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Segment Regeneration in the Vestimentiferan Tubeworm, *Lamellibrachia satsuma*

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The ability to regenerate missing body parts varies among species. To elucidate the evolution of regenerative capability, an understanding of the regeneration mechanisms of diverse organisms is required. We focus on vestimentiferan tubeworms, which have a body plan that is unique among annelids. We found that the vestimentiferan *Lamellibrachia satsuma* is able to regenerate its posterior body parts, but not its anterior body parts. Based on observations of live specimens, we defined five stages in the process of posterior regeneration. The morphogenesis was observed in detail by a series of sections and scanning electron microscopy. The most posterior domain of the opisthosome differentiated from the blastema, while the anterior domain of the opisthosome regenerated from the remaining trunk region. We also examined the expression pattern of the *engrailed* gene during regeneration, and found that *engrailed* was expressed in the mesodermal cells of each segment.

Key words: Annelida, polychaete, Siboglinidae, morphogenesis, *engrailed*

INTRODUCTION

Wound healing and regeneration capabilities are widely distributed among metazoans, and have been investigated in a variety of animals, including vertebrates and invertebrates (Sanchez Alvarado and Tsonis, 2006; Bely and Nyberg, 2010). The degree of regenerative capability varies significantly even between closely related species, and within the same animal between different organs, tissues, and body parts (Bely, 2006). The phylogenetic distribution of regenerative capability indicates that this ability has been lost (or perhaps gained) multiple times during evolution (Bely, 2006; Bely and Nyberg, 2010). To elucidate the evolution of regenerative capability, the comparison of regeneration ability among various animal models is important (Bely and Nyberg, 2010). Annelids are known to have the greatest capability for regeneration among metazoans, and their regeneration is clearly evolutionarily labile (Bely, 2006). The ability to regenerate both anterior and posterior segments is widespread in annelids, and has been investigated in a variety of taxa (Bely, 2006). However, information on the regenerative capabilities of siboglinid polychaetes, comprising frenulates, moniliferans, *Osedax*, and vestimentiferans (Rouse et al., 2004), remains limited. Among siboglinid polychaetes, regeneration of the posterior body parts in the vestimentiferan tubeworm *Lamellibrachia satsuma* has not been investigated extensively (Miura et al., 1997).

Vestimentiferan tubeworms live in a chitinous tube, and inhabit primarily hydrothermal vents and hydrocarbon seeps (Bright and Lallier, 2010). They lack a mouth, an anus, and a digestive tract. Instead, they harbor chemoautotrophic bacteria in the trophosome and derive their metabolic needs from these bacteria (Cavanaugh et al., 1981; Cavanaugh, 1985). Although they are polychaetes, they lack the characteristic multi-segmented body of the group except in the most posterior end of the body, the opisthosome. Due to the unique morphological features of vestimentiferans, they were previously classified into the independent phylum Vestimentifera (Jones, 1985). Recent morphological and molecular phylogenetic analyses have shown that they are modified sedentary polychaetes belonging to the family Siboglinidae (Kojima et al., 1993; Rouse and Fauchald, 1995, 1997; Black et al., 1997; McHugh, 1997; Halanych et al., 1998, 2001; Kojima, 1998; Rouse et al., 2004). Information on the regeneration ability and the regeneration process in such a morphologically modified animal may provide important insights into the evolution and mechanisms of regeneration. In addition, although regeneration is not a simple recapitulation of embryogenesis, knowledge of morphogenesis during regeneration should help us to understand how the unique body plan of vestimentiferan tubeworms developed.

In the present study, we examined the regenerative capability of the vestimentiferan tubeworm *L. satsuma*. We observed the regeneration process and characterized the morphogenesis. In addition, we examined the expression of the *engrailed* (*en*) gene, which plays an essential role in metazoan segmentation.

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MATERIALS AND METHODS

Animal collection, culture and manipulation

Specimens of *Lamellibrachia satsuma* were collected at a depth of 101 m from Kagoshima Bay with a manipulator of the ROV *Hyper-Dolphin* on R/V *Natsushima* on 31 July 2008, and 12 and 14 April 2012. Animals were maintained in an aquarium containing artificial seawater at 14–15°C (Shinozaki et al., 2010; Miyamoto et al., 2013). Worms were cut with a surgical knife at the vestimental region, trunk, or opisthosome and divided into anterior and posterior fragments. The fragments were cultured in small dishes, and the dishes were put into the same aquarium where the adult worms were maintained. The fragments were observed under a light microscope. We observed at least six individuals for each fragment.

