Morphological characterization of the asexual reproduction in the acorn worm *Balanoglossus simodensis*

Norio Miyamoto[1, 2] and Yasunori Saito[2]

[1]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

[2]Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka, Japan

Corresponding author: Norio Miyamoto

Postal address: Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

Telephone: +81-29-853-4671

Fax: +81-29-853-4671

E-mail: s0830211@u.tsukuba.ac.jp

Short title: Asexual reproduction in *Balanoglossus simodensis*

ABSTRACT

The acorn worm *Balanoglossus simodensis* reproduces asexually by fragmentation and subsequent regeneration from the body fragments. We examined the morphogenesis of its asexual reproduction. At first, we collected asexually reproducing specimens and observed their morphogenesis. Then, we succeeded in inducing the asexual reproduction artificially by cutting the worm at the end of the genital region. The process of morphogenesis is completely the same between naturally collected and artificially induced specimens. The stages during morphogenesis were established on the basis of the external features of the asexually reproducing fragments. The internal features of the fragments were also examined at each stage. In a separate phase of the study, the capacity for regeneration of some body parts was also examined by dividing intact worms into about 10 fragments. Although the capacity for regeneration varied among the different body parts, some fragments regenerated into complete individuals in 1 month. The process of regeneration was the same as that in the asexually produced fragments.

Keywords: asexual reproduction, enteropneust, hemichordate, morphology, regeneration

Introduction

Although the processes of wound healing and regeneration are widely distributed among metazoans, large-scale regeneration of complex body structures occurs only in some invertebrates, such as hydras, planarians, echinoderms, and ascidians (Reviewed in Sánchez Alvarado 2000; Candia Carnevali 2006). The degree of regeneration varies significantly even between closely related species and, within the same animal, between different organs, tissues, and body parts (Bely 2006). Nuclear transplantation studies, however, have shown that the nuclei of differentiated cells contain all the information necessary to produce complete individuals (Wilmut *et al*. 1997; Kato *et al*. 1998). Why, then, is the ability of regeneration so limited in most animals? To answer this question, an integrated understanding of the regeneration mechanisms of diverse organisms is required (Brockes *et al*. 2001; Sánchez Alvarado & Tsonis 2006).

Enteropneusts, or acorn worms, are free-living, vermiform marine invertebrates that mostly live in sediment at the bottom of the sea. About 80 species have been described, and some of them are known to be able to regenerate, more or less, their lost body parts (Spengel 1893; Hill 1894; Willey 1899; Cori 1902; Assheton 1908; Hinrichs & Jacobi 1938; Kirk 1938; Tweedell 1961). Whereas the regeneration of enteropneusts is a well-known phenomenon, its morphological characterization is limited mainly to the external features. Although a few observations of internal features during regeneration have been performed (Dawydoff 1909; Rao 1955; Rychel & Swalla 2008), there is no detailed information on the time course of the regeneration of each organ and tissue. Moreover, because these studies were limited to early stages of regeneration, when only the proboscis regenerated, there is no information on the histological features of the regeneration of more posterior regions, such as the collar and trunk.

In addition to the high regeneration ability of enteropneusts, four ptychoderids were reported to reproduce asexually (Gilchrist 1923; Packard 1968; Petersen & Ditadi 1971). Gilchrist (1923) first reported on asexual reproduction in enteropneusts and revealed that *Balanoglossus proliferans* was not a true species, but an asexually reproducing individual, designated as the branchiogenital individual in the present paper, of *Balanoglossus capensis*. He noted that a bud from *B. proliferans* (i.e., branchiogenital individual of *B. capensis*) regenerated into an intact worm. More recently, Packard (1968) collected various types of specimens of *Balanoglossus australiensis* and concluded that these specimens were asexually reproducing. Petersen and Ditadi (1971) reported on the asexual reproduction of *Glossobalanus crozieri* and observed the process of morphogenesis of collected asexually reproducing fragments and artificially cut fragments. These studies suggested that the process of asexual reproduction seems to be identical among ptychoderids. That is, vegetative fragments were produced from the posterior end of the genital region (Packard 1968 and Fig. 2). However, because the entire process of the fragmentation of the worm body and morphogenesis from the fragments to complete worms was not observed, there is no clear evidence of how the fragmentation takes place. For example, does the fragmentation occur in series or can several fragments bud off at the same time. In addition, a complete description of both the external and internal morphology of asexual reproduction is essential in determining whether asexual reproduction occurs in the same manner as the developmental process from a fertilized egg.

We found that *Balanoglossus simodensis*, a recently described ptychoderid enteropneust (Miyamoto & Saito 2007), undergoes asexual reproduction in the non-breeding season. In the present study, we first observed the time course of asexual

reproduction in naturally collected specimens of *B. simodensis*. We found a method, which is described in detail below, to induce fragmentation in the same manner as fragmentation under natural conditions. In addition, we examined the capacity for regeneration of each body part.

Materials and Methods

Animal collection, culture, and manipulation

Specimens of the acorn worm *B. simodensis* (Fig. 1A) were collected from the lower intertidal zone and the subtidal zone near the Shimoda Marine Research Center, University of Tsukuba, at least once a month in 2005 and 2006. The worms were transported to the marine center and kept in aquariums containing sand from the natural habitat under fresh, running seawater. *Balanoglossus simodensis* attains lengths of up to 10 cm, and worms of more than 4 cm in length were selected for regeneration experiments. Worms were cut with a surgical knife and divided into several different body part fragments. The fragments were cultured in small dishes containing sand from their natural habitat under fresh, running seawater at 21°C to 23°C. The fragments were observed every day during the first month and every few days during the following months under a light microscope.

Histology

For histological observation, some worms were allowed to defecate their gut contents by keeping these worms without sand. The cleaned worms were relaxed in *l*-menthol in filtered seawater for about 1 hr and then fixed in Bouin's fixative. The fixed worms were dehydrated in a graded ethanol-*n*-butanol series and embedded in paraplast (Sigma Chemical Co., St. Louis, USA). These specimens were sectioned at 7 μm, stained with Delafield's hematoxylin and eosin Y, and observed under a light microscope.

Results

In the present paper, as a matter of convenience, we defined the terms of asexual reproduction and regeneration as following: asexual reproduction is the process through a fragmentation of worm by autotomy and subsequent body repair; and regeneration is the process of body repair after an artificial cut. The body repair after the induced fragmentation is also defined as the asexual reproduction. Figures 1 and 2 show the morphology of intact worms of *B. simodensis*.

Fragmentation

In the natural habitat, we found three kinds of fragmented individuals. Fragmented individuals were collected only from June to December, but never at other times when *B. simodensis* undergoes sexual reproduction (January to May). Because these fragments were morphologically identical to the fragments undergoing asexual reproduction in the species described in previous reports, we followed the nomenclature for the three fragments as described by Gilchrist (1923) and Packard (1968). The first fragment is called the “branchiogenital individual” and consists of the anterior half of the worm, which contains the proboscis, collar, branchial region, and genital region (Fig. 1B). The posterior end of the genital region is elongated in this fragment. The second fragment is called the “hepatic individual” and is the posterior half of the worm, which consists of the posterior end of the genital region, the hepatic region, and the caudal region (see Fig. 1C; a hepatic individual regenerating anterior body parts). The third fragment is called the “regenerand” and is a small fragment of the genital region (Fig. 1B). All three kinds of fragments contained yellow granules in their gonads. In *B. simodensis*, the yellow granules were stored in their gonads after a gamete release and were replaced by sperm

or eggs from December. The functions and composition of the yellow granules are still unknown, but they probably relate to the maturation of this species. Although some enteropneust species secrete a large amount of mucus and build distinct burrows (Duncan 1987), *B. simodensis* secretes a smaller amount of mucus than other enteropneusts and does not build any distinct burrows. Despite this situation, a pair of branchiogenital and hepatic individuals along with several regenerands in different regeneration stages were collected from the same mucous mass. This suggests that the regenerands are produced serially from the branchiogenital individual.

To confirm that regenerands bud off from the branchiogenital individual, we kept branchiogenital individuals in aquariums in a laboratory. A few days after transferring the individuals to the lab tanks, we observed that regenerands were produced from the posterior end of the branchiogenital individual through autotomy one by one. We observed 17 regenerands formed from 3 branchiogenital individuals, with a frequency of one regenerand every 1 or 2 days. The average length of the regenerands was 7.3 mm; the minimum length was 4 mm, and the maximum length was 17 mm, when the regenerands were anesthetized.

In the course of examination of the regeneration ability of artificially cut fragments (as mentioned below), we found that autonomous fragmentation can be induced by the following method (the process of fragmentation is shown in Fig. 3): First, a worm that contained yellow granules in the genital region was cut into two pieces at the posterior end of the genital region. Both the anterior and posterior fragments were nearly the same as the two fragments produced by fission, the branchiogenital individual and the hepatic individual (Fig. 3, Step 1). When the anterior half of the cut fragments (the branchiogenital individual) was kept in an aquarium with sand, the posterior end of the

branchiogenital individual elongated (Fig. 3, Step 2). About 1 week after cutting, the first regenerand was produced from the posterior end of the branchiogenital individual by fission (Fig. 3, Step 3). After that, new regenerands were produced every 1 or 2 days. When the genital region of the branchiogenital individual became shorter, the branchiogenital individual stopped producing regenerands and began to regenerate its posterior body part. Finally, all fragments, the branchiogenital and hepatic individuals and the regenerands, regenerated their lost body parts (Fig. 3, Step 4). The total number of produced regenerands varied by each individual, depending on the length of the branchiogenital individual and regenerands. Even in the case of long worms, the number of produced regenerands was small if it produced only long regenerands. Regenerands were never formed when the anterior fragment was kept in an aquarium without sand or when the worm did not contain yellow granules in the gonads. In these cases, the branchiogenital individual began to regenerate only the posterior lost parts soon after the worm was cut.

