博 士 論 文

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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Contents

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*.

Figure I-1

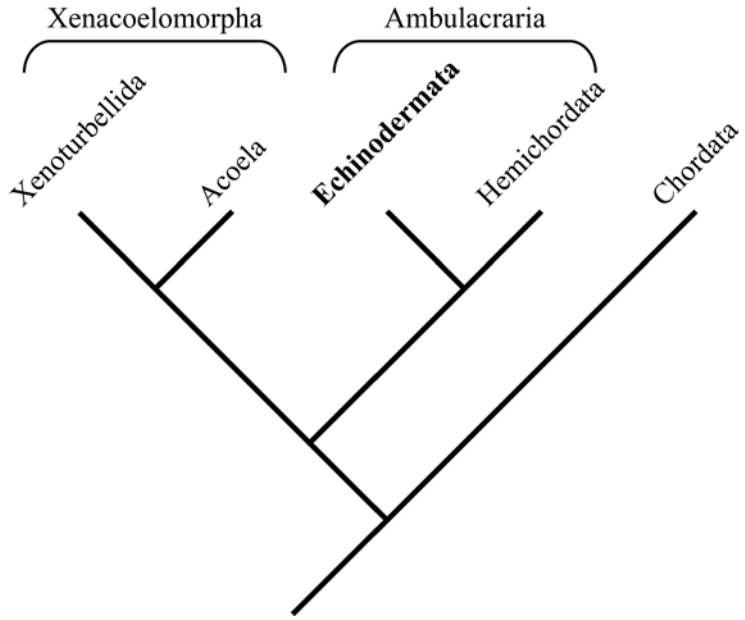

Fig. I-1. Phylogenetic relationships of deuterostomes. Deuterostomes are a monophyletic group of animals that include chordates, hemichordates, echinoderms, acoels, and xenoturbellids (Philippe et al., 2011). Echinoderms and hemichordates are sister taxa, forming a group known as the ambulacaria (Swalla and Smith, 2008).

Figure I-2

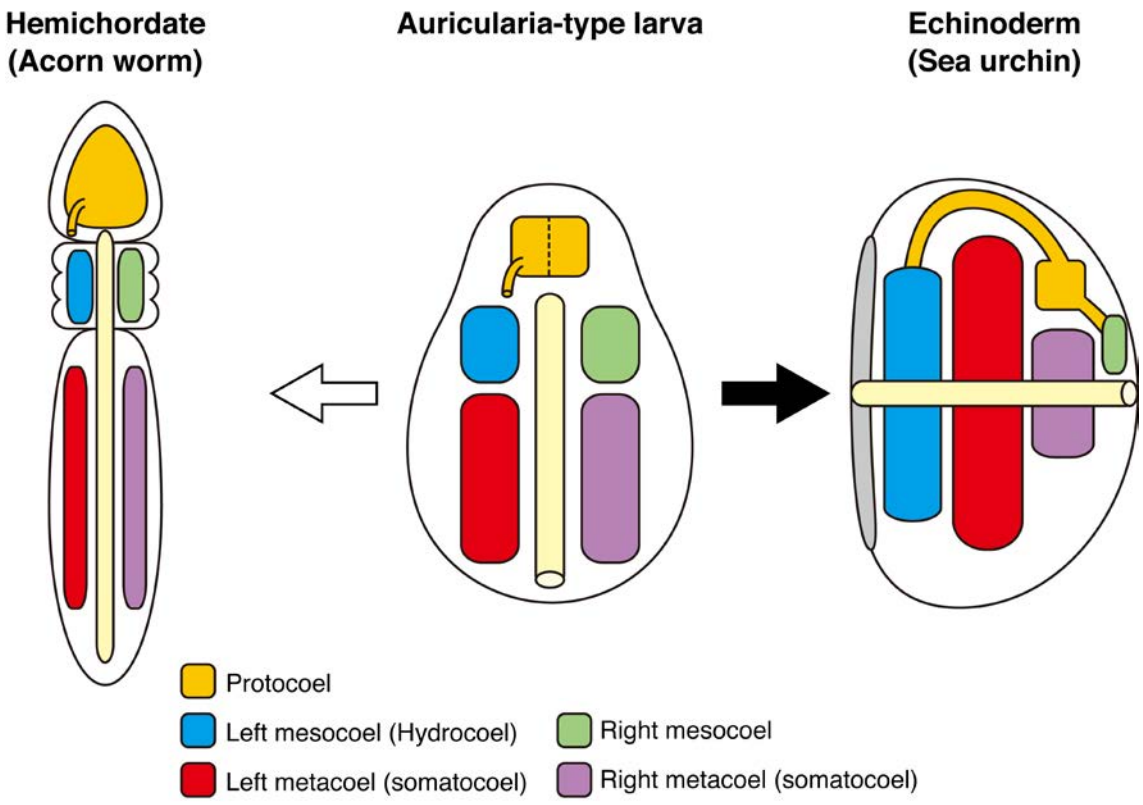

Fig. I-2. The coelomic architectures of the auricularia-type larva, and adult hemichordate and echinoderm. The larva is characterized by having three parts of coeloms along the anteroposterior axis: the protocoel (orange), and a pair of the mesocoels (blue and green) and metacoels (or somatocoels; red 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.

Figure I-3

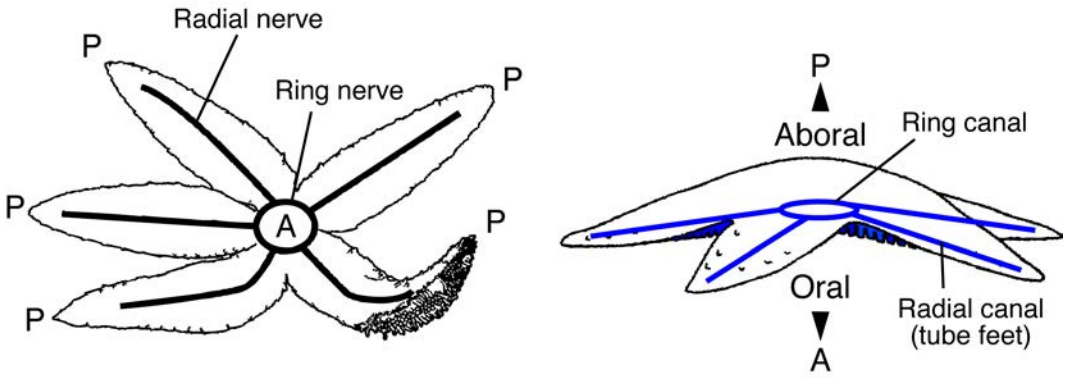

Fig. I-3. Two hypotheses regarding the orientation of the ancestral bilaterian AP axis in the adult echinoderm body plan (modified from fig. 6-16b in Carroll et al., 2005). **Left**: A "rays-as-axes" model is based on the fivefold symmetry of the adult nervous system; five radial nerves connected by 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 considerd as an outgrowth of the water vascular system (Hotchkiss, 1998; Peterson et al., 2000).

Figure I-4

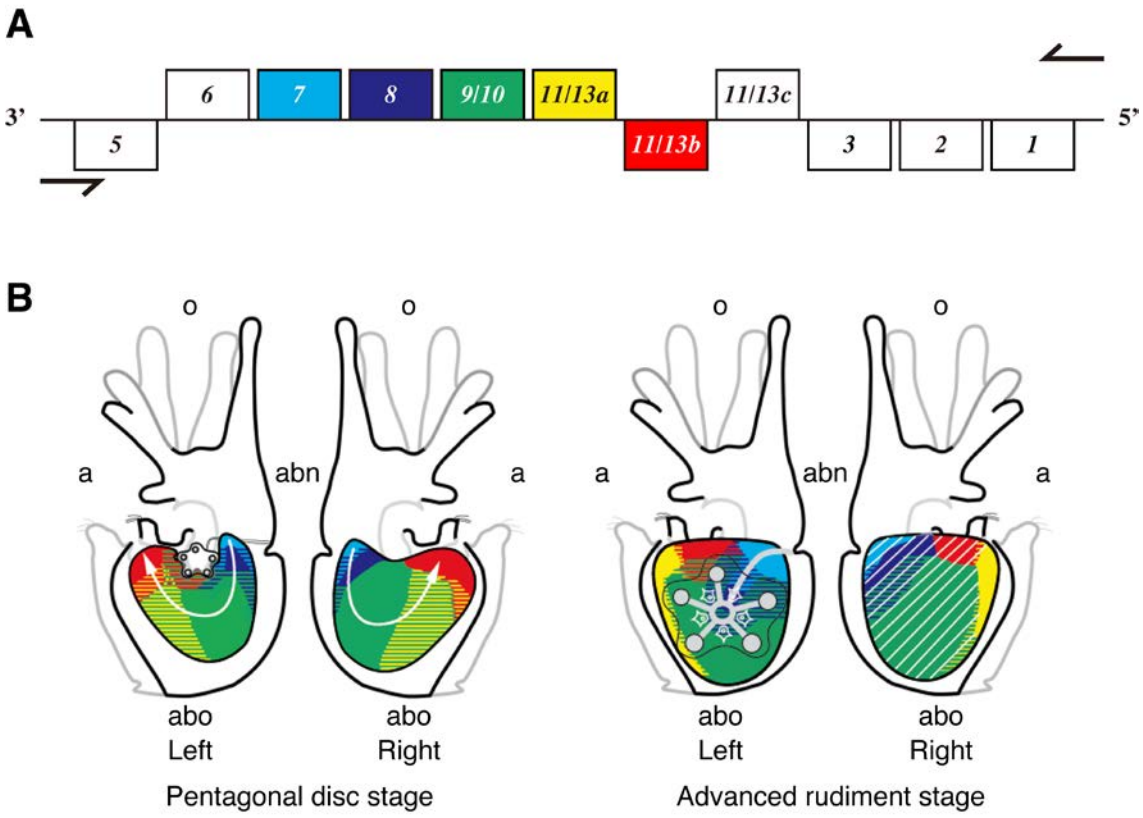

Fig. I-4. **A**: The sea urchin *Hox* cluster (Cameron et al., 2006). Half-arrows indicate the direction of transcription. **B**: Summary of patterns of five medial/posterior *Hox* gene expression in the somatocoels of *S. purpuratus* (modified from Fig. 7 in Arenas-Mena et al., 2000). The expression 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 Kulzur, 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

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 *P. japonica* imago three weeks after metamorphosis, which had been fed the diatom *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 *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 portion 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 section s 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 *Echinarachnius parma*, an indirect-developing sand dollar, is marked along Lovén's axis rather than von Ŭbisch'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

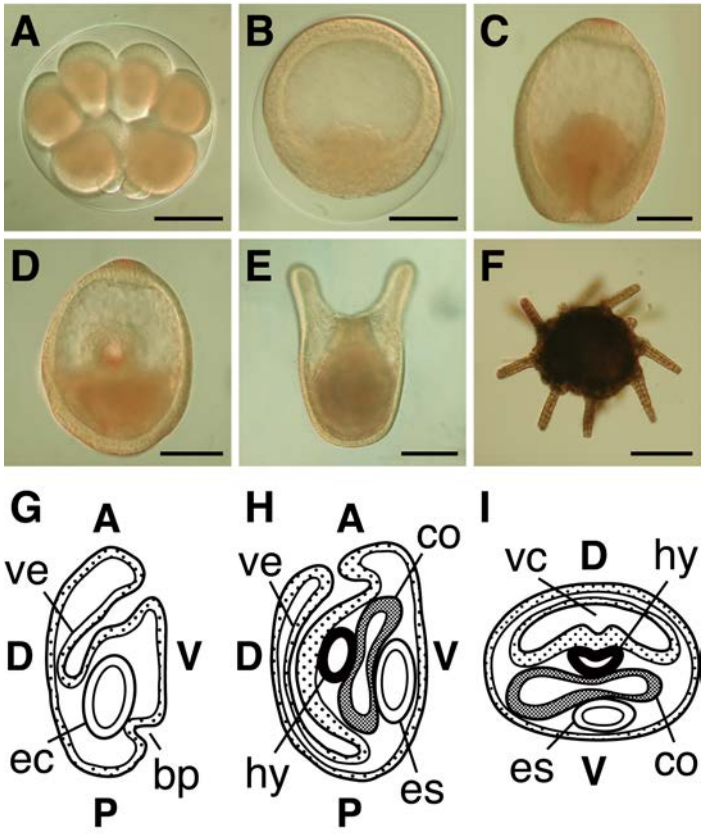

Fig. 1-1. Development of *P. japonica* (**A–F**) and schematic drawings of larvae (**G–I**; modified from fig. 1 in Okazaki and Dan, 1954). **A**: Embryo at the 16-cell stage (2.5 h, lateral view). Micromeres are formed at the vegetal pole. **B**: Blastula before hatching (9 h, lateral view). The primary 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.

Figure 1-2

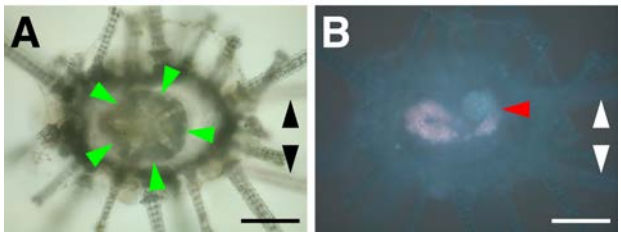

Fig. 1-2. Imago three weeks after metamorphosis fed with diatoms (aboral view). **A**: Bright field image. **B**: Fluorescence image. Green and red arrowheads indicate adult dental elements and anus, respectively. White and black arrowheads show remnant larval postoral rods, traces of the former anterior 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.

Figure 1-3

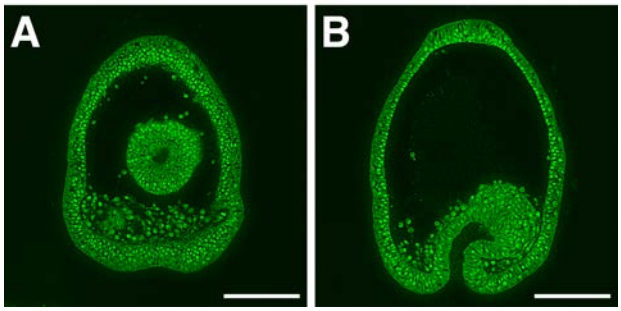

Fig. 1-3. Transverse sections of a gastrula at 16 h along the oral-aboral axis. **A**: Oral-side section. Vestibule invaginates in the oral field. **B**: Medial section. Archenteron leans toward the left side of the embryo. A number of mesenchyme cells have migrated out of the archenteron tip. Scale bar = 100 μm.

Figure 1-4

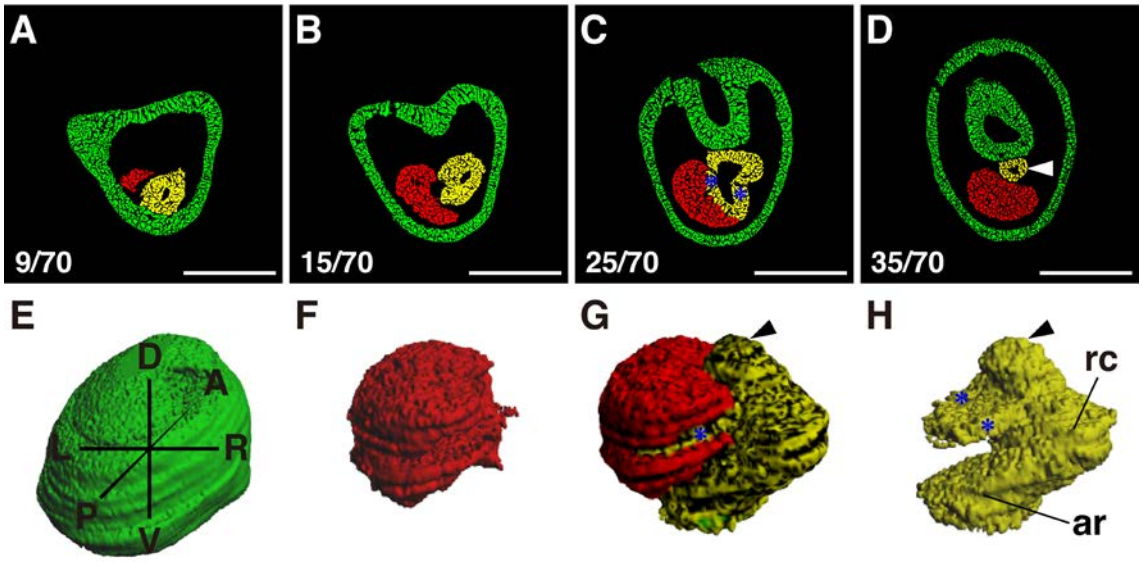

Fig. 1-4. Horizontal sections along the dorsoventral axis (**A–D**) and reconstructed three-dimensional images (**E–H**) of an early prism larva (18 h). (**A–D**) Four of 70 serial sections (3 µm thick) of the larva from the ventral bottom to dorsal top. The ectodermal layer, a mass of mesenchyme cells, 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.

Figure 1-5

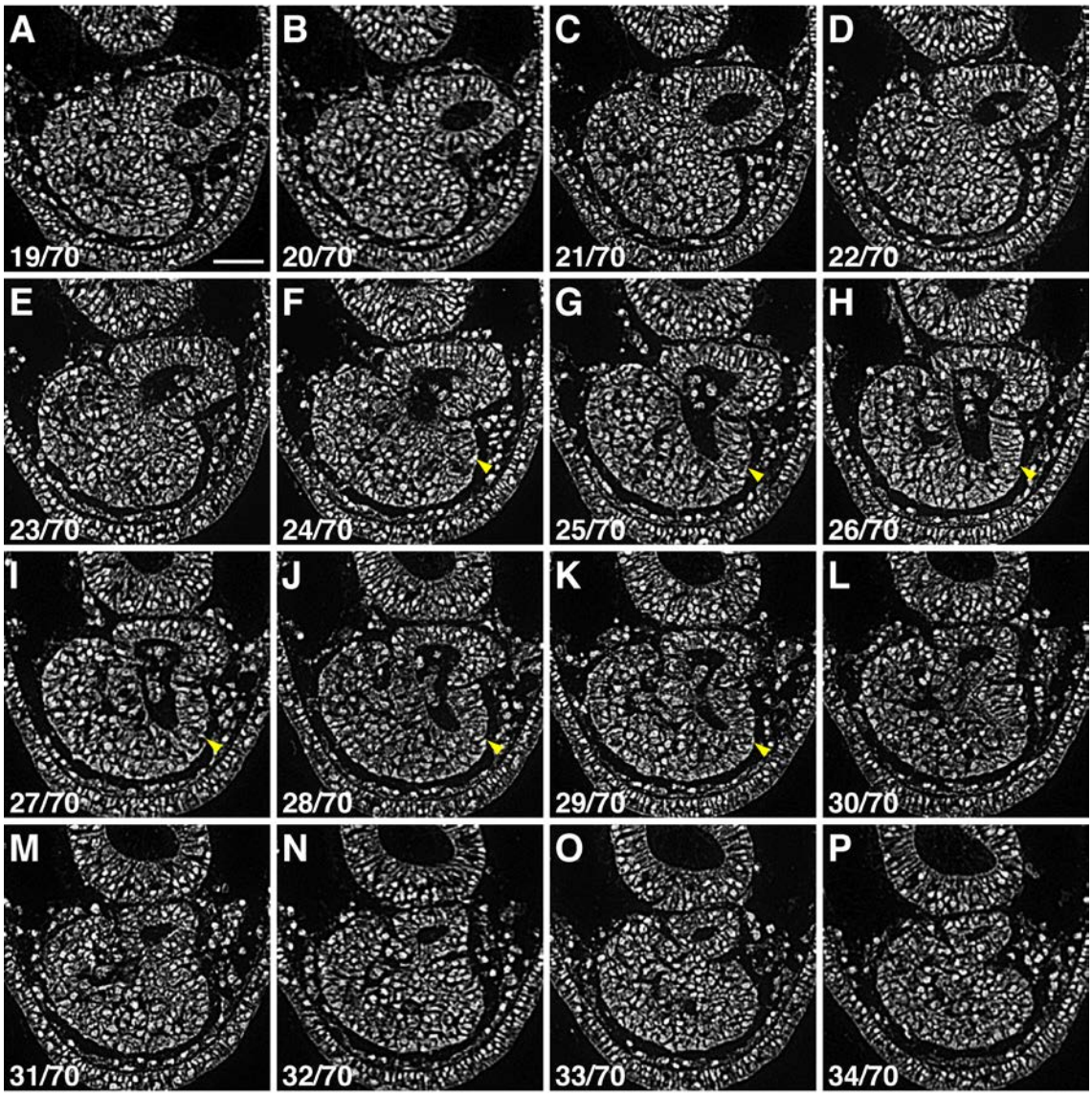

Fig. 1-5. Original horizontal sections along the dorsoventral axis of an early prism larva (18 h). **(A–P)** Sixteen serial sections (from the nineteenth to thirty fourth) of 70 serial sections (3 μm thick) of the larva from the ventral bottom to dorsal top. Arrowheads show an epithelial lobe projected posteriorly from the coelomic pouch. Scale bar = 25 μm.