Histology

For histological observation, worms were relaxed in *l*–menthol in artificial seawater (ASW) for about 1 h and then fixed with Bouin's fixative. The fixed worms were dehydrated in a graded ethanol–*n*–butanol series and embedded in paraplast (Sigma-Aldrich). These specimens were sectioned at 5 μm, stained with Delafield's hematoxylin and eosin Y, and observed under a light microscope. We examined three individuals for each stage.

Scanning electron microscopy

For scanning electron microscopy, animals were fixed with 2.5% glutaraldehyde in ASW at 4°C. Samples were washed in ASW and post-fixed with 2% OsO_4/ASW for 2 h at 4°C. After several washes with distilled water (DW), samples were incubated with 1% aqueous tannic acid (pH 6.8) for 1 h for conductive staining. The samples were again washed with DW, treated with 1% OsO_4/DW for 30 min and washed with DW. The samples were dehydrated in a graded ethanol series. After critical-point drying, the samples were coated with osmium. The coated samples were observed with a scanning electron microscope. We examined three individual for each stage.

Isolation of *engrailed* and molecular phylogenetic analysis

A DNA fragment of the vestimentiferan ortholog of *en* was amplified by PCR. The primers used were en-F TGGCCNGCNTGGGTNTAYTGYAC, en-R1 TGRTTRTANARNCCYGTNGCCAT, en-R2 TTYTGRAACCADATYTTDATYTG. After determining the nucleotide sequences, we performed molecular phylogenetic analyses using PhyML 3.0 (Guindon et al., 2010). Amino acid evolutionary models were selected using Modelgenerator (Keane et al., 2006). The accession number of *en* is AB894551.

In situ hybridization

Animals were fixed with 4% paraformaldehyde (PFA) in a MOPS buffer (0.1 M 3-(N-morpholino) propanesulfonic acid, 0.5 M NaCl) at 4°C overnight. Specimens were dehydrated through an ethanol series up to 80% ethanol, and stored in 80% ethanol at –20°C. Frozen sections (8 μm in thickness) were air dried, washed with phosphate-buffer saline (PBS; 0.1 M, pH 7.4), and fixed with 4% PFA/PBS for 10 min at room temperature (RT). The slides were then washed with PBS and digested with 1 μg/ml proteinase K/PBS for 10 min at RT. After a brief wash with PBS, the samples were post-fixed in 4% PFA/PBS for 10 min at RT. The slides were washed with PBS three times, acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride for 15 min at RT and washed with PBS three times. The slides were prehybridized for at least 1 h in hybridization solution (50% formamide, 5× saline-sodium citrate (SSC), 5× Denhardt's, 100 μg/ml yeast RNA) at 60°C, and hybridized with a DIG-labeled RNA probe at 60°C for at least 16 h. The slides were washed in 50% formamide/2× SSC for 60 min, 2× SSC for 30 min twice, and 0.2× SSC for 30 min twice at 60°C. They were then rinsed twice with PBS, blocked with a 0.5% blocking reagent (Roche, Indianapolis, IN, USA) in PBS for 60 min at RT, and incubated overnight at 4°C with a 1:1500 dilution of anti-DIG-AP antibody (Roche) in a blocking buffer. They were washed with PBS for 30 min six times, transferred into an AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 2% polyvinylalcohol), and were developed by incubating the slides in NBT/BCIP (Roche) in an AP