We think that this artificially induced autotomy is comparable to what occurs in the natural habitat because the size and number of the regenerands and branchiogenitals are quite similar to those found in the mucus of the natural habitat. More importantly, autotomy only occurred when individuals were cut at the posterior end of the genital region and never occurred when cut at different positions. Thus, we decided to observe the details of the asexual reproduction process by using the artificially induced regenerands.

Morphogenesis of the regenerand

First, to define the stages of asexual reproduction, we observed the external

morphology during the growth of the regenerands. Figure 4 shows the external features of each stage. Furthermore, we examined the details of the internal morphology at each stage (Fig. 5). Just after fission, the wound at the anterior end of a regenerand was still open (Figs. 4A and 5A, Stage 1). The wounds at the posterior end of the branchiogenitals and anterior end of the regenerands closed about 1 day after being cut, regardless of being cut artificially or by autotomy through the connection between the epidermis and intestinal epithelium. Thus, the wound at the posterior end of a regenerand had already healed when the regenerand was produced by fission from the branchiogenital individual (Figs. 4B and 5B, Stage 2). After wound healing, a blastema was formed on the dorsal side of the regenerand gut (Fig. 4C, Stage 3). At this stage, the blastema was observed as two projections and was filled with round-shaped cells that were probably undifferentiated cells (Figs. 5C and 6A). In this stage, distinct epithelial cells emerged on the gut side between the epidermis and gut epithelium, which contained vacuoles (Fig. 6A, asterisk indicates the region of the new epithelial cells). This region would be the esophageal epithelium and stomochord in which cells are morphologically identical in an intact worm (Fig. 2B). In an intact worm, a thick layer of nerve fibers forms under the proboscis epidermis (Fig. 2B, C). The epithelial layer of nerve fibers then began to form (Fig. 6A, arrow). The nerve layer was also observed in the blastema of early regeneration stage of *Ptychodera flava* (Rychel and Swalla, 2008). Four days after fission, two projections of the blastema fused into a single projection that became the proboscis (Fig. 4D, stage 4). At this stage, internal structures of the proboscis began to differentiate (Fig. 5D). The proboscis coelom formed as a cavity inside of the proboscis (Fig. 6B). The features of the stomochord cells, containing prominent vacuoles basally and nuclei more apically, became more distinct.

Undifferentiated cells were located on the periphery of the proboscis coelom, associated with the developing nerve layer (Fig. 6B). Although the epidermis of the blastema was more similar to that found in the trunk of the intact worm in the preceding stages (Figs. 2D and 6A), the epidermis became thinner in width than in the previous stage and contained a smaller number of vacuoles (Fig. 6B). This is because *de novo* epidermal cells probably differentiated in this stage and the epidermal cells have not contained vacuoles yet.

Six days after fission, the proboscis began to move and the collar began to form (Fig. 4E, Stage 5). The proboscis pore and pericardium began to form and the stomochord protruded into the proboscis (Fig. 5E). Undifferentiated cells were associated with the membranes of the proboscis pore and pericardium (Fig. 6C). The proboscis began to move because the longitudinal musculature of the proboscis developed in this stage (Fig. 6D). The first gill slits began to form on the dorsal side of the gut lumen, but gill pores were not yet open. Eight to 10 days after fission, the proboscis and collar grew and the first gill pores opened (Fig. 4F, Stage 6). The proboscis skeleton began to form on the ventral side of the stomochord (Figs. 5F and 6E, F). The gill slit opened through the branchial sac, and the tongue bar, which is a process facing the branchial sac from the dorsal part of the pharynx, developed (Fig. 7A, B). The collar pores, which are the openings of the mesocoel, or the collar coelom, opened to the first branchial sacs at the same time as the first gill pores opened (Fig. 7C-E). In this stage, the epidermis of the regenerating proboscis contained vacuoles that were found in the proboscis of intact worms (Figs. 2C and 5F). All organs in the proboscis were now formed, and in the following stages these organs grew gradually.

The collar grew from stage 5 to stage 9 with both sides fusing at the dorsal and

ventral midlines, and the collar nerve cord formed on the dorsal side. In stage 5 the collar was formed from the lateral edges, and the proboscis was adjacent to the trunk (Fig. 4E). In the cross section, the collar coelom was observed in the lateral sides, but there is no collar structure in the dorsal midline (Fig. 8A) The lateral portions of the collar grew and expanded to the dorsal side in stage 6 (Figs. 4F and 8B). About 2 weeks after fission, both ends of the collar touched each other at the dorsal midline and began to fuse in the middle of the contact area (Figs. 4G and 8C, D, Stage 7). From there, the fusion of the collar extended both anteriorly and posteriorly in a zipper-like manner to produce the tube structure of the collar nerve cord. In contrast to the dorsal side, a tube was not formed on the ventral side. The portions of the collar that extended from the lateral edges first fused at their posterior ends in stage 5. Then, the fusion area expanded anteriorly and was almost complete in stage 7. There were two or three pairs of gill pores in stage 7. About 20 days after fission, the dorsal side of the collar fused (Fig. 4H, Stage 8). The tube separated from the rest of the ectoderm, and the collar nerve cord formed in this stage (Fig. 8E). About 1 month after fission, the proboscis and collar gradually became opaque (Fig. 4I, Stage 9), and the hepatic saccules began to form on the dorsal side of the trunk (Fig. 9A, B). Several hepatic saccules formed at the same time. Two months after fission, the appearance of the regenerands was almost indistinguishable from intact worms. The external and internal features of regeneration are summarized in Table 1.

Regeneration ability of various body parts

As mentioned above, the three kinds of asexual fragments (i.e., the branchiogenital, hepatic, and regenerand) contained gonads. To reveal the relationship between the

presence of gonads and the ability to form a whole body from a small fragment, we examined the regeneration ability of the following body parts when the fragments were produced by artificial cutting. Abbreviations of each body fragment are as follows: proboscis (P), collar (C), branchial region (B), genital region (G), hepatic region (H), caudal region (Ca), proboscis and collar (PC), proboscis, collar, and branchial region (PCB), genital, hepatic, and caudal regions (GHCa), and hepatic and caudal regions (HCa). Results are summarized in Table 2. We concluded that the fragments that contained the branchial or genital regions, where gonads are present, could regenerate completely. Although fragments P, C, and PC healed their cut surfaces in 1 day, they did not regenerate their lost parts; however, they were able to survive for more than 2 months as fragments. In fragment PC (Fig. 10A-C), the gut lumen closed at the cut surface about 1 week after being artificially cut. In contrast to the regenerating fragments, the blastema did not form, and a small number of mesenchymal cells associated with the cut surface were observed (Fig. 10C). Fragments B and G regenerated both anterior and posterior parts in the same manner as that seen in the regenerands. Fragment GHCa also regenerated anterior parts. In fragments B, G, and GHCa, the wounds healed 1 day after cutting, as seen in the regenerands. It took about 4 to 6 days before the blastema formed after cutting, whereas the blastema was formed 1 day after wound healing in the regenerands. After blastema formation, the regeneration processes of fragments B and G occurred in the same manner as that of regenerands. The fragment GHCa also regenerated the lost anterior part in the same manner. Fragment PCB only regenerated its posterior part and did not produce regenerands. Instead it produced hepatic saccules one week after being cut. It is notable that branchiogenital individuals, which stopped producing regenerands, similarly

regenerated hepatic saccules 1 week after the last regenerand production. In fragments H, Ca, and HCa, some regenerated but others did not. When the anterior end of the gut lumen closed after wound healing, the fragment did not regenerate its lost body parts (Fig. 10D). In these fragments, the cut surface was covered by epidermal cells without vacuoles (Fig. 10E, F, arrow), and a small number of mesenchymal cells associated with the ectoderm, but no blastema-like structures, formed (Fig. 10E, F, arrowheads). Although some of these fragments formed a blastema-like projection at the anterior end, the blastema-like projection did not differentiate into the proboscis. In contrast, if the anterior end of the gut lumen did not close after wound healing, the fragment formed a blastema and regenerated the lost anterior parts. Fragments B, G, PCB, and GHCa could grow to form complete worms about 2 months after being cut, whereas fragments H, Ca, and HCa could not because of a high death rate and slow growth in these fragments. Fragments that contained gonads, such as B, G, PCB, and GHCa, formed new gonads after regeneration. In fragments H, Ca, and HCa, which did not contain gonads, we did not observe new gonadal formation.

Discussion

Asexual reproduction in enteropneusts

Asexual reproduction was previously reported in four enteropneust species, *B. capensis*, *B. australiensis*, *Glossobalanus minutus*, and *G. crozieri* (Gilchrist 1923; Packard 1968; Petersen & Ditadi 1971). However, the whole process of asexual reproduction was not described in any of these species. We observed the whole process of asexual reproduction, fragmentation, and subsequent morphogenesis of specimens undergoing asexual reproduction collected from their natural habitat. We succeeded in artificially inducing asexual fragmentation in a process that was basically the same as that of naturally collected worms. The present observation complements the process of asexual reproduction predicted from previous observations. That is, first, a worm divides itself into two fragments at the posterior end of the genital region, and the anterior of these two fragments—the branchiogenital individual—produces regenerands from its posterior end. The mechanism and induction stimulus of fission are still unknown. Packard (1968) suggested that breakage at the end of the genital region seemed to occur for two reasons. First, this region is a natural weak point of balanoglossids. Second, the posterior half of the body of an intact worm is anchored in the substratum by food and sand in the alimentary canal of the hepatic and caudal regions. Consequently, when the anterior body part contracts rapidly, the worm divides at the posterior end of the genital region. In *B. simodensis*, the initial fission of an intact worm and the formation of regenerands occurred only when worms were kept in a substratum, such as sand or mud. In contrast, worms did not undergo reproductive autotomy when they were not kept in the substratum. These findings also indicate that the substratum plays an important role in the fission of enteropneusts. However, this

hypothesis cannot explain other findings in the present work that worms without gonadal contents never underwent fragmentation and that regenerands were produced from the genital region without an anchor-like structure. Moreover, the fact that all hepatic individuals contained a piece of the genital region indicates that the position of initial fission is non-randomly. In the natural habitat, we only found asexually reproducing worms in a non-sexual reproductive season. These series of findings suggest that asexual reproduction in *B. simodensis* is not a simple result of mechanical factors. Other factors, such as seasonal changes in hormones, nervous system, nutritional condition may be involved.