Figure 1-6

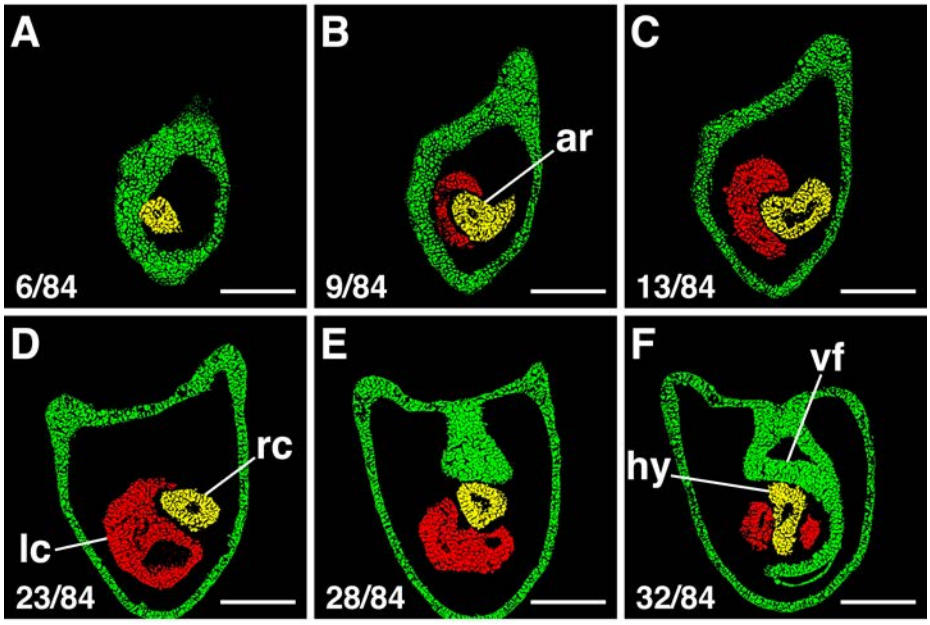

Fig. 1-6. Horizontal sections along the dorsoventral axis of an early pluteus larva (24 h). (**A–F**) Six of 84 serial sections (3 μm thick) of the larva from the ventral bottom to dorsal top. The ectodermal layer, the left coelom developed from the mesenchyme plus an epithelial lobe, and archenteron-derived 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

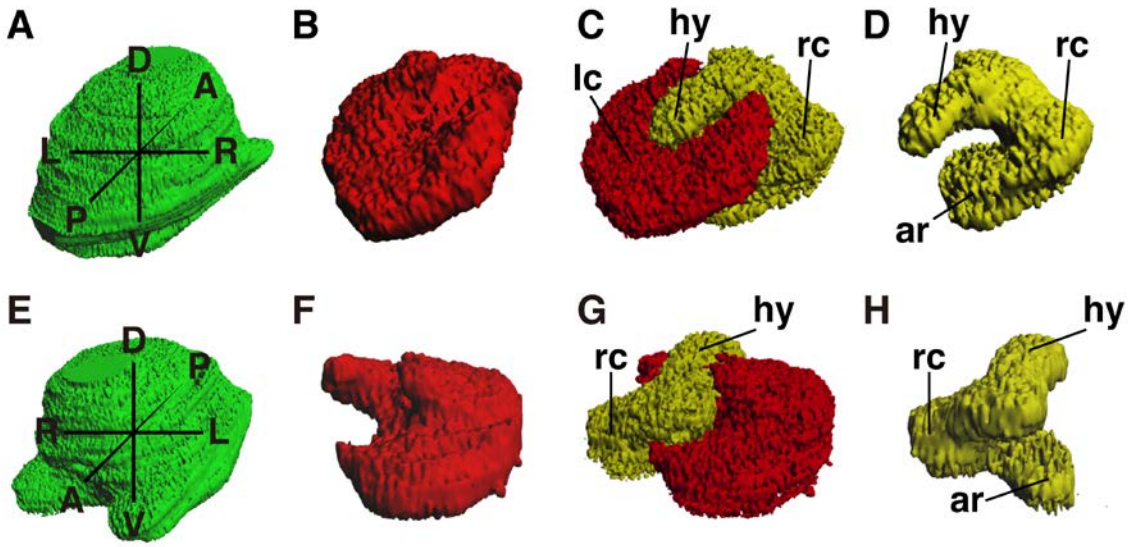

Fig. 1-7. Three-dimensional images of an early pluteus larva (24 h) reconstructed from serial sections. (**A–D**) Images viewed from the right-posterior and slightly dorsal side of the larva. (**E–H**) Images viewed from left-anterior and slightly dorsal side. Color code is the same as in Fig. 1-6. The 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.

Figure 1-8

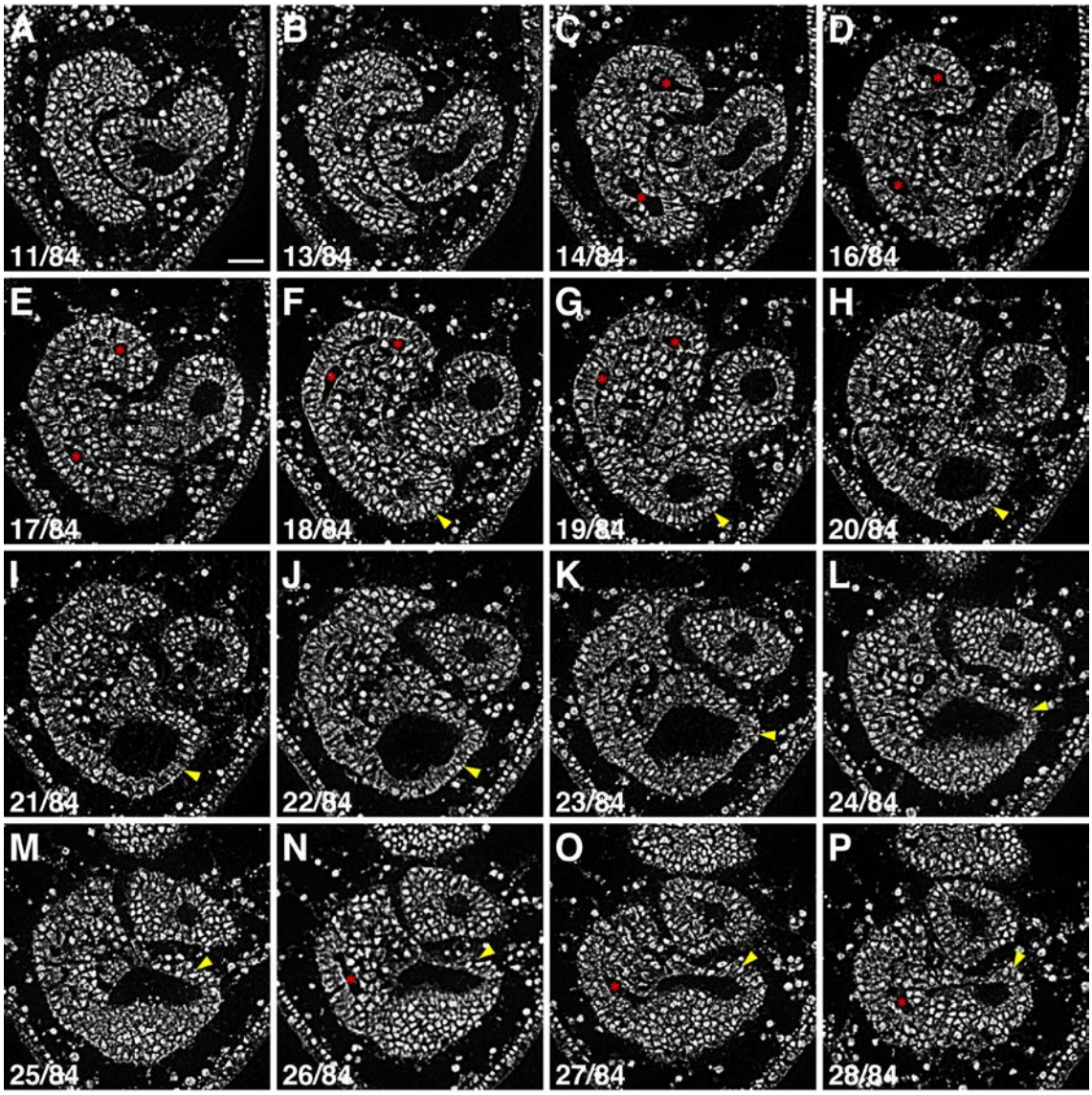

Fig. 1-8. Original horizontal sections along the dorsoventral axis of an early pluteus larva (24 h). **(A–P)** Sixteen serial sections (from the eleventh to twenty eighth) of 84 serial sections (3 μm thick) of the larva from the ventral bottom to dorsal top. Arrowheads show a part of the left coelom 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.

Figure 1-9

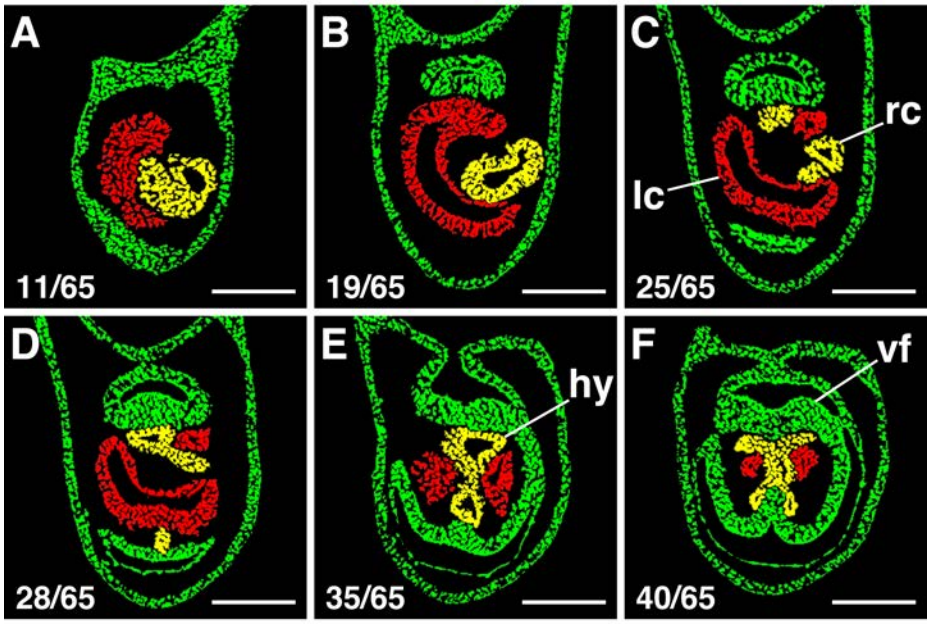

Fig. 1-9. Horizontal sections along the dorsoventral axis of a pluteus larva (28 h). (**A–F**) Six of 65 serial sections (2.5 μm thick) of the larva from the ventral bottom to dorsal top. Color code is the same as in Fig. 1-6. **A**: Eleventh section from the ventral bottom (11/65). **B**: Nineteenth 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.

Figure 1-10

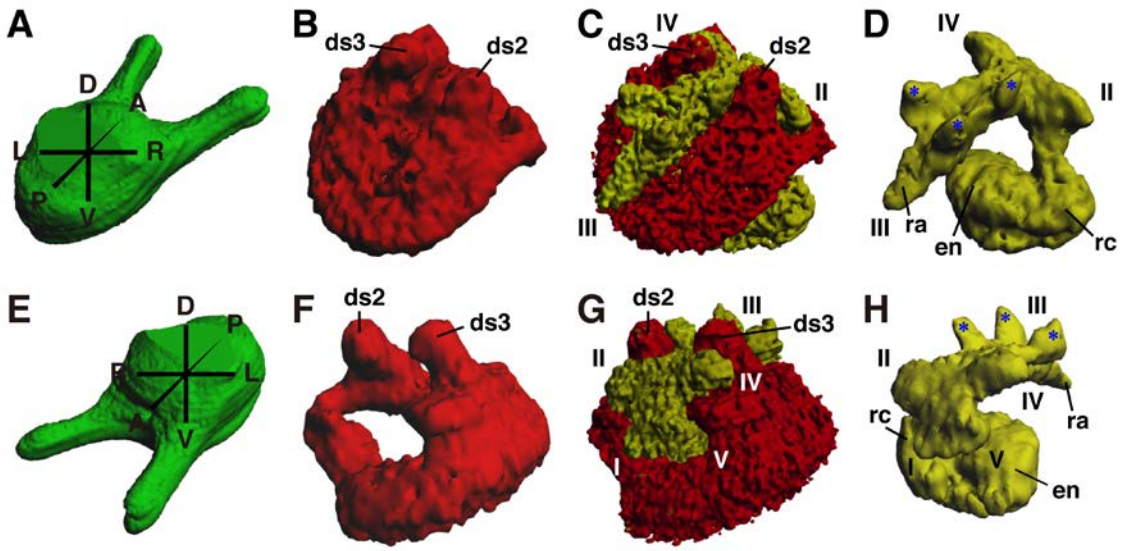

Fig. 1-10. Three-dimensional images of a pluteus larva (28 h) reconstructed from serial sections. (**A–D**) Images viewed from the right-posterior, slightly dorsal side of the larva. (**E–H**) Images viewed from left-anterior on the slightly dorsal side. Color code is the same as in Fig. 1-6. The formerly 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

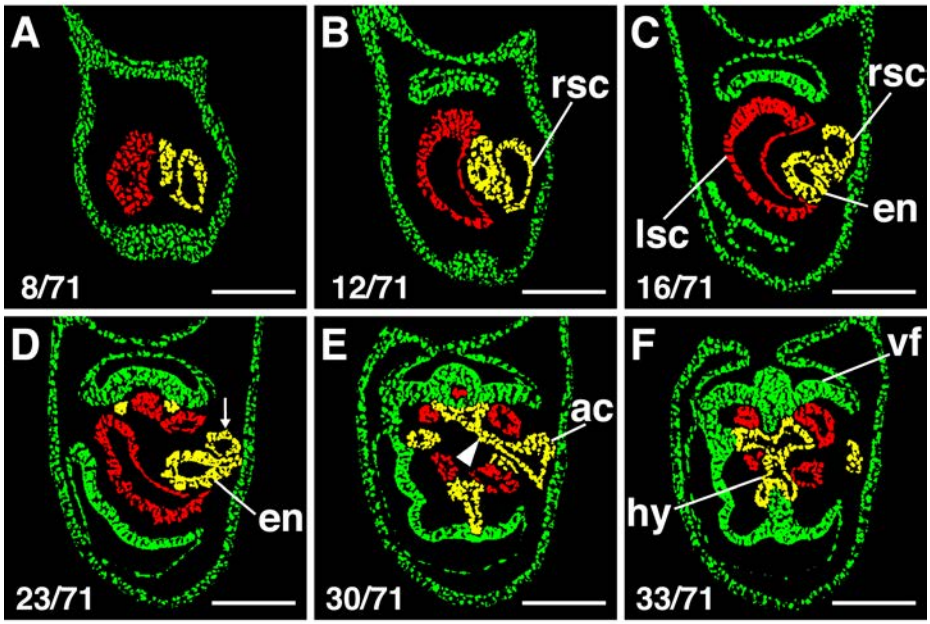

Fig. 1-11. Horizontal sections along the dorsoventral axis of a pluteus larva (32 h). (**A–F**) Six of 71 serial sections (2.5 µm thick) of the larva from the ventral bottom to dorsal top. Color code is the same as in Fig. 1-6. **A**: Eighth section from the ventral bottom (8/71). **B**: Twelfth section (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

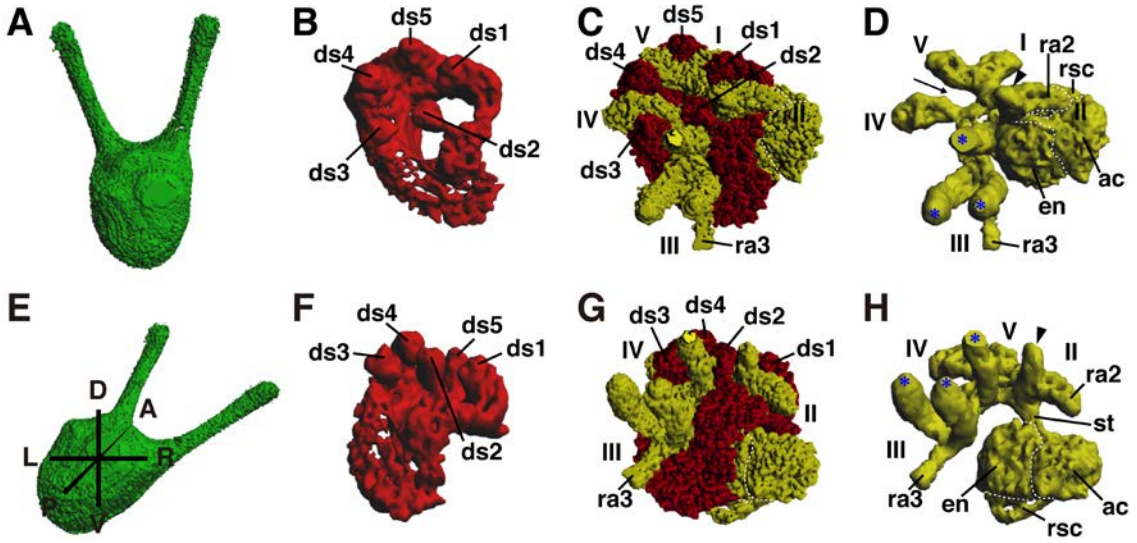

Fig. 1-12. Three-dimensional images of a pluteus larva (32 h) reconstructed from serial sections. (**A–D**) Images viewed from the dorsal side of the larva. (**E–H**) Images viewed from the right-posterior and slightly dorsal side. Color code is the same as in Fig. 1-6. The five lobes of the hydrocoel 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 amburaclum 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 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 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 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. 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 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 *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 *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* expressions is insufficient in both the developmental stages and complements.

The sand dollar *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 *Hox* genes from *P. japonica*, and the expression patterns were examined by whole-mount in situ hybridization. In addition to *Hox* genes, *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 survay, I found that *Hox1*, *Hox3*, *Hox5*, *Hox11/13b*, and *Otx* were pentamerally expressed in either the ambulacral or interambulacral regions, whereas *Hox7*, *Hox8*, *Hox9/10*, *Hox11/13a*, and *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.

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'-GGCCGTGCCCGCAGTAACTCGATTTTAG-3';

PjHox1-5'-RACE-reverse-2, 5'-CATGGCGATGGAGGGATGCATATGATGG-3';

PjHox1-3'-RACE-forward, 5'-CCGCCATGTTGGGACTCAATGAAACG-3';

PjHox2-5'-RACE-reverse, 5'-CCCCCGGGTCTATCAGTTGCCACAAG-3';

PjHox2-5'-RACE-reverse-2, 5'-TGAGGTATGTGACCCATTACCCTGAC-3';

PjHox2-3’-RACE-forward, 5’-TCGACCCCGTCGGATCGAG-3’;

PjHox2-3’-RACE-forward-2, 5’-GAGATCGCCGACTTCCTGGAGCTGT-3’;

PjHox3-5’-RACE-reverse, 5’-GGAAAGTCCTATCGGAGTGATGCG-3’;

PjHox3-5’-RACE-reverse-2, 5’-CCAAAGAGAAATGACTCGCCAACATGC-3’;

PjHox3-3’-RACE-forward, 5’-GACCTCGGAGGGTCGAAATGG-3’;

PjHox5-5’-RACE-reverse, 5’- CCAGGAGGTGTGACGGTAGC -3’;

PjHox5-5’-RACE-reverse-2, 5’- GCTTCCTGGCTGATAAGTTGTGAGATGC -3’;

PjHox5-3’-RACE-forward, 5’- CCATTTCAACCGATATCTCACCCGACGT -3’;

PjHox5-3’-RACE-forward-2, 5’- CCACGCTCTCGGACTCACG -3’;

PjHox6-5’-RACE-reverse, 5’- CCACCCCACGTCCGTGATTTCCTTG -3’;

PjHox6-5’-RACE-reverse-2, 5’- GTCCCGTGTTCCCTCTTCCACTTCATC -3’;

PjHox6-3’-RACE-forward, 5’- TCGCACAGAGTCTCGGTCTCAGC -3’;

PjHox7-5’-RACE-reverse, 5’- CCCTGGTAGCCACTCGCGGTATTGTG -3’;

PjHox7-5’-RACE-reverse-2, 5’- CCTCTCCCGATCCTTCCTCTTTGTTC -3’;

PjHox7-3’-RACE-forward, 5’- TAACGCGACGACGACGGATCGAAC -3’;

PjHox7-3’-RACE-forward-2, 5’- TCAGCCACCTCCTCGGCTTG -3’;

PjHox8-5’-RACE-reverse, 5’- CGAGTCACTGTCATTGTCCTCGCCTTTG -3’;

PjHox8-5’-RACE-reverse-2, 5’- CATCCTCACAGTTCTTTCCCTCCTCTTCAC -3’;

PjHox8-3’-RACE-forward, 5’- CATTTCAACCGCTACCTGACGCGGAAG -3’;

PjHox8-3’-RACE-forward-2, 5’- GACGCATCGAGATCGCACAAGCTGTTT -3’;

PjHox9/10-5’-RACE-reverse, 5’- GGACCCGATTGTGTTGGCTTGG -3’;

PjHox9/10-5’-RACE-reverse-2, 5’- CTCTCCATGGGATGCCAATCAACATACG -3’;

PjHox9/10-3’-RACE-forward, 5’- GATCTGGTTCCAGAATAGGCGGATG -3’;

PjHox11/13a-5’-RACE-reverse, 5’- CTGCCAAAGGCTATCGAGTACTGTG -3’;

PjHox11/13a-5’-RACE-reverse-2, 5’- GGGGATTGTGATGCGCAAGGTGGTGATG -3’;

PjHox11/13a-3’-RACE-forward, 5’- GCGCATCACAATCCCCATCATTTGC -3’;

PjHox11/13b-5’-RACE-reverse, 5’- GGTGGTGGTGGTGATGGGCGTGATG -3’;

PjHox11/13b-5’-RACE-reverse-2, 5’- GAGGATGGTGATGCTGTTGATGCG -3’;

PjHox11/13b-3’-RACE-forward, 5’- GCCGGACCAAGATGTCACAGG -3’;

PjHox11/13c-5’-RACE-reverse, 5’- CTGGAGCTACGACTACACAACACTACCAG -3’;

PjHox11/13c-5’-RACE-reverse-2, 5’- CCTTTTCTCGTTCCAGCTGCTCCTGTTC -3’;

PjHox11/13c-3’-RACE-forward, 5’- GTACCTGACGAGGGACCGAAGGAATAG -3’; and

PjHox11/13c-3’-RACE-forward-2, 5’- GACCGAAGGAATAGGATTTCCGAGGCAT -3’.

