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Authors: Chen, Qiran, Takagi, Masako, Mogi, Makoto, Kikuchi, Miki, Saito, Yudai, et al.

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External Asymmetry and Pectoral Fin Loss in the Bamboo Sole (*Heteromycteris japonica*): Small-Sized Sole with Potential as a Pleuronectiformes Experimental Model

Qiran Chen[1], Masako Takagi[1], Makoto Mogi[1], Miki Kikuchi[1], Yudai Saito[1], Shunya Nakamura[1], Hayato Yokoi[1], Tadahisa Seikai[2], Susumu Uji[3]*, and Tohru Suzuki[1†]

[1]*Laboratory of Marine Life Science and Genetics, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*
[2]*Faculty of Marine Biology, Fukui Prefectural University, Obama 917-0003, Japan*
[3]*National Research Institute of Aquaculture, Fisheries Research Agency, Minami-Ise, Mie 516-0193, Japan*

Pleuronectiform fish develop marked external asymmetry in eye location and skin color at metamorphosis. The bamboo sole, *Heteromycteris japonica*, also exhibits loss of the pectoral fins at metamorphosis. Because of its small body size, short generation time, and long spawning season, we focused on the bamboo sole as an experimental model to investigate metamorphic asymmetry and pectoral fin loss during development. In the present study, we utilized a small-scale culture system to evaluate bamboo sole larvae and larval development, and a microinjection system for fertilized eggs. The culture system described here uses an 18 L culture tank for rotifers (the first diet for larvae) and 5 L plastic beakers for larval culture. Under this system, most larvae completed metamorphosis, including one-eye migration and pigmentation of the ocular side, by 23 days post-fertilization (dpf) at 25°C. Larvae at density of 120–150 per liter were grown from hatching to 23 dpf with a survival ratio of 60–75% per beaker. Pectoral fins, including coracoid and disk cartilage, formed but were completely lost in late metamorphosis without formation of proximal radials and fin rays. The microinjection system designed here is adequate for the bamboo sole and allows injection of 100 one-cell-stage embryos per day. We expect that the culture and microinjection systems described here will facilitate the use of the bamboo sole as an experimental model organism in developmental biology.

Key words: Pleuronectiformes, sole, metamorphosis, larval development, pectoral fin loss, microinjection

INTRODUCTION

Metamorphic development of species of Pleuronectiformes, including flounders, turbots, and soles (refer to Fig. 1A), is characterized by transition from an externally symmetric to a left-right asymmetric arrangement of eye location and skin color, making them attractive as model organisms in endocrinology and developmental biology research. Enhancement of metamorphosis by thyroid hormones, correlation between the Nodal pathway and the lateralization of eye-sidedness, and the migration route of the adult-type chromatophores that color the ocular side have been reported (Inui and Miwa, 1985; Suzuki et al., 2009; Washio et al., 2013). Recently, it was suggested that differences in illumination result in a retinoic acid gradient between the bilateral skins, which leads to ocular-side pigmentation (Shao et al., 2017). Obtaining a more complete understanding of the system that controls metamorphic development in the order Pleuronectiformes will require investigations at both the molecular and cellular levels.

Studies along this line of research have hitherto focused on large species commonly used in aquaculture, such as Japanese flounder (*Paralichthys olivaceus*), summer flounder (*Paralichthys dentatus*), and turbot (*Scophthalmus maximus*) (Brinon et al., 1993; Inui and Miwa, 1985; Schreiber and Specker, 1998). A disadvantage of using these species in developmental biology research is their seasonally limited spawning, which occurs primarily in the spring. In addition, the application of developmental engineering techniques such as genetic modification and gene knockout is difficult in these large-sized fish because sexual maturation requires several years. The bamboo sole (Fig. 1B), by contrast, reportedly reaches sexual maturation at about 7 cm body length in one year, and its spawning season is relatively long, lasting nearly six months (Ochiai, 1966). In addition to asymmetric development, the bamboo sole exhibits unique

* Corresponding author. E-mail: uji@fra.affrc.go.jp
† Corresponding author. E-mail: toru.suzuki.a8@tohoku.ac.jp
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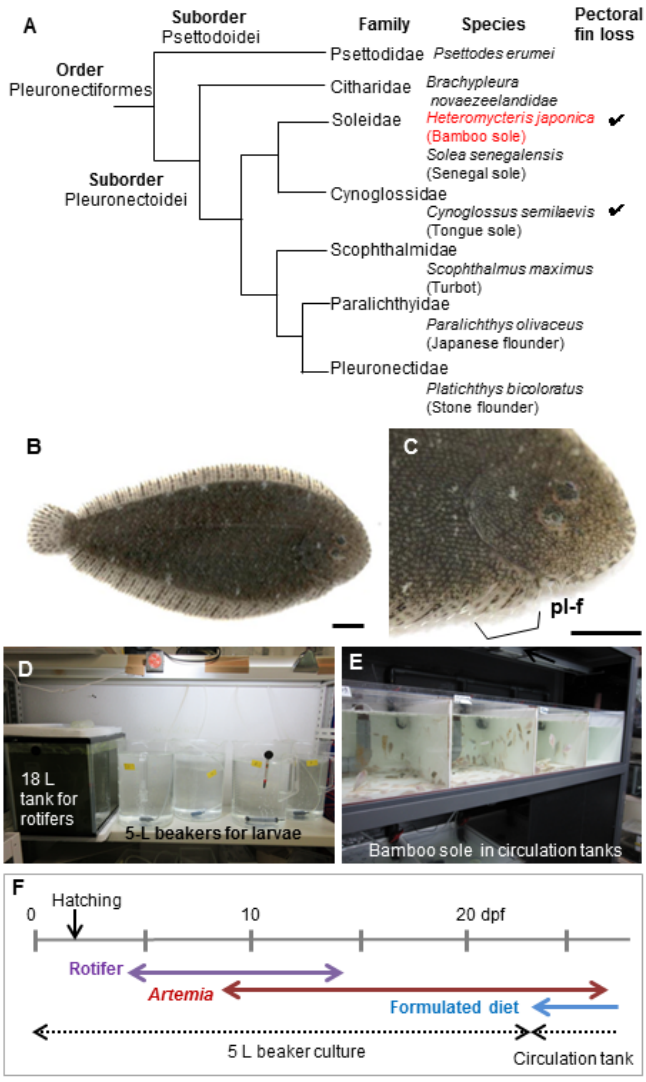