Regeneration in enteropneusts

Gonadal contents perform an essential role in the asexual reproduction and regeneration in *B. simodensis*. Regeneration of several enteropneusts was previously reported: *Glossobalanus minutus* (Dawydoff 1909), *G. crozieri* (Petersen & Ditadi, 1971), *P. flava* (Rao 1955; Nishikawa 1977; Rychel & Swalla 2008), and *Saccoglossus kowalevskii* (Tweedel 1961). The regeneration pattern seems to vary among taxa of the class Enteropneusta. In the family Ptychoderidae, which consists of the genera *Glossobalanus*, *Balanoglossus,* and *Ptychodera*, small fragments of a worm tend to be able to regenerate anterior body parts. There is, however, a controversy as to whether anterior-half fragments can regenerate their lost posterior body parts (Affirmation: Gilchrist 1923; Packard 1968; Petersen & Ditadi 1971. Negation: Dawydoff 1909; Rao 1955; Nishikawa 1977). According to our observations in *B. simodensis*, fragments that contain the branchiogenital or genital region can regenerate their lost posterior parts, such as hepatic saccules. Furthermore, recent report showed that anterior fragments of *P.*

flava regenerated posterior parts when worms were kept under a good condition (Humphreys *et al.* 2010) For these reasons, we believe that species of the family Ptychoderidae, which have high regeneration abilities, can regenerate their posterior parts from anterior fragments containing the genital region, if they are kept in a satisfactory condition.

In enteropneusts, the cells that participate in blastema formation and morphogenesis during regeneration have not been identified. In hydras and planarians, numerous totipotent cells are distributed throughout the body and play important roles in regeneration (Baguñà 1998; Bosch 1998). In annelids, although there are a small number of neoblast-like cells, regeneration is mainly achieved through dedifferentiation and cell activation (Thouveny & Tassava 1998; Myohara *et al*. 1999). Echinoderms regenerate from coelomocytes and dedifferentiated cells (Candia Carnevali 2006). Vertebrates regenerate through dedifferentiation and cell activation without the contribution of multipotent stem cells (Stocum 1998). In *B. simodensis* we could not detect any neoblast-like cells that were round with a large nucleus in intact worms. Benito and Pardos (1997) examined the ultrastructure of *G. minutus*, which reproduces asexually, and also did not find neoblast-like cells. Recently, Rychel and Swalla (2008) examined cell proliferation and apoptosis in the ptychoderid *P. flava*, and showed that mesenchymal cells contribute to form regenerating structures. They also reported that the mesenchymal cells are associated with the nerve layer and predicted that hemichordates may have nerve-dependent regeneration. Our results show that cells contributing to regeneration are distributed in the whole trunk region. However, together with the fact that all naturally produced hepatic individuals contained the genital region, the low regeneration ratio of fragments without the genital region shows

that the contents of the genital region perform essential roles in regeneration.

Asexual reproduction and metamorphosis

In *B. simodensis*, morphogenesis during asexual reproduction basically followed that of the normal developmental process during metamorphosis. Although the body plans of regenerands and larvae are different, order of morphogenesis and tissue movement are similar between the asexual reproduction and metamorphosis. The stomochord differentiated from the anterior part of the esophageal endoderm, the posterior part of which differentiated into the esophageal epithelium during both asexual reproduction and normal development (Miyamoto and Saito, unpublished data). In *P. flava*, however, the stomochord is formed from the ventral side of the proboscis ectoderm during regeneration, whereas it seems to develop from the endoderm during metamorphosis (Rao 1955; Ruppert *et al*. 2004; Rychel & Swalla 2008). This difference in stomochord formation between the two species seems to be due to differences in their early regeneration processes. In *B. simodensis*, the mouth never closed during regeneration, and the esophagus differentiated between the epidermis of the blastema and the gut epithelium. Then, the stomochord differentiated from the anterior part of the esophageal epithelium. In contrast, in *P. flava*, the mouth closed and re-opened after the formation of the blastema (Rao 1955; Rychel & Swalla 2008). Because the stomochord formed through the invagination of the ventral part of the blastemal epidermis, the stomochord forms from ectodermal tissue in *P. flava*. The glomerulus and the proboscis skeleton formed following stomochord invagination. This process is identical in both the normal development and morphogenesis of regenerands. However, the timing of pericardium formation differs between these two types of morphogenesis. Because the

pericardium develops in the larval stage, it is present in the early metamorphic stage when the stomochord begins to invaginate. In contrast, the pericardium develops in stage 5 when the stomochord has already invaginated into the proboscis during the morphogenesis of regenerands.

We observed the morphological aspects of asexual reproduction and examined the regeneration abilities of each body part in *B. simodensis*. Asexual reproduction and regeneration ability are considered to be related phenomena because the fissiparous process is carried out through the fragmentation of an intact animal and subsequent regeneration from the small fragments (Sánchez Alvarado 2000; Bely & Wray 2001). Since morphogenesis of asexually produced fragments is basically the same as the regeneration of cut or injured fragments in *B. simodensis*, we believe that these two types of morphogenesis are comparable. Moreover, because we can easily obtain a large number of specimens using asexual reproduction, this system is very useful for studying the regeneration of enteropneusts. The present study provides a basis for understanding the regeneration mechanisms of enteropneusts. Knowledge of the regeneration of enteropneusts should provide important insight into the mechanism and the evolution of regeneration in deuterostomes. Furthermore *B. simodensis* is a promising model organism for studying regeneration and developmental biology in enteropneusts because this species is the only one whose asexual reproduction and normal development have been observed under laboratory conditions.

Acknowledgments

We thank Hiroshi Wada for critical reading of the earlier version of the manuscript, and the staffs of Shimoda Marine Research Center (University of Tsukuba) for their kind assistance and hospitality during this work. MN was supported by the Sasakawa Scientific Research Grand from The Japan Science Society (19-411), and is a recipient of a JSPS Research Fellow (20·639). This study is contribution No. xxx from Shimoda Marine Research Center.

References

Assheton, R. 1908. A new species of *Dolichoglossus*. *Zool. Anz.* **33**, 517–520.

Baguñà, J. 1998. Planarians. In *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans* (Ed. P. Ferretti & J. Géaudie), pp. 135–165. John Wiley and Sons, New York.

Bely, A. E. 2006. Distribution of segment regeneration ability in the Annelida. Integr *Comp. Biol.* **46**, 508–518.

Benito, J. & Pardos, F. 1997. Hemichordata. In *Microscopic Anatomy of the Invertebrates*, Vol. 15 (Ed. F. W. Harrison & E. E. Ruppert), pp. 15–101. Wiley-Liss, New York.

Bosch, T. C. G. 1998. Hydra. In *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans* (Ed. P. Ferretti & J. Géaudie), pp. 111–134. John Wiley and Sons, New York.

Brockes, J.P., Kumar, A. & Velloso, S. P. 2001. Regeneration as an evolutionary variable. *J. Anat.* **199**, 3–11.

Candia Carnevali, M. D. 2006. Regeneration in echinoderms: Repair, regrowth, cloning. *Invert. Surv. J.* **3**, 64–76.

Cori, C. I. 1902. Über das Vorkommen des *Polygrordius* und *Balanoglossus* (*Ptychodera*) im Triester Golfe. *Zool. Anz.* **25**, 361–365.

Dawydoff, C. 1909. Regenerationsprozrss bei den Enteropneusten. *Z. Wiss. Zool.* **93**, 237–304.

Duncan, P. B. 1987. Burrow structure and Burrowing activity of the funnel-feeding enteropneust *Balanoglossus aurantiacus* in Bogue Sound, North Carolina, USA. *Mar. Ecol-Evol. Persp.* **8**, 75–95.

Gilchrist, J. D. F. 1923. A form of dimorphism and asexual reproduction in *Ptychodera capensis* (Hemichordata). *J. Linn. Soc. Lond.* **35**, 393–398.

Hill, J. P. 1894. On a new species of Enteropneusta (*Ptychodera australiensis*) from the coast of New South Wales. *Proc. Linn. Soc. N. S. W.* **10**, 1–42.

Hinrichs, O. & Jacobi, L. 1938. *Saccoglossus pygmaeus*, eine neue Enteropneustenart aus der suedlichen Nordsee. *Zool. Anz.* **121**, 25–32.

Humphreys, T., Sasaki, A., Uenishi G., Taparra, K., Arimoto, A. & Tagawa, K. 2010. Regeneration in the hemichordate *Ptychodera flava*. *Zool. Sci.* **27**, 91-95.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. & Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. *Science*

282, 2095–2098.

Kirk, H. B. 1938. Notes on the breeding habits and the early development of *Dolochoglossus otagoensis* Benham. *Trans. Proc. R. Soc. N. Z.* **68**, 49–50.

Miyamoto, N. & Saito, Y. 2007. The morphology and development of a new species of *Balanoglossus* (Hemichordata: Enteropneusta: Ptychoderidae) from Shimoda, Japan. *Zool. Sci.* **24**, 1278–1285.

