinoderms as axes, because only
*Hox1* and *Hox11/13b* are expressed in the ambulacra, and their domains are largely overlapped along the proximodistal axis of the ray. It is rather plausible to consider that *Hox1* and *Hox11/13b* are involved in regional specification of the adult ectoderm. *Hox3* and *Hox5* appear to be associated with formation of the dental sacs and/or spines. In other words, *Hox1, Hox3, Hox5*, and *Hox11/13b* appear to be co-opted for development of the echinoderm-specific surface domains and structures. Intriguingly, the four *Hox* genes are all inverted in orientation in the *S. purpuratus Hox* cluster, suggesting that the evolution of echinoderm morphological novelties may have been accompanied by, or precipitated by disorganization of the *Hox* cluster.
Introduction

Deuterostomes are a monophyletic group of animals that include chordates, hemichordates, echinoderms, acoels, and xenoturbellids (Fig. I-1; Philippe et al., 2011). Echinoderms and hemichordates are sister taxa, forming a group known as the ambulacaria (Swalla and Smith, 2008). Typical members of the ambulacraria adopt indirect development with an auricularia-type larva (Nakano et al., 2003). The larva is characterized by tripartite coeloms along the anteroposterior (AP) axis: the protocelel, and a pair of the mesocoels and metacoels (or somatocoels) (Fig. I-2; Peterson et al., 2000). Adult hemichordates inherit fundamental body plan from the larva. In contrast, echinoderms transform from a bilateral larva into a pentaradial juvenile; the origin of the unique and highly derived body plan of echinoderms remains a puzzle.

Echinoderms include sea lilies, starfishes, brittle stars, sea cucumbers, and sea urchins. In the typical sea urchin development, adult mouth will open on the left side of the larva. Thus, the mouth-to-anus axis turns at a right angle to the left during metamorphosis. The left mesocoel is called the hydrocoel, because it develops into the echinoderm-specific water vascular system, together with overlying ectoderm, called the vestibule. The adult rudiment is a complex of tissues, which is composed of the vestibule, hydrocoel, and left somatocoel. During the rudiment formation, the left somatocoel comes to lie under the hydrocoel, while the hydrocoel encircles the future esophagus and develops five extensions, known as the primary lobes. Each lobe gives rise to a radial canal with tube feet (podia), forming a ray called the ambulacrum.
With respect to the ancestral AP axis in adult echinoderms, two major models have been proposed (Fig. 1-3). A “rays-as-axes” model is based on the fivefold symmetry of the adult nervous system; five radial nerves connected by a central ring nerve. From the analogy to the central nervous system of chordates, each echinoderm ‘arm’ represents a duplication of the bilaterian AP axis (Raff, 1996). In contrast, a “rays-as-appendages” model regards the adult oral-aboral axis as an ancestral AP axis; each ‘arm’ is considered as an outgrowth of the water vascular system (Hotchkiss, 1998; Peterson et al., 2000).

The Hox gene complex encodes a family of transcriptional regulatory proteins with a highly conserved role in patterning along the AP axis in bilaterians. Hox genes usually occur in a single cluster on the chromosome; the order of each gene in the cluster corresponds to its expression domain along AP axis (collinearity). However, the Strongylocentrotus purpuratus Genome Project revealed unusual gene order and organization in the sea urchin Hox cluster. When compared to the chordate Hox cluster, (1) the anterior-most three Hox genes (Hox1, Hox2, and Hox3) are translocated to the 5’ end of the cluster, in an inverse orientation, (2) it lacks Hox4, and (3) Hox5 and Hox11/13b are inverted in situ (Fig. I-4A; Cameron et al., 2006).

The expression patterns of Hox genes have been reported in three echinoderm species. The S. purpuratus larva at rudiment formation stages has a U-shaped digestive tract, which is flanked by a pair of the somatocoels on either side. In the somatocoels, five Hox genes ordered in the cluster, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b, are expressed collinearly along the mouth-anus axis (Fig. I-4B; Arenas-Mena et al., 2000). Similarly, in the somatocoels of the sea lily Metacrinus rotundus, Hox5, Hox7, Hox8, and Hox9/10 are expressed in their numeric order along the AP axis of the larva (Hara et al., 2006). In contrast,
translocated \textit{Hox3} is expressed in the dental sacs protruding from the left somatocoel between the primary podia (Arenas-Mena et al., 1998). On the other hand, in the vestibula larva of \textit{Holopneustes purpurescens}, a direct developing sea urchin, \textit{Hox5} and \textit{Hox11/13b} are expressed in the vestibule that gives rise to the adult ectoderm (Morris and Byrne, 2005). In either case, however, information on the \textit{Hox} expressions is insufficient in both the developmental stages and complements.

The sand dollar \textit{Peronella japonica (Pj)} is a direct-type developing echinoid. The embryo develops into an abbreviated pluteus larva and then metamorphoses on day three without feeding. The developmental process was described in the part 1. In the present study, I cloned the full-length cDNAs of eleven \textit{Hox} genes from \textit{P. japonica}, and the expression patterns were examined by whole-mount in situ hybridization. In addition to \textit{Hox} genes, \textit{orthodenticle (Otx)} gene was cloned and examined the expression pattern, which is involved in the fundamental processes of anterior neural patterning and sensory organ formation in animals (Hirth and Reichert, 1999; Tomsa and Langeland, 1999; Acampora et al., 2001; Arendt et al., 2001; Lowe et al., 2003; Zuber et al., 2003; Castro et al., 2006). Through a comprehensive survey, I found that \textit{Hox1, Hox3, Hox5, Hox11/13b}, and \textit{Otx} were pentamerally expressed in either the ambulacral or interambulacral regions, whereas \textit{Hox7, Hox8, Hox9/10, Hox11/13a,} and \textit{Hox11/13b} were expressed in the left somatocoel in their numeric order. Based on the expression patterns, I discuss evolution of echinoderms as well as the ancestral AP axis in echinoderms.

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Materials and Methods

Animals, Embryos, and Larvae

Adult *P. japonica* were collected in Matsushima beach, Noto Island, Ishikawa, Japan. Gametes were obtained by intracoelomic injection of 0.5 M KCl. Embryos and larvae were cultured in plastic dishes at 24°C in Marine Art SF-1 artificial seawater (Tomita Pharmaceutical, Tokushima, Japan).