Fig. 1. Phylogenetic position of the bamboo sole (*Heteromycteris japonica*) in the Pleuronectiformes, adult form, and small-scale culture of rotifers and the sole larvae and juveniles. **(A)** Major families of Order Pleuronectiformes, indicating the phylogenetic position of the bamboo sole (cf, Hoshino, 2001). **(B)** Right lateral of bamboo sole adult form, approximately 1.5 years old. **(C)** Head and pectoral region at higher magnification. Note presence of pelvic fin (pl-f), but lack of pectoral fins. Scale bar, 10 mm. **(D)** Culture system used in this study, including an 18-L tank for rotifers and four 5-L plastic beakers for larvae. **(E)** Juveniles in circulation tanks. **(F)** Feeding schemes at different developmental stages.

appendage development at metamorphosis: pectoral fin loss (Minami, 1981; Uji et al., 2013). The adult bamboo sole lacks pectoral fins (Fig. 1C). We therefore focused on the bamboo sole as a potential experimental model among the Pleuronectiformes, substituting for Japanese flounder, for investigating the development of its asymmetry, and also as model for the evolution of fin loss. We previously established a culture system that enables the production of fertilized eggs for use in experiments (Uji et al., 2013). Here, we report the development of a small-scale culture system that enables the study of bamboo sole asymmetric development and fin loss in an ordinary laboratory. We also demonstrate an efficient microinjection system we developed for fertilized eggs.

MATERIALS AND METHODS

Culture of rotifers

Rotifers, the first diet for sole larvae, were purchased from a pet shop (Nikkai-Center, Tokyo, Japan). Marine Art Hi artificial seawater (SW) salts, one bag per 500 L, (Tomita Pharmaceutical, Tokushima, Japan) were used for rotifer and larval culture. All the salts in the bag were dissolved in 500 L of tap water, and adjusted to 32‰ (100% SW). *Nannochloropsis*, a dietary phytoplankton for rotifers, was purchased from MarineTech Inc. (Aichi, Japan).

Commercially obtained rotifers (about 1000 individual per ml; two packs of 700 ml) were filtered using a plankton net and then suspended in 80% SW (diluted with tap-water) to a concentration of 1000 per ml. They were then transferred into an 18 L plastic tank for culture and kept at approximately 28°C using a sheet-type heater set under the tank (Fig. 1D). Air was supplied from air pump using an air stone. Five milliliters of a solution of *Nannochloropsis* was given to the rotifers once per day. As the rotifers increased in number, the density was adjusted to about 1000 per ml by adding 80% SW salts twice per day until the total volume in the tank reached 18 L, after which the larval culture was started. The density of rotifers was counted using the following method. One ml of solution was taken from the culture tank, diluted 10 times with SW, and after mixing and addition of one drop of Lugol's solution, the number of rotifers per one ml of diluted solution was counted under stereomicroscope. During larval culture, water in the tank was renewed once per week while filtering out all of the rotifers.

Larval culture

Larvae were cultured in 5-L plastic cylinder-type beakers (Fig. 1D). The schedule of feeding and rearing larvae is summarized in Figure 1E. Fertilized eggs were collected from tanks containing 100 matured sole, at the National Research Institute of Aquaculture (NRIA), Mie, Japan, as described elsewhere (Uji et al., 2013). The water temperature of the tanks was maintained at 23°C. Fertilized eggs were sent by commercial carrier to arrive the next day at the laboratory in Sendai, Miyagi, Japan. At delivery, the embryos were in the late-somite stage, one day post-fertilization (dpf). Soon after delivery, the larvae were transferred into 5-L beakers filled with 5 L of SW and placed in the laboratory. The water temperature was maintained at around 25°C by the laboratory's air conditioning. Air was supplied from an air pump using an air stone, adjusting the air flow to a level that forced all of the larvae to float in the beakers without swarming to the wall (Fig. 1D). Four beakers were used in this experiment: two for determining survival ratio and another two for fixation and morphologic observation. The density of embryos was adjusted to about 10 per 50 ml.