To isolate partial fragments of *PjOtx*, PCR cloning experiment was performed using cDNA mentioned above as a template. Degenerate primers toward the *Otx* gene were designed based on conserved sequences of the homeodomain. Primer sequences are as follows: Otx-forward, 5’- AARMGNMGNAAYMGNACNACNTT -3’ coding for KKRRNRTTF; and 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:

PjOtx-5’-RACE-reverse, 5’- GCTGGTTGCGCGTTGCTCATGGTATAG -3’;

PjOtx-5’-RACE-reverse-2, 5’- GCGGGACTCCAGATGCTGTTGTTGTTAG -3’;

PjOtx-3'-RACE-forward, 5'- GTCGAACCAGATATCCAGACATCTTCATG -3'; and *PjOtx*-3'-RACE-forward-2, 5'- GTGGCGATGAAGATTAATCTACCAGAGTC -3'.

The PCR products were cloned into the pTA2 vector (TArget 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; MmHoxc12, 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% H_2O_2 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 $MgCl_2$, 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

(Carl Zeiss, Oberkocken, Germany). 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 pluteusu 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. I-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 expresson 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 amburacla 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 ambulacrarian 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.

Figure 2-1

A : PjHox1

sequence size : 1800 bp

CDS : 188 – 1390 (1203 bp)

amino acids : 400 aa

```
   1  AGATTGTTTT TTTACCCGAG CCGGCGTGGC GAGGCGCCCA CCTAGCTTGC GAGACGAACA AACCAACAAA TCATTTATCA TTACGACCCA ACGTCGACAG  100

 101  TCAGCTAGGA GTAGCTGAGA ATCTGAACAT TTACGACTCC ACTTATTTGT TTCGATTGTG GGACGTTAGA AACACAGTAG ACACTACATG GAGAAATCGT  200
   1                                                                                                 M  E  K  S    4

 201  CGCCCACTTC AGCCGCCAGT GCACCGTACG CTGACTACAC CTCCGCAGAT CAAGCCAGCA TGTACACGGG CGTACGAAGC CTAGATTATT GCAGCTCCTC  300
   5  S  P  T  S  A  A  S  A  P  Y  A  D  Y  T  S  A  D  Q  A  S  M  Y  T  G  V  R  S  L  D  Y  C  S  S  S   38

 301  TGCTATATCG CAGGGTGTCG GACTCTACCA AAACCACGCC AAGAGTTACG GTGGGTTGGA CACTCAGGGG TTCGATGGGC ACCTGGTACC GGGGATAGGC  400
  39  A  I  S  Q  G  V  G  L  Y  Q  N  H  A  K  S  Y  G  G  L  D  T  Q  G  F  D  G  H  L  V  P  G  I  G   71

 401  GTGCAAGGTA GCCCCAACTC GTATTCGCAT CTCGACAGTC GACATCGATT GCATGATTAT ACTCCGCCAT CGACCTGTAA TTCAATGTCG AGCATGAACT  500
  72  V  Q  G  S  P  N  S  Y  S  H  L  D  S  R  H  R  L  H  D  Y  T  P  P  S  T  C  N  S  M  S  S  M  N  104

 501  ACGTTTACAA CCGATCGTCG GCCTATGCCT ACCCGAGCTC TGTTAATCAC TATGGCAATT CAGACATCGA TTACCCACAC ATTCACAAGC ACGTACGGAG  600
 105  Y  V  Y  N  R  S  S  A  Y  A  Y  P  S  S  V  N  H  Y  G  N  S  D  I  D  Y  P  H  I  H  K  H  V  R  S  138

 601  CCCGACATCA GCCGTGTCGT GTGCGACTTC GGGTGTCAAC TTCAACGGTA GCTACAACAG CGAGTCGCCG TCGCTAACAA ACCTTGATTC ACCGTCACCG  700
 139  P  T  S  A  V  S  C  A  T  S  G  V  N  F  N  G  S  Y  N  S  E  S  P  S  L  T  N  L  D  S  P  S  P  171

 701  ACATCGTGTG CACTAGGTTC GGGTCAGGAG CCGACCACTG GACAGCCGCT GGGCTCTAGT GGTGGTGGCT TGGATGATGA CGGAAGGGGA GTCTCGATGG  800
 172  T  S  C  A  L  G  S  G  Q  E  P  T  T  G  Q  P  L  G  S  S  G  G  G  L  D  D  D  G  R  G  V  S  M  204

 801  CCAACGGACG ATCGCCGACG TCCAGTTCGA ACGGAGACCC GCAGTCACCC TCGGGAGATG CTGGACTCTA CAAGTGGATG AGAATCAAAA GAAACCCTCC  900
 205  A  N  G  R  S  P  T  S  S  S  N  G  D  P  Q  S  P  S  G  D  A  G  L  Y  K  W  M  R  I  K  R  N  P  P  238

 901  TAAAACAGTG AAAGCTGGTG AGTTTACAAC GAATGCAGCA GCAAACAACA ACGGAAGAAC AAACTTCACC AACAAACAAT TGACTGAGTT AGAGAAAGAG 1000
 239  K  T  V  K  A  G  E  F  T  T  N  A  A  A  N  N  N  G  R  T  N  F  T  N  K  Q  L  T  E  L  E  K  E  271

1001  TTTCATTTCA ACAAATATCT GACGAGAGCA AGGAGAATAG AGATTGCCGC CATGTTGGGA CTCAATGAAA CGCAGGTCAA AATCTGGTTC CAAAATCGAA 1100
 272  F  H  F  N  K  Y  L  T  R  A  R  R  I  E  I  A  A  M  L  G  L  N  E  T  Q  V  K  I  W  F  Q  N  R  304

1101  GGATGAAGGA GAAGAAAAAG ATGAAGGAAT GCATCCCATC TCAGCACCAC TCTCACTCTA TGTCCCACCT CCATCATATG CATCCCTCCA TCGCCATGAC 1200
 305  R  M  K  E  K  K  K  M  K  E  C  I  P  S  Q  H  H  S  H  S  M  S  H  L  H  H  M  H  P  S  I  A  M  T  338

1201  AACGCCATCA TCAATATCTG TTCATCAGGG TTCTCCACCA CTTACACACT TCACTGGATC GCTTCCGGGT CAAACTGTCA TTTCCGGTGG GTCACCACTC 1300
 339  T  P  S  S  I  S  V  H  Q  G  S  P  P  L  T  H  F  T  G  S  L  P  G  Q  T  V  I  S  G  G  S  P  L  371

1301  GGCGGGGACG GCGATCTAAA ATCGAGTTAC TGCGGGCACG GCCATCTTAA CCACTCGGGT TCAGCGATGA TAATAACAAA TGGGACATAA GGTGCGCCAT 1400
 372  G  G  D  G  D  L  K  S  S  Y  C  G  H  G  H  L  N  H  S  G  S  A  M  I  I  T  N  G  T  *               400

1401  TTAAATCGAT TTAGATAGAA CTATTTATTT CAGAGACTAA AGACACTAAG GAACTTTTGT ATTAACTGTG CGATTGGTTC ACCTTTGATG TAAGATATGT 1500
1501  GCAGCTCTAT TATTTATAAA CAAGTAAAAA GTTTTAATGG CGCCCTTTGA GGTGCAACGT GCTGACTGCT CGTTGCAGGA AGATCGAAAA GTGACATGGT 1600
1601  AAAACTGTAC AAGAGTGATC ACAACTTTAA CTTGGACGAT ACGATTAAAT CATTGAACAT AAAAGGGGGT GACGTTGCCA GGGTTTCGGT GGGGGTGGGG 1700
1701  CGAAAAAGTT TTTAACGATT TTCCCCCGAA AAAAAATCCC GACTGTAGGT GTAATTTTTC CATATGTTTA CATTTATTTC CTACTTTTTT AGGTGGAAAG 1800
```

B : PjHox2

sequence size : 1876 bp

CDS : 563 – 1399 (837 bp)

amino acids : 278 aa

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 101  CAAACAACTG TTCAGCTGCC TGCCGTCAAT AAATAACAAA TAATCATGCA CTTGCTATGC AACAAGTCAA CCTCAATATA AATCCATTTC ATTCAAGTTA  200
 201  TATAAAAGCG GAAATATTGT TAAAACTCGC AAATTGCATA CAAATATGAA TTGTTTCGTT TTTGGAGTTC TCCTATAAGC TAAAATGATT ATTCTGCTTC  300
 301  TTCTTGTTAT TTTTATTTAT TTATTGATTT TCTTTGCTTC AAGGCACGGT TAATTATCAG ACATTCAAGC AGGTGGGAAA TTATTTTCCA GAGAGCAACA  400
 401  ACTTCCGTTC ATTTCATGAC CATGCTTTGT TCACATTTCA CCATTCCGGA CGCTGTCGCT GTTTTATTAA GTATGTGTTA ATTTCTGTTA AAATAGTTGG  500

 501  GCAATATACT GAAAACTATT GCATGCAGAA TATTGTAGCT GAAGGCAGCT ACAGTTCACT ACATGTGTCG CACCCCTATT CATCAAATGT TTCCTCGCCA  600
   1                                                                  M  C  R  T  P  I  H  Q  M  F  P  R  Q   13

 601  GCGCACACAC TCCGACACCT TTCCGGCAAC CTGTGATTTA GCAGGGATCT CTACTTGGGC TCCTTATTGG GATGCGACGA GTTCAGAGGT CACGGGGTCG  700
  14   R  T  H  S  D  T  F  P  A  T  C  D  L  A  G  I  S  T  W  A  P  Y  W  D  A  T  S  S  E  V  T  G  S   46

 701  TTTCCCCCAG GAGCGATGGG GAAGACATCG TCTCCCTCAA GATCTGGGAC TCTATCTACT CATCCCTCAA TATTCAGTTC TTCTTCGGTG GCGTCGTTGA  800
  47   F  P  P  G  A  M  G  K  T  S  S  P  S  R  S  G  T  L  S  T  H  P  S  I  F  S  S  S  S  V  A  S  L   79

 801  ATTCAACATT GACATTGTCA TCATTGACAG AATTGTCCTC GCTGTTATTA ACGACGCCAT CGCCATCAGC ATCATCATCA TCACCATTAT CAACAGTTGC  900
  80  N  S  T  L  T  L  S  S  L  T  E  L  S  S  L  L  L  T  T  P  S  P  S  A  S  S  S  S  P  L  S  T  V  A  113

 901  ATCATCCTCT TCATCTCCAT CGTCATTAAT ACCACTATCA CCGGAAGAAT TTACATTTGT CCCGTCGTGC GCGTTTGCAG CTGGTAAGGA TCTGCTGGGA 1000
 114   S  S  S  S  S  P  S  S  L  I  P  L  S  P  E  E  F  T  F  V  P  S  C  A  F  A  A  G  K  D  L  L  G  146

1001  AATGAGGCCT CGGTGAGTCA GGAGAAACCG GTATCGACCC AGCAGAATAT CCCAACGGTT CGGCTGCCAG AGTATCCATG GATGGAAGAC ACACCAACGG 1100
 147   N  E  A  S  V  S  Q  E  K  P  V  S  T  Q  Q  N  I  P  T  V  R  L  P  E  Y  P  W  M  E  D  T  P  T  179

1101  TTGAGTTTGA AGCCTCAGGA TCGACCAAAA ACAGCAAAGA GAATCATACA TCAGCAAGTG AACGTAGCCA CCATCGAATA CGGACCGCGT TCACAACCAC 1200
 180  V  E  F  E  A  S  G  S  T  K  N  S  K  E  N  H  T  S  A  S  E  R  S  H  H  R  I  R  T  A  F  T  T  T  213

1201  TCAACTTCTA GAGCTCGAGC ACGAGTTCCG ACTCAATAAA TACCTCTGTC GACCCCGTCG GATCGAGATC GCCGACTTCC TGGAGCTGTC AGAAAGACAG 1300
 214   Q  L  L  E  L  E  H  E  F  R  L  N  K  Y  L  C  R  P  R  R  I  E  I  A  D  F  L  E  L  S  E  R  Q  246

1301  GTCAAAATAT GGTTCCAAAA TAGACGCATG AAGCAGCGAA AACTGGAATC CAAATCACGT GACCAAAAAC GTCAGGGTAA TGGGTCACAT ACCTCATAAT 1400
 247  V  K  I  W  F  Q  N  R  R  M  K  Q  R  K  L  E  S  K  S  R  D  Q  K  R  Q  G  N  G  S  H  T  S  *   278

1401  CAGCGAATTA TAATGAGTCG AATCGTAAGA AGATGTATAT TGACCATCCA CCTATGGTAT ATGTCTATGG TGTCGGTTGT TTTCTTGATC CACGGCTTGT 1500
1501  GGCAACTGAT AGACCCGGGG GTTCGACAGT ATGTTACAAT TTTCAAGGAA TATTTGATTG GTTGATCAAA ATCAGCGGGA ATTTGGATTT TTGAAAGAAA 1600
1601  AAAAAATCCA AATTCTGCAG AACCAAGGTT CATCGAAAAT AACTAAGATC ATAAAAAAAC TCAAAAATAT CAGCTGTCCT TAATATTGGC TTTCGAAAAA 1700
1701  AAAGAAATTA TTATTATATA AACATCTTTG TACATATCCA TCCATATTAT TTCCATGTTA ATTTGATGTG TTCATAATAT GAACACATCA AAGTTATAAT 1800
1801  ATTATTATAA TACTGTTATT TGTTACTGCT GTTATTTGTT ATTTGTTACT TCCAAACGCA TAAACTACCT TATGTG                          1876
```

C : PjHox3

sequence size : 1594 bp

CDS : 52 – 1170 (1119 bp)

amino acids : 372 aa

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   1                                                        M  Q  A  V  E  Y  Y  E  R  G  P  A  I  Y  G  A    16

 101  ATTATTTCAC CGGGCAAACT GGAACGGCTA ATGGGTACGT CTATGGCGCC AGCACAGGGC ACCACCACCA CCAGCAATAT TACTACCACG ATCAGGGGCA   200
  17  D  Y  F  T  G  Q  T  G  T  A  N  G  Y  V  Y  G  A  S  T  G  H  H  H  H  Q  Q  Y  Y  Y  H  D  Q  G  Q    50

 201  ATCCGCCTTG AATGGTAGCG GTGGCTACCA TGGACTCGAC CCGAGAGAGG CAGCAGAGGC GACGGGATTG TACGGGGCTG GTGAGAGCCC CGACCGGGCG   300
  50    S  A  L  N  G  S  G  G  Y  H  G  L  D  P  R  E  A  A  E  A  T  G  L  Y  G  A  G  E  S  P  D  R  A    83

 301  AGCTCTGGTG GAGCAGGTGG TAGTGGAGGG GGTGATACTG CTGGAGGGTC GTGTGGGGAA CAGGATATTT GTGGAGGGAT GCTGAATTGT CGAACAGGTG   400
  84   S  S  G  G  A  G  G  S  G  G  G  D  T  A  G  G  S  C  G  E  Q  D  I  C  G  G  M  L  N  C  R  T  G    116

 401  CCGGTATACC CGGTCAGGGA CCCAAGGCCA TCTATCCCTG GATGATGGAA TCACGACAGA ACACCAGACA GAAGAAGCTG CCCGAATGCG GTGTTCTCGA   500
 117  A  G  I  P  G  Q  G  P  K  A  I  Y  P  W  M  M  E  S  R  Q  N  T  R  Q  K  K  L  P  E  C  G  V  L  D   150

 501  CCAAGACGAA AAGAAGAAAC CCCTGACAGT ACTAGCACCC GAAGCTCCCC TAGTTAGCAT GACTTCTACC AACACCGCAG GGGCTGGTCC CGGACAAACT   600
 151    Q  D  E  K  K  K  P  L  T  V  L  A  P  E  A  P  L  V  S  M  T  S  T  N  T  A  G  A  G  P  G  Q  T   183

 601  ACTTCTTCAG TTCCGGCTGG CGTAATTACA ACTAATACCA CTGCAAATGG GGTCAGCCCG CGCTCGGACA GCAGCAGCAG TAGCGGCCCA ACAAACACGT   700
 184   T  S  S  V  P  A  G  V  I  T  T  N  T  T  A  N  G  V  S  P  R  S  D  S  S  S  S  S  G  P  T  N  T   216

 701  CAAACACGGG GAGCAGCAGC GGGGGAAAGT CCGAGGGGAA CGGTGGGAGT AGTCCTAAAC GGAATCGCAC AGCTTTCACC AGCGCCCAAC TTGTTGAACT   800
 217  S  N  T  G  S  S  S  G  G  K  S  E  G  N  G  G  S  S  P  K  R  N  R  T  A  F  T  S  A  Q  L  V  E  L   250

 801  CGAAAAGGAG TTCCATTTCA ACCGGTATTT GTGCCGACCT CGGAGGGTCG AAATGGCTAA ATCACTGAGC CTAACCGAAA GACAAATAAA AATATGGTTT   900
 251    E  K  E  F  H  F  N  R  Y  L  C  R  P  R  R  V  E  M  A  K  S  L  S  L  T  E  R  Q  I  K  I  W  F   283

 901  CAAAATCGCA GGATGAAATA CAAGCGAGAC ATGAAGGAAA GCGAGCGAGC CGAGCGAGGG ATCAAAGTAT CGGACTCTGT GTTAGCACGA CAGGCGGAGC  1000
 284   Q  N  R  R  M  K  Y  K  R  D  M  K  E  S  E  R  A  E  R  G  I  K  V  S  D  S  V  L  A  R  Q  A  E   316

1001  GGGCAGGGCA TGTTGGCGAG TCATTTCTCT TTGGTTACCA CCCCAGTACC TCCTCGCATC ACTCCGATAG GACTTTCCGC GGGGCACCGC CCTATTGTCA  1100
 317  R  A  G  H  V  G  E  S  F  L  F  G  Y  H  P  S  T  S  S  H  H  S  D  R  T  F  R  G  A  P  P  Y  C  Q   350

1101  GTACTCAACA TACTCGTGTA GTGCACAAGA ACAATCACTG GAGGCCACGC CCCACCTAAC ACATTTGTAA AGTTTATATT GACTTTTTTT TCAAATTTTG  1200
 351    Y  S  T  Y  S  C  S  A  Q  E  Q  S  L  E  A  T  P  H  L  T  H  L  *                                   372

1201  ACTTTCAGGA TGTAAATGCT TAAAAATGCT CTGTACAGAA GAAACTTCAA CTTGTTTGGA GCTGACAGTA TTGACTTTCT GGCGATAAAC TTCAATAATT  1300
1301  CTGTCTCTAT GCATAATAAC TTATTTATTA TAACATTGAA ATACTTACTG AAATGTCTGT AAATGTACTA TACTTAAATG TAAATAGTTC AATTTTAACC  1400
1401  CTCATCCCAT GGTGAATGGG TGGGTTGTTG GGGGGGGGGG GGATTCGGCA ACGAGAGAAA CTCTTGCCGC CACGTTACAT GACCTTTTTA TTGCGAATGT  1500
1501  AGTTTAATTT TAATTTAATA ATTGAAATAA ATTATGCTGA AAAATAAAAA GGTTTCCGCT AATTTTTTTG GTCAAAAATA AAATCCAAAA TTCG        1594
```