Cloning of eleven *Hox* and *Otx* genes from *P. japonica*

Hano et al. (2001) reported eleven *Hox* gene fragment sequences of *P. japonica*. To obtain the full-length cDNA of *Hox* genes, rapid amplification of the 5’ and 3’ cDNA ends (RACE) was performed using the GeneRacer Kit (Invitrogen, Carlsbad, CA). Template cDNA was synthesized from a mixture of the total RNA of blastulae (8 h) and pluteus larvae (40 h), which was purified using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan), MagExtractor -RNA- (TOYOBO, Osaka, Japan), and DNase I (Takara Bio Inc., Shiga, Japan). The 5’- and 3’-fragments were amplified by PCR using KOD FX DNA polymerase (TOYOBO) and the following primers:

*PjHox1*-5’-RACE-reverse, 5’-GGCCGTGCCGCCGAGTAACTCTGATTTTGA-3’;

*PjHox1*-5’-RACE-reverse-2, 5’-CATGGCGATGGAGGGATGATGTGTG-3’;

*PjHox1*-3’-RACE-forward, 5’-CCGCCATGTTGGACTCAATGAAACG-3’;

*PjHox2*-5’-RACE-reverse, 5’-CCCCCGGTCTATCATGTTGCAACAAGG-3’;

*PjHox2*-5’-RACE-reverse-2, 5’-TGAGGTATGTGACCCATTACCCTGAC-3’;
PjHox2-3’-RACE-forward, 5’-TCGACCCCCGTCGGATCGAG-3’;
PjHox2-3’-RACE-forward-2, 5’-GAGATCGCCGACTTCTGGAGCTGT-3’;
PjHox3-5’-RACE-reverse, 5’-GGAAAGTCCTATCGGAGTATGCG-3’;
PjHox3-5’-RACE-reverse-2, 5’-CCAAAGAGAAATGACTCGCCAACATGC-3’;
PjHox3-3’-RACE-forward, 5’-GACCTCGGAGGGTCGAATGG-3’;
PjHox5-5’-RACE-reverse, 5’- CCAGGAGGTGTGACGTTAGC -3’;
PjHox5-5’-RACE-reverse-2, 5’- GCTTCTGGCTGATAAGTGTGAGTGC -3’;
PjHox5-3’-RACE-forward, 5’- CCATTCAACCGATATCTCAACCCGACGT-3’;
PjHox5-3’-RACE-forward-2, 5’- CCACGCTCTCGGACTCAG -3’;
PjHox6-5’-RACE-reverse, 5’- CCACCCACGTCGGATTTCTTG -3’;
PjHox6-5’-RACE-reverse-2, 5’- GTCCCGTGTTTCCCTCTTCACTC -3’;
PjHox6-3’-RACE-forward, 5’- TCGCACAGAGTCTCGGTCTCAGC -3’;
PjHox7-5’-RACE-reverse, 5’- CCCTGGTAGCCACTCGCGGTTG -3’;
PjHox7-5’-RACE-reverse-2, 5’- CCTCTCCCGATCCTTCCTTTTTC -3’;
PjHox7-3’-RACE-forward, 5’- TAACGCGACGACGACGATCGAAC -3’;
PjHox7-3’-RACE-forward-2, 5’- TCAGCCACCTCCTCGGCTTG -3’;
PjHox8-5’-RACE-reverse, 5’- CGAGTCATCTGTCATTGTCTCGCTTTGG -3’;
PjHox8-5’-RACE-reverse-2, 5’- CATCCTCACAGTCTTTCTCTCTCTCTCCAC -3’;
PjHox8-3’-RACE-forward, 5’- CATTTCACCCGCTACCGTGACGGAAG -3’;
PjHox8-3’-RACE-forward-2, 5’- GACGCACTCGAGATCGCACAAGCTGTGT -3’;
PjHox9/10-5’-RACE-reverse, 5’- GGACCAGATGTGTTGGGCTTTG -3’;
PjHox9/10-5’-RACE-reverse-2, 5’- CTTCATGGGATGCAATCAACATCG -3’;
To isolate partial fragments of \textit{PjOtx}, PCR cloning experiment was performed using cDNA mentioned above as a template. Degenerate primers toward the \textit{Otx} gene were designed based on conserved sequences of the homeodomain. Primer sequences are as follows: \textit{Otx}-forward, 5’- AARMGNMGAAYMGNACNNTT -3’ coding for KKRRNRTTF; and \textit{Otx}-reverse, 5’- TYTGRAACCAIACYTGIAC -3’ coding for VQVWFQN. Partial sequences were amplified by PCR using GoTaq DNA Polymerase (Promega, Madison, WI). To obtain the full-length cDNA, RACE was performed using the GeneRacer Kit (Invitrogen). The 5’- and 3’-fragments were amplified by PCR using KOD FX DNA polymerase (TOYOBO) and the following primers:

\textit{PjOtx}-5’-RACE-reverse, 5’- GCTGAGTTCGGGATCGCCTTGGTATAGTAT -3’;

\textit{PjOtx}-5’-RACE-reverse-2, 5’- GCGGAGCTCCAGATGCTTGTAT -3’;
The PCR products were cloned into the pTA2 vector (TATarget Clone -plus-; TOYOBO) and sequenced using a Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA) and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic analysis of Hox genes