The schedule of feeding larvae is summarized in Figure 1F. Feeding with rotifers was begun at 4 dpf, removing 2 L of solution daily from the 18 L rotifer culture tank, replacing the removed volume with 2 L of fresh 80% seawater. Rotifers in the 2 L of solution were filtered using a net and suspended in 500 ml of SW, which was given to the larvae in four beakers during the daytime by dividing 3-4 times so as to adjust the density of rotifers in the beaker to around 10 per ml. This cycle of culture maintained the rotifer density at around 1000 per ml in the 18-L tank and was sufficient for rearing a total of 2000 sole larvae in four 5-L beakers. *Nannochloropsis* solution (1 ml) was daily added to the larval tank as nutrition for the rotifers. The larvae were fed with rotifers up to 14 dpf.

Artemia larvae from Vietnam hatched and as many of the egg shells as possible were removed before the *Artemina* nauplii were fed to the larvae from 8 to 28 dpf. Before feeding, newly hatched *Artemia* larvae were incubated overnight with Super Capsulated Powder (SCP) as a nutrient supplement (Pacific Trading Co. Ltd., Fukuoka, Japan), following the manufacturer's instructions. After washing with SW, *Artemia* larvae were given to the sole larvae.

The number of larvae in two beakers was counted once every three days to determine the survival ratio. Larvae were distributed evenly within a beaker by strong aeration, and the number of larvae was counted in a 50 ml sample. This was repeated, and the mean value of three counts was recorded. Larvae were returned to the beaker after counting.

After metamorphosis, juveniles were reared in a circulation tank (Iwaki, Japan; Fig. 1E). From 23 dpf, juveniles were fed with a specific formulated diet, Otohime B-1 (Marubeni Nisshin Feed Co, Tokyo, Japan). All experiments using bamboo sole were approved by the Tohoku University Committee of Animal Experiments.

Morphologic observations

Larvae were anesthetized with 0.01% MS-222 for photography using a Leica DFC500 digital camera (Leica, Wetzlar, Germany) attached to a Leica MZ16F stereomicroscope. For staging and skeletal staining, 60–70 larvae were collected from two beakers designated for fixation sampling. They were fixed with 4% paraformaldehyde in phosphate-buffered saline, transferred to methanol, and stored at –20°C until use. For skeletal observation, samples were stained with neutral Alcian blue and Alizarin red solution and bleached with 3% hydrogen peroxide solution in 1% KOH (Walker and Kimmel, 2007).

Microinjection

Glass needles were prepared from GD1 glass capillaries (Narishige, Tokyo, Japan) using a vertically mounted PC-10 puller (Narishige) at a heater level of 80 and with a weight of 250 g. The tip of the glass needle was sharpened using an EG-400 micro-grinder (Narishige). Injection was carried out using an MMN-8 micromanipulator (Narishige) and hydraulic manual microinjector (Cell-