D : PjHox5

sequence size : 2006 bp

CDS : 285 – 1136 (852 bp)

amino acids : 283 aa

```
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 101  TTGTATCTTA GTAACTGTTG TGTGTATATC AAACTATTTG TTCGAGGTTG TGAGATAGAC CAGACTCGGT CTGTCGGTGC GTCCATACGT CAACCAGAGG  200

 201  TTGTACCTAC AAATCACTTG TCAGTCTCTT GTCCCGTTCT TGGGATCTCT TGTCGTTCAG CAAGGAAATA GACATAAAAC ACAAATGAGT TCGTATTTCG  300
   1                                                                                          M  S  S  Y  F    5

 301  CGCACCCGAT GGCTGGAGGT GACTTCCACC ACTCTGGCGG GTCAAATTAC CCACAGCAAG CCGTTGGTAG CTATGCCCTA ACGGCTGAAG GAGCTCAACA  400
   6  A  H  P  M   A  G  G   D  F  H   H  S  G  G   S  N  Y   P  Q  Q   A  V  G  S   Y  A  L   T  A  E   G  A  Q  H   39

 401  CGCAACACAT CGACACCAGC ACTACCCTCC CGCCAACGCC GTAACTTCCC CGGCCGGGAG CTACGCCTAT CCGTACTCAG CCGAAGACGG GTTGCACCAC  500
  40    A  T  H   R  H  Q   H  Y  P  P   A  N  A   V  T  S   P  A  G  S   Y  A  Y   P  Y  S   A  E  D  G   L  H  H   72

 501  GGTTTGCAGA ACGGGTACTA CACCATGGAA GGCGCAACGG GGATGGATGC GACCACGGCG ATCAACCACC ACCACCACGC AGCATCAGTA AACTCCTCAT  600
  73   G  L  Q   N  G  Y  Y   T  M  E   G  A  T   G  M  D  A   T  T  A   I  N  H   H  H  H  A   A  S  V   N  S  S   105

 601  GTGGGTACAC GGTGGATCCG TACAGTCAAA CATCACCAGG AATGGGGGCT ATGCCTCGAA GGAAGACCAG CCCGGACACC CCTCCCATCA CCCTCTCGGA  700
 106  C  G  Y  T   V  D  P   Y  S  Q   T  S  P  G   M  G  A   M  P  R   R  K  T  S   P  D  T   P  P  I   T  L  S  D   139

 701  CTGCGGATCC AACGCAAGTC CACCAGCAAA CATCAACAAC AATAACAACC ACCATCATGG GTCAATGAAG GGGGCTACGA AGGGGCAGAG CAATGAACAG  800
 140    C  G  S   N  A  S   P  P  A  N   I  N  N   N  N  N   H  H  H  G   S  M  K   G  A  T   K  G  Q  S   N  E  Q   172

 801  ATTTATCCCT GGATGAGAAG GATACACTCC ACTACAGCTG CGACAGCGGT GAACGGAACA GAACCAAGCA AGAGATCGAG GACTGCCTAC ACCCGCTACC  900
 173  I  Y  P   W  M  R  R   I  H  S   T  T  A   A  T  A  V   N  G  T   E  P  S   K  R  S  R   T  A  Y   T  R  Y   205

 901  AGACCCTCGA ACTCGAAAAG GAGTTCCATT TCAACCGATA TCTTACCCGA CGTCGACGGA TCGAGATCGC CCACGCTCTC GGACTCACGG AGAGGCAGAT 1000
 206  Q  T  L  E   L  E  K   E  F  H   F  N  R  Y   L  T  R   R  R  R   I  E  I  A   H  A  L   G  L  T   E  R  Q  I   239

1001  CAAAATTTGG TTTCAAAATC GCCGGATGAA ATGGAAGAAG GAACACAATG TTAAAAGCAT CTCACAACTT ATCAGCCAGG AAGCAGCGGC TAACCTCGCC 1100
 240    K  I  W   F  Q  N   R  R  M  K   W  K  K   E  H  N   V  K  S  I   S  Q  L   I  S  Q   E  A  A  A   N  L  A   272

1101  GGAGCTACCG TCACACCTCC TGGTGCAATG CGCTGATAAA GCTGCTGCAA ATTTGTCCAT TTGACTCGTA TTGTTCGCAC AGTTCGTACA CTACGCACAT 1200
 273   G  A  T   V  T  P  P   G  A  M   R  *                                                                      283

1201  ACATGCAGAA GCCAGCCCGG CGCAGCGGGC TAGACCGCAT CGATATAATG ACTATTATCG TGGCCGGAGG TGACGGCAGA CTTGGGTACG CAGCGCGTCG 1300
1301  TTGAACTTGA TCGACTCTGA TGGATGTGGT GTTAATATCA GCATCTCAGT GTAAACCAAT TACTTTCATT AGTGGCACAG TGTGAATTGA CCCCCGGGGG 1400
1401  TCCATTGAGG CCTGTACAGA ATCCTCGTTA ATGACCCTCC AAAAAAGCAC CCTAAACGCG GATAAAGGAT ATCTTATTTT GATACCCTTT TCGCGTATTC 1500
1501  GCGGATAAAG AATAGGTAAT TTTGATACCC TTTCGCGAAT TTGAAGGGTT AATTTTGATA CTCTTTTCCT CGTATTTTTT TGAAATTGTG TTTTTGATAC 1600
1601  CCTTTCCCCC AATTTTCCCG ATTTTGACAC CCTAAACGCG TTACGCGCGT ATCGCGCCGG ACCGTGAATT TGATACCCTT TTCACGCTTT GACAAGGATG 1700
1701  CTGTACAGGC TCCAATGGCA CATACCCCCC CCCCCCGGGA ACTGACTAAT GTATCATTCA CTTCACACCA AAAGTATAGT ATCACAAAAT GTGAATTTAT 1800
1801  TGACTGCACC GTATATTGAT ATTGAAAAGT GTACAGCACA ATGTTTGTAA GATAAGAAAT ATTTTATCAC ACCTTGTTTA AAGGTTCATA TAGAGGGATG 1900
1901  TTGAAATGGG CAGTCCAGTA ACTTAAGTTT TCTTTTTATA TAGGTAATAT TTGAAAGAAC ATCACGAGCC CGTAATGCGT TGAAAATTAA ATGCATTATT 2000
2001  ATTATC                                                                                                       2006
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E : PjHox6

sequence size : 2573 bp

CDS : 283 – 1122 (840 bp)

amino acids : 279 aa

```
   1  ATAAAAAAAC CTCCACCACT TTCGCTGCGT CGAACTAAAA TGTGAAAGTA CCCACCACTT CTCGCAGCTC TTCTATTAAC TCATCTGTCA TCACATTATT  100
 101  ATTTTTAAAT ACAGTTTTTA TGTAACTTTG CCGCCATTTT GTCGCGGGTT AAAATAGTGA TTACTTGAAG CAGTATCGAG TGATTTTGTA TGTTTATTTA  200

 201  ATACATTGTC AGTTACTTTG AATGAGAAAC TCCGAATGTG AATTTTACAT TAGAAATTTG GAAAACAAGT ATTGACATCA TAATGAGCGT GTACTACACG  300
   1                                                                                          M  S  V  Y  Y  T    6

 301  GATTACCACC ACCAGGGCAG TGGGATGAAC TCACGATCAG CGAGCGACAG CTGGCTATCC ACCCCGACCG CACCCCGTGG ATCCGTCGAC TCCTCTCCGC  400
   7   D  Y  H   H  Q  G  S   G  M  N   S  R  S   A  S  D  S   W  L  S   T  P  T   A  P  R  G   S  V  D   S  S  P     39

 401  TCCTCGCCTC CCTAAACGAG CTGACACCAT CAGCATCTCA CCATCAGTCA TCCAGTCAGC ATTTTTCTCA CCCATCACAG AACAATCTTG ACTCCACCTT  500
  40   L  L  A  S   L  N  E   L  T  P   S  A  S  H   H  Q  S   S  S  Q   H  F  S  H   P  S  Q   N  N  L   D  S  T  F   73

 501  CTCGTTCTTT ATGCCACAAT TCTACCCCCA TCACGGGCCT GACTCTTATG GTGGTCCATC AACCAAGAAT TCATTAAATC CATACCACGG CGCATCAAGT  600
  74    S  F  F   M  P  Q   F  Y  P  H   H  G  P   D  S  Y   G  G  P  S   T  K  N   S  L  N   P  Y  H  G   A  S  S  106

 601  GGAGCATCAT CGTCAACTTC CGCCGGCGGG CCCGGGACGC ACGGCGTCAT TACCCCGTAT TCGTCATCGC TCTATGACCA GTGTAAGCAA AATGAACTCG  700
 107   G  A  S   S  S  T  S   A  G  G   P  G  T   H  G  V  I   T  P  Y   S  S  S   L  Y  D  Q   C  K  Q   N  E  L   139

 701  GACCGACTTC GTACTTGGCA TTCAAGGCCA GCGATTACCA GAAACACGAC ATGGAGTCGT CAACGGACAG AGAGATCAGA GAGCTGTCAT CAGCTGCTAG  800
 140  G  P  T  S   Y  L  A   F  K  A   S  D  Y  Q   K  H  D   M  E  S   S  T  D  R   E  I  R   E  L  S   S  A  A  R  173

 801  GTGTGGCAAG GACATGGATG GAGATAAAAT GGCGTTCTAT CCGTGGATGA AGAGCATAAG TCCCACCTCG GACGGCAAGC GGGGACGGCA GACGTACACT  900
 174    C  G  K   D  M  D   G  D  K  M   A  F  Y   P  W  M   K  S  I  S   P  T  S   D  G  K   R  G  R  Q   T  Y  T  206

 901  CGACAGCAGA CTCTCGAGCT CGAGAAGGAG TTTCACTTCA GTCGTTACGT GACGCGAAGG AGACGCTTCG AGATCGCACA GAGTCTCGGT CTCAGCGAAC 1000
 207  R  Q  Q   T  L  E  L   E  K  E   F  H  F   S  R  Y  V   T  R  R   R  R  F   E  I  A  Q   S  L  G   L  S  E  239

1001  GTCAGATCAA GATCTGGTTT CAAAATCGGC GGATGAAGTG GAAGAGGGAA CACGGGACGA ACTGCACCAT GACGAACCAC CAGGAACAGA TGCCAAGCAT 1100
 240  R  Q  I  K   I  W  F   Q  N  R   R  M  K  W   K  R  E   H  G  T   N  C  T  M   T  N  H   Q  E  Q   M  P  S  M  273

1101  GGCAGACTTC ATCGGGTCGT AATCGCCGAT GTCGAGTGAC TTACTCGGAG ACAGGGACCG TTACAATATC GAGACGCTTT GAAAAGCTGA TATAATTTAA 1200
 274    A  D  F   I  G  S   *                                                                                    279

1201  ACGTTAAAAT ACAAACAAAG TCTCGAGTAA ATGGCGACAA AAATTCGTTA GAATTGTCAA GGAAATCACG GACGTGGGGT GGGTGCTTGC CATACCCCTA 1300
1301  TAAATTACAT TTTTACATTT TGTCGACAAC AACCCACCCC CCCCCCCCAC AACCACCAAA TCATTATATT GTTACACCAT TGGGTTACAA AGTTAAACAG 1400
1401  CAAAACATGC GCCACTGGGA TGTAAACCCC AAGGTGGTGT TACTCGGGGG TATCTGCCAT TGAAGCCTGT ACAGCATCCT CGTAAATGGC ACTCTGAAAA 1500
1501  GCAGCCTATT CGCAGCTAAA GATCGTCTTA CATTCAAGAG GACGCGGTTC AATATTGCGC GGGAATGAGG ATGGTGCACG CATTCCATTG AAAAGCACTG 1600
1601  ATATGGCAAT TCCATAGTTG CCGAGGTATT GTGCAATACT TGATGGTTAT TGCATAATTC TGATCACATG TCGAAGAGAC TTTAATACCA AGGCATGATC 1700
1701  TATAGAGTTG AGAGCGTAAA ATTGCAAGGG GTGGAAAGAC CTGGGGACCA TTATCACCGA GAAAGGGTCT ATACATAAAG ACACTCGTTA CTCCAAGTTT 1800
1801  TTATCAATAA AAAAACACAC TTAAGTTATG CATATGAATT GTCCTAAATG GTTGGTGGGC ACCAAATAAG ATTCTTTCCT ATATATTTAA ATATCTTCAG 1900
1901  TTAATATATC AGTAATATTC AAAGAACTTG GGGGCGTCAT TTCACGAAGA AAAGTCTCTA CGTAGTAAAT GTCACCGGTA TGACTGGCTT GAGTCACTCA 2000
2001  TAAGTCTATG GATATTTTGT ACAAACCGTT TTTTTTTTTT TGCGCAACAA CTGTGAAATA TATAGAGGCA ATGTGCCCCT GTGTCTCTGG AGGCGAAGGA 2100
2101  CCTTATTTTT TTTTCATTGT CGCCTCGCCG ACGTTGCCCC CTCCCCCACC CCTGGCTTCA ACCTCCCCGA ATGTCCCGCC TCCCACCCCC ACCCCCCCCC 2200
2201  CCCCCCATTT GAAAATCCTG GTGGCGCCGC TGTGAGAGAC TCGTGTCACT GGTAAGTTTG GATTATGTAC ATACCGGTGA TGTTTAGAGA CCAACATGTG 2300
2301  AATCTATGAT ATCTAAGAAA TGATACACAC AAAAATAAAA TAAAGGCGGG GCCTAATCCC TACGCCCAGC CCCTTTCCCC TTCCATCCGG AATGCACGGA 2400
2401  ATTTAATGTT GATTAAACCA AACAGTTTTA ACGTTGATTT GATGTACAGT ATTAAATATC TTTGACATAT TTTAAATTGT AAAATATATC TTATTTATCA 2500
2501  TAATTTCAAT ACATATTATT TACTTAAATG TGAAATGTTT GTGTGACGAA TAAAGAGATT TTACATAAAA GGG                            2573
```

F : PjHox7

sequence size : 2419 bp

CDS : 530 – 1492 (963 bp)

amino acids : 320 aa

```
   1  AAAACTCGCA CGCTATTGGT CAATTCGTTA GTTCATTGGG TGGAATAGAA AAAGAGGGAA AAAATGTGGT GGAACTTTAG GTTTGGATAA ATGGCTGAAA  100
 101  CCTTTCCAGA CAAGGTTCAG AGTACACATA GTTCAAACAT CATTAGTTGA TCGAGATGAA CACAGCGTAC ATTTCAAAGT TAAGACCAAT TTACTAGTCA  200
 201  AATTGTGTAA TTTATGTATG GCATCCAGTT GAAGAGCTTT GTGACTATGA ACAATGTCTG TATAGCAGTA TTCTTTAGTT GGATTGTTGG AGACTTTTTT  300
 301  CTGGCATATT TCTTTTAACA CCTGCATATG AATGCAAACT GATCTGCTTG ATATACAACA TAATTTTTTT TTTGTTTAGA GAGATTGGAT AAGAGGACTT  400
 401  TAACAACCAA AAAATCATAA CTTTGACTTT GAACCTGAGT TTATTGTTTT ATTGCAAGTT CAATTGACAT CTTTGACTGG TTTATGGTAG TATTTATCTC  500

 501  ACCGCCTCAT CAACCTCCTC AAGATCAACA TGAGTTCTAG TTCATATTTC GTGAACTCCG CGTTCTTTTC AGCCTACCCC CACTCTGGGG ATCAATACTA  600
   1                            M  S  S  S  S  Y  F  V  N  S  A  F  F  S  A  Y  P  H  S  G  D  Q  Y  Y   24

 601  CCCCTCCCCG GCCGGAGCCA CTGGGTACGA ACTGTCGTCA TGCGCTTTTA GCAAGAATCA GAAATCGACC ACCTACTCGA CTAGTACCTC GCCGACACTC  700
  25    P  S  P  A  G  A  T  G  Y  E  L  S  S  C  A  F  S  K  N  Q  K  S  T  T  Y  S  T  S  T  S  P  T  L   57

 701  CAGGCTACGG TGACAACCAA GCAATCGGTC ACGCAGCAAA TCGGCACTAC GCCAACCGCG GCCTCTTTCT ACAGCCACGG CGGAGGTTCC TTGTCGAACT  800
  58   Q  A  T  V  T  T  K  Q  S  V  T  Q  Q  I  G  T  T  P  T  A  A  S  F  Y  S  H  G  G  G  S  L  S  N   90

 801  TCCCCACGAC AGTTGGATAT GGTGACCACA CAGTCAGCCC AGCTTCGTAC GGGTCGATGT CACAGCCTCT TTCGCCCACC AGTTCATGGG AGGCATCTGC  900
  91  F  P  T  T  V  G  Y  G  D  H  T  V  S  P  A  S  Y  G  S  M  S  Q  P  L  S  P  T  S  S  W  E  A  S  A  124

 901  CCGCATGCCA GCCAGCTACA ACTCGCCGGC GTGGGGTACA ACGGCAGCTG AGCTGGCGGG AGACGGGGGC TACCGGTCTA GGGTGAATGC TCTAGCCGCC 1000
 125    R  M  P  A  S  Y  N  S  P  A  W  G  T  T  A  A  E  L  A  G  D  G  G  Y  R  S  R  V  N  A  L  A  A  157

1001  GGTACTGGGT GCTTAGTATC AGCCGCAGCG GAGCCAAATA ATAACCACAT TTCGCATCAA GTTATGTCCC CCTGCAAGTC GTCCGCTGGG TACCCGTGGA 1100
 158   G  T  G  C  L  V  S  A  A  A  E  P  N  N  N  H  I  S  H  Q  V  M  S  P  C  K  S  S  A  G  Y  P  W  190

1101  TGCCCGTGTC AGGTCCCAAT GTCGGACTGG AGGTCGGCCG GAAGAGATGT CGTCAGACGT ACACTCGCTA CCAGACACTC GAACTCGAGA AGGAGTTCCA 1200
 191  M  P  V  S  G  P  N  V  G  L  E  V  G  R  K  R  C  R  Q  T  Y  T  R  Y  Q  T  L  E  L  E  K  E  F  H  224

1201  CTTCAACCGC TACCTAACGC GGCGACGACG GATCGAACTC AGCCACCTCC TCGGCTTGAC AGAACGACAG ATCAAGATCT GGTTTCAAAA CAGAAGGATG 1300
 225    F  N  R  Y  L  T  R  R  R  R  I  E  L  S  H  L  L  G  L  T  E  R  Q  I  K  I  W  F  Q  N  R  R  M  257

1301  AAGTACAAGA AGGAGAGCAA GAACAAAGAG GAAGGATCGG GAGAGGGCGA GGGTGAGAAT GAGAGCGAGT CTACGGTGGC TGCATCGGCT GAGGGCGTAA 1400
 258   K  Y  K  K  E  S  K  N  K  E  E  G  S  G  E  G  E  G  E  N  E  S  E  S  T  V  A  A  S  A  E  G  V   290

1401  CACATAATAC TGGAGTCACG GTGCTGGAGA AACCAGCGTC GCTTATGCTA CACGTAGATG ATACGGTGGG ACTAAACGTT CGGCATTCAT GAACGAAACA 1500
 291  T  H  N  T  G  V  T  V  L  E  K  P  A  S  L  M  L  H  V  D  D  T  V  G  L  N  V  R  H  S  *            320