Seventy-two amino acid residues (60 residues of the homeodomain plus both the N-terminal and C-terminal flanking six residues) from eighty Hox genes, the mouse Mus musculus (Mm), the amphioxus Branchiostoma floridae (Bf), the hemichordates Balanoglossus simodensis (Bsim) and Saccoglossus kowalevskii (Sk), the sea lily Metacrinus rotundus (Mr), and the sea urchins Strongylocentrotus purpuratus (Sp) and P. japonica Hox genes, were edited, aligned, and analyzed by neighbor-joining (NJ) method with Clustal W using three engrailed genes as an outgroup (Thompson et al., 1994). The bootstrap analysis was performed with 1,000 replications. Accession numbers of MmHox, BfHox, BsimHox, SkHox, and MrHox proteins are as follows: MmHoxa1, NP_034579; MmHoxa2, NP_034581; MmHoxa3, NP_034582; MmHoxa5, NP_034583; MmHoxa6, NP_034584; MmHoxa7, NP_03458; MmHoxb8, NP_034591; MmHoxa9, NP_034586; MmHoxa10, NP_032289; MmHoxa11, NP_034580; MmHox12, NP_034593; MmHoxa13, NP_032290; BfHox1, BAA78620; BfHox2, BAA78621; BfHox3, P50901; BfHox5, ABX39489; BfHox6, CAA84518; BfHox7, ABX39491; BfHox8, ABX39492; BfHox9, ABX39493; BfHox10, CAA84522; BfHox11, AAF81909; BfHox12, AAF81903; BfHox13, AAF81904; BfHox14, AAF81905; BsimHox1,
BAH96544; BsimHox2, BAH96545; BsimHox3, BAH96546; BsimHox4, BAH96547; BsimHox5, BAH96548; BsimHox6, BAH96549; BsimHox7, BAH96550; BsimHox8, BAH96551; BsimHox9/10, BAH96552; BsimHox11/13a, BAH96553; BsimHox11/13b, BAH96554; BsimHox11/13c, BAH96555; 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; MrHox1, BAF43721; MrHox2, BAF43722; MrHox4, BAF43723; MrHox5, BAF43724; MrHox7, BAF43725; MrHox8, BAF43726; MrHox9/10, BAF43727; MrHox11/13c, BAF43728. SpBase IDs of SpHox genes are as follows: SpHox1, SPU_017352; SpHox2, SPU_012252; SpHox3, SPU_027568; SpHox5, SPU_005169; SpHox6, SPU_005171; SpHox7, SPU_005170; SpHox8, SPU_002630; SpHox9/10, SPU_002633; SpHox11/13a, SPU_002632; SpHox11/13b, SPU_002631; SpHox11/13c, SPU_000388 (SpBase, http://www.spbase.org/SpBase/).

Whole-mount in situ hybridization (WMISH)

DIG-labeled RNA probes were synthesized using either T3 or T7 RNA Polymerases (Roche, Indianapolis, IN) and DIG RNA Labeling Mix (Roche) from full-length cDNAs of PjHox genes, except for Hox6. The probe of Hox6 and Otx were synthesized from the 5'-RACE and 3'-RACE clone, respectively. Embryos and larvae were fixed with Fixative III (4% paraformaldehyde in 32.5% ASW, 162.5 mM NaCl, 32.5 mM MOPS, pH 7.0) and Fixative I (4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS, pH 7.0), respectively, at 4°C overnight.
(Minokawa et al., 2004). Fixed specimens were washed with MOPS buffer (0.1 M MOPS, 0.5 M NaCl, 0.1% Tween 20, pH 7.0) five times and stored at -20°C in 70% ethanol.

WMISH was performed according to the method of Hibino et al. (2004) with some modifications. Specimens were washed with PBST (PBS containing 0.1% Tween 20) once and then bleached with 0.3% H₂O₂ in PBST at room temperature for 30 min. After being bleached, the specimens were washed with PBST three times and then incubated in prehybridization buffer (50% formamide, 5× SSC, 100 µg/ml yeast RNA, 50 µg/ml heparin, 1% Tween 20) at 50°C for 3 hours. Hybridization was performed in the prehybridization buffer containing 0.2 µg/ml of each probe at 50°C for 7 days. After hybridization, the specimens were washed twice in solution I (50% formamide, 5× SSC, 1% SDS) at 50°C for 20 min, twice in solution II (50% formamide, 2× SSC, 1% SDS) at 50°C for 20 min, once in solution III (2× SSC, 0.1% Tween 20) at room temperature for 5 min, once in solution III at 37°C for 20 min, once in solution III at 50°C for 20 min, twice in solution V (0.2× SSC, 0.1% Tween 20) at 50°C for 20 min, and finally twice in PBST at room temperature for 15 min. After being washed, specimens were incubated in blocking buffer [0.5% blocking reagent (Roche) in 0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl] at room temperature for 60 min and then incubated at 4°C overnight in blocking buffer with 1/2,000 volume of anti-DIG-AP (Roche). Before immunodetection, the specimens were washed three times with TNMT buffer [0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, 0.05 M MgCl₂, 0.1% Tween 20] at room temperature for 10 min.

Immunodetection was performed according to the method of Minokawa et al. (2004) with a modification: the reaction was stopped by adding PBST instead of MOPS buffer. The specimens were observed in 50% glycerol in PBS with a fluorescence microscope Axioplan 2
In hybridization with sense probes, no signal above the background levels was detected with any of the genes studied.

Sectioning

The specimens that were examined by WMISH were dehydrated in an ethanol series and acetone, embedded in Technovit 8100 (Heraeus Kulzur, Hanau, Germany), and cut serially into 7 µm thick sections on a microtome LEICA RM 2255 (Leica, Nussloch, Germany). Sectioned larvae were counter-stained with Nuclear Fast Red (MERCK, Darmstadt, Germany). The sections were observed with a fluorescence microscope BZ-9000 (KEYENCE, Osaka, Japan).
**Result**

**Isolation and re-identification of Hox genes**

Eleven Hox gene fragments have been cloned from *P. japonica* (Hano et al., 2001). The full-length cDNAs were isolated by RACE. Fig. 2-1 shows cDNA sequences and deduced amino acid sequences of eleven Hox genes. All Hox proteins had the homeodomain in the C-terminal side (red boxes in Fig. 2-1). The hexapeptide motif is conserved among *Antp*-class and *lab*-class genes (Burglin, 1994). In *P. japonica*, all Hox proteins but Hox11/13b had the motif (blue boxes in Fig. 2-1).

Fig. 2-2 shows a neighbor-joining tree constructed from the Hox protein sequences in the mouse *Mus musculus* (Mm), amphioxus *Branchiostoma floridae* (Bf), hemichordates *Balanoglossus simodensis* (Bsim) and *Saccoglossus kowalevskii* (Sk), sea lily *Metacrinus rotundus* (Mr), and sea urchins *S. purpuratus* (Sp) and *P. japonica* (Pj). Fig. 2-3 shows alignments of sequences of hexapeptide motifs, homeodomains, and the C-terminal flanking regions of Hox1, Hox2, Hox3, Hox5, Hox6, Hox7, and Hox9/10 from the mouse, amphioxus, and sea urchins. Gray boxes in Fig. 2-3 indicate paralog-characteristic residues conserved between *Drosophila* and vertebrate Hox members (Sharkey et al., 1997). Fig. 2-4 shows alignments of sequences of hexapeptide motifs, homeodomains, and the C-terminal flanking regions of ambulacraria-specific Hox genes, Hox11/13a, Hox11/13b, and Hox11/13c, from sea urchins and hemichordates.