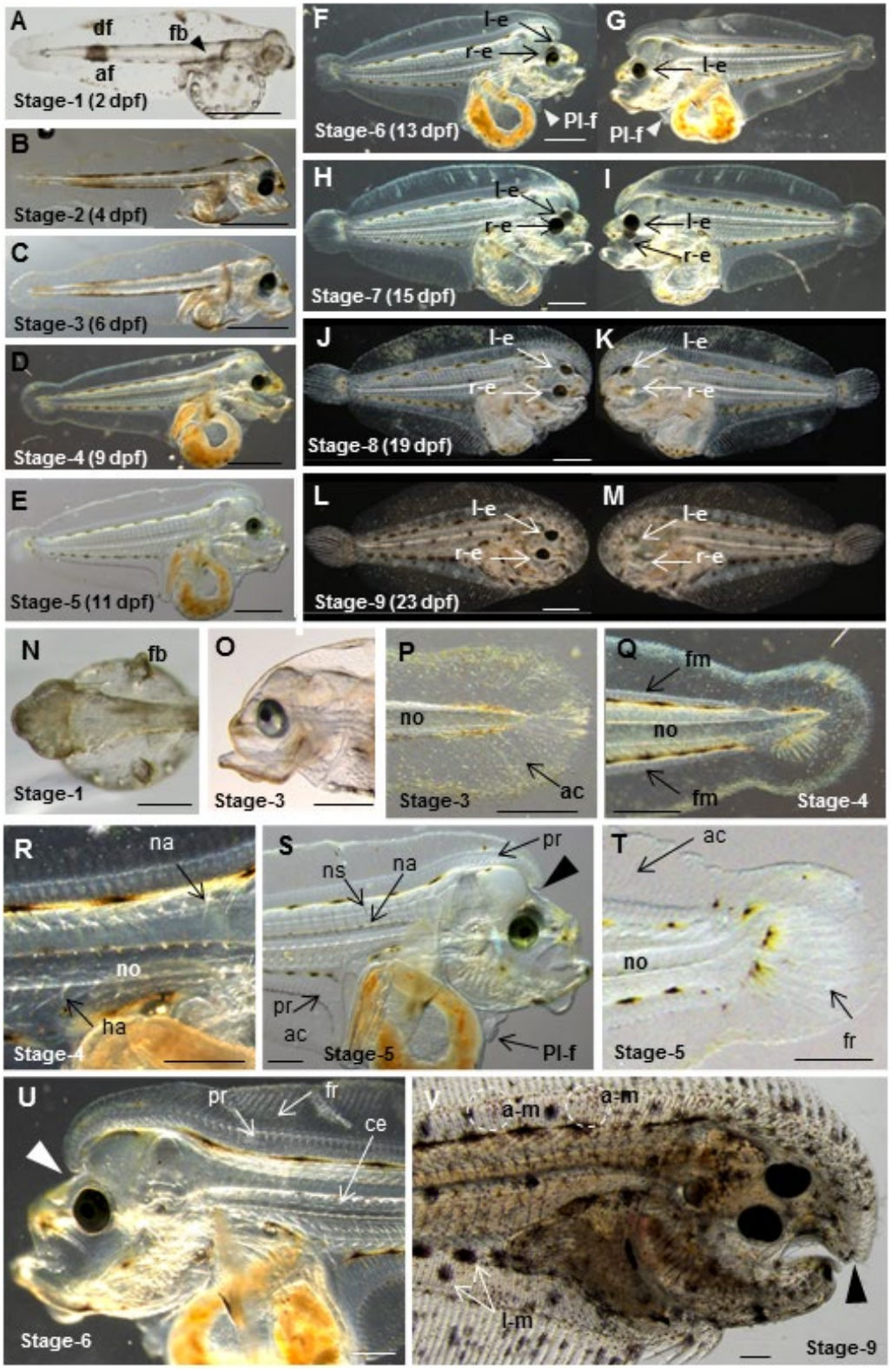


Fig. 2. Larval development of the bamboo sole. **(A–E)** Right lateral view at stages 1 to 5. **(F–M)** Right and left lateral views at stages 6–9. **(N–V)** Higher magnifications of larvae at stages indicated. The morphological features of each stage are summarized in Table 1. Arrowheads in **(S)**, **(U)**, and **(V)** mark anterior end of extending dorsal fin. ac, actinotrichia; af, anal fin fold; a-m, adult-type melanophore; df, dorsal fin fold; fb, pectoral fin bud; fm, fin muscle; ha, hemal arch; hs, hemal spine; l-e, left eye; l-mlarval-type melanophore; na, neural arch; ns, neural spine; no, notochord; pl-f, pelvic fin; pr, proximal radial; r-e, right eye. Scale bars, 0.5 mm in A–L; 0.2 mm in N–V.

Tram Oil; Eppendorf, Hamburg, Germany). Under natural light conditions, fertilized eggs can be collected between 14:00 and 18:00. Fertilized eggs collected from the spawning tank were placed in a 100 ml glass beaker on ice and then transferred to the experimental room. Individual eggs floating in the beaker were aspirated using a 200 μl tip attached to a pipette, which was placed under the stereomicroscope for microinjection (Fig. 7D, E). FITC-labeled mock morpholino-oligo (Gene-Tools, Philomath, USA) was injected into the embryos. FITC fluorescence was detected using a Leica MZ16F stereomicroscope at fluorescence mode using a filter for specific for FITC.

RESULTS

Larval culture system

The rotifer culture as described above was sufficient to rear 2,000 the bamboo sole larvae through metamorphosis. Cylinder-type beakers are more suitable than cubic tanks for culturing larvae, as aeration produces a uniform water current. Using this system, the survival ratio of larvae from the beginning of culture was around 70% at 23 dpf (n = 830/1160, n = 730/1100), when most larvae had completed metamorphosis.

Staging of larvae

Development of the bamboo sole larvae was characterized by anterior extension of the dorsal fin, downward protrusion of the intestines, and degeneration of the pectoral fins, as well as eye migration and pigmentation of the ocular side, common features among the Pleuronectiformes (Fig. 2). Larval development was classified into stages 1–9. The morphological criteria for staging are summarized in Table 1. Caudal, dorsal, and anal fin development, ventral extension of the intestines, and vertebral column formation serve as good markers for staging until stage 5, just before the start of eye migration. Fin muscles, which are well developed along the base of the dorsal and anal fins in the Pleuronectiformes, begin to form at stage 4 (Fig. 2Q); their early development has been described in detailed in a previous paper (Uji et al., 2013). The centra of the bamboo sole forms by membranous ossification (Fig. 5F, G), which is common to zebrafish (Fleming et al., 2004), medaka (Inohaya et al., 2007), fugu (Kaneko et al., 2016) and Japanese flounder (Wu et al., 2016). Formation of centra begins at stage 6 (Fig. 2U).

Stages 5–9 correspond to metamorphosis, for which the position of the migrating left eye and extending anterior dorsal fin, as well as the pigmentation of the ocular-side skin, serve as good markers.

The left eye begins migration at stage 6, arriving at the midline of the head at stage 8 and settling in the final right-lateral position at stage 9 (Fig. 2F–M). The anterior dorsal fin begins extension at stage 5 and reaches the upper jaw at stage 8 (Fig. 2E–K, S, U). It finally fuses with the dorsal side of the head at stage 9, just after the left eye arrives at the left-lateral position (Fig. 2L, M, V). Adult-type melanophores, which are markedly smaller than larval-type melanophores, appear on the ocular side at stage 9, establishing the bilateral asymmetry of eye location and skin color (Fig. 2L, M).

Figure 3 shows the ratio of larvae in each of the stages during this rearing experiment. About 73% (n = 49/67) of the larvae reached stage 9 by 23 dpf; larvae with abnormality in pigmentation, eye location and dorsal fin were counted as stage 9, when they had settled on the bottom and the morphology of the intestines had progressed from larval protruding form to juvenile form. Tracing the metamorphosis individually by keeping 3–5 larvae in 500-ml beakers indicated that eye migration covering stages 6–9 took 8–10 days.