1501  TTCCGATCAG CTTAAAGGTG ACGTCATAAC GGACGCCGTG CGGTACTACT ACACTGCAAA TGGTTTGTGC ACAATTCAAT TATCACACAA TACCGCGAGT 1600
1601  GGCTACCAGG GGATGATTTT GTTTTTTGTT TTTTGTTGTT GTTTTTTTGT GCAATCATGC GCGATTTTTG TCCAGTCACC ACGCAGAGCT GTCAGACTAC 1700
1701  CGCACAGCAT TCGATATGGG AGAGACTTTC TACAGATATT TAAATAATGA TACTGTAGTA TCATAGTATA CCTCATGTAC TGTCAAATTC TGTGTTTTTT 1800
1801  TTTTTTTTTA ACATCATGTG AACATTTGAG AAGCAGAAGA AGACACTGTA TAGTGTGAAA CAACTTTCAG TATTGTGTTA AAGCAATTAT GGTGTGGTGT 1900
1901  GTGTAATATC ACCAGAGGTT GTATATATTT ATTCCCTACG TGACATGTAT ATCGTGTATA TAACACTTTG TCTAGTCCTA AAATGTTTGT ATAGATAGAT 2000
2001  ACTATGTTTT AAAAAATGTT CTTGTGTGTA CTATAGTACA GTATAGATAA GATTTATGAA TGGCTGAGTA TACTATTAAA ACCAAACAAT ATTAAAGAGC 2100
2101  ACACAGGTAT GACATGTGTG TCGAAATGAC ATGCGCATAA TTATTGATGC CTGCAGATTT GGTCCATAAT TTGGTGGGTA ATAATGATAT ACAACTATTC 2200
2201  TAACATTGTC AACTGTATAT CATTTGGGTC ATTTATGTCA CGTTTATAGC GTACGCGCAT GCGCTATAAT AGACCTTATG TATTGACGTC ACTGATTCAC 2300
2301  GTCACTGACT GTAGAAGGGC GCCGCATTGG TTTCGTTGTT CAGAAATTAA GCCCCTACGG ATAATCACAA AAAGTGCGCG CTGCCCAATG TGCATTTTCA 2400
2401  GTAGAAAAAT CAGCTTTCC                                                                                      2419
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G : PjHox8

sequence size : 2004 bp

CDS : 87 – 1070 (984 bp)

amino acids : 327 aa

```
   1  ACTAAATCAG ACGAGAATCT TTGGAGCGGA TCGAATTGGC TCATAAAGGC ACAGCTAACG AGGTTACAAA TCAATCTTCA ACTCAGATGA GCTCAATGTT  100
   1                                                                                          M  S  S  M  F    5

 101  TCTCAACTCG CTGTTCAGCA AGTACCAGCC GGGTGAGTCT TGGTTCCCTA GCGGGTTCGA CCCGGCTGTG TCATCCCCAA GTAGGAGCTG CAAGACAGGG  200
   6    L  N  S  L  F  S  K  Y  Q  P  G  E  S  W  F  P  S  G  F  D  P  A  V  S  S  P  S  R  S  C  K  T  G   38

 201  GTTGGTGATC ACTCGATGGC AGCCACAGCA GCCCCAGTGG GATGGAGGGG AGTACACGGG GGCGCTGCAC ATGCCCATGC CTCGGCGACG GGGGCTTACG  300
  39   V  G  D  H  S  M  A  A  T  A  A  P  V  G  W  R  G  V  H  G  G  A  A  H  A  H  A  S  A  T  G  A  Y   71

 301  CCTCTTTTGA TTTCACCAAC TCGAACTATT ACACCTCTGG GAGCATTCCT ACCACGTTTG CTACATCCTA CGGGTACCAG GCATGCGGCT TCGCCACCAG  400
  72  A  S  F  D  F  T  N  S  N  Y  Y  T  S  G  S  I  P  T  T  F  A  T  S  Y  G  Y  Q  A  C  G  F  A  T  S  105

 401  CTGCCCTCAG ACCCCCTCTA TTCAAAATGG GGGCTACCCT GACCAGTATT CAGCATTCGC CCATCACCAT AATGGCTACG CCACCCACCC GACAGCGACC  500
 106    C  P  Q  T  P  S  I  Q  N  G  G  Y  P  D  Q  Y  S  A  F  A  H  H  H  N  G  Y  A  T  H  P  T  A  T  138

 501  ACTTACCCCA CAGCATGGAG TCCGTCTCAA AAAGATGCAG CTGGGTTTAT AAGTCCTGTC AACGGGATCC CAGGAGCATT CTCAGCCGTA ACTCCGGGGC  600
 139   T  Y  P  T  A  W  S  P  S  Q  K  D  A  A  G  F  I  S  P  V  N  G  I  P  G  A  F  S  A  V  T  P  G   171

 601  AGGACATTGG TAGCTTGACG CATCATGACC TGAAGGTGGT GGATTGCCAC TTGGACTCTA AGCGGGATGA GGATGGTGAG CCGGTGTATA AGGGACCAGT  700
 172  Q  D  I  G  S  L  T  H  H  D  L  K  V  V  D  C  H  L  D  S  K  R  D  E  D  G  E  P  V  Y  K  G  P  V  205

 701  CTATAACTGG ATGAAAATAC CAGGAACGCA AATTATCGGC ACGGACCGCA AGCGAGGAAG GCAGACGTAC ACGAGAACGC AGACGCTGGA ACTCGAGAAG  800
 206    Y  N  W  M  K  I  P  G  T  Q  I  I  G  T  D  R  K  R  G  R  Q  T  Y  T  R  T  Q  T  L  E  L  E  K  238

 801  GAGTTCCATT TCAACCGCTA CCTGACGCGG AAGCGACGCA TCGAGATCGC ACAAGCTGTT TGCTTGACAG AACGACAGAT CAAGATCTGG TTTCAGAACC  900
 239  E  F  H  F  N  R  Y  L  T  R  K  R  R  I  E  I  A  Q  A  V  C  L  T  E  R  Q  I  K  I  W  F  Q  N  271

 901  GCAGGATGAA GTGGAAGAAG GAGCGACAAC GAGATGGACA TGATGATGAC GCTGATGACG AGGGTGAAGA GGAGGGAAAG AACTGTGAGG ATGAAGACGA 1000
 272  R  R  M  K  W  K  K  E  R  Q  R  D  G  H  D  D  D  A  D  D  E  G  E  E  E  G  K  N  C  E  D  E  D  D  305

1001  CGATAAAAGC TACAGCGTCA AAGGCGAGGA CAATGACAGT GACTCGGTGA AAGAAATAAA CAATCGCTAG CCGAGATATC GTTTCGGACT AAATATTTTG 1100
 306    D  K  S  Y  S  V  K  G  E  D  N  D  S  D  S  V  K  E  I  N  N  R  *                                    327

1101  ATCAGGTCAA TACTGGTAAT AGGACGCCAT AACTATATTG AATCATAAAA TTTTTACAGA TTTTTTTTAA TAAAATAAAC TGTGATTCAT TGCCTCTTGG 1200
1201  TGCAGCTTCA TGAAGTAGAA GTTGTAGAAC AATGACGTGG AGCTAGGGTG ACAATGAAAT GGTAATCACT TTGGCGTACA ATTTGGTAGG AGATTCGTGA 1300
1301  ATATAATTCG GCTTTCCTAC ATGACGTCAT GCGCATAACT ACAAGTTAGG CTTCTGTGAG TAAAACAAAA TAGTAATTTC AAGTGCATTA TTATTGTTGG 1400
1401  ACTGCACAAT TACCCATTGA TCAGGTGGCG AAAAAGTAAT TTGGAACTCA CTACTATATG TACACTCGTT TCAATAACAG AAAACAGAAA CCTGAAATGA 1500
1501  AAGGGGTGTT TTTTAGATCA TCTGATGATC TAATCAATTA GAATCGTAAA TTTAGCTTAA TAATATGGTC TGCTACCATA GACACTGTGT ATTAAAAGCG 1600
1601  GCTCCAGATG TAGCGGGTAT GAGTTTCATG CGCCATGAGA ACGTAGATGG CGAAAACTAT TGTCCCGATG ATTTGGCACC TTTAAAGGTT CCCTAAAAGG 1700
1701  TTCTAAAGGT TTCATAAAGG TTATAAACAC TCACCTTGGA ACGTTTTTAG GTTCTTCATA GAACCTTTTA AGGTTCTTGA GCCCTTAAAA AACCTTTTGG 1800
1801  GAGGGTTCCA AATGCGAACC TTTACCATGA CCTTTGACAA AATTTTAAGT CATAATTTTG GCTGCTGCCG GGGCATATAG TGTTTCACAG ACACATTTTT 1900
1901  TTTTTTTTTT TTGGGGGGGG GGTATTTTTT GTGTATTTAT TTGTTTTGTT ATAACAATTT TTGCAAAGCC TTGACAGTGG TATAAACAAA GATGAACAAA 2000
2001  TTGT                                                                                                       2004
```

H : PjHox9/10

sequence size : 1922 bp

CDS : 477 – 1556 (1080 bp)

amino acids : 359 aa

```
   1  AACCAGACTT GGTCCTCCAA GTAGTACTGG ATGCAAGCGC TGCAGTTAAC TGGTAGCATG TAACTTGGCG CTTTATTCAT CACATTTCGT CACCTTCAGG   100
 101  TCGCTGTTGC AGTACTGATC ACCAAGTTAT TTTCATTCAT TTGCAAAGCG CATCAACGTG AATGTTTTCT CATTTTTCCA AATAGTTTAA CTCTTTGAAT   200
 201  TAGCCCTATC TGCAGTCATC CCAGGTGTTG TATTTTAAAT TTAAACACGA GTAAAAAAAT AGTTGATGAA GATCGATCGA CAACGACACA ACGCATCCTG   300
 301  CTTCAATAGT GTTCAGTCGA GGCATCGGGA TACCCAATTC CACATTTCAG GCATCAAGCA TATTGGTGGT TTGCATTGAG TTTATCACCG ATAGCAAAGT   400

 401  GTGGAGTGTG GGCAGGGCAC CCAAGCATAA TAAAAACTTA TGGACATAAA AACCAGAGGT GATAAATAAA ACAAGAATGA CGACCTCGGC CTTCTGCGTA   500
   1                                                                                  M  T  T  S  A  F  C  V    8

 501  AACTCACTGA TCAATGGAGG TGATCCGGCG GCTAGCTTGA AAGGGGCTGT CCAGTCCGAG ATCAATTCCA ACATCATCCA CGGATCGTCG AAATGTTCAC   600
   9  N  S  L  I  N  G  G  D  P  A  A  S  L  K  G  A  V  Q  S  E  I  N  S  N  I  I  H  G  S  S  K  C  S     41

 601  TGGCATCCAA GTTTGCAGAC TCGTCCATCC ACCATTCGTC AACCGTGCCG GCGACCACGG GGAGTGGGCT TCCAGCCGTT CCGCAGGTGT CGGCGCCGAG   700
  42  L  A  S  K  F  A  D  S  S  I  H  H  S  S  T  V  P  A  T  T  G  S  G  L  P  A  V  P  Q  V  S  A  P  S     75

 701  CATGTATCAC ATACCAGGTG ATCAAAACAG CAGTTACGCC ACCCCCTGGT TTTACTCTGC CGATCACCAC ACCTACTCAA CGATGTCCAT GGCGATGCCG   800
  76    M  Y  H  I  P  G  D  Q  N  S  S  Y  A  T  P  W  F  Y  S  A  D  H  H  T  Y  S  T  M  S  M  A  M  P    108

 801  GCTGTCGCCG ATGATTTCGA CAACGCGTAC CACTACAGCA TGGGTGCTGC AAAGAACTTT GAAAACGGTT TGATGACGAC GTCGGGAGGT AGTTTTTACT   900
 109  A  V  A  D  D  F  D  N  A  Y  H  Y  S  M  G  A  A  K  N  F  E  N  G  L  M  T  T  S  G  G  S  F  Y     141

 901  CCATCGGCCG ACATCAGACA TACGACAGGT ACGCAAACCA TGCCAATTCC GTCTATCATC ATCCGTCCGC TCACTCTACC CCGATACCAG TCAACCTCAC  1000
 142  S  I  G  R  H  Q  T  Y  D  R  Y  A  N  H  A  N  S  V  Y  H  H  P  S  A  H  S  T  P  I  P  V  N  L  T    175

1001  CACCGCCGCA ACTCTCCCGA CAAAATACAT CACGAGTGAT GGTAGCACCG CTGCTATCAG CGGTAGCAGC CCACCTCAGA CTGCTGCTCA TCATGTCATA  1100
 176    T  A  A  T  L  P  T  K  Y  I  T  S  D  G  S  T  A  A  I  S  G  S  S  P  P  Q  T  A  A  H  H  V  I    208

1101  CTGGAGGAGA AGTACAGGGG AACAGCGGGC TATGACTCCT TCAGCTGCCC GAAGGACAGT AGCAAGACAG CGATTGACGC TAGAGGAGGC GAAATTGGTC  1200
 209  L  E  E  K  Y  R  G  T  A  G  Y  D  S  F  S  C  P  K  D  S  S  K  T  A  I  D  A  R  G  G  E  I  G    241

1201  ACTCGTACAC GGGGTCGAAT GCTGGATCAC CCATCTCGTT CGCCAACAAA GAATCACCGA GGTCAGACAC GAAGGAGAAT GACCTCATTG ACGACTGTGA  1300
 242  H  S  Y  T  G  S  N  A  G  S  P  I  S  F  A  N  K  E  S  P  R  S  D  T  K  E  N  D  L  I  D  D  C  D    275

1301  TGATGACGAA AAGATGAAGA ACGGCGACAC GCCCAACTGG TTATCGGCGA CGTCGGGACG CAAGAAACGA TGTCCGTACA CCAAGTTCCA GACGCTGGAG  1400
 276    D  D  E  K  M  K  N  G  D  T  P  N  W  L  S  A  T  S  G  R  K  K  R  C  P  Y  T  K  F  Q  T  L  E    308

1401  CTGGAGAAGG AATTCCTCTT CAACATGTAC TTGACCCGAG ATCGACGCCT CGACATCGCA CGGATGTTGA GCCTGACCGA GCGCCAAGTC AAGATCTGGT  1500
 309  L  E  K  E  F  L  F  N  M  Y  L  T  R  D  R  R  L  D  I  A  R  M  L  S  L  T  E  R  Q  V  K  I  W    341

1501  TCCAGAATAG GCGGATGAAA ATGAAGAAGC AAAACCGAGC TCAGAATTAC CCATAAACAC TGATAATTTT TAGCAAAGAC AATCCTAAAA CTATATCAAT  1600
 342  F  Q  N  R  R  M  K  M  K  K  Q  N  R  A  Q  N  Y  P  *                                                    359

1601  AATATTTAAT TATTTTCTAA AATTATTACG TATGTTGATT GGCATCCCAT GGAGAGAACG TCTGGCAGAC TCTGTCTCTG CTTGCCAAGT TGATTATTGT  1700
1701  TCGGGCCCAA GCCAACACAA TCGGGTCCAA TCACGGCGCG CGTAGCTTAG CTAAGAGTAT GTTGTTGTTG TTGTGTTTGG TAGGGTTCGG CATTCTTCGT  1800
1801  TAACAAAATG GCGGAGGAAA ATCACACCCT CTATCATGAC GTAGTGTCTT TAACATGTGG TTTTTAAACC AAGGAATATA ACAGAAAAGG ACCCATATGG  1900
1901  TAATTTTGAC ATTTTATTTT GC                                                                                      1922
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I : PjHox11/13a

sequence size : 1746 bp

CDS : 368 – 1393 (1026 bp)

amino acids : 341 aa

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 101  TCATTTTCAT TTGTTTACCC GAGGGAGGAT AATTTTATGA CGCTATAGCC CTGGCTTGCC GAATTTCGTC AACCTCTCAG CCCCTTCATG CAAAGGACAT  200
 201  TGAGGAGAGC GTGGCCAATA TCTAGAAGCG GTAGACGAAT TCGTGGTTGT TACGTGTACT ATTTGAACCG AATTTTAGGA CGGGAATAAA ATTTTATGTT  300
 301  CAGTGGGAAA TAAAAGAGGA ACTCGTCGTT TGACGCGAGG TTTTTGGGTC ATTTGCTCTT ATCTATTATG GAGGGGATTC AAGCACCGCG ACCCTATGCC  400
   1                                                                    M  E  G  I  Q  A  P  R  P  Y  A   11

 401  AGTATCGGTT CATTTTACGA CACCGTTTCA AATGGCAATA ATAATAGTTA CGGCCTTTCA CATGGCGGTA GTTATGTCGG TTTTATGGGT GGTTTTTCCT  500
  12   S  I  G  S  F  Y  D  T  V  S  N  G  N  N  N  S  Y  G  L  S  H  G  G  S  Y  V  G  F  M  G  G  F  S   44

 501  ACCACAGCAG CACGACTTGC CTTCGACGTC AACCCAGTCC TGCAGCCAGC GGAAGCGGCG CGGGAGGTGA AGGCAGTGGT GGTGGTGGCG GCGGGGATGA  600
  45   Y  H  S  S  T  T  C  L  R  R  Q  P  S  P  A  A  S  G  S  G  A  G  G  E  G  S  G  G  G  G  G  D  D   78

 601  TGGTGACGTG ACGCCAACGA ACTGCCGATC GACATCGGCT GTTGCACCAT CCAGTGGTGG TCATCCCTGG TCGTCAACGC CGACACACCA TGGGGAGAAG  700
  79   G  D  V  T  P  T  N  C  R  S  T  S  A  V  A  P  S  S  G  G  H  P  W  S  S  T  P  T  H  H  G  E  K  111

 701  ACAGGAGGAC ACTTTCCCTT CAACTACCAG GACATGTTCA CCTCAGGAGC CCATCAGGGG TCTCCGACGT CATCGGGACA TCATGGGGTG ACTACGCCAA  800
 112   T  G  G  H  F  P  F  N  Y  Q  D  M  F  T  S  G  A  H  Q  G  S  P  T  S  S  G  H  H  G  V  T  T  P  144

 801  GCAAGCTTGG GTTCAGCGGA GCAGTGACTG GACGGCCAAC GGGCAGTAAC GGGGACGATG ATGCTTCAAG ACACAATAGT GACTCAGACC AAAGCACCGG  900
 145   S  K  L  G  F  S  G  A  V  T  G  R  P  T  G  S  N  G  D  D  D  A  S  R  H  N  S  D  S  D  Q  S  T  G  178

 901  CCACCAGACA GGCTCAAACA GCTACACTTG GATGGCACCT CCACCCAACG TCCGCACTCG CAAAAAGCGG AAGCCCTACA CTAAGTTCCA AACCTTTGAA 1000
 179   H  Q  T  G  S  N  S  Y  T  W  M  A  P  P  P  N  V  R  T  R  K  K  R  K  P  Y  T  K  F  Q  T  F  E  211

1001  CTGGAGAAGG AGTTCCTTTA CAACATGTAC CTCACGCGTG ATCGGCGCTC GCATATCGCC CGCGCTCTCA GTCTCACGGA GCGCCAGGTC AAGATATGGT 1100
 212   L  E  K  E  F  L  Y  N  M  Y  L  T  R  D  R  R  S  H  I  A  R  A  L  S  L  T  E  R  Q  V  K  I  W  244

1101  TTCAGAACAG ACGCATGAAG CTTAAGAAGA TGCGCGCACG AGAGGAAAAC GAGCGCAAGC ACCATCCGCA CGCACTCCAA CAACACCATC ACCACCTTGC 1200
 245   F  Q  N  R  R  M  K  L  K  K  M  R  A  R  E  E  N  E  R  K  H  H  P  H  A  L  Q  Q  H  H  H  H  L  A  278

1201  GCATCACAAT CCCCATCATT TGCATCCGCA CCACATGGAC GTGAAGGGAG GTTCGGAGCA CTACACACCC GATATGTTTC ACAAGACAAG CGCGTTGCAC 1300
 279   H  H  N  P  H  H  L  H  P  H  H  M  D  V  K  G  G  S  E  H  Y  T  P  D  M  F  H  K  T  S  A  L  H  311

1301  CACCATGGGT ATGGTGGACT TCATGACGCA AATCCTCTAT CGGAATTACA GCAGCACGGA CTCGTACAAC ACCACATTGT TGCCTCGATG TAGTGCCAGA 1400
 312   H  H  G  Y  G  G  L  H  D  A  N  P  L  S  E  L  Q  Q  H  G  L  V  Q  H  H  I  V  A  S  M  *            341

1401  AACTTTTGTT ATACTGACTA GAAATTAACA TACATCGAAT GGAGACAGCA GAATAAATGT GAAATCGATA TTTAGTCTTA TGCTACTCAC GATCTGATGA 1500
1501  TTGAGTGGAA ACTGAAAAAT TGTGTCAAGT TTATTTGTTT ATGGTTCATT TTTTTTTTCA GTAATGCACG TCGCCAAGTC ACAATAAGTC ACAGTACTCG 1600
1601  ATAGCCTTTG GCAGATTTGC AACAAGAATG TCATCCATAG TCGTGCAAAG TCGTCGTTGT CGCTCTCATC AGACTCATAA TTATCTTTTT GTCAATATTT 1700
1701  TGTCATATTA ATACCGTAAA TACAATAAAA TGTAAGTGTT AGTATG                                                          1747
```

J : PjHox11/13b

sequence size : 1562 bp

CDS : 306 – 1331 (1026 bp)

amino acids : 341 aa

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 101  TCACACACAA GTACGGAACA CACTCTAACC ACAAGATCAT CCTAATCTAG TTGATCTTGA CTACTTATAG GTCTAGCTGG GATTGGTCGT AACACTCATT   200
 201  ATAATATTTC ACCTTGCGTT AGGGTCGTTA CTCGGCATTT ACGCTCTGAC GGGTTTCCGC TGACTGAATT TTATAGGGGG TGACTCGGTA TAGTTTGTGG   300

 301  GTACGATGCA GCTTAGTATG GAACAGGCCT GGTCAGCTCA GAGATCAAGT GGAATGGATA GCTGTGCAGC AGAATATAGG AGCATATCCA ATCCTATGAA   400
   1        M  Q  L  S  M  E  Q  A  W  S  A  Q  R  S  S  G  M  D  S  C  A  A  E  Y  R  S  I  S  N  P  M  N  32

 401  TGGACTTTAT GCTTCAAGTA GGCAACGGAC AACTTGTAAC ACCATGGTCA GCGTTAACAC CCCAAGCGCG GAACAGATAA GCGCCTTTCA TTACTCCTAC   500
  33    G  L  Y  A  S  S  R  Q  R  T  T  C  N  T  M  V  S  V  N  T  P  S  A  E  Q  I  S  A  F  H  Y  S  Y  65