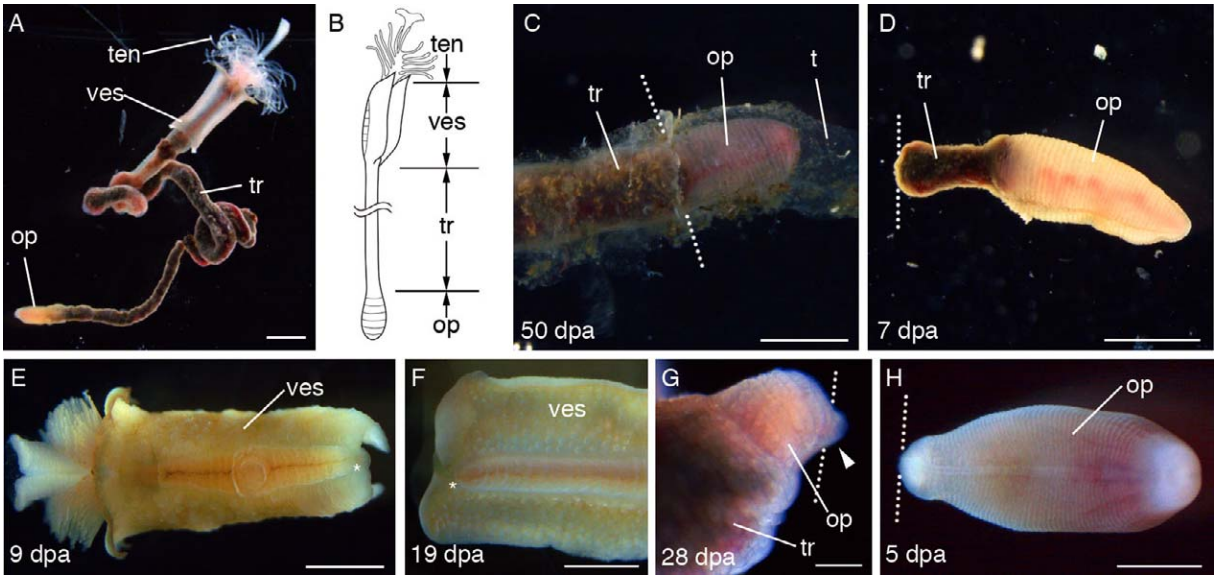

Fig. 1. Anatomical overview and regeneration ability of *L. satsuma*. **(A)** A worm removed from a tube shows four body regions. **(B)** Schematic illustration of *L. satsuma*. **(C)** The posterior region of a 50 dpa (days post-amputation) animal showing the regenerated opisthosome. **(D)** A 7 dpa animal shows that a fragment containing the trunk and the opisthosome does not demonstrate anterior regeneration. **(E)** An anterior fragment cut at the vestimental region does not regenerate posterior parts. **(F)** A posterior fragment cut at the vestimental region does not regenerate anterior parts. **(G)** A 28 dpa animal cut at the opisthosome showing the blastema formation. **(H)** An opisthosomal fragment does not regenerate anterior parts. Dotted lines and asterisks indicate cut surfaces. **op**, opisthosome; **t**, tube; **ten**, tentacular region; **tr**, trunk; **ves**, vestimental region. Scale bars: **(A)**, **(C–F)**, **(H)** = 1 mm; **(G)** = 200 μm.

buffer until a signal was visible. The reaction was stopped in PBS, post-fixed in 4% PFA/PBS overnight, and washed with PBS. Nuclei were counter-stained with DAPI and mounted with 80% glycerol. The prepared slides were observed under a light microscope. We examined three individuals for each stage.

RESULTS

Gross anatomy of *L. satsuma*

The body of *L. satsuma* consists of four distinct regions: the tentacular, vestimental, trunk, and opisthosomal regions (Fig. 1A, B). In the tentacular region, two obturacula and numerous tentacles project from the anterior end of the body. The vestimental region follows the tentacular region, and has dorsolateral wings throughout its length. This region contains the brain, the heart, the excretory organs, and openings of the reproductive system. The trunk is the longest region of the body and contains reproductive organs and the trophosomes within which the symbionts exist. The opisthosomal region is the posterior-most portion of the body, and is the only segmented region of vestimentiferans.

We examined the regeneration ability of each body part by cutting worms at the vestimental region, the trunk, or the opisthosome. When we cut animals into two pieces at the trunk, the anterior fragments, which contained the tentacular, vestimental, and trunk regions, regenerated their respective posterior body parts (Fig. 1C). In contrast, the posterior fragments containing the trunk and opisthosomal regions did not regenerate their respective anterior body parts (Fig. 1D). When we cut animals at the vestimental region, both fragments healed the cut surface. However they did not regenerate their lost parts (Fig. 1E, F). After cutting animal at the opisthosome, anterior fragments regenerated posterior parts (Fig. 1G), but posterior fragments did not regenerate anterior parts (Fig. 1H).