Table 1. Staging of bamboo sole larval development.

Stage	Morphologic characteristics
1	Just after hatching. Large yolk. Pectoral fin bud. Eyes begin pigmentation. Mouth before opening.
2	Yolk has disappeared. Eyes are pigmented. Mouth has opened. First feeding. Pectoral fins have formed with disc and coracoid cartilage.
3	Guanine deposition is apparent in eyes. Constriction of proximal caudal fin has begun. Actinotrichia, fine fibrous fibers, apparent in caudal fin.
4	Bending of notochord to dorsal direction has started in caudal fin. Muscles supporting dorsal and anal fins have begun to form. Intestines beginning to protrude downward. Neural and hemal arches formed as cartilagenous skeleton.
5	Bending of notochord apparent in caudal fin. Fin rays have started to form in caudal fin. Proximal radial cartilage has started to form in anterior parts of dorsal and anal fins. Dorsal fin has started extending anteriorly. Actinotrichia have appeared in dorsal and anal fins. Downward protrusion of intestines is more prominent. Cartilage of neural and hemal arches has been replaced by calcified bone. Neural and hemal spines have formed. Pelivic fins have started to form.
6	Left eye has just started migration. Anterior dorsal fin has reached position in front of tectum. All proximal radial cartilage has formed. Fin rays have started to form in dorsal and anal fins. Extension of skeletal muscle toward dorsal and ventral directions is marked. Centra have started to form by membraneous ossification.
7	Left eye is migrating to left lateral. All fin rays have formed in dorsal and anal fins. Anterior dorsal fin has reached near the nose. Larvae have started settlement on the bottom.
8	Left eye has migrated to midline. Anterior dorsal fin has reached near upper jaw. Downward protrusion of intestines has shortened. Pectoral fins begin to decrease in size.
9	Left eye has finished migration. Adult-type melanophores have appeared on ocular-side skin. Anterior dorsal fin has fused with dorsal side of head. Pectoral fin cartilage degenerates. Downward protrusion of intestines becomes negligible.

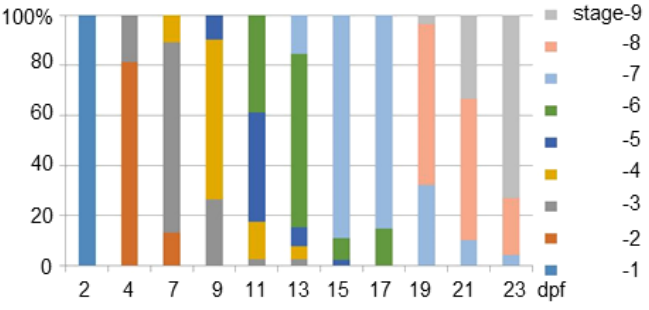


Fig. 3. Ratio of larval stages during the rearing experiment. A total of 60–70 larvae were assayed on each day post-fertilization (dpf).

Abnormal metamorphic development

Developmental abnormality of the juveniles cultured in this system was examined at 5 months after fertilization. One hundred-fifty juveniles were selected randomly from approximately 300 juveniles surviving in the circulation tanks. In this culture experiment, the ratio of externally normal juveniles (Fig. 4A, B) was 49% (n = 74/150), while the other juveniles exhibited some morphological anomalies. Four types of morphological anomaly were correlated with eye position and skin color. Type 1 anomaly is left-right reversal, in which the eye position and skin color were completely reversed relative to normal development (n = 2/150) (Fig. 4C, D). Type 2 anomaly is characterized by incomplete eye migration, in which the left eye was retained in the left-lateral position in conjunction with an absence of adult-type melanophores: complete pseudoalbinism (n = 7/150) (Fig. 4E, F). Type 3 anomaly is characterized by incomplete eye migration and partial pseudoalbinism of the right-side skin (n = 18/150) (Fig. 4G, H). Type 4 anomaly involved partial pseudoalbinism of the right (ocular)-side skin, with normal eye location (n = 33/150) (Fig. 4I, J). In both types 3 and 4 anomalies, partial pseudoalbinism occurred in the abdominal region (Fig. 4G, I). These four types of anomaly were accompanied by incomplete fusion of the dorsal fin to the upper jaw (Fig. 4C–J). Juveniles with incomplete fusion of the dorsal fin, while exhibiting normal eye location and pigmentation, constituted the fifth type of anomaly (n = 14/150) (Fig. 4K, H).