 501  CCCATGTACA ACTCACCTAC CGAATCGAGC CTTGCCGCCT CAACTGCTGG GGAGGAGATA CCGGGAAGTG TGGTATCACC GGCAGCCGCA GCAGCGGCGG   600
  66   P  M  Y  N  S  P  T  E  S  S  L  A  A  S  T  A  G  E  E  I  P  G  S  V  V  S  P  A  A  A  A  A  A  98

 601  CGTGGAATGG AGGAGAAATA CGGAGCGGAG CGGCAGCCCA CGGGGCAACT CCCCGGCCTT ACCCAAACAC CTTTGGGAAT ACGTTCCTAA CTCACGCGGG   700
  99  A  W  N  G  G  E  I  R  S  G  A  A  A  H  G  A  T  P  R  P  Y  P  N  T  F  G  N  T  F  L  T  H  A  G  132

 701  TTCGCCACCC CATCATCCAC ATATGGCCCA TCAGCAGCAG TACATGAGTG CCAACGCCTA TGGCTCCCCA TATGGACTTC ACAACTCTGC CTACCCCCTC   800
 133    S  P  P  H  H  P  H  M  A  H  Q  Q  Q  Y  M  S  A  N  A  Y  G  S  P  Y  G  L  H  N  S  A  Y  P  L  165

 801  GATATGACAG GTGGTGCCAC GTCTTGCAGC CTGCTCGCCA CTTTTACTAC CACTCCCCGA CGCACGAAAC GACGTCCATA TTCCAAGCTC CAGATTTACG   900
 166   D  M  T  G  G  A  T  S  C  S  L  L  A  T  F  T  T  T  P  R  R  T  K  R  R  P  Y  S  K  L  Q  I  Y  198

 901  AACTCGAGAA GGAGTTTCAG TCCAACATGT ACCTGACTCG AGATCGCCGG ACCAAGATGT CACAGGCCCT CGACCTCACG GAGCGACAGG TCAAGATCTG  1000
 199  E  L  E  K  E  F  Q  S  N  M  Y  L  T  R  D  R  R  T  K  M  S  Q  A  L  D  L  T  E  R  Q  V  K  I  W  232

1001  GTTTCAGAAT CGAAGAATGA AGATGAAAAA GCTGAATGAT AGAGAGAAGA GTAAACAATC CAAGAAGTCG TCTTCGAGTT CATCAGCGTC GTCGTCATCA  1100
 233    F  Q  N  R  R  M  K  M  K  K  L  N  D  R  E  K  S  K  Q  S  K  K  S  S  S  S  S  S  A  S  S  S  S  265

1101  TCGACGGATG GATCTTCCCT TACCTCATCA GCAACGCCAG CATCAGTTTC GTCCGGACAT GTGCCGACGT CATCATCATC ATCGTCGTCG TACACCACTG  1200
 266   S  T  D  G  S  S  L  T  S  S  A  T  P  A  S  V  S  S  G  H  V  P  T  S  S  S  S  S  S  S  Y  T  T  298

1201  CGTCGCAACA CCACCCGCAT CAACAGCATC ACCATCCTCA TCATCACGCC CATCACCACC ACCACCATAA TCCCCACCCG ACAGCCCTCC ATCCCGCGCA  1300
 299  A  S  Q  H  H  P  H  Q  Q  H  H  H  P  H  H  H  A  H  H  H  H  H  H  N  P  H  P  T  A  L  H  P  A  H  332

1301  TCCAGTAGGT CACCAGGCAC TCATACGGTG AGGGAAAAAT AAAACAATTT GAAGAACAAT ATATAAAATT ATAACATCTT ATTAAGGCCA CAAACTTTCA  1400
 333    P  V  G  H  Q  A  L  I  R  *                                                                             341

1401  AAGAACAAAA CAGCCCGATT GACCTTTTGT ATACAGCCAC ATTGGATTTA TATTATGTTT CAATATAGTG TAATGTTTTG TATGATGGTA TATTATGGAC  1500
1501  AATCTTTCTG AGAAATTAAT TTCTCTGTGT GTGAATAACA TTACTTTTCT ACTTTGAAAT AT                                          1562
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K : PjHox11/13c

sequence size : 2707 bp

CDS : 1427 – 2308 (882 bp)

amino acids : 293 aa

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 101  GCAGGACAAT TAATGTTTTA TTTGGATAAT TAATCTGGAC ACGCAAAAAA TATTACCCGG ACACTCAAGT GATATTTTCT GGACACTCAA ATATTATTGT  200
 201  CTGGACAGTA GGACACTCAA ATTTTATTAC ATGGACACTC AAATATTATT ATCTGATATA TGCAATATTA CCTGGAAACT CAATTCTTAT TATCTCGAAT  300
 301  ATCATTAATC AGTATACTAA TTTTATATTT TGTGATTAAT TCAAATTGTG TTCCATAATT TTGGATAATT CACTTTTATA TCATATTGGA TAATATTTTC  400
 401  TGATTATTAC GGATTATCAA CCCCATTTTT TTTTTACTAC TCTAACCTTA AATGCATGAA AATTGAATAA ACTGGGAATT CAACACTGGA TGAATGGTTG  500
 501  TCTTAAAAAA TATGCAAAAA TCGAAAAAAA ATGAATAAGT AAGAGATATT TATTTTATCA ATTACTTGTA CACCATCGGC AACTGTACAC CAACAATTCA  600
 601  ACAAAACTTT CGGGAAATTA TTGTATGTTT CTGTTGAAAA TTGGCATAGT ATATAACTAC AACTTCTGGC GAAAAGGCGA ATGAAGAAAA CCACTGACAA  700
 701  CTCAACTGCA TTATTACGTA ACTTTGGATT TTCAAAAACG AATTTTATGA ACTATGTATC AAGGTTAACA TTATGTGCAA TACATTCTAC CCACCCTCTC  800
 801  CACTAGTCTT CCCGGATATA GCCCACTCAG GTGCTGACAG GGCTTTAAGC CACATATCAC TCTCCACTAG TCTTGGTTAT TCAAGTTATG CGTTCGCTTC  900
 901  CCAGTTCGCT TTCAGCAGTG TAGCACCTTT ATGCTAGAGT TTATGGAATC ATTTAAAAAA CAATTAAATA CATTAAGAGA ACTACTTTCA TTACACATTT 1000
1001  TTAACCATTT CAGACGATAA AACTTTTTTT TCTTCAGTCC ACAAGGGGTT AATGGGTAAC CAACTTGTTT CTGTTCAATA CAATTTATTA AAAAGTATAT 1100
1101  TGTCTTCAAA GTGTTTTCAA AATCTTCAAA GAGATTTAAT TTATAAATAG GTGAGTGTTA ATAGGAATGG TCACACTATT GTTTGGAATA TTTATAATAA 1200
1201  CAATCAGTTA CAAATATAAT TATTAAACTG ATATGGACAT ATCGTCTGTT TCATAAAAAA ATATTCATTA AGGATGATTG AATCTAACAG TCAAAGAAAT 1300
1301  TACGATATAT AAACAAAAAA TAAGGGTTAT AAGCCCGGTT TTAAGCCTCT GGAGAAAATT CCTTGAAAAA TATAGAGTTA GTCCGCATAC AGATAACTTT 1400

1401  GGGCATTGTG TGTTTTGAAG AGTGAAATGC AAAGTTACAC CTCAGAACCT TGGGCGCAAA TTATTACCAC GATGAACGCG CCTAAATCCG CATCGTCGTC 1500
   1                          M  Q  S  Y  T  S  E  P  W  A  Q  I  I  T  T  M  N  A  P  K  S  A  S  S  S   25

1501  ATCCACGGCA TCGATAACCG CTCACGCTCA TCATCATTTT CCATCTCAAC GCTCAGAGAC TGCTCTCGGG CATCTTACGG CGTCGCACGC CGCCACCATG 1600
  26   S  T  A  S  I  T  A  H  A  H  H  H  F  P  S  Q  R  S  E  T  A  L  G  H  L  T  A  S  H  A  A  T  M   58

1601  CCGCTGACAC ACCGATACGG ATACTACAAT AATCTTGACG GGCCGACAAC GACTGCCAGT TCAATCTCGC CTTACGTCGA CGATTTCTCG GCCTGGAACA 1700
  59   P  L  T  H  R  Y  G  Y  Y  N  N  L  D  G  P  T  T  T  A  S  S  I  S  P  Y  V  D  D  F  S  A  W  N   91

1701  CAATCGATTT CTCTTCGTCG GCAGCGCATG GTCATCCGAC GCCTTCATCT CGTGCTTCGT TCGCGGCGGC CGCAGCAGCA GTACACCACG TTGGTCACTT 1800
  92  T  I  D  F  S  S  S  A  A  H  G  H  P  T  P  S  S  R  A  S  F  A  A  A  A  A  A  V  H  H  V  G  H  F  125

1801  CCACCACCCT AGCGGGCCTG CCCCGGCCAC GCCAACGCCC ATCAACCCCA ACATGGTCAT GGGGGTTGGA GGTCAGCACA GGGAGTCGTT ATTGACAGCA 1900
 126   H  H  P  S  G  P  A  P  A  T  P  T  P  I  N  P  N  M  V  M  G  V  G  G  Q  H  R  E  S  L  L  T  A  158

1901  GCTGCAGCAG CTCACGTGCA CGCCGCGGCC GCCGGCGCAG CAACCTTCGG TATGGCTCAT CGTCATCCCG CGTCTTTTGG TTCTGGTAGT CATGCCACTA 2000
 159   A  A  A  A  H  V  H  A  A  A  A  G  A  A  T  F  G  M  A  H  R  H  P  A  S  F  G  S  G  S  H  A  T  191

2001  ACTGGGTCTC CCCTTCATCA TCCAGTTCGA GTAGGAGAAC GAAGCGTCGT CCGTACTCCA AGCTGCAGAT CATCGAGCTC GAGAAGGCCT TCCAAGAAAA 2100
 192  N  W  V  S  P  S  S  S  S  S  S  R  R  T  K  R  R  P  Y  S  K  L  Q  I  I  E  L  E  K  A  F  Q  E  N  225

2101  CATGTACCTG ACGAGGGACC GAAGGAATAG GATTTCCGAG GCATTGAACC TGAGTGAGAG ACAGGTTAAG ATCTGGTTCC AAAATCGAAG AATGAAGATG 2200
 226   M  Y  L  T  R  D  R  R  N  R  I  S  E  A  L  N  L  S  E  R  Q  V  K  I  W  F  Q  N  R  R  M  K  M  258

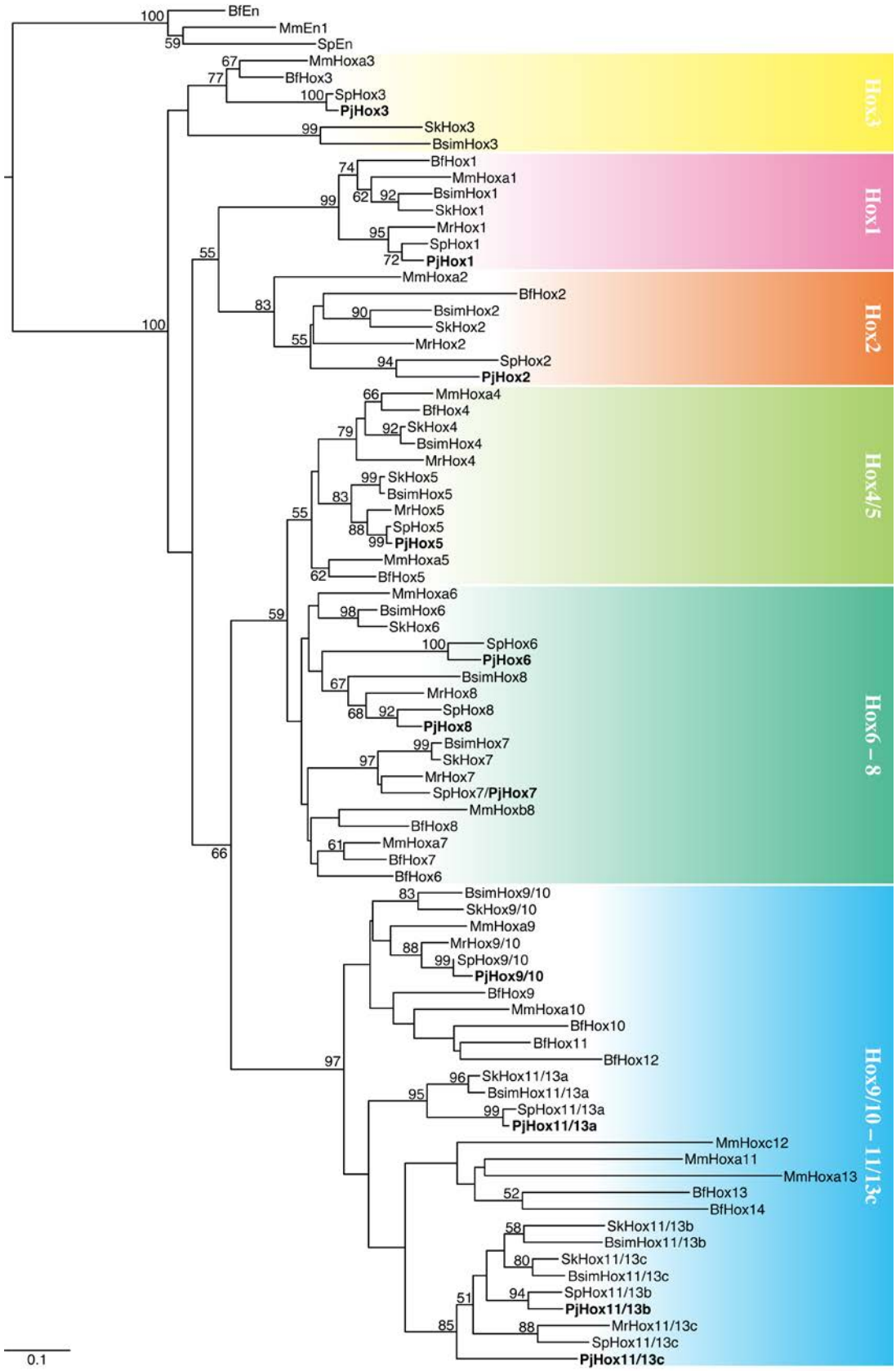
2201  AAGAAACTAA CGGAAAGAGA AAGAACGGAA CAGGAGCAGC TGGAACGAGA AAAGGCGAGC CTTGGAGCAA AATATTTCTT GAACACGGGT CATATGCCTG 2300
 259   K  K  L  T  E  R  E  R  T  E  Q  E  Q  L  E  R  E  K  A  S  L  G  A  K  Y  F  L  N  T  G  H  M  P   291

2301  TGCCATGATT CTTTTGTTTT CTTTCTTTTT AATTGTATAT AAATCCATGT TTAATAATAT ACGTAATGAA GTGATTGTTT CTATAATGTG TGTTTTTATA 2400
 292  V  P  *                                                                                                293