Myohara, M., Yoshida-Noro, C., Kobari, F. & Tochinai, S. 1999. Fragmenting oligochaete *Enchytraeus japonensis*: A new material for regeneration study. *Dev. Growth Differ.* **41**, 549–555.

Nishikawa, T. 1977. Preliminary report on the biology of the enteropneust, *Ptychodera flava* Eschscholtz, in the vicinity of Kushimoto, Japan. *Publ. Seto Mar. Biol. Lab.* **23**, 393–419.

Packard, A. 1968. Asexual reproduction in *Balanoglossus* (Stomochordata). *Proc. R. Soc. Lond. B Biol. Sci.* **171**, 261–272.

Petersen, J.A. & Ditadi, A. S. F. 1971. Asexual reproduction in *Glossobalanus crozieri* (Ptychoderidae, Enteropneusta, Hemichordata). *Mar. Biol*. **9**, 78–85.

Rao, K. P. 1955. Morphogenesis during regeneration in an enteropneust. *J. Anim.*

Morphol. Physiol. India **1**, 1–7.

Rychel, A. L. & Swalla, B. J. 2008. Anterior regeneration in the hemichordate *Ptychodera flava*. *Dev. Dyn.* **237**, 3222–3232.

Ruppert, E. E., Fox, R.S. & Barnes, R. D. 2004. Introduction to the Deuterostomia and Hemichordata. In *Invertebrate zoology*. pp. 857–871. Brooks/cole-Thompson Learning, Belment.

Sánchez Alvarado, A. 2000. Regeneration in the metazoans: Why does it happen? *Bioessays* **22**, 578–590.

Sánchez Alvarado, A. & Tsonis, P. A. 2006. Bridging the regeneration gap: Genetic insights from diverse animal models. *Nat. Rev. Genet.* **7**, 873–882.

Spengel, J. W. 1893. Die Enteropneusten des Golfes von Neapel und der angrensenden Meeresabschnitte. *Fauna Flora Golf Neapel, Monogr.* **18**, 1–758.

Stocum, 1998. Bridging the gap: Restoration of structure and function in humans. In *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans* (Ed. P. Ferretti & J. Géaudie), pp. 411–450. John Wiley and Sons, New York.

Thouveny, Y. & Tassava, R. A. 1998. Regeneration through phylogenesis. In *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans* (Ed. P. Ferretti

& J. Géaudie), pp. 9–43. John Wiley and Sons, New York.

Tweedel, K. S. 1961. Regeneration of the enteropneust, *Saccoglossus kowalevskii*. *Biol. Bull.* **120**, 118–127.

Willey, A. 1899. Enteropneusta from the South Pacific, with notes on the West Indian species. *Willey's Zoological Results* **3**, 223–324.

Wilmut, I., Schieke, A. E., McWhir, J., Kind, A. J. & Compbell, K. H. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.

Figure Legends

Figure 1. Overview of the acorn worm *Balanoglossus simodensis* and its regenerating fragments. **A:** An intact worm. **B:** Branchiogenital individual (lower, big fragment) and regenerand (upper, small fragment). **C:** Regenerating hepatic individual. br, branchial region; c, collar; cr, caudal region; gr, genital region; hr, hepatic region; hs, hepatic saccule; p, proboscis.

Figure 2. Internal morphology of intact worms. **A**, Scheme of the longitudinal section of the proboscis, collar and anterior end of the trunk. **B–D:** Sections of the boxed areas in A. Asterisks in C and D indicate vacuoles of the proboscis and trunk epidermal cells. Epidermal cells in intact worms contain prominent vacuoles to secrete mucus. c, collar; cc, collar coelom; eph, pharyngeal epithelium; gl, gut lumen; glm, glomerulus; gs, gill slit; lm, longitudinal musculature; mo, mouth; nl, nerve layer; p, proboscis; pc, proboscis coelom; prc, pericardium; ps, proboscis skeleton; stc, stomochord; t, trunk; tc, trunk coelom

Figure 3. Scheme showing the process of asexual reproduction in *B. simodensis*. An intact worm divides into two fragments: the branchiogenital individual and the hepatic individual (Step 1). The posterior end of the branchiogenital individual elongates (Step 2) and produces regenerands from the posterior end in series (Step 3). Each fragment regenerates into an intact worm (Step 4).

Figure 4. External morphogenesis during regeneration of regenerands in *B. simodensis*. Anterior is at left, and the dorsal view is presented. **A:** Stage 1. Just after fission: the wound is still open. **B:** Stage 2. One day after fission: the wound is healed. **C:** Stage 3. Two days after fission: a blastema with two projections has formed. **D:** Stage 4. Four days after fission: the two projections of the blastema have fused to become a single projection. **E:** Stage 5. Six days after fission: the proboscis has differentiated (from a single projection of the blastema) and the collar primordia are formed at the lateral edges. **F:** Stage 6. Eight days after fission: the inset shows the newly formed gill pores (arrow). **G:** Stage 7. Two weeks after fission: the collar begins to fuse on the dorsal side. Inset 1 shows the proboscis pore (arrow). Inset 2 shows the gill pore (arrow). **H:** Stage 8. Twenty days after fission: the collar has fused. **I:** Stage 9. One month after fission: the proboscis and collar have begun to become opaque. b, blastema; c, collar; g, gonad; gs, gill slit; p, proboscis.

Figure 5. Internal morphogenesis during regeneration of regenerands in *B. simodensis*. Anterior is at left and dorsal is towards the top. **A:** Stage 1. Arrows indicate wounds. **B:** Stage 2. Arrows indicate closed wounds. **C:** Stage 3. A blastema filled with undifferentiated cells has formed. **D:** Stage 4. Proboscis organs begin to differentiate. **E:** Stage 5. The proboscis pore has formed. **F:** Stage 6. The proboscis skeleton has formed on the ventral side of the stomochord. **G-I:** From stage 7 through stage 9, each organ in the proboscis grows gradually. c, collar; g, gonad; gl, gut lumen; glm, glomerulus; gs, gill slit; lm, longitudinal muscle; nc, nerve cord; p, proboscis; pc, proboscis coelom; pp, proboscis pore; prc, pericardium; ps, proboscis skeleton; stc, stomochord.

Figure 6. Detail of regeneration of the proboscis. **A:** High magnification of stage 3 blastema filled with undifferentiated cells (inset). New type of epithelium differentiated between the blastema ectoderm and gut epithelium (asterisk). The arrow indicates the developing nerve layer. **B:** High magnification of the proboscis in stage 4. The proboscis organ is developing. The region of the future stomochord and pharyngeal epithelium (asterisk) is expanded. Inset indicates the developing nerve layer. **C:** Developing proboscis in a stage 5 regenerand. **D:** High magnification of the proboscis in stage 5 showing developing longitudinal musculature. **E:** Pharyngeal region of a stage 6 regenerand. **F:** Boxed area in **E** showing the proboscis skeleton developing on the ventral side of the stomochord. gl, gut lumen; glm, glomerulus; lm, longitudinal musculature; nl, nerve layer; pc, proboscis coelom; pp, proboscis pore; prc, pericardium; ps, proboscis skeleton; stc, stomochord.

Figure 7. The first gill slit and collar pore in a stage 6 regenerand. **A:** Gill slits (boxed area) formed on the dorsal part of the gut lumen. Anterior is to the left and dorsal is towards the top. **B:** High magnification of the boxed area in **A** showing a developing tongue bar. **C-E:** Series of sections showing that the collar pore opens to the branchial sac of the first gill slit. bs, branchial sac; c, collar; cc, collar coelom; cp, collar pore; gl, gut lumen; gp, gill pore; p, proboscis.

Figure 8. Nerve cord formation during regeneration of regenerands in *B. simodensis*. Dorsal is at top. **A:** Stage 5. **B:** Stage 6. **C:** Stage 7. Anterior part of the collar in which both sides have not fused. Arrows in A-C indicate portions of the collar that expanded from the lateral edges. Arrowheads in A-C indicate the dorsal midlines. **D:** Stage 7. Middle part of the collar in which both sides have began to fuse (arrow). **E:** Stage 8. The nerve cord has separated from the rest of the ectoderm. **F:** The cross section of an intact worm. cc, collar coelom; ep, epidermis; gl, gut lumen; nc, nerve cord; stc, stomochord.

Figure 9. Formation of hepatic saccules during regeneration of regenerands in *B. simodensis*. **A:** Dorsal view of a stage 9 regenerand. **B:** Longitudinal section of a stage 9 regenerand. Anterior is at left. Arrowheads show regenerating hepatic saccules. **C**, **D:** Dorsal view and longitudinal section of intact worms. gl, gut lumen.

Figure 10. Morphology of non-regenerated fragments. **A-C:** Fragment of the proboscis and collar. Arrow in **A** indicates the cut surface. **B:** Longitudinal section of **A** shows that the gut is closed and the blastema did not form. Anterior is to the left and dorsal is towards the top. **C:** High magnification of the boxed area in B. Although a small number of mesenchymal cells were observed, no blastema-like structure formed. **D-F:** Fragment of the caudal region. **D:** Ventral view. Gut lumen is closed, and the asterisk indicates where the gut opening was. **E:** Longitudinal section of **D**. Anterior is to the left and dorsal is towards the top. **F:** High magnification of the boxed area in **E**. Arrow shows the undifferentiated state of the ectoderm covering the cut surface. A small number of mesenchyme cells are associated with the cut surface (arrowheads), and no blastema-like structure was observed. c, collar; gl, gut lumen; p, proboscis.