The phylogenetic analysis clearly indicated that ten out of eleven *P. japonica* Hox genes were orthologous to *S. purpuratus* Hox1, Hox2, Hox3, Hox5, Hox6, Hox7, Hox8, Hox9/10,
Hox11/13a, and Hox11/13b, respectively (Fig. 2-2). This assignment was also supported by a presence of the paralog-characteristic residues, as shown in Figs. 2-3 and 2-4. The last one Hox gene was expected to be Hox11/13c. However, the gene did not form either the sea urchin or echinoderm Hox11/13c clade, although it belonged to the ambulacrarian Hox11/13b/Hox11/13c group (Fig. 2-2). Therefore, I tentatively designated this gene PjHox11/13c, since PjHox11/13b and SpHox11/13b were clearly orthologous. Given right designation, PjHox11/13c was diversified in the P. japonica lineage for unknown reason.

Temporal expression patterns of Hox genes

I examined expression patterns of Hox genes of P. japonica at 14 developmental stages, from the fertilization to metamorphosis: the fertilized egg (0 h), cleavage-stage embryo (4 h), early blastula (6 h), mesenchyme blastula (8 h), hatched mesenchyme blastula (10.5 h), early gastrula (13 h), mid gastrula (15 h), early prism larva (18 h), prism larva (21 h), early pluteus larva (24 h), and pluteus larva at 36 h, 48 h, 60 h, and 72 h.

Fig. 2-5 shows expression patterns of all Hox genes but Hox2 revealed by WMISH. Hox2 cDNA was cloned using cDNA derived from total RNA of blastulae (8 h) and pluteus larvae (40 h); however, Hox2 expression was not detected. Most Hox genes had been expressed by the pluteus larva stage at 36 h, and the expressions began to fade after 60 h. In most animal species, temporally collinear expressions of Hox genes are observed (McGinnis and Krumlauf, 1992). However, such expressions were not observed in P. japonica.
Conserved embryonic Hox gene expressions

In indirect-type developing sea urchins, expressions of Hox7 and Hox11/13b at embryonic stages have been reported. In the *Hemicentrotus pulcherrimus* blastula and gastrula, Hox7 is expressed in the aboral ectoderm (Ishii et al., 1999), whereas, in *S. purpuratus*, Hox11/13b is activated in the vegetal plate of the blastula, and then the expression is restricted to a torus encircling the blastopore at the pluteus larva stage (Arenas-Mena et al., 2006).

Fig. 2-6 shows expression patterns of Hox7, Hox11/13b, and Hox11/13c at embryonic stages of *P. japonica*. Like in indirect developers, Hox7 was activated in the aboral ectoderm by the hatching mesenchyme blastula (10.5 h), and the expression was maintained until the pluteus larva at 72 h (Fig. 2-5; Fig. 2-6A–C). Expression of Hox11/13b was detected in the vegetal plate of the early blastula (6 h), and then the expression was restricted to a torus encircling the blastopore in the early pluteus larva at 24 h (Fig. 2-5A, B; Fig. 2-6D–F). Thus, embryonic expressions of Hox7 and Hox11/13b were conserved between *P. japonica* and indirect developers. In addition, Hox11/13c was expressed in cells at the vegetal pole of the early blastula (6 h) (Fig. 2-6G). The descendants ingressed as mesenchyme cells, in which the signal faded out by the mid gastrula (15 h) (Fig. 2-5A; Fig. 2-6H, I).

Pentamerous expressions of Hox1, Hox11/13b, and Otx in the ambulacral region

Hox1 is one of three Hox genes translocated in an inverse orientation in the *S. purpuratus* Hox cluster (Fig. I-4A; Cameron et al., 2006); however, the expression has not been reported yet. In the vestibula larva of *Holopneustes purpurescens*, Hox11/13b is expressed in ambulacral regions of the vestibule (Morris and Byrne, 2005).
Fig. 2-7 shows the expression patterns of *Hox1*. *Hox1* was activated in the posterior region of the vestibule and cells at the vegetal side of vestibular opening in the early prism larva at 18 h (blue and light blue arrowheads, respectively, in Fig. 2-7B). As the vestibule extended posteriorly, the former *Hox1* domain expanded posteriorly (blue arrowheads in Fig. 2-7C, D). In the early pluteus larva at 24 h, the expression faded in the center of the vestibule (blue arrowheads in Fig. 2-7E, F); the expression was restricted to distal regions of the ambulacra II, III, and IV in the vestibular floor at 36 h (blue arrowheads in Fig. 2-7G, H). On the other hand, the latter *Hox1* domain developed into the anterior region of the vestibular floor, in which *Hox1* expression was maintained until the early pluteus larva (24 h) (light blue arrowheads in Fig. 2-7C–F). In the pluteus larva at 36 h, the expression was restricted to the ambulacra I and V (light blue arrowheads in Fig. 2-7G, H). Thus, *Hox1* was pentamerally expressed in the ambulacra I–V in the vestibular floor that gives rise to adult ectoderm.

Like *Hox1*, in the early pluteus larva at 24 h, *Hox11/13b* started to be expressed in the posterior region of the vestibule, which will develop into the ambulacrum III (blue arrowheads in Fig. 2-9T, U). The expression domain was expanded to the ambulacra I–V in the pluteus larva at 36 h (Fig. 2-5B). This observation on *Hox11/13b* expression patterns is consistent with that by Morris and Byrne (2005).

In sea urchin embryos and larvae, *Otx* has been reported to be expressed in the archenteron, ciliary cells, and vestibular floor (Gan et al., 1995; Mitsunaga-Nakatsubo et al., 1998; Nielsen et al., 2003; Morris and Byrne, 2005). *Otx* was expressed in the enteric sac, ciliary band, and center of the vestibular floor in the early pluteus at 24 h (Fig. 2-7I, J); the expression domain in the vestibular floor expanded pentamerally in the pluteus larva at 36 h.
(yellow arrowheads in Fig. 2-7K, L). The Otx domain in the vestibular floor corresponds to the region that overlies the hydrocoel and primary lobe (Fig. 1-7; Fig. 1-12), and Morris and Byrne (2005) assigned the Otx domain to the adult nervous system. Since Otx regulates neural fate specification in a variety of developmental aspects (Hirth and Reichert, 1999), Otx probably has a conserved role in development of the adult nervous system.