2401  ATGTGCTCGT TTCAAAATTT TGTGACCTCA TTAATTTGCA ATTAACCCAT CATACACTGG TAGTGTTGTG TAGTCGTAGC TCCAGGTAGC CTTGGTGGTA 2500
2501  GCTTTAAACA AATTTTGATA TTAATAGTAC ACCAAGGCTA CAGGACACAG CCATTTAATT CAGAGGGACT GTTAAAATGT GAGTTAAAAT TGCTCCAAAA 2600
2601  TGTGGTCAAG ATTGTCTTCT TTTGGCCCAT AATGTGCTGC ATCAATTCTT TTAACCATGT TCTTTCAGAT TATCTAAGTT AAATTCCACA AAAATCGCTG 2700
2701  ATCATTT                                                                                                2707
```

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.

Figure 2-2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BsimHox1
SkHox1
MrHox1
SpHox1
PjHox1
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BfHox2
BsimHox2
SkHox2
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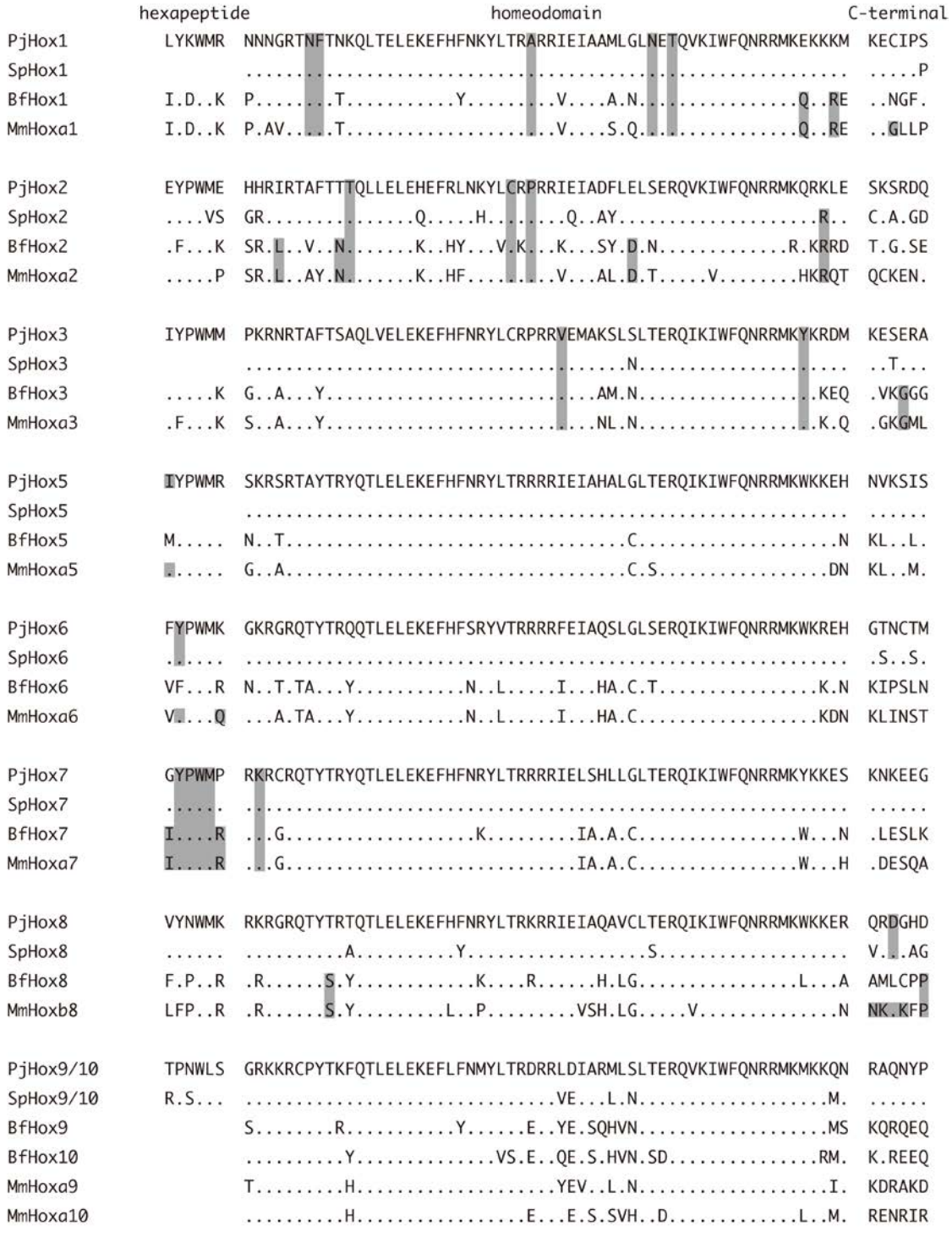
SpHox2
PjHox2
MmHoxa4
BfHox4
SkHox4
BsimHox4
MrHox4
SkHox5
BsimHox5
MrHox5
SpHox5
PjHox5
MmHoxa5
BfHox5
MmHoxa6
BsimHox6
SkHox6
SpHox6
PjHox6
BsimHox8
MrHox8
SpHox8
PjHox8
BsimHox7
SkHox7
MrHox7
SpHox7/PjHox7
MmHoxb8
BfHox8
MmHoxa7
BfHox7
BfHox6
BsimHox9/10
SkHox9/10
MmHoxa9
MrHox9/10
SpHox9/10
PjHox9/10
BfHox9
MmHoxa10
BfHox10
BfHox11
BfHox12
SkHox11/13a
BsimHox11/13a
SpHox11/13a
PjHox11/13a
MmHoxc12
MmHoxa11
MmHoxa13
BfHox13
BfHox14
SkHox11/13b
BsimHox11/13b
SkHox11/13c
BsimHox11/13c
SpHox11/13b
PjHox11/13b
MrHox11/13c
SpHox11/13c
PjHox11/13c
Hox3
Hox1
Hox2
Hox4/5
Hox6 – 8
Hox9/10 – 11/13c
0.1

Fig. 2-2. Neighbor-joining tree constructed from 80 Hox proteins using three engrailed protains 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.

Figure 2-3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homeodomain
C-terminal
PjHox1 LYKWMR NNNGRTNFTNKQLTELEKEFHFNKYLTRARRIEIAAMLGLNETQVKIWFQNRRMKEKKKM KECIPS
SpHox1 ....P
BfHox1 I.D..K P.....T.....Y.....V....A.N.....Q..RE ..NGF.
MmHoxa1 I.D..K P.AV....T.....V....S.Q.....Q..RE ..GLLP
PjHox2 EYPWME HHRIRTAFTTTQLLELEHEFRLNKYLCRPRRIEIADFLELSERQVKIWFQNRRMKQRKLE SKSRDQ
SpHox2 ....VS GR.....Q.....H.....Q..AY.....R.. C.A.GD
BfHox2 .F...K SR.L..V..N.....K..HY...V.K...K...SY.D.N.....R.KRRD T.G.SE
MmHoxa2 .....P SR.L..AY.N.....K..HF.....V...AL.D.T.....V.....HKRQT QCKEN.
PjHox3 IYPWMM PKRNRTAFTSAQLVELEKEFHFNRYLCRPRRVEMAKSLSLTERQIKIWFQNRRMKYKRDM KESERA
SpHox3 .....N..... ..T...
BfHox3 .....K G..A...Y.....AM.N.....KEQ .VKGGG
MmHoxa3 .F...K S..A...Y.....NL.N.....K.Q .GKGML
PjHox5 IYPWMR SKRSRTAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALGLTERQIKIWFQNRRMKWKKEH NVKSIS
SpHox5 ....
BfHox5 M..... N..T.....C.....N KL..L.
MmHoxa5 ..... G..A.....C.S.....DN KL..M.
PjHox6 FYPWMK GKRGRQTYTRQQTLELEKEFHFSRYVTRRRRFEIAQSLGLSERQIKIWFQNRRMKWKREH GTNCTM
SpHox6 ..... .S..S.
BfHox6 VF...R N..T.TA...Y.....N..L.....I...HA.C.T.....K.N KIPSLN
MmHoxa6 V....Q ...A.TA...Y.....N..L.....I...HA.C.....KDN KLINST
PjHox7 GYPWMP RKRCRQTYTRYQTLELEKEFHFNRYLTRRRRIELSHLLGLTERQIKIWFQNRRMKYKKES KNKEEG
SpHox7 ....
BfHox7 I....R ...G.....K.....IA.A.C.....W...N .LESLK
MmHoxa7 I....R ...G.....IA.A.C.....W...H .DESQA
PjHox8 VYNWMK RKRGRQTYTRTQTLELEKEFHFNRYLTRKRRIEIAQAVCLTERQIKIWFQNRRMKWKKER QRDGHD
SpHox8 ....A.....Y.....S..... V...AG
BfHox8 F.P..R .R.....S.Y.....K....R.....H.LG.....L...A AMLCPP
MmHoxb8 LFP..R .R.....S.Y.....L..P.....VSH.LG.....V.....N NK.KFP
PjHox9/10 TPNWLS GRKKRCPYTKFQTLELEKEFLFNMYLTRDRRLDIARMLSLTERQVKIWFQNRRMKMKKQN RAQNYP
SpHox9/10 R.S... .....VE...L.N.....M. .....
BfHox9 S.....R.....Y.....E..YE.SQHVN.....MS KQRQEQ
BfHox10 .....Y.....VS.E..QE.S.HVN.SD.....RM. K.REEQ
MmHoxa9 T.....H.....YEV..L.N.....I. KDRAKD
MmHoxa10 .....H.....E...E.S.SVH..D.....L..M. RENRIR

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 ancelet *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

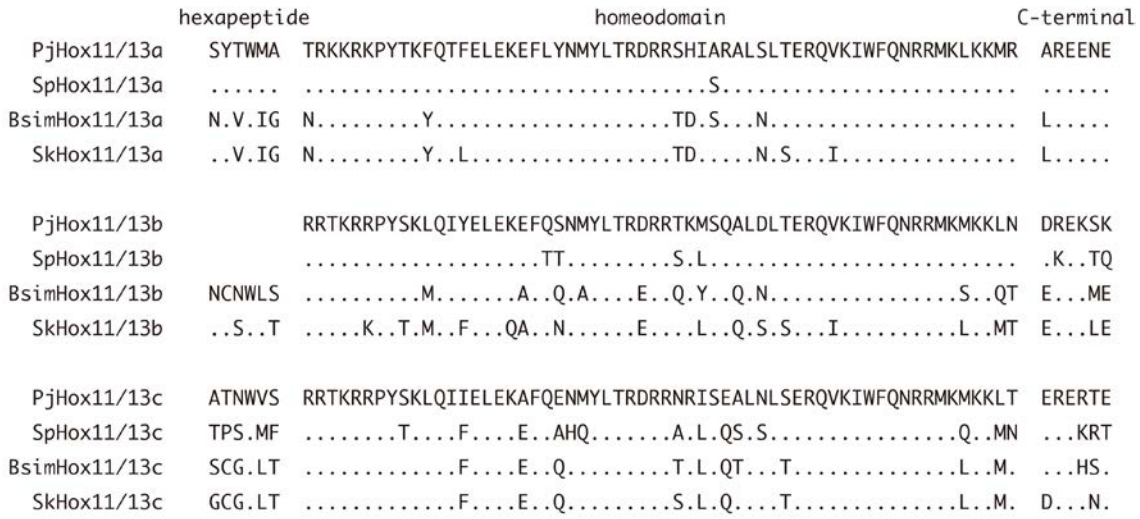
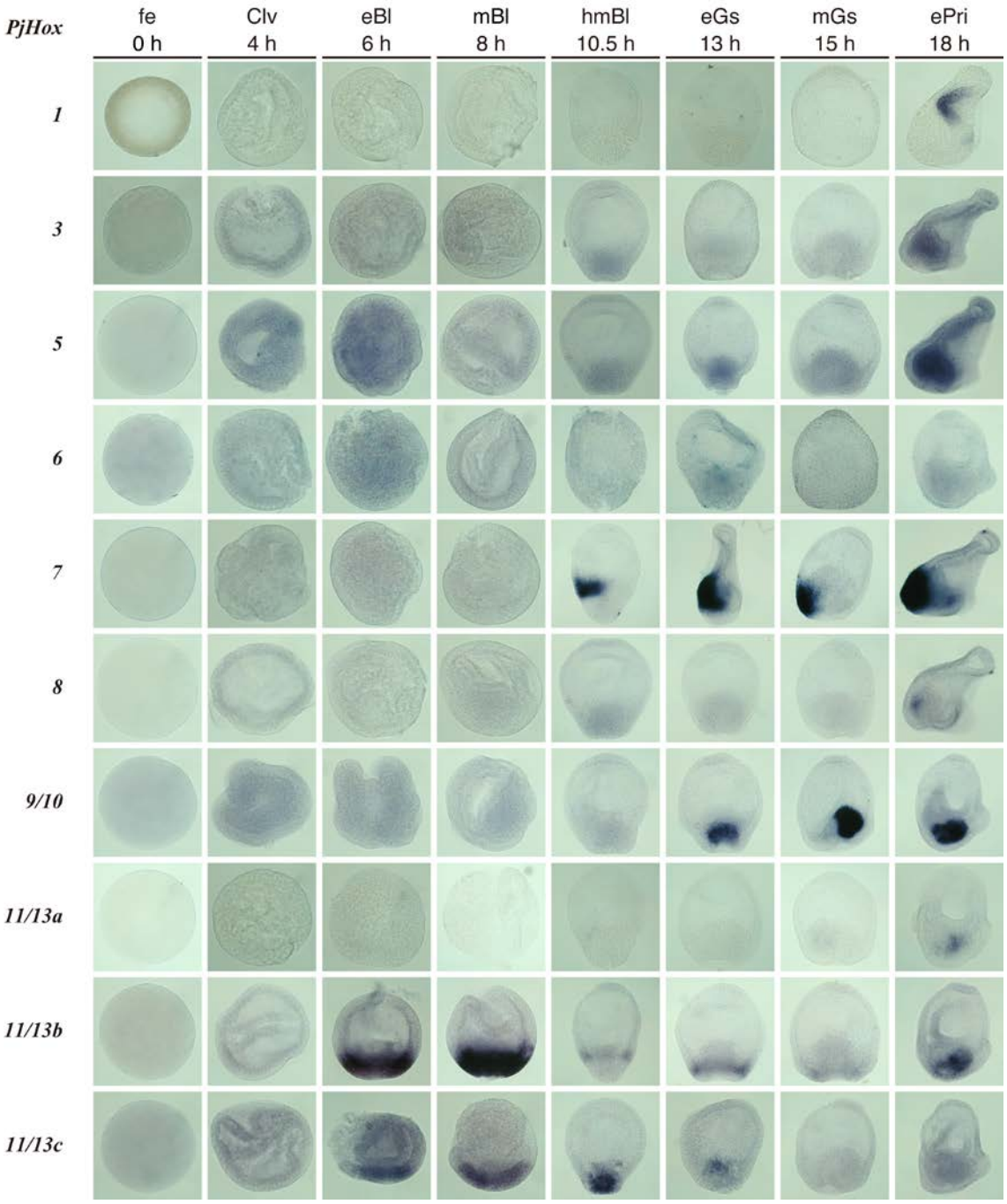


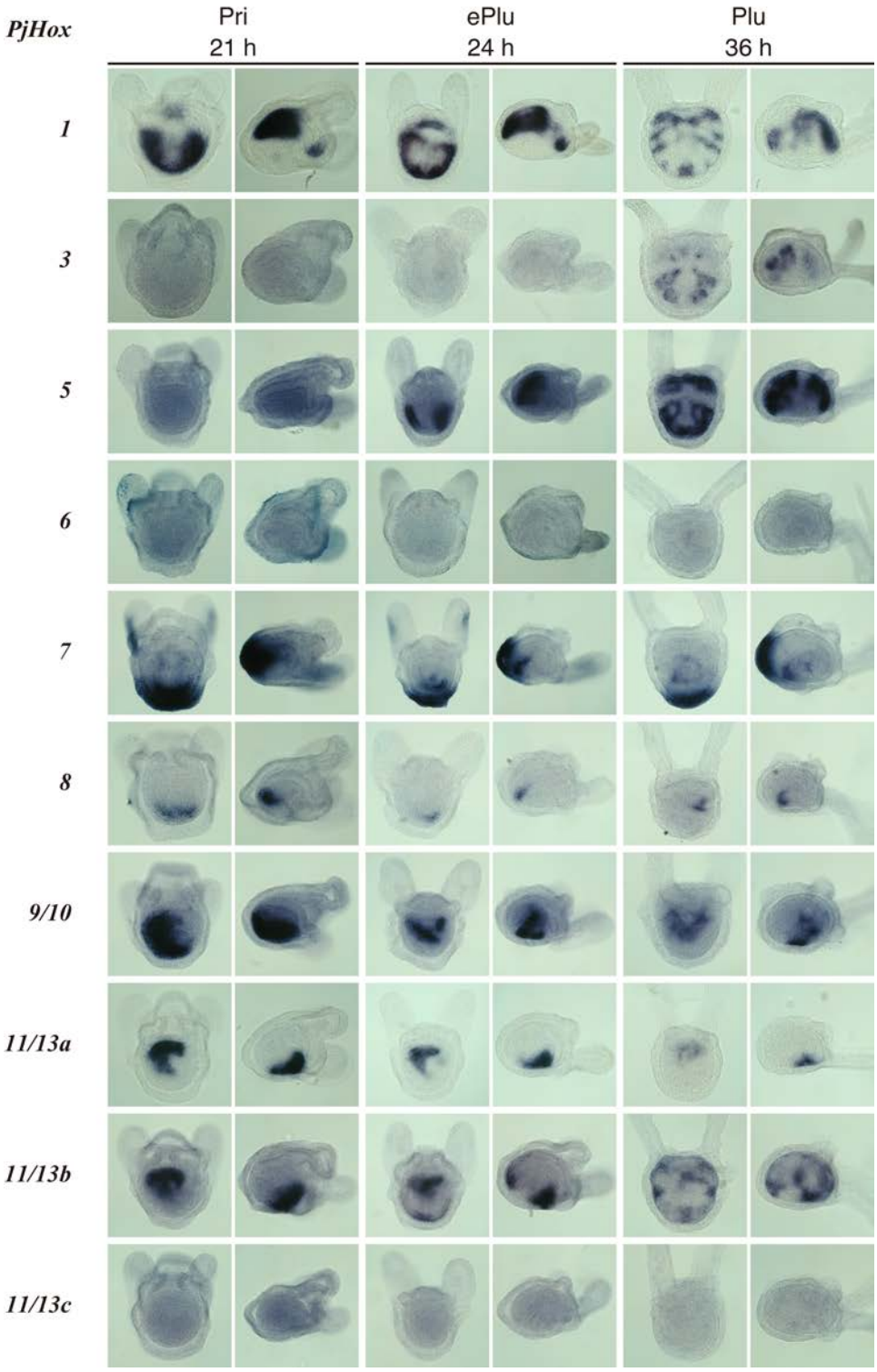
Fig. 2-4. Alignments of sequences of hexapeptide motifs, homeodomains, and the C-terminal flanking regions of ambulacraria-specific *Hox* genes, *Hox11/13a, Hox11/13b,* and *Hox11/13c* from sea urchins *S. purpuratus* and *P. japonica*, and hemichordates *Balanoglossus simodensis* (Bsim) and *Saccoglossus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

Figure 2-5

A : 0–18 h



B : 21–36 h



C : 48–72 h

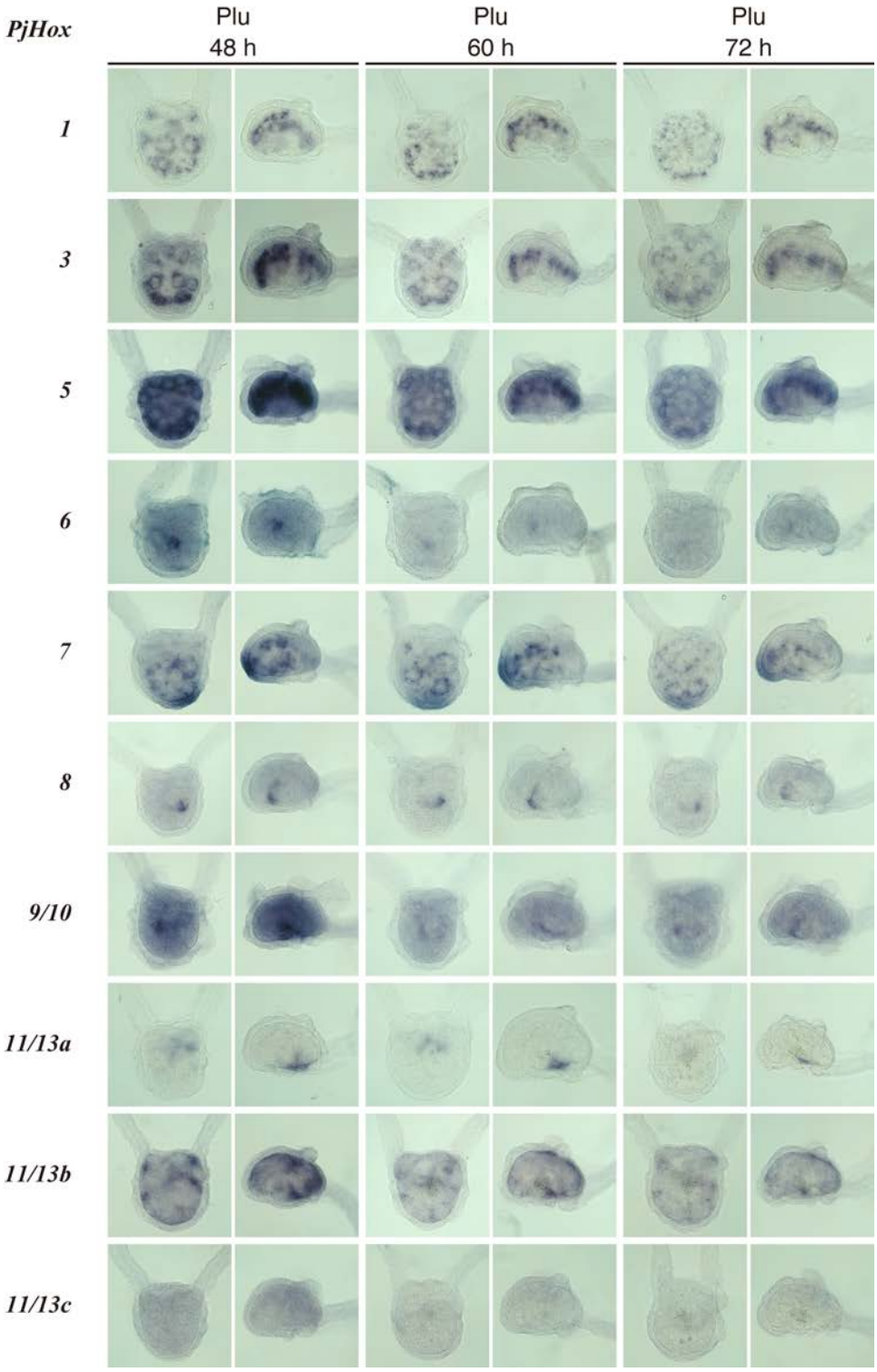


Fig. 2-5. Expression patterns of *Hox* genes revealed by WMISH. WMISH was performed on the eleven *Hox* genes (*Hox1, Hox2, Hox3, Hox5, Hox6, Hox7, Hox8, Hox9/10, Hox11/13a, Hox11/13b, Hox11/13c*) at 14 developmental stages, from fertilization egg to metamorforsis. **A**: From the fertilized egg at 0 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

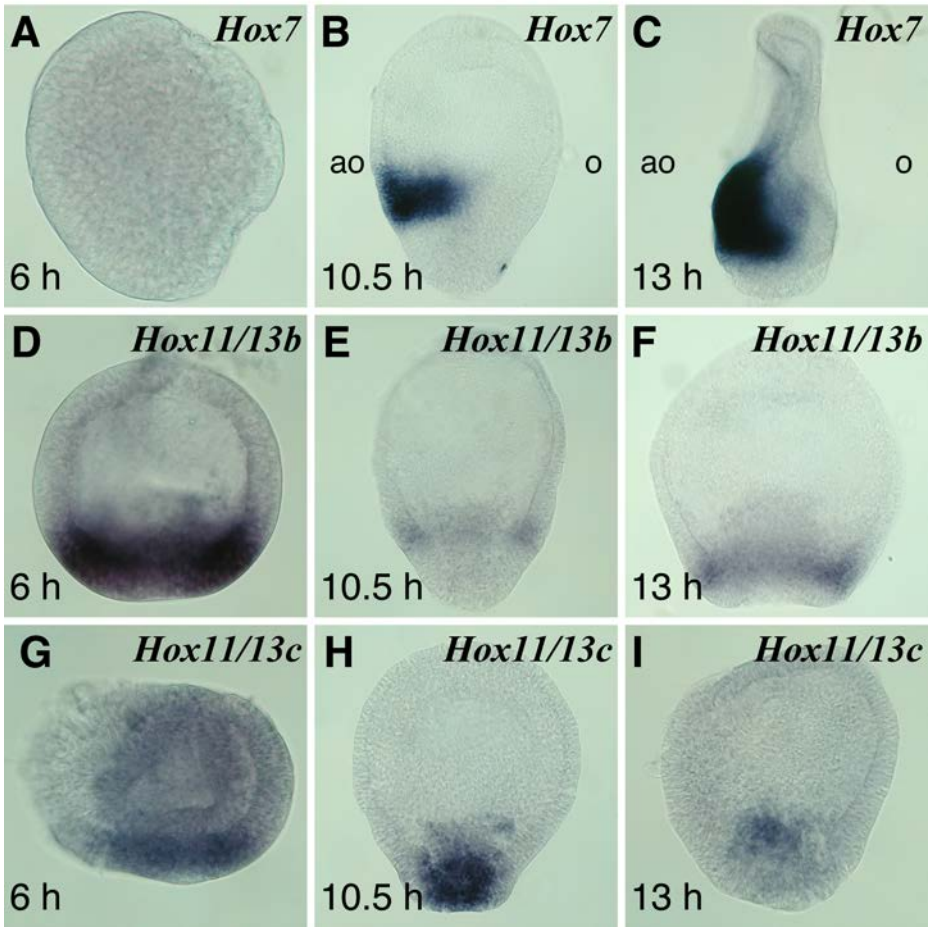


Fig. 2-6. Expression patterns of *Hox7*, *Hox11/13b*, and *Hox11/13c* at embryonic stage. Spatial expression of *Hox7* (**A–C**), *Hox11/13b* (**D–E**), and *Hox11/13c* (**G–I**) was examined by WMISH. Images of all embryos are shown as lateral views. 6 h, early blastula at 6 h; 10.5 h, hatched mesenchyme blastula at 10.5 h; 13 h, early gastrula at 13 h; ao, aboral; o, oral.