Pectoral fin formation and degeneration

In juvenile bamboo sole, no pectoral fin skeleton was observed (Fig. 5A, B). Pectoral fin buds, however, appeared at stage 1 (Fig. 2A) and formed coracoid and fin disk cartilage at stage 2 (Fig. 5C–E). Actinotrichia, which are fine fibrils essential for fin skeletal formation (Duran et al., 2011), are located in the proximal-distal direction along the outside of the fin disk cartilage, forming membranous tissue that was not stained by Alcian blue (Fig. 6A). The pectoral fin began to degenerate between stages 8 and 9, during late metamorphosis (Fig. 5F). This is in stark contrast to Japanese flounder, in which segmented adult-type pectoral skeleton is produced from the coracoid and fin disk cartilage and fin rays are formed (Fig. 5J, K). During pectoral fin degeneration, both the coracoid and fin disk cartilage decreased in size, the actinotrichia were lost, and no fin rays

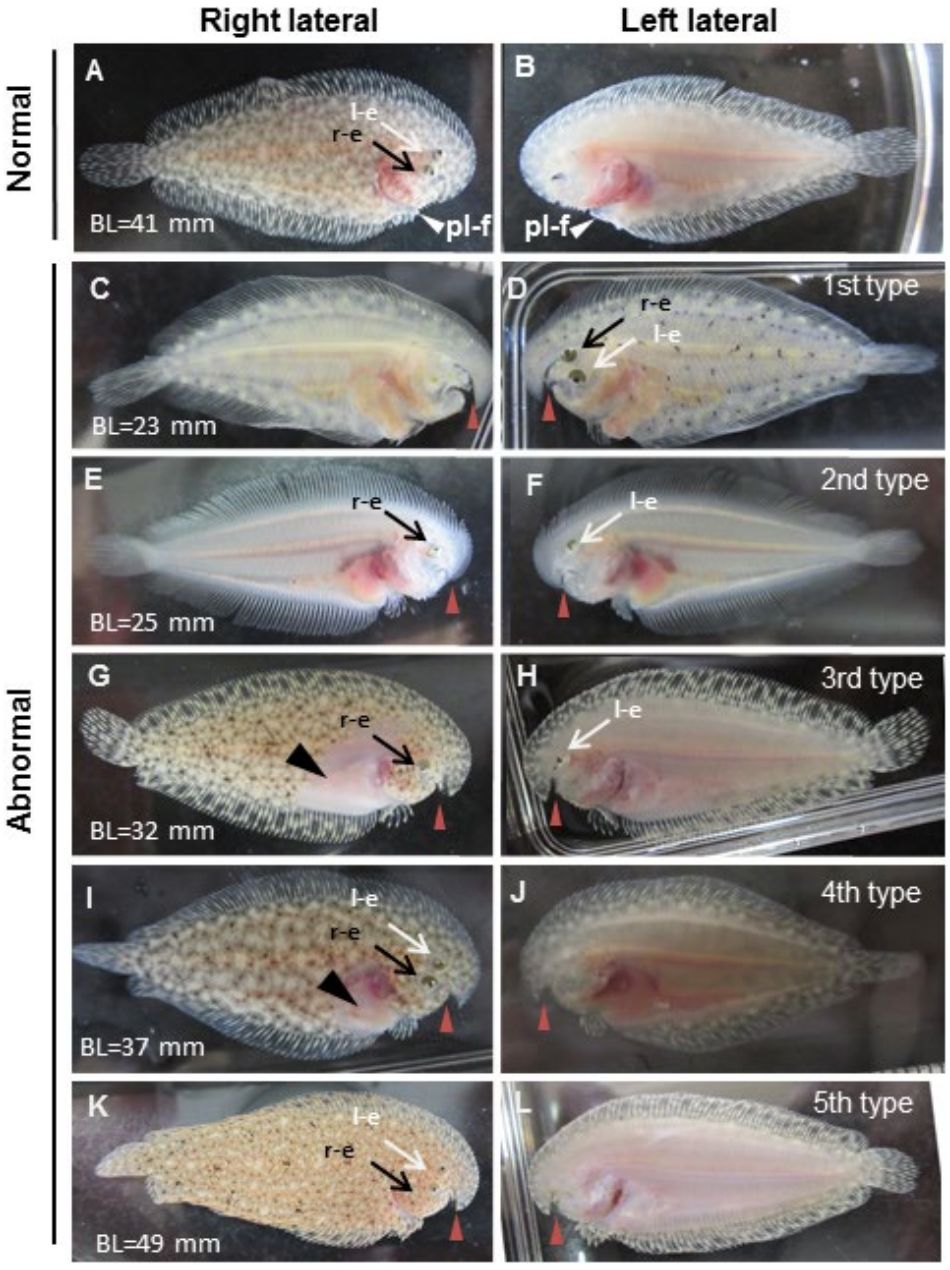


Fig. 4. Externally normal and abnormal juveniles observed in the rearing experiment. **(A, B)** Normal juvenile. **(C, D)** Type 1 anomaly: left-right reversal. **(E, F)** Type 2 anomaly: incomplete left-eye migration and absence of metamorphic pigment cells (complete pseudoalbinism). Red arrowheads mark incomplete fusion of anterior dorsal fin with upper jaw. **(G, H)** Type 3 anomaly: incomplete left-eye migration and partial pseudoalbinism in right-lateral skin (black arrowhead). **(I, J)** Type 4 anomaly: normal eye location and partial pseudoalbinism in right-lateral skin (black arrowhead). **(K, L)** Type 5 anomaly: incomplete fusion of anterior dorsal fin with upper jaw (red arrowhead), while left-eye migration and pigmentation being normal. l-e, left eye; pl-f, pelvic fin; r-e, right eye. BL, total body length.