Morphogenesis during regeneration

To study the segment regeneration, we observed the morphogenesis during the posterior regeneration after cutting at the trunk. Just after amputation, the trophosome evaginated from the wound opening (Fig. 2A, A'). At five days post-amputation, the wound was still open. The trophosome remained evaginated, but became smaller (Fig. 2B, B′). In most cases the wound healed about 10 days after cutting (Fig. 2C, C′). At this stage, very thin epidermal cells covered the cut surface (Fig. 2C′, arrow), but these cells did

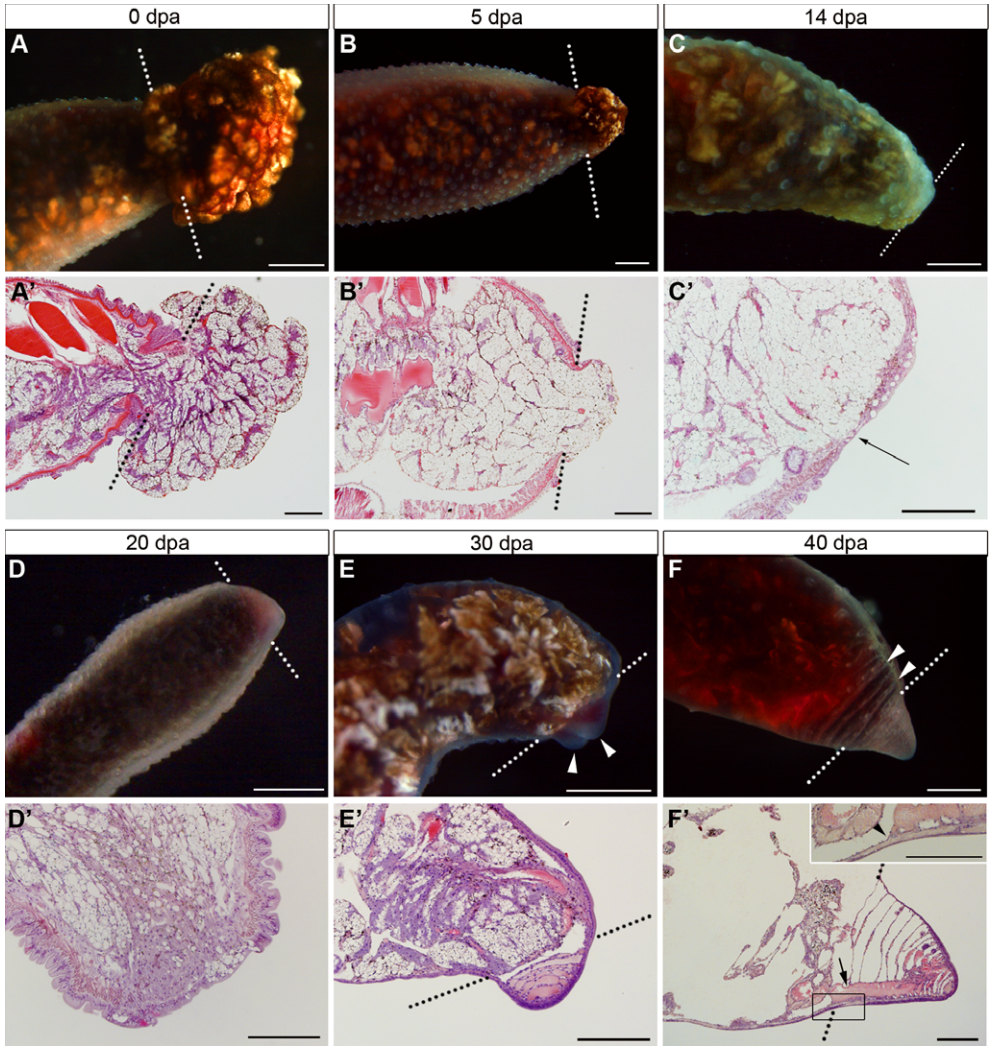

Fig. 2. Morphogenesis during the posterior regeneration of *L. satsuma*. **(A)** Just after cutting. The wound was open and internal tissues were evaginated. **(A′)** A longitudinal section of a 0 dpa animal. **(B)** A 5 dpa animal with a still-open wound. The evaginated trophosome was decreasing in size. **(B′)** A longitudinal section of a 5dpa animal. **(C)** A 14-dpa worm showing that the wound was healed. **(C′)** A longitudinal section of a 14-dpa animal. The wound was closed. Trophosome cells were present beneath the closed epidermis. **(D)** A 20-dpa individual. The blastema had formed. **(D′)** A longitudinal section of a 20-dpa animal. Mesodermal cells, which seem to be undifferentiated were present beneath the cut surface. **(E)** A 30-dpa animal showed two projections of the blastema. **(E′)** A longitudinal section of a 30-dpa animal. Septa started to form in the blastema. **(F)** A 40-dpa animal. The blastema fused to become a single projection and formed segments. Segmentation occurred in the epidermis anterior to the cut surface (arrowheads). **(F′)** A longitudinal section of a 40-dpa animal. Clear segments formed in the blastema. No septa were observed anterior to the cut surface. The blood vessel was continuous from the trunk (arrow). The inset is a high magnification image of the boxed area. The ventral nerve cord was continuous from the trunk (arrowhead). Dotted lines indicate the cut surfaces. Scale bars = 500 μm.

not contain vacuoles. There was no papilla or pyriform gland in the healed area. Bacteriocytes were present immediately beneath the healed epidermis, and there was no aggregation of mesenchymal cells (Fig. 2C'). About three weeks post-amputation, a regenerating blastema formed at the posterior end of the body (Fig. 2D). The blastema was white to pink in color and did not contain dark-colored bacteriocytes (Fig. 2D, D′). Although