Figure 2-7

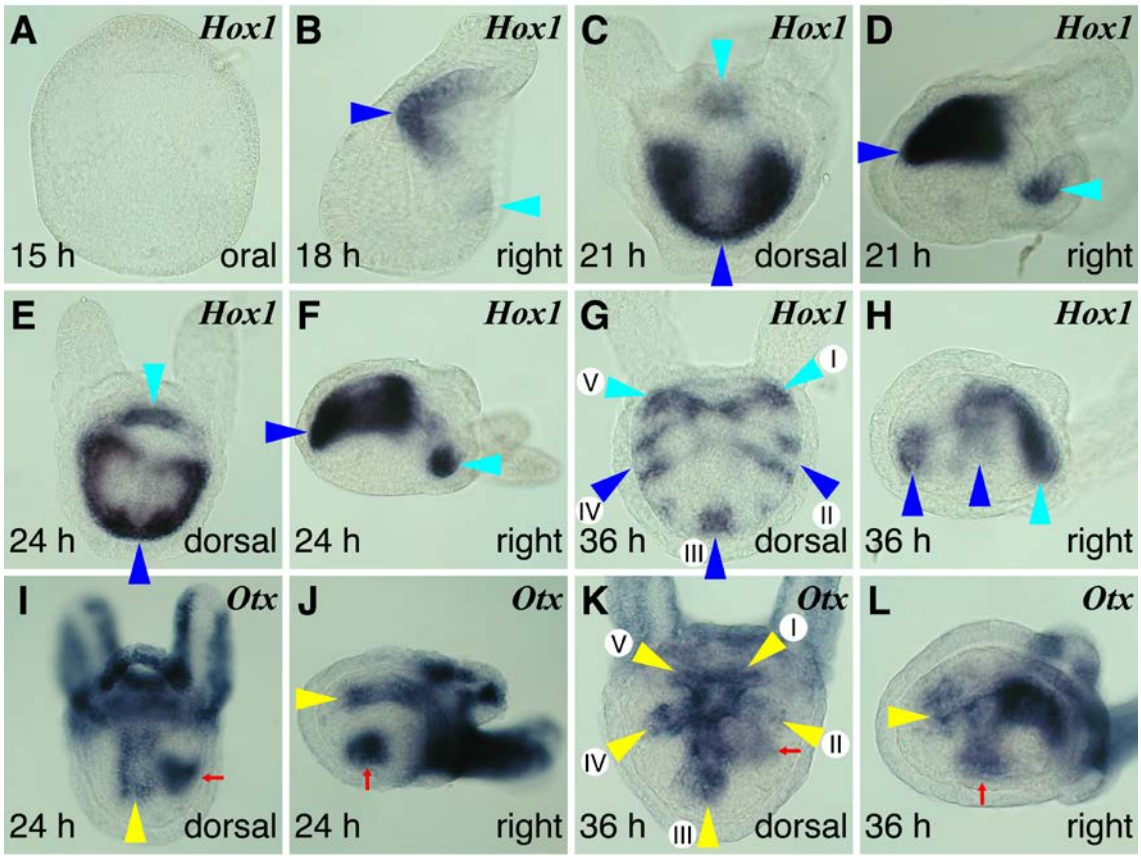


Fig. 2-7. Expression patterns of *Hox1* and *Otx*. Spatial expression patterns of *Hox1* (**A–H**), and *Otx* (**I–L**) were examined by WMISH. (**A**) Oral view, (**B, D, F, H, J, L**) right lateral view, and (**C, E, G, I, K**) dorsal view. Blue arrowheads indicate expression domains of *Hox1* in posterior 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 ambulracra 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.

Figure 2-8

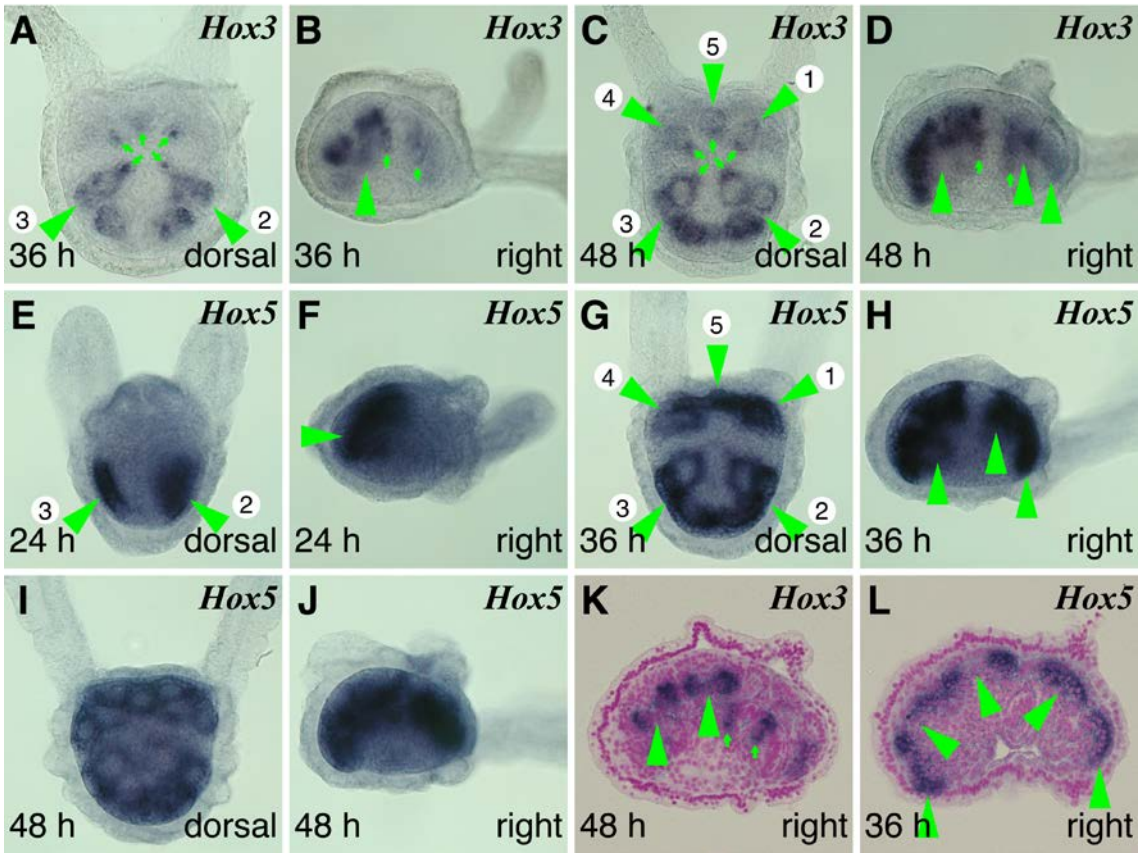


Fig. 2-8. Expression patterns of *Hox3* and *Hox5*. Spatial expression patterns of *Hox3* (**A–D**), and *Hox5* (**E–J**) were examined by WMISH. (**K, H**) Longitudinal sections of *Hox3* or *Hox5* stained larvae. (**A, C, E, G, I**) Dorsal view. (**B, D, F, H, J, K, L**) right lateral view. Green arrowheads 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

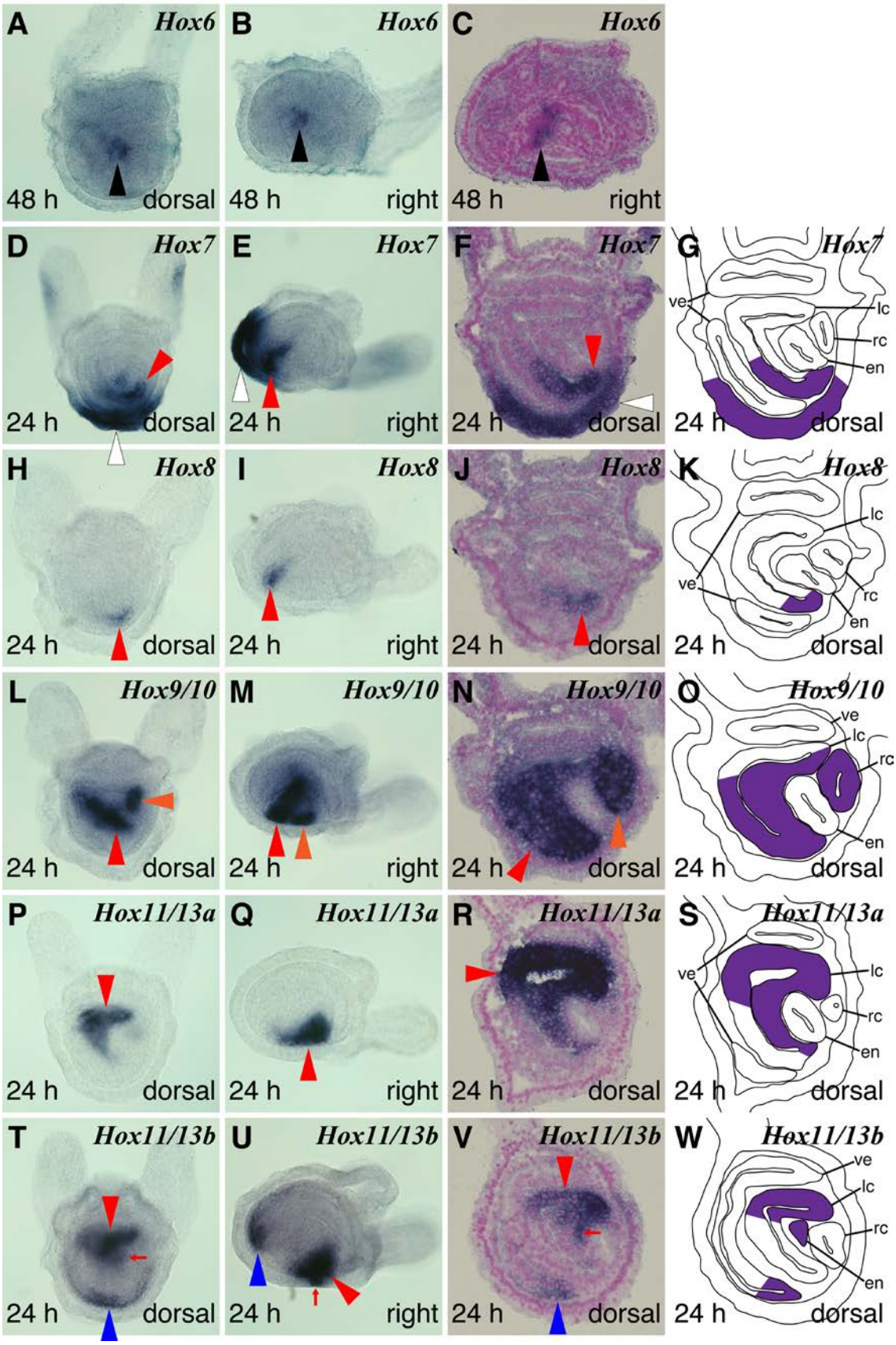

A Hox6
48 h dorsal
B Hox6
48 h right
C Hox6
48 h right
D Hox7
24 h dorsal
E Hox7
24 h right
F Hox7
24 h dorsal
G Hox7
ve
lc
rc
en
24 h dorsal
H Hox8
24 h dorsal
I Hox8
24 h right
J Hox8
24 h dorsal
K Hox8
lc
ve
rc
en
24 h dorsal
L Hox9/10
24 h dorsal
M Hox9/10
24 h right
N Hox9/10
24 h dorsal
O Hox9/10
ve
lc
rc
en
24 h dorsal
P Hox11/13a
24 h dorsal
Q Hox11/13a
24 h right
R Hox11/13a
24 h dorsal
S Hox11/13a
ve
lc
rc
en
24 h dorsal
T Hox11/13b
24 h dorsal
U Hox11/13b
24 h right
V Hox11/13b
24 h dorsal
W Hox11/13b
ve
lc
rc
en
24 h dorsal

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.

Figure 2-10

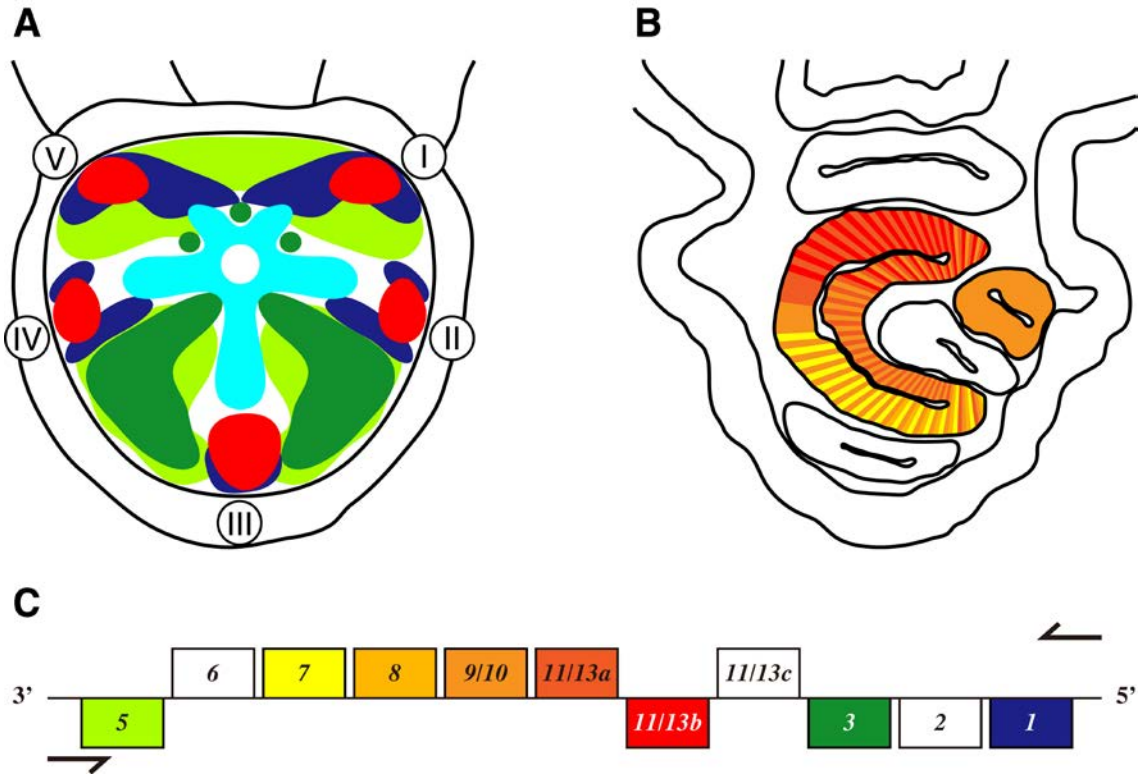


Fig. 2-10. Summary of *Hox* and *Otx* expression patterns in *P. japonica*. **A**: Expression patterns of *Hox1*, *Hox3*, *Hox5*, *Hox11/13b*, and *Otx* in the ambulacra and interambulacra. **B**: Somatocoelar expression patterns of *Hox7*, *Hox8*, *Hox9/10*, *Hox11/13a*, and *Hox11/13b*. **C**: The 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 pentamerous shape, chronographically, the ectodermal *Hox* expression precedes the mesodermal morphology. During the development of *P. japonica*,

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*.'.

Figure D-1

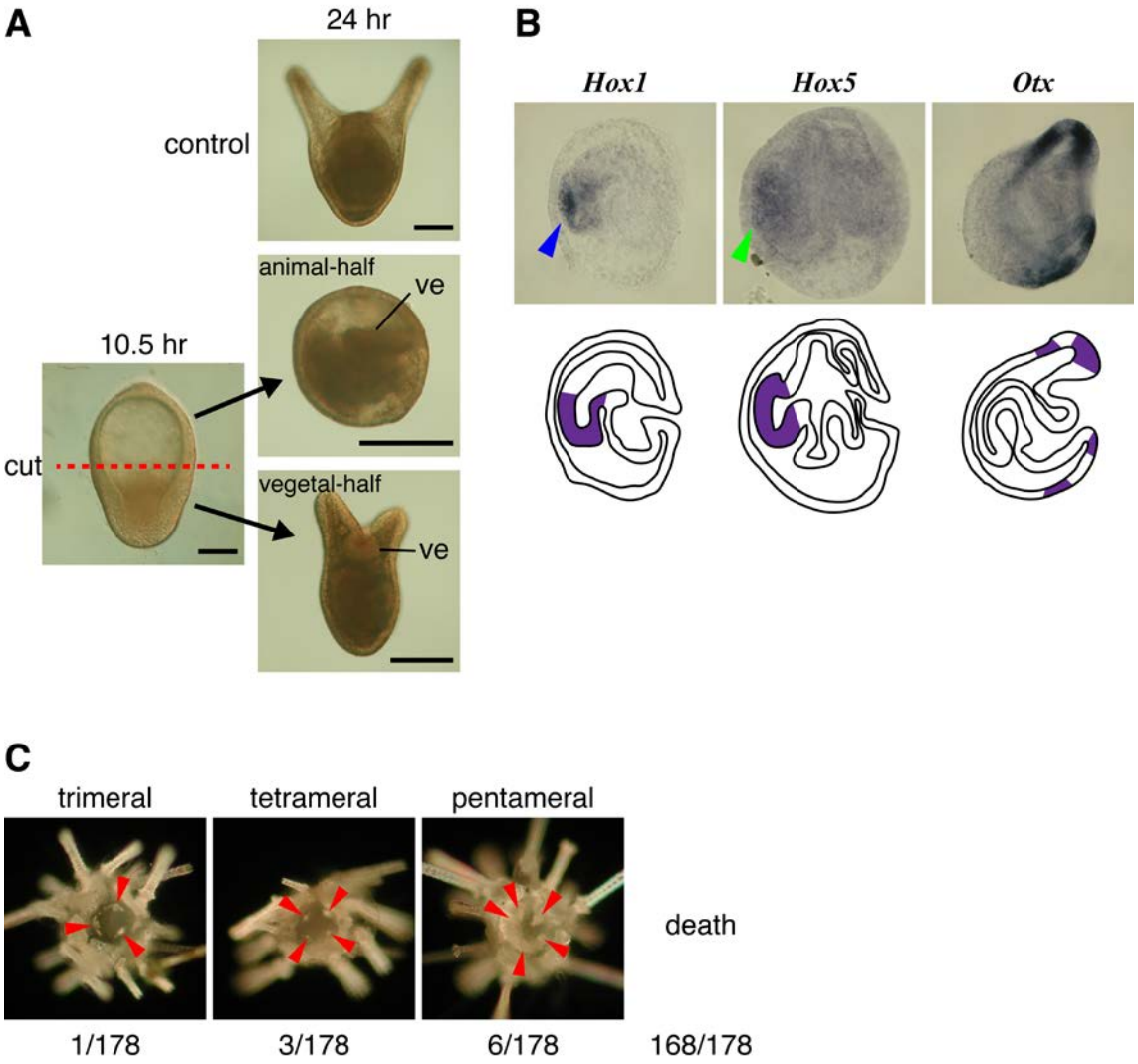


Fig. D-1. **A**: Phenotypes of animal and vegetal halves. **B**: *Hox1*, *Hox5*, and *Otx* expression patterns in blastulae developed from animal halves at 24 h. Blue and green arrowheads indicate *Hox1* and *Hox5* domain, respectively. **C**: Juveniles developed from vegetal halves. Red arrowheads indicate 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.

Acknowledgments

First of all, I would like to express my sincere appreciation to my supervisor, Professor Masaaki Yamaguchi, Kanazawa University, for his kind instruction, invaluable suggestions, and repeated encouragement to accomplish this study.

I would like to express my gratitude to Emeritus Professor Shonan Amemiya, University of Tokyo, and Associate Professor Takuya Minokawa, Tohoku University, for technical suggestions and invaluable discussions. I also gratefully acknowledge Associate Professor Toshihiro Yamada, Kanazawa University, for technical suggestions, and Assistant Professor Daisuke Kurokawa, University of Tokyo, for technical advice and encouragement. I would like to be profoundly grateful to Assistant Professor Makoto Urata, Hiroshima University, for helpful discussions, and Dr. Mizuki Aihara, Kaisei high school, for supports for microsurgical experiment in General discussion.

I thank Professor Yuichi Sasayama, Kanazawa University, for allowing me to use the fluorescence microscope, BZ-9000, and Dr. Akihito Omori, University of Tokyo, and Ms. Natsu Katayama, Kanazawa University, for technical advice on the microtomy. I also thank Professor Hiroshi Wada, University of Tsukuba, for helpful discussions, and his laboratory members for their relationships.

Here I wish to acknowledge the following members in our laboratory. I am grateful to Dr. Kosuke Shiomi and Dr. Atsuko Yamazaki for their kind technical suggestions, valuable discussions, repeated encouragements, and relationships. I would like to thank Rika Kawabata and Naoyuki Takahashi for their help, friendship and kindness. I also thank other colleagues,

Hidewo Nakata, Keiko Minemura, Hiroki Ishiai, Takahiro Oyama, Mitsuyoshi Kagawa, Yousuke Furuzawa, and Youichi Nishii for their relationships.

Finally, I would also like to extend my gratitude to my mother, Yoshiko Tsuchimoto for their long-time support.

This research was partially supported by Japan Society for the Promotion of Science (JSPS).

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