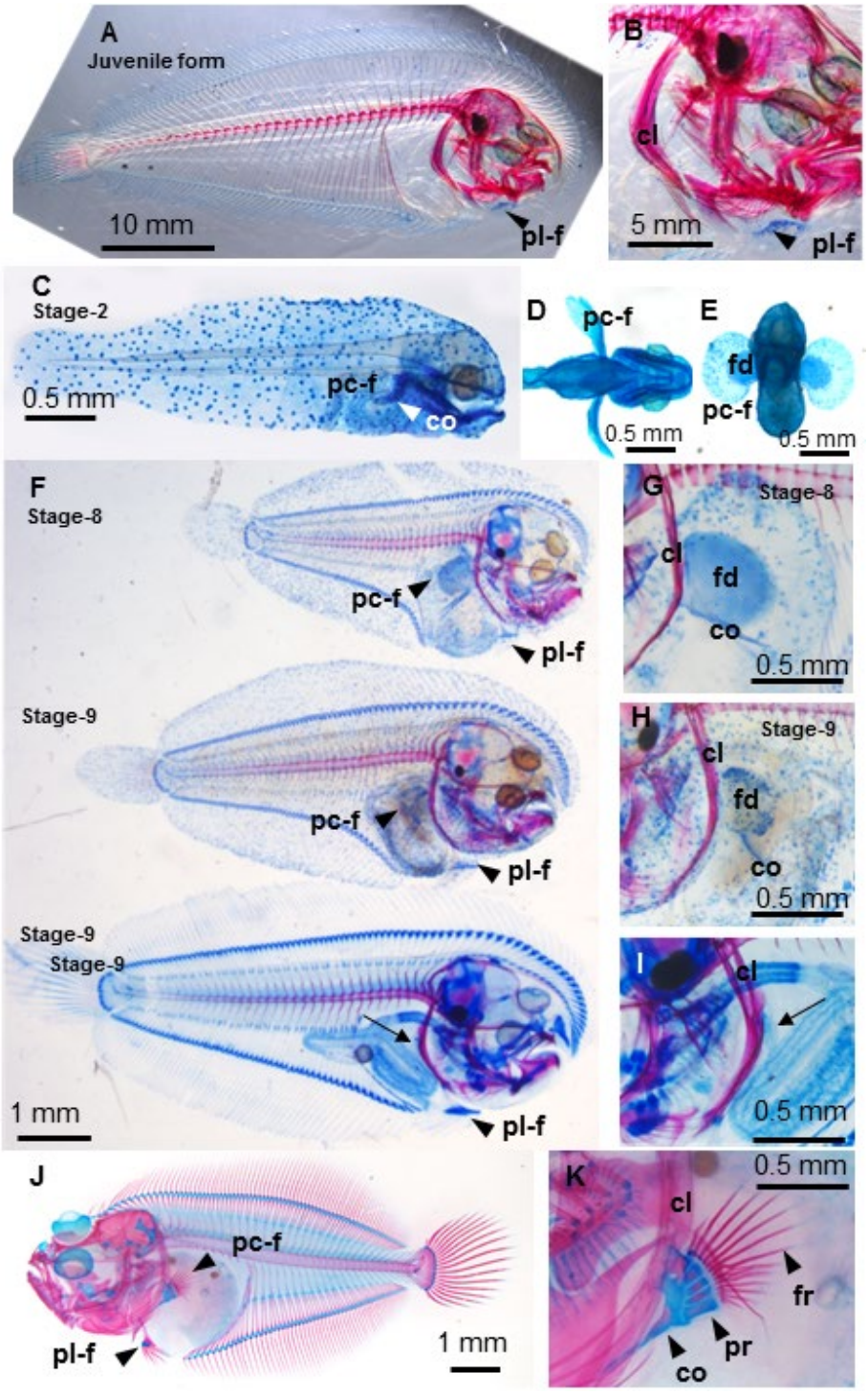


Fig. 5. Pectoral fin formation and degeneration in bamboo sole larvae. Samples were stained with acid-free Alcian blue and Alizarin red double staining (Walker and Kimmel, 2007). **(A, B)** Skeletal system in a juvenile and magnification of pectoral region, respectively. In the bamboo sole, fin rays of dorsal, anal and pelvic fins are not stained by Alizarin red, in contrast to strong staining in Japanese flounder as shown in J. **(C–E)** Stage 2 larva. Lateral, ventral, and anterior view, respectively. **(F)** Stage 8 and 9 larvae in lateral view. **(G–I)** Magnification of pectoral region of larvae shown in **(F)**. Arrows in **(F)** and **(I)** mark vestigial cartilage of pectoral fin. **(J, K)** Lateral view of Japanese flounder larva and magnification of pectoral region, respectively. Fin disk cartilage has been segregated to proximal radials. cl, cleithrum; co, coracoid cartilage; fd, fin disk cartilage; fr, fin ray; pc-f, pectoral fin; pl-f, pelvic fin; pr, proximal radials.

formed (Fig. 5G–I, Fig. 6). In late stage 9, the cartilage began to degenerate (Fig. 5F, I). In contrast to the pectoral fins, the pelvic fins formed between stages 5 and 6 (Fig. 2E–G, S) and were maintained in the juveniles (Fig. 5A, B).