some epidermal cells of the blastema contained smaller vacuoles, the morphology of the cells was distinct from that of intact epidermal cells (Fig. 2D′). The epidermal cells of the blastema were thin and there was no papilla or pyriform gland. In contrast, the epidermis of the remaining tissue of the trunk consisted of thick and columnar-shaped cells, which formed continuous pleats (Fig. 2D′). Many mesodermal cells were present in the blastema and the cells were surrounded by connective tissue (Fig. 2D′). At one month post-amputation, the blastema formed two projections laterally (Fig. 2E, arrowheads). Although there was no sign of segmentation in the epidermis in this stage, segmented septa were observed in sections (Fig. 2E, E′). The mesodermal cells were associated with the septa in a segmented manner (Fig. 2E′). Until this stage, the epidermal cells were flat, with few papillae and pyriform glands (Fig. 2E′). About 40 days after amputation, the blastema developed into a single projection (Fig. 2F). In this stage, segments were observed as an external feature (Fig. 2F). We found that repeated rows of setae differentiated in the epidermis anterior to the cut surface (Fig. 2F, arrowheads). Sections in this stage showed that mesodermal septa existed only in the region posterior to the cut surface (Fig. 2F′). The blood vessel of the regenerated opisthosome was continuous from that of the pre-existing trunk (Fig. 2F′, arrow). The ventral nerve cord of the opisthosome was also continuous with the trunk nerve cord (Fig. 2F′, arrowhead). The external and internal features of regeneration are summarized in Table 1.

Scanning electron microscopy

We also observed morphogenesis by scanning electron microscopy. In intact worms, the opisthosome was subdivided into two regions. The anterior region, which occupied about four fifths of the opisthosome, had a single row of setae (Fig. 3A, B, arrowheads). In the posterior region, there were no setae (Fig. 3C). The epidermis of the trophosome contained numerous papillae and the opening of the pyriform glands (Fig. 3B). In the healed wound stage, there was no sign of blastema formation (Fig. 3D). In this stage, wrinkles of the epidermis started to extend and the surface of the epidermis became smoother (Fig. 3D). In the stage of blastema formation (about three weeks after amputation), the trophosome epidermis was almost completely smooth, and the

Table 1. Stages of regeneration in *Lamellibrachia Satsuma*.