Tables

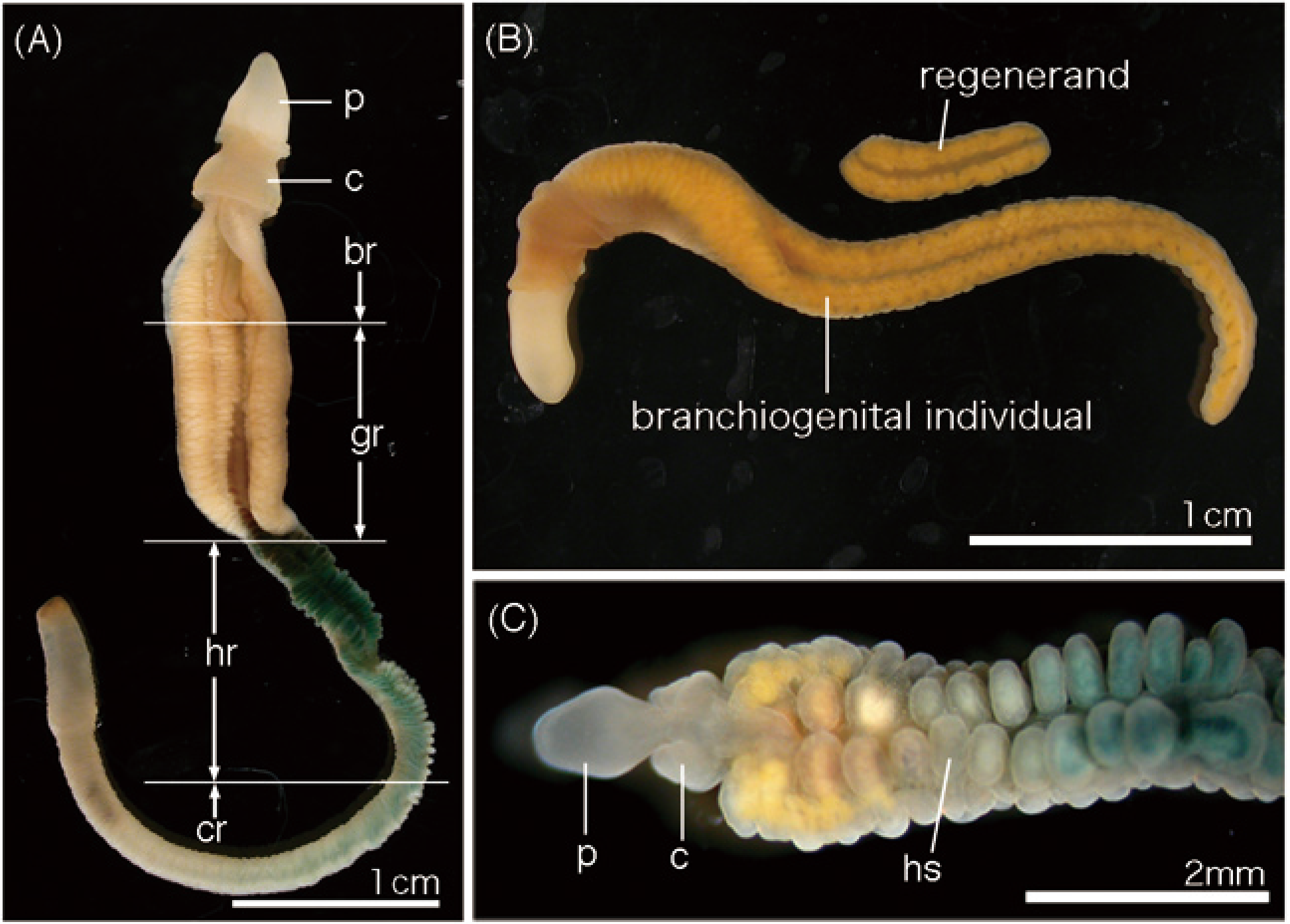
Table 1. The Stages of Regeneration in *Balanoglossus simodensis* (21°C–23°C)

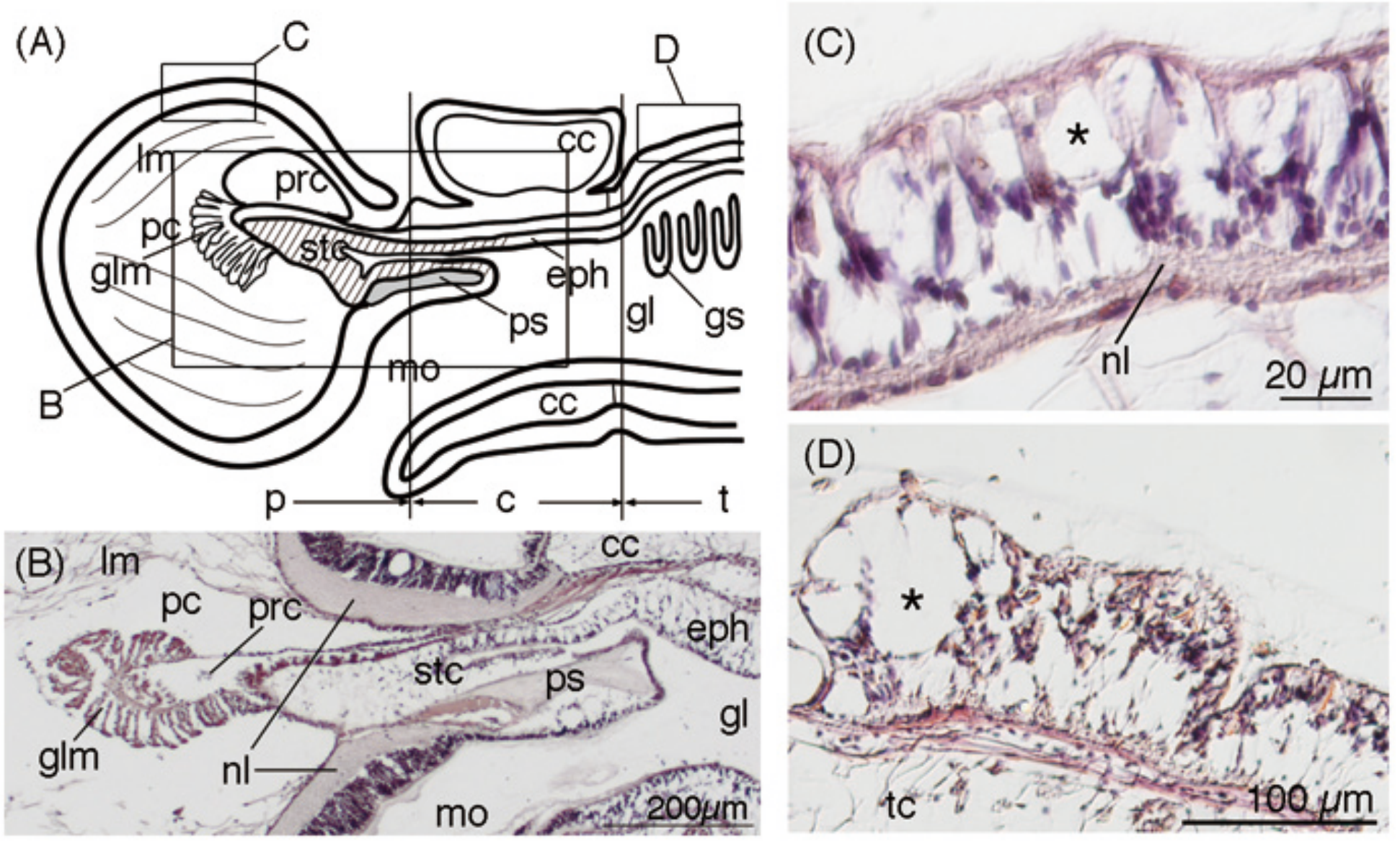
Stage no.	Days after fission	External features	Internal features
1	0	Fragmentation	Fragmentation
2	1	Wound healing	Wound healing
3	2	Two-projection blastema	Blastema filled with undifferentiated cells
4	4	One-projection blastema	Formation of glomerulus and stomochord
5	6	Movement of proboscis; collar formation	Formation of proboscis pore, pericardium, and proboscis musculature
6	8–10	First gill pores	Formation of proboscis skeleton
7	14	Beginning of collar concrescence	Formation of collar cord
8	20	Completion of collar concrescence	↓
9	30	Formation of hepatic saccules	Formation of hepatic saccules

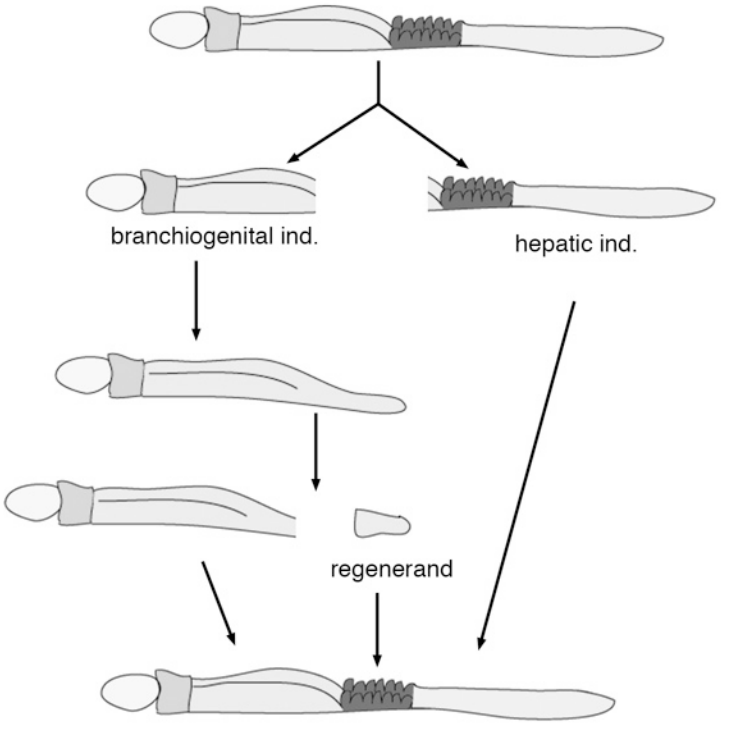
Table 2. Regeneration Ability of Each Body Part of *Balanoglossus simodensis*