**Pentamerous expressions of Hox3 and Hox5 in the interambulacral region**

Fig. 2-8 shows expression patterns of Hox3 and Hox5. Expressions of these genes started in the posterior side of the larva, followed by the anterior expansion. Hox3 was expressed in five dental sacs and the spine rudiments in the interambulacra 2 and 3 in the pluteus larva at 36 h (Fig. 2-8A, B); at 48 h, the expression expanded to spine rudiments in the interambulacra 1, 4, and 5 (green arrowheads in Fig. 2-8C, D).

Hox5 was activated in the interambulacra 2 and 3 regions of the vestibular floor in the early pluteus larva at 24 h (Fig. 2-8E, F). In the pluteus larva at 36 h, Hox5 started to be expressed in the interambulacra 1, 4, and 5, whereas earlier Hox5 domains in the interambulacra 2 and 3 were restricted to the spine rudiments (Fig. 2-8G, H). At 48 h, Hox5 domains in the interambulacra 1, 4, and 5 were restricted to the spine rudiments (Fig. 2-8I, J). Although Hox3 and Hox5 were similar in the expression patterns, cells expressing Hox3 and Hox5 were different; Hox3 was expressed in mesenchyme cells in the spine rudiments (green arrowheads in Fig. 2-8K), whereas Hox5 was in ectodermal cells in the rudiments (green arrowheads in Fig. 2-8L).
Expressions of five medial/posterior Hox genes in the somatocoel

In *S. purpuratus*, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b are collinearly expressed in a pair of the somatocoels along the mouth-anus axis (Fig. 1-4B; Arenas-mena et al., 2000).

Fig. 2-9 shows the expression patterns of Hox6, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b in *P. japonica*. Like in *S. purpuratus*, five Hox genes, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b, were expressed in the C-shaped left somatocoel clockwise in their numeric order, when viewed from the dorsal side of larvae (oral side of adults) (Fig. 2-9; Fig. 2-10). However, unlike *S. purpuratus*, only Hox9/10 was expressed in the right somatocoel. I describe detailed expression patterns of untraslocated Hox genes in below.

*Hox6* was activated in inner thick cell layer in the pluteus larva at 48 h (black arrowheads in Fig. 2-9A–C); the expression faded out by 72 h (Fig. 2-5C).

*Hox7* transcripts were detected in posterior end of the left somatocoel and aboral ectoderm in the early pluteus larva at 24 h (Fig. 2-9D–G). The expression in the left somatocoel faded out by 72 h (Fig. 2-5). In the posterior region of the vestibule, Hox7 started to be expressed in the pluteus larva at 48 h, and the domain expanded anteriorly in the pluteus larva at 72 h (Fig. 2-5C).

*Hox8* was expressed in the posterior end of the left somatocoel of the early pluteus larva at 24 h (Fig. 2-9E–K). The domain was smaller than Hox7 domain (Fig. 2-9G, K). The signal was gradually reduced until 72 h (Fig. 2-5B, C).

*Hox9/10* was activated in the archenteron tip in the early gastrula at 13 h (Fig. 2-5A). The region will generate the left somatocoel via mesenchyme cells (Fig. 1-3; Fig. 1-4), in which
*Hox9/10* expression was maintained in the prism larva at 21 h (Fig. 2-5B). At the early pluteus stage (24 h), the signal of *Hox9/10* was detected from the central to posterior region of the left somatocoel (red arrowheads in Fig. 2-9L–O). In addition, the right somatocoel started to express *Hox9/10* (orange arrowheads in Fig. 2-9L–O); the expression faded out by 48 h, whereas the expression in the left somatocoel was gradually down-regulated until 72 h (Fig. 2-5C).

In the early prism larva at 18 h, *Hox11/13a* were activated in a part of mesenchyme cells that will develop into the left somatocoel (Fig. 1-4; Fig. 2-5A). In the early pluteus larva at 24 h, *Hox11/13a* was expressed in the anterior to central region of the left somatocoel (Fig. 2-9P–S). The expression faded out by 72 h (Fig. 2-5C).

In addition to the embryonic and vestibular expressions of *Hox11/13b* (Fig. 2-5B; Fig. 2-6), a subset of mesenchyme cells expressed *Hox11/13b* in the early prism larva at 18 h, like *Hox11/13a* (Fig. 2-5A). In the early pluteus larva at 24 h, *Hox11/13b* was expressed in the anterior region of the left somatocoel (Fig. 2-9T–W). The expression was maintained in the pluteus larva at 36 h (Fig. 2-5B); the expression was gradually reduced until 72 h (Fig. 2-5C).
Discussion

For the first time, here I showed comprehensive expression patterns of Hox genes during the development of *P. japonica*. Hox7, Hox11/13b, and Hox11/13c are activated at embryonic stages. Hox1 and Hox11/13b are pentamerally expressed in the ambulacra I–V in the vestibular floor that give rise to adult ectoderm (Fig. 2-10A). In contrast, Hox3 and Hox5 are expressed in the interambulacra 1–5 (Fig. 2-10A); Hox3 and Hox5 appear to be associated with formation of adult dents and spines. Finally, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b are expressed in the left somatocoel clockwise in their numeric order, when viewed from the adult oral side (Fig. 2-10B).

The ancestral AP axis resides in the somatocoel in echinoderms

Both in *S. purpuratus* and *P. japonica*, five Hox genes, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b, are expressed in the left somatocoel; this collinear/numeric-order expression is clockwise, when viewed from the adult oral side (Fig. 2-10B). Furthermore, in the see lily *M. rotundus*, Hox5, Hox7, Hox8, and Hox9/10 are expressed in a pair of the somatocoels in their numeric order along the AP axis (Hara et al., 2006). Since sea lilies and sea urchins are considered to be the most basal and derived groups in echinoderms, respectively (Wada and Satoh, 1994), this somatocoelar expression of medial/posterior Hox genes is probably conserved in echinoderms, although an expressing paralog set differs each other. This strongly suggests that the ancestral AP axis resides in the somatocoel of echinoderms. As for the paralog set, there are at least two explanations: a distinct set or an apparently different
but potentially identical set between sea urchins and sea lilies. The former regards the difference as functional orthologs; paralogs play functionally equivalent roles (Greer et al., 2000). In the latter, the Hox5-expressing region of the right somatocoel will develop into the chamber organ associated with sea lily-specific stalk (Hara et al., 2006); expressions of the same set might be conserved in the rest of the somatocoel.