Stage	Days post-amputation	External features	Internal features
1	0–10	Wound healing	Trophosome evagination
2	14	Wound closure	No mesodermal cell aggregataion
3	20	Blastema formation	Mesodermal cell aggregation
4	30	No segmention in epidermis Two-projection blastema	Septa formation
5	40	Epidermis segmentation One-projection blastema	Segmentation

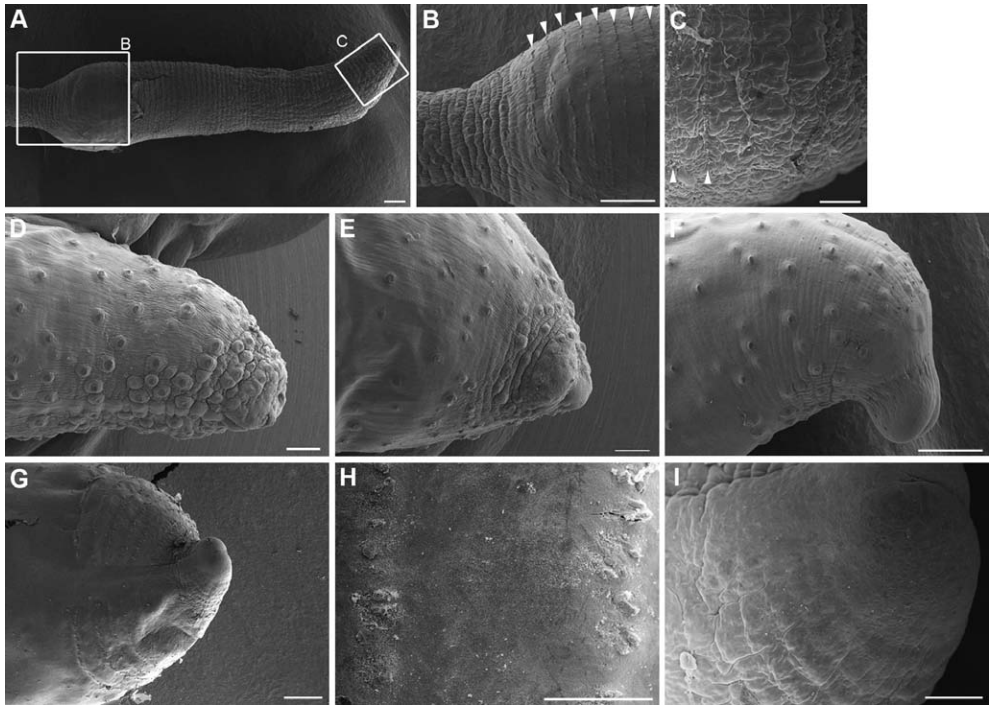

Fig. 3. Scanning electron microscopy of *L. satsuma* regeneration. **(A)** The opisthosome of an intact animal. **(B)** The boxed area labeled **B** in **(A)**. The anterior region of the opisthosome has a single row of setae in each segment. **(C)** The boxed area labeled **C** in **(A)**. The posterior region of the opisthosome has no seta. **(D)** A 14-dpa animal. The wound was closed. No sign of blastema formation. **(E)** A 23-dpa animal shows that the blastema formed as two projections. **(F)** A 30-dpa animal. The blastema developed, but there was no sign of segmentation in the epidermis. **(G)** A 40-dpa animal. The blastema developed into a single projection. The segments were observed. **(H)** A high magnification of the anterior region of a 40-dpa animal. A single row of setae was observed in each segment. **(I)** The posterior region of the regenerating opisthosome. Segments without setae were observed. Arrowheads indicate the rows of setae. Dotted lines indicate the cut surfaces. Scale bars: **(A)**, **(B)**, **(D–G)** = 200 μm; **(C, H, I)** = 50 μm.