	Total no. of examined individuals	Healing	Blastema	Anterior	Posterior	Gonad
P	13	13	NA	NA	0	0
C	10	10	0	0	0	0
B	8	8	6	6	6	6
G	16	16	16	16	16	16
H	34	34	18	15	18	ND
Ca	34	34	3	2	4	0
PC	13	13	NA	NA	0	0
PCB	11	11	NA	NA	11	11
GHCa	11	11	11	11	11	11
HCa	36	36	23	10	29	ND

Numbers in the table indicate the number of individuals that exhibited regeneration. ND indicates “no data.” NA indicate “not applicable.” The column headings are as follows: Healing = wound healing; Blastema = blastema formation at the anterior end of a cut fragment; Anterior = regeneration of anterior body parts, such as the proboscis, collar, and gill slits; Posterior = regeneration of posterior body parts, such as the anus; Gonad = gonad maturation in regenerating fragment. P, proboscis; C, collar; B, branchiogenital region; G, genital region; H, hepatic region; Ca, caudal region; PC, proboscis and collar; PCB, proboscis, collar, and branchiogenital region; GHCa, genital, hepatic, and caudal


(A)
p
c
br
gr
hr
cr
1 cm
(B)
regenerand
branchiogenital individual
1 cm
(C)
p
c
hs
2mm


(A)
C
D
lm
prc
cc
pc
stc
glm
eph
ps
gl
gs
mo
B
cc
p
c
t
(B)
lm
pc
prc
cc
eph
stc
ps
gl
glm
nl
mo
200μm
(C)
*
nl
20 μm
(D)
*
tc
100 μm