Left and right somatocoels give rise to the oral and aboral coeloms of adults, respectively. In the body cavity, the digestive tract is suspended by the mesenteies, mesodermal membranes formed by epithelial fusion between the oral and aboral coeloms (Hyman, 1955), and the juvenile digestive tract largely turns clockwise in the body cavity, when viewed from the oral side (Fig. 1-2). Given that the somatocoelar Hox expression controls differentiation of the digestive tract, the collinear/numeric-order Hox expression in the somatocoel support a “rays-as-appendages” model, although the oral-aboral (mouth–anus) axis is twisted.

In P. japonica, only Hox9/10 was expressed in the right somatocoel, whereas five Hox genes of S. purpuratus are expressed there, like in the left somatocoel. Since, in the sea lily M. rotundus, four Hox genes are expressed both in the left and right somatocoels, the left dominant expression is probably a derived character of P. japonica. P. japonica exclusively skips bilateral phases in coelom formation, and develops smaller right somatocoel than left one (Part 1). This unusual coelom formation may result in the left dominant expression, because, in S. purpuratus, expressions of five Hox genes in the right somatocoel are down-regulated as the developmental stage proceeds (Fig. I-4; Arenas-Mena et al., 2000).
Co-option of Hox genes for the evolution of echinoderm morphological novelties

The water vascular system is a major synapomorphy of echinoderms, which consists of the ring canal and the radial canal, and communicates with the external medium through the stone canal and madreporite. The radial canal sends numerous small projections (podia or tube feet) to the exterior. The surface of an echinoderm is divided into two areas, ambulacra, at which the podia project to the exterior and interambulacra between the ambulacra.

In *P. japonica*, *Hox1*, *Hox11/13b*, and *Otx* were pentamerally expressed in ectoderm of the ambulacra (Fig. 2-10). Morris and Byrne (2005) demonstrated using the sea urchin *Holopneustes purpurescens* that *Hox11/13b* is expressed in the distal regions of the ambulacla I–V, while *Otx* is in the nervous system, including the nerve ring, radial nerves and neuroepithelium around podia. My observations are completely consistent with those of Morris and Byrne (2005), suggesting that *Hox1* and *Hox11/13b* are involved in regional specification of the adult ectoderm, whereas *Otx* controls development of the adult nervous system in echinoderms; thus, not supporting the ‘rays-as-axes’ model.

The surface of an echinoderm is typically covered with spines (whence the name Echinodermata from the Greek *ecino*, spiny, and *derma*, skin); the calcareous biomineralization is another synapomorphy of echinoderms. *Hox3* was expressed in the dental sac rudiment, which accords with the result of Arenas-Mena et al. (1998). Furthermore, *Hox3* and *Hox5* were expressed in the spine rudiments; *Hox3* in mesenchyme cells, while *Hox5* in ectoderm. Thus, *Hox3* and *Hox5* seem to be associated with formation of the dental sacs and spines. Indeed, *Ets1* is expressed in the rudiments, a marker gene of skeletogenic cells in larvae and adults (Gao et al., 2008).
Hox genes are often co-opted for the evolution of morphological novelties. In tetrapods, Hox genes regulate limb development; three temporal phases of Hox gene expression regulates the elaboration of three distinct elements of the limb (Nelson et al., 1996). In cephalopods, a combination of Hox gene expressions controls brachial identity (Lee et al., 2003). Given co-option or recruitment of Hox genes for echinoderm- or ecinoid-specific morphologies, there is a critical distinction; both in tetrapods and cephalopods, Hox genes are utilized to generate new appendages, in addition to the original role in patterning along the primary axis. In sea urchins, however, Hox genes are used to pattern or cover the body surface at the expense of the original function, because Hox1, Hox3, Hox5, and Hox11/13b do not involved in the axial patterning at all. Intriguingly, the four Hox genes are all inverted in orientation in the S. purpuratus Hox cluster.

**Organization of Hox gene complex and evolution of echinoderms**

Based on the Hox complements from the sea lily (Hara et al., 2006), feather star (Mito and Endo, 2000), starfish (Mito and Endo, 1997; Long et al., 2000, 2003), and sea cucumber (Méndez et al., 2000), the common ancestor of echinoderms is inferred to have had three anterior, five medial, and four posterior Hox genes. Furthermore, we showed that the hemichordate, that is a sister taxon of echinoderms, has an identical Hox set (Urata et al., 2009). Thus, the ambulacranian ancestor probably had a full complement of 12 Hox genes. The Saccoglossus kowalevskii Genome Project announced exciting news: the hemichordate has a completely organized Hox cluster, like chordate ones (Gerhart, 2009, plenary lecture in 42nd annual meeting for JSDB). It is important to note that Hox genes are expressed in the numeric
order, that is, collinearly along the AP axis in *S. kowalevskii* (Aronowicz and Lowe, 2006). These data lead me to suggest that the evolution of echinoderm morphological novelties, including teeth and spines, may have been accompanied by, or precipitated by disorganization of the *Hox* cluster. Information on the organization of *Hox* clusters of echinoderm groups as well as *P. japonica* will reveal the evolution of the *Hox* cluster in echinoderms, which may provide an insight into another way to build a completely different adult form.
B : PjHox2

sequence size : 1876 bp
CDS : 563 – 1389 ( 837 bp )
amino acids : 278 aa
C : PjHox3

sequence size : 1594 bp
CDS : 52 – 1170 (1119 bp)
amino acids : 372 aa

64
**D : PjHox5**

sequence size : 2060 bp  
CDS : 285 – 1136 ( 852 bp )

amino acids : 283 aa
E : PjHox6

sequence size : 2573 bp
CDS : 283 – 1122 ( 840 bp )

amino acids : 279 aa
G : PjHox8

sequence size : 2004 bp
CDS : 87 – 1070 ( 984 bp )
amino acids : 323 aa
Fig. 2-1. Nucleotide and deduced amino acid sequences of *PjHox* genes. The eleven *PjHox* gene sequences are shown: *PjHox1* (A), *PjHox2* (B), *PjHox3* (C), *PjHox5* (D), *PjHox6* (E), *PjHox7* (F), *PjHox8* (G), *PjHox9/10* (H), *PjHox11/13a* (I), *PjHox11/13b* (J), and *PjHox11/13c* (K). Red and blue boxes indicate the homeobox and hexapeptide sequences, respectively. Underlines and black boxes also indicate the in-frame stop codons and predicted polyadenylation signal sequences, respectively.
Fig. 2-2. Neighbor-joining tree constructed from 80 Hox proteins using three engrailed proteins as an outgroup. Bootstrap values $>50\%$ are shown at the respective nodes. The scale bar indicates 0.1 amino acid substitutions per position in the sequence.
<table>
<thead>
<tr>
<th>Hox</th>
<th>Hexapeptide</th>
<th>Homeodomain</th>
<th>C-terminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>PjHox1</td>
<td>LYKMR</td>
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<td>KECIPS</td>
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Fig. 2-3. Alignments of sequences of hexapeptide motifs, homeodomains, and the C-terminal flanking regions of Hox genes from the mouse Mus musculus (Mm), the anelet Branchiostoma floridae (Bf), and the sea urchin Strongylocentrotus purpuratus (Sp) and P. japonica. Dots show residues identical with P. japonica sequence. Gray boxes indicate paralog-characteristic residues conserved between Drosophila and vertebrate Hox members (Sharkey et al., 1997).
### Figure 2-4