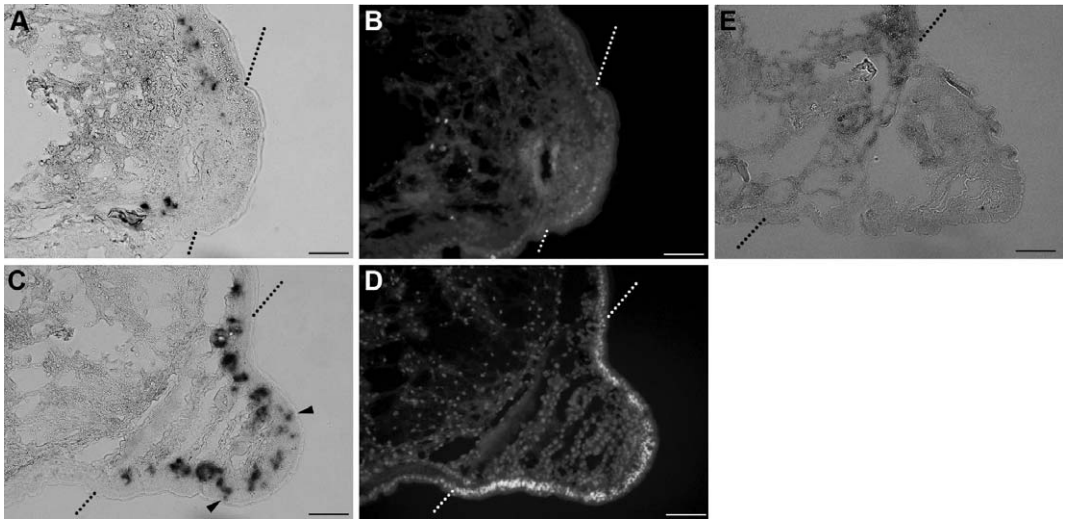

Fig. 4. Expression of *engrailed* during regeneration. **(A)** A longitudinal section of a 20-dpa animal. Expression of *en* was detected in the mesodermal cells anterior to the cut surface. No *en* expression was observed in the blastema. **(B)** DAPI staining of the same section. **(C)** A longitudinal section of a 35-dpa animal. Expression of *en* was detected in the mesodermal cells located where the septa attached to the epidermis. A few *en*-positive cells were observed in the epidermis (arrowheads). **(D)** DAPI staining of the same section. **(E)** A negative control showed no staining. Dotted lines indicate the cut surfaces. Scale bars = 50 μm.

distribution of the papillae and the pyriform glands was scattered (Fig. 3E). The blastema formed as two projections, as described above (Fig. 3E) SEM observations showed no segmentation of the external features in this stage (Fig. 3E). About one month after amputation, the blastema grew, but no segmented structure was observed by scanning electron microscopy (Fig. 3F). The blastema was almost a single projection with a shallow groove at the dorsal midline (Fig. 3F). At 40 days after regeneration, single rows of chaetae were observed in the epidermis anterior to the cut surface as observed under a light microscope (Figs. 2F and 3G, H). We found repeated structures in the epidermis posterior to the cut surface, but no chaetae were observed (Fig. 3G, I). There was no segment or shallow groove at the dorsal midline at the posterior end of the blastema (Fig. 3G, I).

Expression of *engrailed*

Spatiotemporal expression of *en* was observed during segment regeneration. At 12 days after amputation, which is the stage of wound healing, *en* expression was detected in mesodermal cells located under the epidermis (Fig. 4A, B). Expression was not detected in the blastema cells. At 35 days after amputation, *en* was expressed in the mesodermal cells where the septa were attached to the epidermis (Fig. 4C, D). A few locations of *en* expression were detected in the ectoderm (Fig. 4C, arrowheads). We did not detect any positive signals using an *en* probe for *Osedax japonicus* (Fig. 4E).

DISCUSSION

Regeneration ability

The present study shows that the vestimentiferan *L. satsuma* can regenerate the posterior region of the body, the opisthosome, from a fragment containing the tentacular, vestimental, and trunk regions. In contrast to this posterior regeneration, posterior fragments did not regenerate the anterior body parts. This result shows that the capability of regeneration in this species varies depending on which body parts are lost. The regeneration ability of the lost body region has been investigated in wide range of annelid taxa (Bely, 2006). Among annelids, regeneration of the posterior segment after amputation is common (Bely, 2006). This may be due to the fact that posterior regeneration is similar to adult growth by segment addition (Bely and Wray, 2001). Many annelids have the capability of anterior regeneration, but this ability is less common than posterior regeneration (Bely, 2006). In the regeneration of *L. satsuma*, the blood vessel and the nervous system of the newly-formed opisthosome are continuous with those of the trunk tissues. Furthermore, the opisthosome secretes the tube. These results suggest that the regenerated structures are functionally integrated to the pre-existing tissues. Compared with the simple structure of the posterior end of the body, it may be difficult to reorganize the highly complex anterior region containing the brain, heart, and nephridia from the trunk. Further investigation of the molecular mechanisms of regeneration will provide information about the differences in the regenerative ability of these organisms along the anteroposterior axis. To better understand regeneration among the siboglinid polychaetes, it is necessary to gain knowledge of regeneration not only in the close relatives of *L. satsuma*, but also in the other siboglinids, such as frenulates, moniliferans, and *Osedax*.