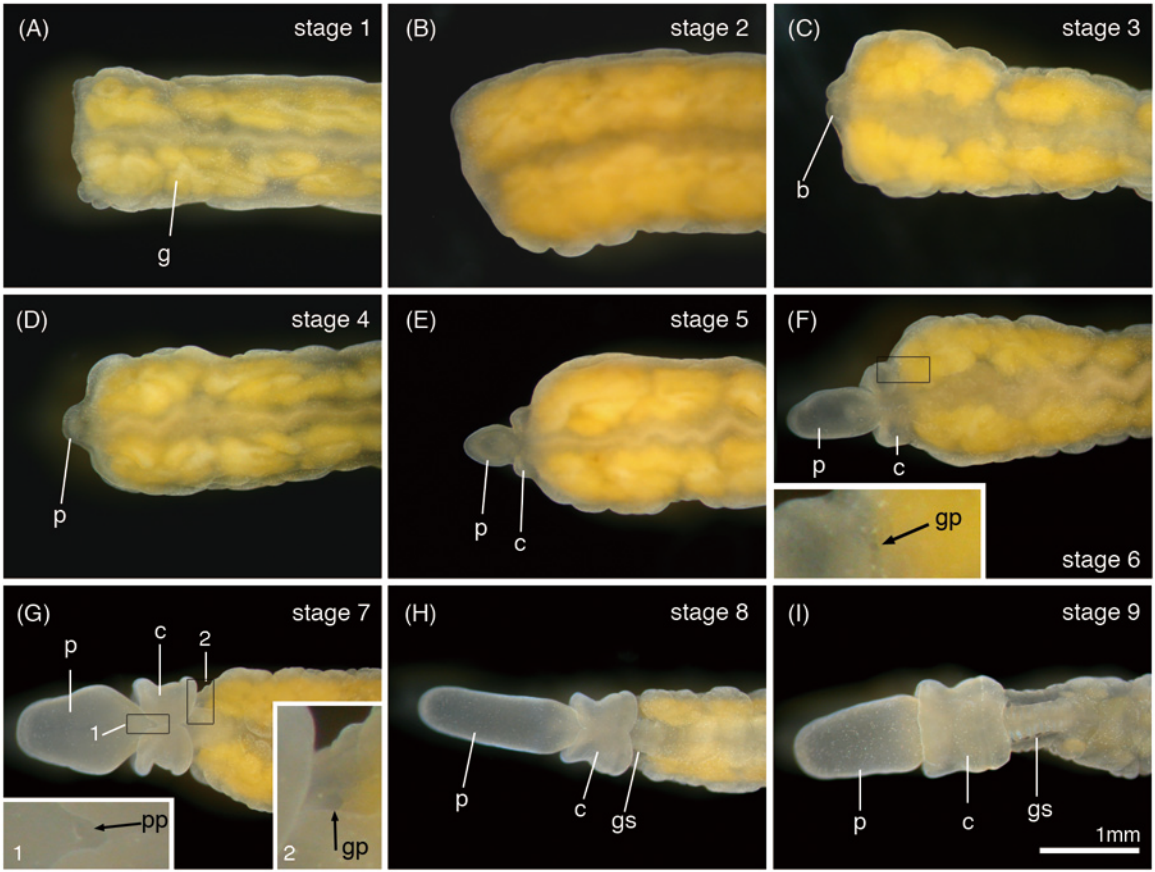


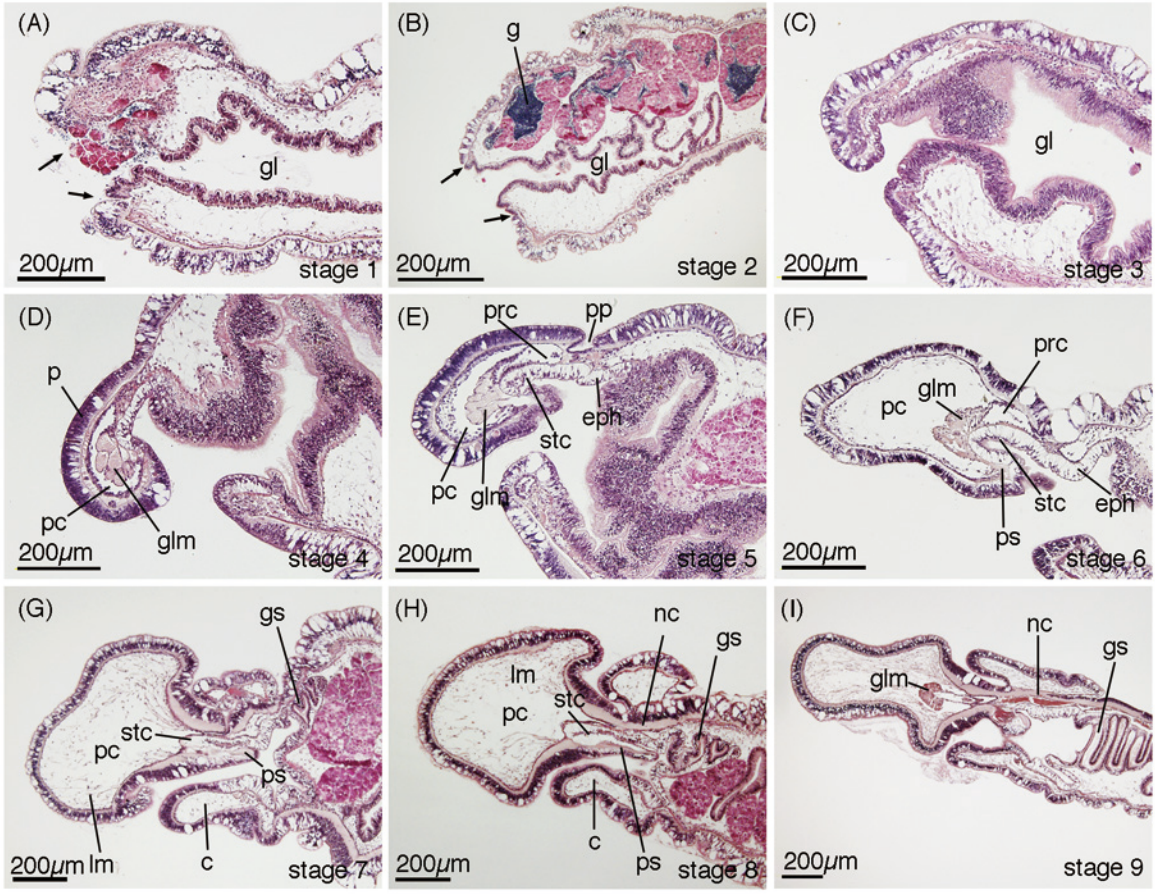
Step 1
autotomy or
artificial cut

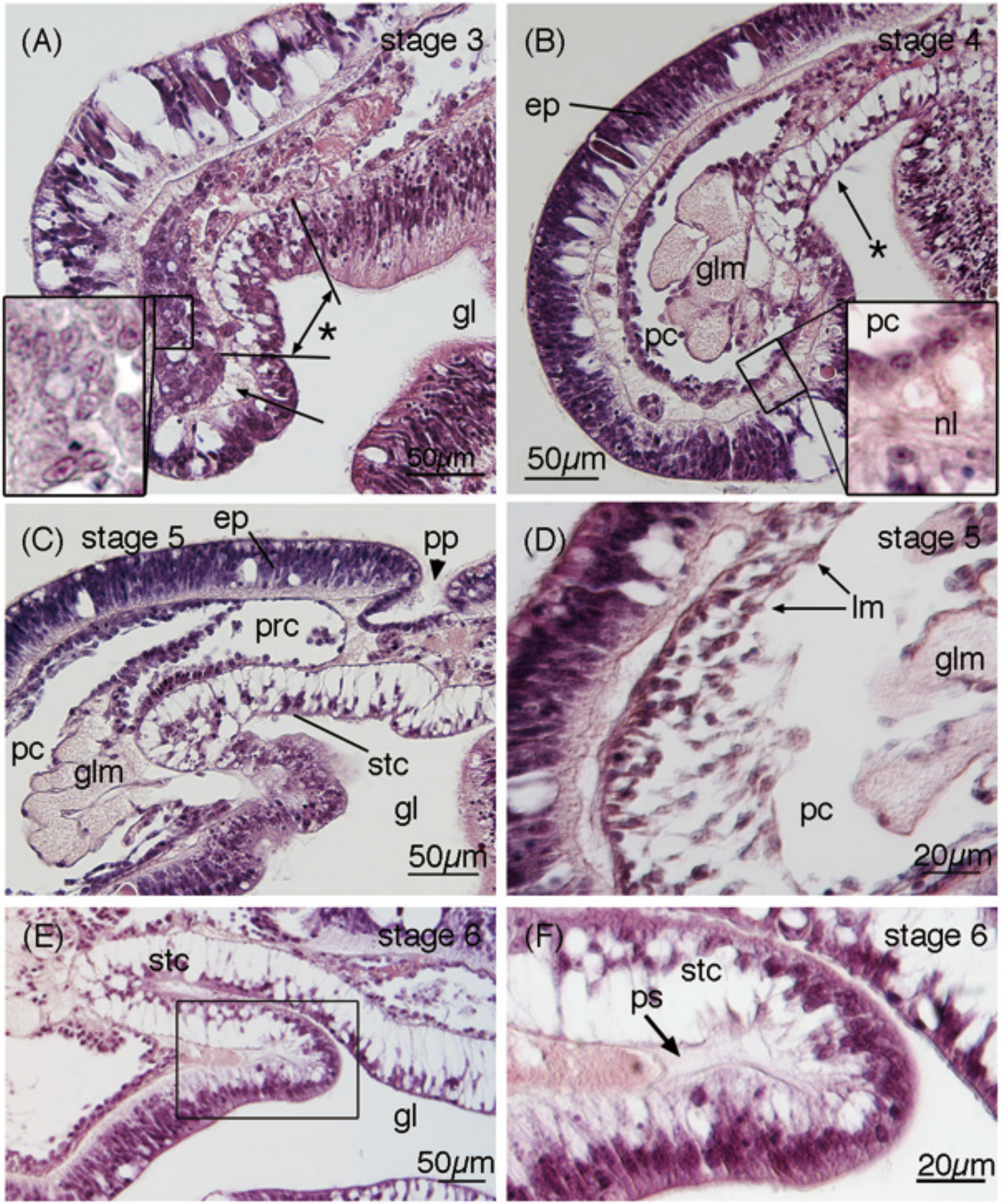
Step 2
elongation

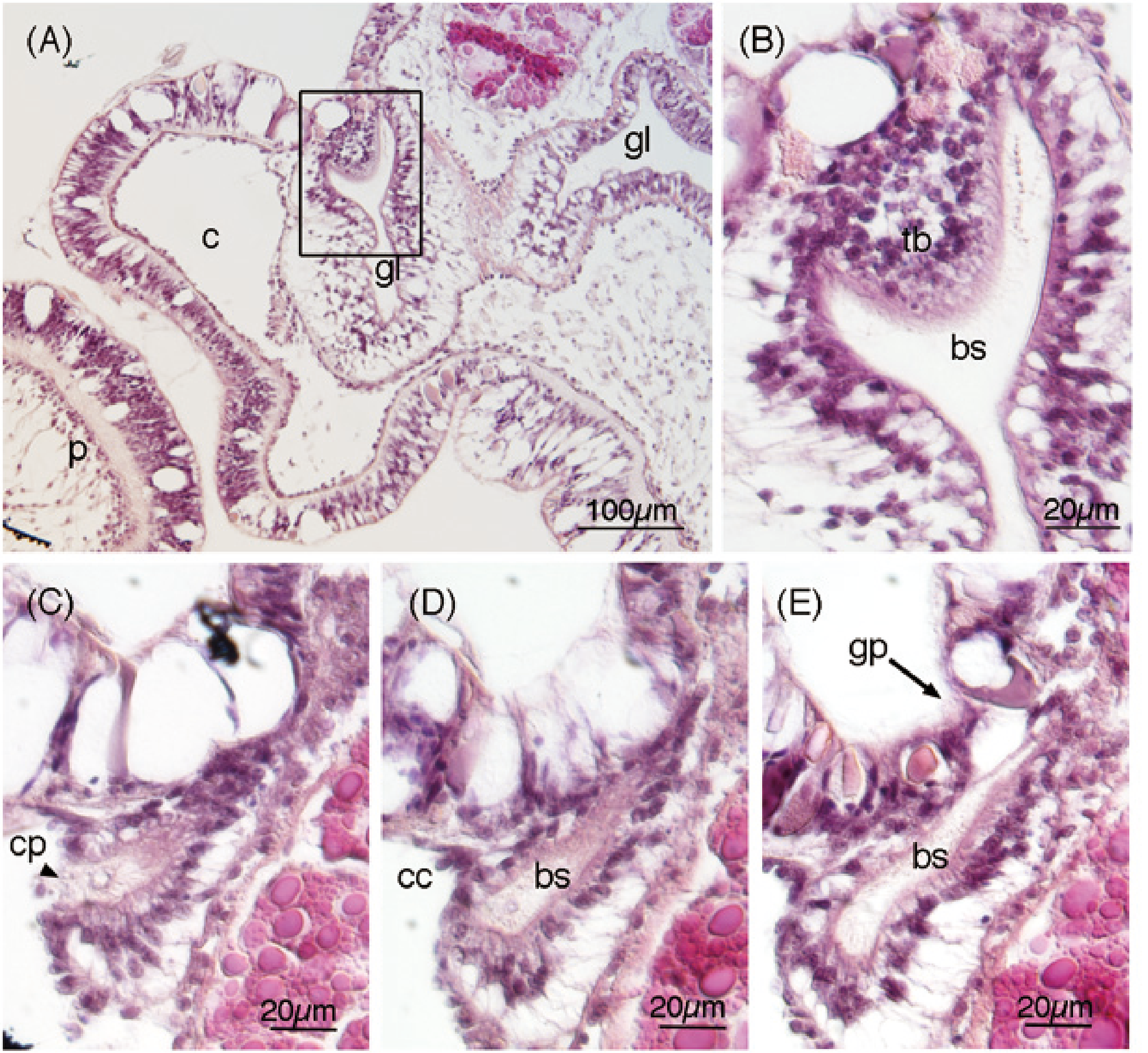
Step 3
fragmentation
through autotomy

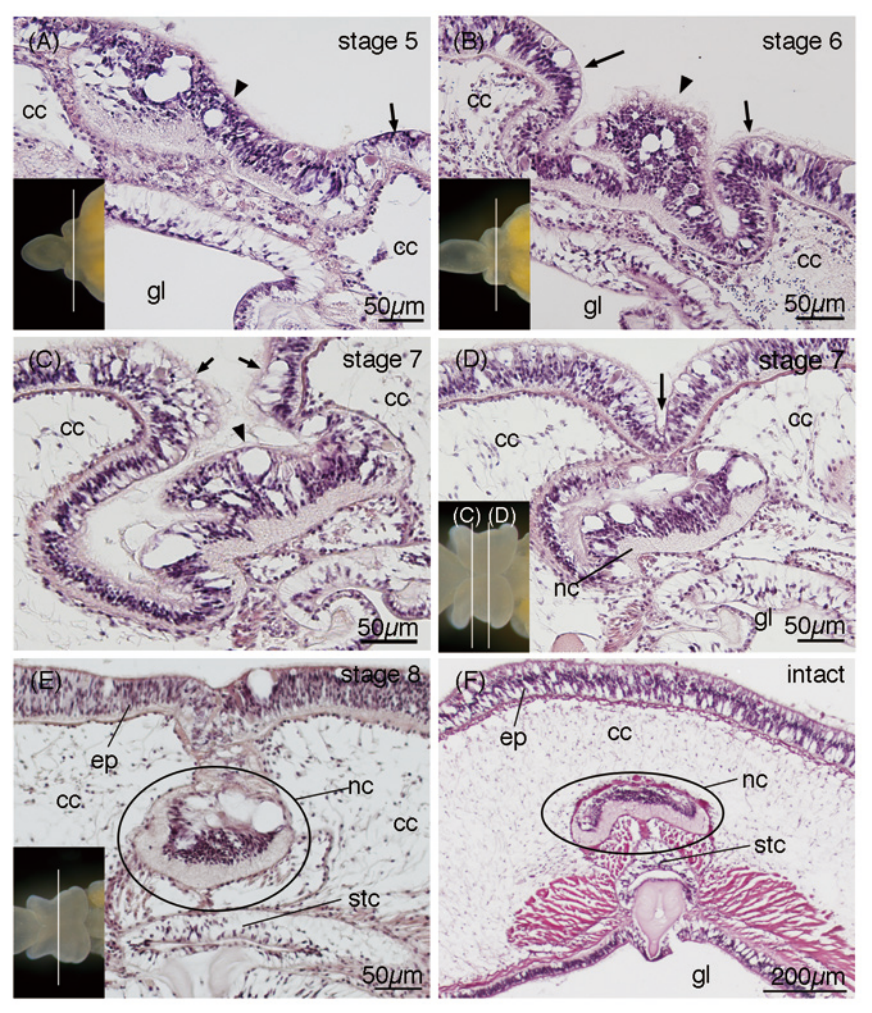
Step 4
regeneration

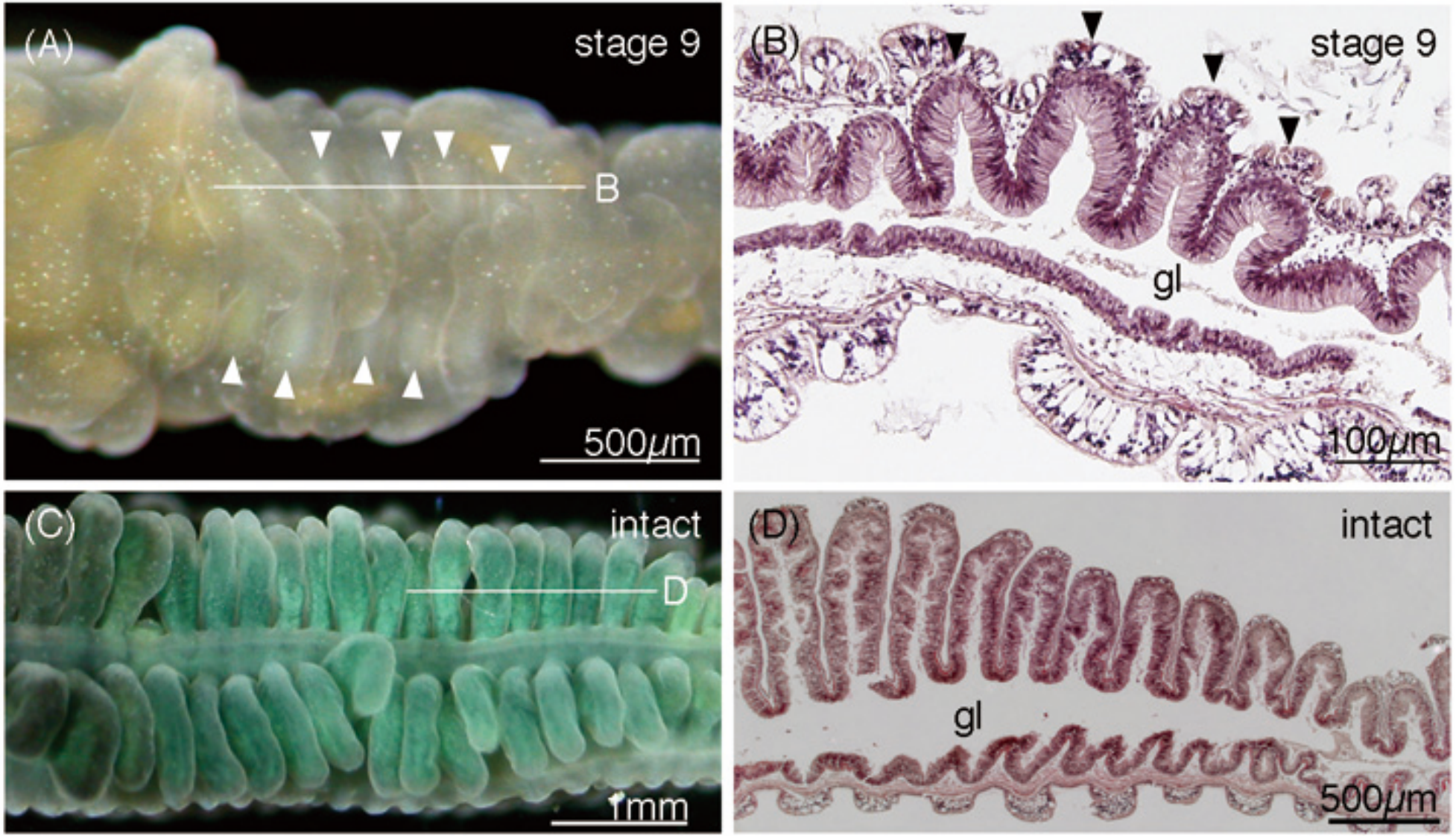

(A)
stage 1
g
(B)
stage 2
(C)
stage 3
b
(D)
stage 4
p
(E)
stage 5
p
c
(F)
p
c
gp
stage 6
(G)
stage 7
p
c
2
1
pp
1
2
gp
(H)
stage 8
p
c
gs
(I)
stage 9
p
c
gs
1mm

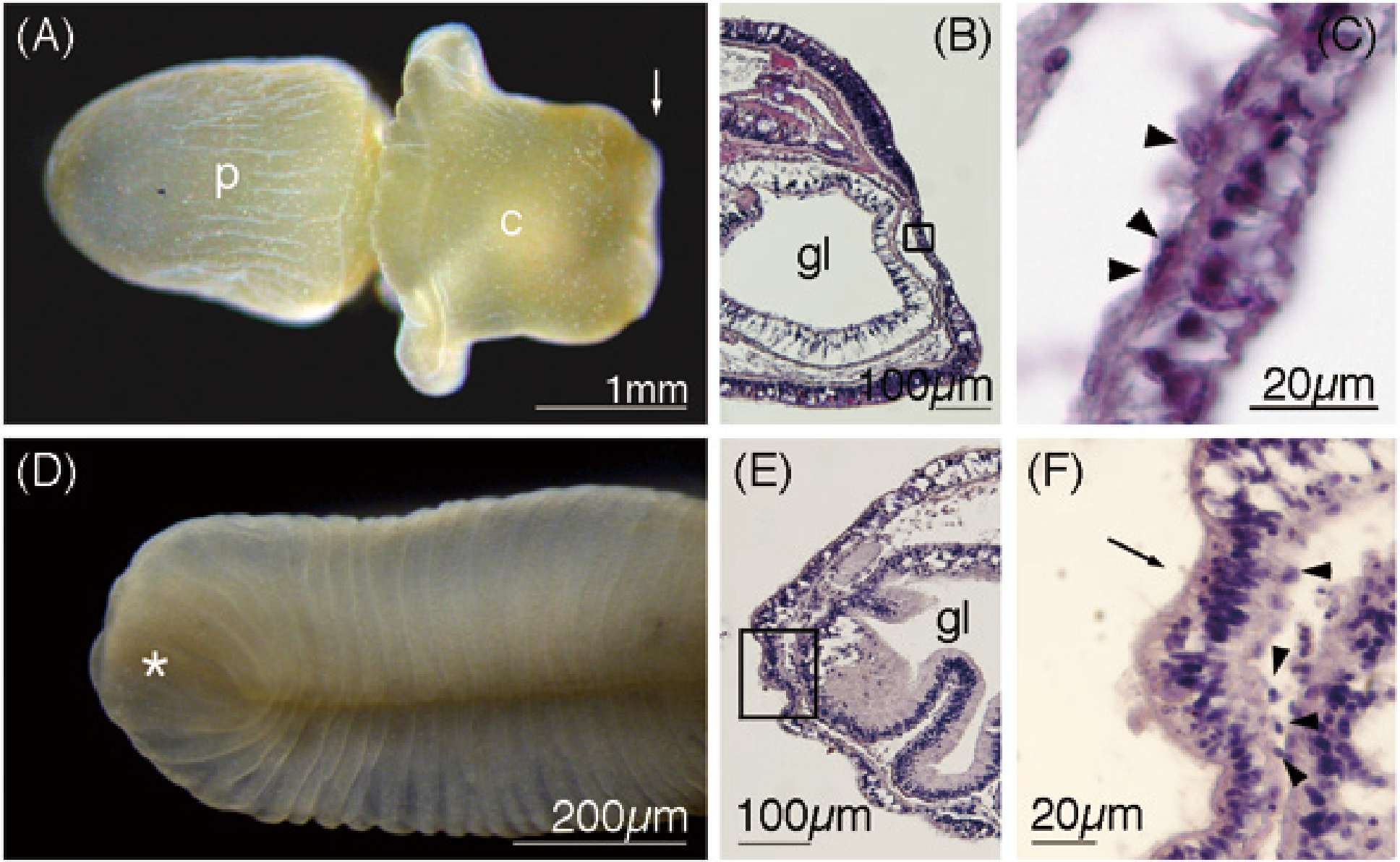

(A)
gl
200μm
stage 1
(B)
g
gl
200μm
stage 2
(C)
gl
200μm
stage 3
(D)
p
pc
glm
200μm
stage 4
(E)
prc
pp
eph
stc
pc
glm
200μm
stage 5
(F)
prc
glm