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**Fig. 2-4.** Alignments of sequences of hexapeptide motifs, homeodomains, and the C-terminal flanking regions of ambulacaria-specific Hox genes, Hox11/13a, Hox11/13b, and Hox11/13c from sea urchins *S. purpuratus* and *P. japonica*, and hemichordates *Balanoglossus simodensis* (Bsim) and *Saccoglossus kowalevskii* (Sk). Dots show residues identical with *P. japonica* sequence.
### Figure 2-5

#### A: 0–18 h

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<th>mBl 8 h</th>
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<th>eGs 13 h</th>
<th>mGs 15 h</th>
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Fig. 2-5. Expression patterns of Hox genes revealed by WMISH. WMISH was performed on the eleven Hox genes (Hox1, Hox2, Hox3, Hox5, Hox6, Hox7, Hox8, Hox9/10, Hox11/13a, Hox11/13b, Hox11/13c) at 14 developmental stages, from fertilization egg to metamorphosis. A: From the fertilized egg at 0 h to early prism larva at 18 h. B: From the prism larva at 21 h to pluteus larva stage at 36 h. C: The pluteus larva from 48 h to 72 h. fe, fertilized egg; Clv, cleavage; eBl, early blastula; mBl, mesenchyme blastula; hmBl, hatched mesenchyme blastula; eGs, early gastrula; mGs, middle gastrula; ePri, early prism larva; Pri, prism larva; ePlu, early pluteus larva; Plu, pluteus larva.
**Figure 2-6**

Expression patterns of *Hox7*, *Hox11/13b*, and *Hox11/13c* at embryonic stage. Spatial expression of *Hox7* (A–C), *Hox11/13b* (D–E), and *Hox11/13c* (G–I) was examined by WMISH. Images of all embryos are shown as lateral views. 6 h, early blastula at 6 h; 10.5 h, hatched mesenchyme blastula at 10.5 h; 13 h, early gastrula at 13 h; ao, aboral; o, oral.
Fig. 2-7. Expression patterns of *Hox1* and *Otx*. Spatial expression patterns of *Hox1* (A–H), and *Otx* (I–L) were examined by WMISH. (A) Oral view, (B, D, F, H, J, L) right lateral view, and (C, E, G, I, K) dorsal view. Blue arrowheads indicate expression domains of *Hox1* in posterior region of vestibule (B), or ambulacra II–IV (C–H). Light blue arrowheads indicate expression domains of *Hox1* in cells at the vegetal side of vestibular opening (B), or ambulacrac I and V (C–H). Yellow arrowheads indicate expression domains of *Otx* in vestibular floor (I–L). Red arrows indicate expression domains of *Otx* in enteric sac (I–L). I–V, ambulacra I–V; 15 h, middle gastrula at 15 h; 18 h, early prism larva at 18 h; 21 h, prism larva at 21 h; 24 h, pluteus larva at 24 h; 36 h, pluteus larva at 36 h.
Fig. 2-8.  Expression patterns of Hox3 and Hox5.  Spatial expression patterns of Hox3 (A–D), and Hox5 (E–J) were examined by WMISH.  (K, H) Longitudinal sections of Hox3 or Hox5 stained larvae.  (A, C, E, G, I) Dorsal view.  (B, D, F, H, J, K, L) right lateral view.  Green arrowheads indicate expression domains of Hox3 and Hox5 in vestibular floor.  Green arrows indicate expression domains of Hox3 in dental sacs.  1–5, interambulacra 1–5; 24 h, pluteus larva at 24 h; 36 h, pluteus larva at 36 h; 48 h, pluteus larva at 48 h.
Figure 2-9
Fig. 2-9. Expression patterns of Hox6, Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b. Spatial expression patterns of Hox6 (A, B), Hox7 (D, E), Hox8 (H, I), Hox9/10 (L, M), Hox11/13a (P, Q), and Hox11/13b (T, U) were examined by WMISH. (C, F, J, N, R, V) Longitudinal sections of the stained larvae. (G, K, O, S, W) Illustrated images of the sections. Images of all larvae are shown as dorsal view, except for images of those indicated in the panel as right lateral view (right). Black arrowheads indicate expression domains of Hox6 in inner thick cell layer. Red, orange, blue, and white arrowheads indicate expression domains in the left somatocoel, right somatocoel, vestibule, and larval ectoderm, respectively. Red arrows indicate expression domains in enteric sac. en, enteric sac; lc, left somatocoel; rc, right somatocoel; ve, vestibule; 24 h, pluteus larva at 24 h; 48 h, pluteus larva at 48 h.
Fig. 2-10. Summary of Hox and Otx expression patterns in *P. japonica*. A: Expression patterns of Hox1, Hox3, Hox5, Hox11/13b, and Otx in the ambulacra and interambulacra. B: Somatocoelar expression patterns of Hox7, Hox8, Hox9/10, Hox11/13a, and Hox11/13b. C: The architecture of the sea urchin Hox cluster. (A, B) The expression domains are depicted using the same colors than the genes, represented in the sea urchin Hox cluster (C), and light blue indicates expression domain of Otx. (C) Half-arrows indicate the direction of transcription. I–V, ambulacra I–V.
General Discussion

In the present study, the following observations were obtained for the developmental processes and expression patterns of Hox genes in sand dollar *Peronella japonica*.

**Part 1**

1. The left somatocoel develops by both schizocoely and enterocoely from the archenteron tip.
2. The hydrocoel, stone canal, axocoel, and right somatocoel form by enterocoely from the archenteron.
3. The coelom formation arranges the coelomic compartments directly along the adult oral-aboral axis by skipping the initial bilateral phases.
4. *P. japonica* retains ancestral asymmetry along the left-right axis in the location of the adult rudiment.