Posterior segment regeneration from non-segmented region

The basic body plan of annelids is multi-segmented, with each segment usually separated by septa. Recent phylogenetic analyses have shown that vestimentiferan tubeworms are a group of polychaetes, and they have subsequently been assigned to the monophyletic group of the family Siboglinidae (Kojima et al., 1993; Rouse and Fauchald, 1995,

1997; Black et al., 1997; McHugh, 1997; Halanych et al., 1998, 2001; Kojima, 1998; Rouse et al., 2004). Considering that siboglinids are derived from polychaetes, a new question is how the siboglinid body plan evolved from the polychaete body plan. One of the most striking differences between the siboglinid and polychaete body plans is that siboglinids lost the typical multi-segmented body except for their posterior end, the opisthosome. Our previous study of the neuroanatomy of the vestimentiferan *L. satsuma* revealed a rope-ladder-like nervous system in the trunk region, despite the loss of segments (Miyamoto et al., 2013). This suggests that there are distinct developmental mechanisms for the repeated structure of the nervous system and segmentation. To understand the evolutionary loss of segmentation, the molecular mechanisms underlying segment formation and the development of the non-segmented region should be investigated. However, the current knowledge of the development of vestimentiferan tubeworms is limited. Embryonic and larval development of a few species has been reported (Young et al., 1996; Miura et al., 1997; Marsh et al., 2001; Miyake et al., 2006). Some authors have described the morphology of post-settlement juvenile stages of tubeworms from fortuitously collected specimens (Southward, 1988; Jones and Gardiner, 1989; Nussbaumer et al., 2006). To examine the developmental mechanisms of segmentation, it is necessary to study many specimens of each developmental stage. Thus, we do not have a good model species for the developmental biology of vestimentiferan tubeworms. In the present study, we show regeneration of the posterior segment from the non-segmented region. Although regeneration is not a simple replication of development, knowledge of regeneration will aid in our understanding of morphogenesis.

Expression of *en* during the regeneration of *L. satsuma* in a metameric manner implies that the gene is somehow involved in segmentation. Expression patterns of *en* are well-studied among annelids. In the polychaete *Platynereis dumerilii*, *en* is expressed in the ectodermal stripes along the anterior edge of segments during embryonic development and posterior regeneration (Prud'homme et al., 2003). The similar expression patterns of *en* in polychaetes and arthropods suggest a conserved role for segmentation (Prud'homme et al., 2003). Other polychaetes such as *Capitella teleta*, *Hydroides elegans*, and *Chaetopterus* sp., however, show expression patterns of *en* distinct from that in *P. dumerilii* (Seaver et al., 2001; Seaver and Kaneshige, 2006). In *L. satsuma* regeneration, *en* was expressed in mesodermal cells adjacent to the ectoderm. The mesodermal expression of *en* in *L. satsuma* is similar to that in *C. teleta* (Seaver and Kaneshige, 2006). Although *en* was expressed in spots in *C. teleta* (Seaver and Kaneshige, 2006), *en* expression was detected broadly along the attachment of the epidermis and septa in *L. satsuma*. These differences in *en* expression patterns among polychaetes suggest the presence of developmental system drift in polychaete segmentation. In addition to the *en* expression, cellular and molecular mechanisms of segmentation in polychaetes have been investigated (de Rosa et al., 2005; Seaver and Kaneshige, 2006; Saudemont et al., 2008; Thamm and Seaver, 2008; Dray et al., 2010; Janssen et al., 2010; Gazave et al., 2013; Niwa et al., 2013). A better understanding of vestimentiferan segmentation and comparison the results with that in other polychaetes should provide important insight into not only the evolution of the vestimentiferan body, plan but also the evolution of segmentation in polychaetes.

The present study provides a basis for understanding the regeneration of vestimentiferan tubeworms. A large number of specimens are necessary for further investigation of regeneration and segmentation. Because *L. satsuma* is the shallowest-living species among vestimentiferans (82 m in depth), with a habitat close to shore (Miura et al., 1997), we can collect them relatively more easily than the other species. Conventional culture systems are available for this species (Miyake et al., 2006; Shinozaki et al., 2010). In addition, the regeneration of *L. satsuma* can be examined multiple times using one individual. The regeneration of *L. satsuma* is a promising model system for the evolution of regeneration and the vestimentiferan body plan.

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