pc
stc
eph
ps
200μm
stage 6
(G)
gs
stc
pc
ps
200μm
lm
c
stage 7
(H)
nc
gs
lm
pc
stc
c
ps
200μm
stage 8
(I)
nc
gs
glm
200μm
stage 9


(A)
stage 3
gl
*
50μm
(B)
stage 4
ep
glm
pc
*
pc
nl
50μm
(C) stage 5
ep
pp
prc
pc
glm
stc
gl
50μm
(D)
stage 5
lm
glm
pc
20μm
(E)
stage 6
stc
gl
50μm
(F)
stage 6
stc
ps
20μm


(A)
gl
c
gl
p
100μm
(B)
tb
bs
20μm
(C)
cp
20μm
(D)
cc
bs
20μm
(E)
gp
bs
20μm


(A)
stage 5
cc
cc
gl
50μm
(B)
stage 6
cc
cc
gl
50μm
(C)
stage 7
cc
cc
50μm
(D)
stage 7
cc
cc
(C) (D)
nc
gl
50μm
(E)
stage 8
ep
cc
nc
cc
stc
50μm
(F)
intact
ep
cc
nc
stc
gl
200μm


(A)
stage 9
B
500μm
(B)
stage 9
gl
100μm
(C)
intact
D
1mm
(D)
intact
gl
500μm


(A)
p
c
1mm
(B)
gl
100μm
(C)
20μm
(D)
*
200μm
(E)
gl
100μm
(F)
20μm