**Part 2**

1. Embryonic expression of *Hox7* and *Hox11/13b* are conserved between the indirect and direct developing echinoids.
2. *Hox11/13c* is activated transiently in the vegetal cells at embryonic stage.
3. *Otx, Hox1, Hox3, Hox5*, and *Hox11/13b* are expressed in ambulacral regions, spine rudiments, and/or dental sacs.
4. The somatocoelar expression of *Hox7, Hox8, Hox9/10, Hox11/13a*, and *Hox11/13b* is detected clockwise in the numeric order, when view from the larval dorsal side.
In part 1, I showed the formation processes of coelomic compartments which are enclosed by the water vascular system or which become the main body cavities in *P. japonica*.

In part 2, I showed that the collinear/numeric-order *Hox* expression in the somatocoels is a trait of the ancestral AP axis that supports a “rays-as-appendages” model, although the oral-aboral axis is twisted but that *Hox1, Hox3, Hox5*, and *Hox11/13b* may be co-opted for the evolution of echinoderm morphological novelties. Furthermore, I suggested that the evolution of echinoderm morphological novelties may have been accompanied by, or precipitated by disorganization of the *Hox* cluster.

Here, I discuss the origin of the pentameral body plan. The body plan is the most important echinoderm-characteristic feature, and the elucidation of the origin is required to reveal the formation processes of the pentameral body plan.

**The origin of the pentameral body plan**

The pentameral body plan of echinoderms is formed in the rudiment consisting of the vestibular ectoderm and mesodermal compartments, the hydrocoel and left somatocoel. During metamorphosis, the hydrocoel and overlying ectoderm generate the water vascular system, while the somatocoel generates the dental elements and oral coelom. In the rudiment, a series of reciprocal interactions between the ectoderm and mesoderm, and among the ectodermal or mesodermal tissues must take place to make adult structures. However, the origin of the pentameral body plan is wrapped in mystery.

In respect to the advent of pentameros shape, chronographically, the ectodermal *Hox* expression precedes the mesodermal morphology. During the development of *P. japonica*,

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the pentameral morphology emerges in larvae from 28 h to 32 h. Two dental sacs protrude from the left somatocoel at 28 h (in the interambulacra 2 and 3; Fig. 1-10), followed by the other three at 32 h (in the interambulacra 4–1; Fig. 1-12). In the ambulacra, three lobes branch out from the hydrocoel at 28 h, followed by the other two at 32 h. On the other hand, signs of the pentamerous expression of Hox genes start from 18 h to 24 h. At 18 h, Hox1 starts to be expressed in two domains, the vestibule and the vegetal cells, and the expressions are restricted to the ambulacra II–VI and V–I, respectively at 21 h (Fig. 2-7B, C). Hox5 expression starts at 24 h in the interambulacra 2 and 3 of the vestibule (Fig. 2-8E). The expression prior to the morphology raise a possibility that pentaradiality may be pre-patterned in the vestibular ectoderm.

It is generally accepted that mesoderm leads inductive events, rather than ectoderm. In P. japonica larvae, Otx is pentaradially expressed in the presumptive nervous system, which makes contact with five lobes of the hydrocoel (Fig. 2-7K), suggestive of a inductive signal(s) from the hydrocoel. The five lobes are interdigitated with five dental sacs projected from the left somatocoel (Fig. 1-12). In the left somatocoel, five dental sacs are largely assigned to distinct Hox domains: dental sacs in the interambulacra 1–5 to Hox7–Hox11/13b domains, respectively. This observation suggests that there may exist five domain characterized by distinct Hox genes, which may underlie a pentameral body plan of echinoderms.

To examin which encloses the prototype of pentaradiality, ectoderm or mesoderm, I constructed animal and vegetal halves by dissecting hatched blasturae (10.5 h) equatorially with a glass needle and examined the phenotypes and gene expressions in the embryoids (Fig. D-1).

Animal halves developed into blastulae, in which a vestibule formed at 24 h (Fig.
D-1A).  Hox1 and Hox5 were expressed in the vestibule, but Otx was not (Fig. D-1B).  This observation indicates that the Hox expressions are autonomous, whereas Otx expression depends on a signal(s) from the hydrocoel.  This autonomous Hox expression suggests a presence of prototype of pentaradiality in ectoderm; however, several hours later, the vestibule underwent apoptosis.

On the other hand, vegetal halves developed into pluteus-like larvae with immature vestibules at 24 h (Fig. D-1A).  On day 10, a minority of the larvae metamorphosed into juveniles with trimerous, tetramerous, or pentamerous dental elements (Fig. D-1C).  This perturbation of pentaradiality appears to be inconsistent with the idea of the somatocoelar origin of petaradiality, since the vegetal half includes whole endomesoderm.

Phenotypes of animal and vegetal halves indicate that reciprocal interactions between ectodermal vestibule and mesodermal coeloms are essential for the formation of adults.  As for the pentaradial base of echinoderm body plan, I prefer the pre-pattern in ectoderm to the Hox-sector in somtocoel from a vast number of WMISH images of regulatory genes as well as Hox genes I have observed.

Echinoderms have the unique pentameral body plan, although echinoderms are included in bilaterians.  The evolution of echinoderms has attached the interest of many scientists, but remain largely unsolved.  One of the reasons is that it takes one month–two months to metamorphose in typical echinoderms.  The present study has showed that studies on P. japonica can provide invaluable information for not only the evolution of echinoderms, but formation processes of the pentameral body plan as well.  Further studies on P. japonica
such as the function and control of $Hox$ genes, architecture of $Hox$ cluster, and origin of the pentameral body plan promise to provide insights into the evolution of echinoderms and to reveal the formation processes of pentameral body plan.

Hyman (1955) said ‘I also here salute the echinoderms as a novel group especially designed to puzzle the zoologists.’.
Fig. D-1.  A: Phenotypes of animal and vegetal halves.  B: *Hox1*, *Hox5*, and *Otx* expression patterns in blastulae developed from animal halves at 24 h.  Blue and green arrowheads indicate *Hox1* and *Hox5* domain, respectively.  C: Juveniles developed from vegetal halves.  Red arrowheads indicate dental elements.  One, three, and six vegetal halves developed into juveniles with trimerous, tetramerous, or pentamerous dental elements, respectively.  ve, vestibule.  Scale bar = 50 µm.
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