Microinjection of one-cell-stage embryos

Fertilized eggs of the bamboo sole (Fig. 7A, E) were 1 mm in diameter, and the chorionic membrane was sufficiently transparent to observe initiation of cell division, which at 23°C occurred approximately 20 min after fertilization. By keeping the glass beaker containing the embryos on ice (Fig. 7B), the water temperature was cooled to around 8°C, and cell division could be delayed for 60 min without significantly affecting the survival ratio until hatching (93%, compared with 100% in controls) (Fig. 7C). At the level of stereo-microscopy, no developmental anomalies of hatched larvae were observed among those developed from cooled fertilized eggs. Although the one-cell stage could be prolonged further to 90 min, the survival ratio until hatching decreased to 55% (Fig. 7C).

As fertilized eggs were floating at the water surface, placing the embryonic cell downwards, it was possible to hold an egg at the tip of a 200 μl tip of a pipette by gentle aspiration, so as to keep the cell orientation (Fig. 7D, E). The pipette tip with the egg was easily observed under the stereomicroscope (Fig. 7E) for microinjection. Up to 100 one-cell-stage embryos per hour could be microinjected. The survival ratio of the injected embryos was 50–60% just after injection and 17–23% at the late-somite stage. When FITC-labeled mock morpholino-oligo was injected at the one-cell stage using the above system, FITC-fluorescence was detected throughout the embryonic body (Fig. 7F, G).

DISCUSSION

Small-scale culture of bamboo sole larvae

Preparation of a sufficient number of rotifers with high nutrient

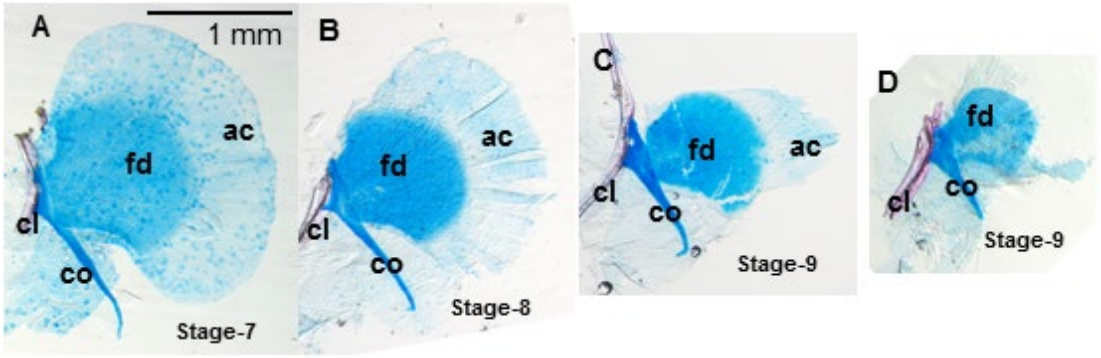
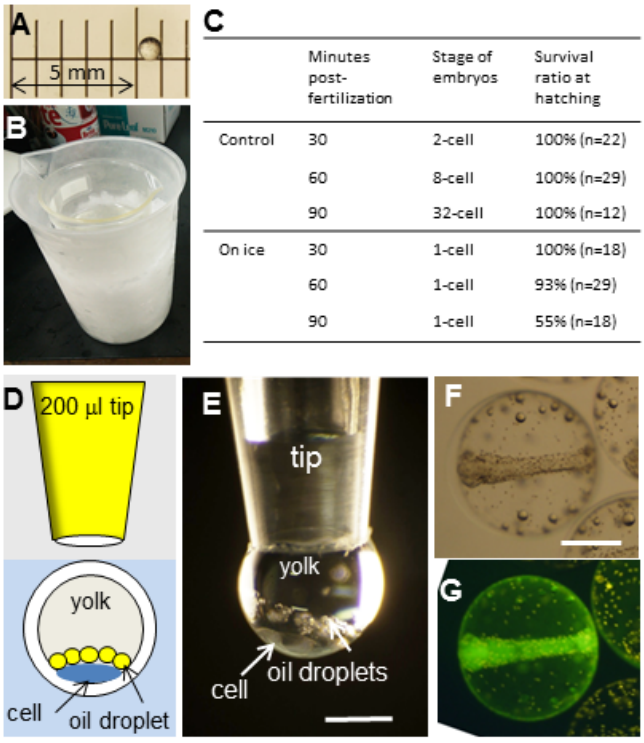


Fig. 6. Degeneration of the pectoral fin cartilaginous skeleton. **(A)** Stage 7. **(B)** Stage 8. **(C, D)** Stage 9. ac, actinotrichia; cl, cleithrum; co, coracoid cartilage; fd, fin disk cartilage. Scale bar, 1 mm (all figures).



	Minutes post-fertilization	Stage of embryos	Survival ratio at hatching
Control	30	2-cell	100% (n=22)
	60	8-cell	100% (n=29)
	90	32-cell	100% (n=12)
On ice	30	1-cell	100% (n=18)
	60	1-cell	93% (n=29)
	90	1-cell	55% (n=18)

Fig. 7. Microinjection system for injection of one-cell-stage bamboo sole embryos. **(A)** Fertilized egg. **(B)** Prolongation of one-cell stage. Glass beaker (100 ml) containing SW and fertilized eggs was placed on crushed ice. **(C)** Survival ratio of embryos after cooling on ice. Embryos just after fertilization were kept at 23°C (control) or on ice for 30, 60, or 90 min, after which they were maintained at 22°C; the survival ratio was determined after hatching. **(D)** Scheme of attaching egg to a pipette tip. **(E)** One-cell-stage embryo attached to a pipette tip under a stereomicroscope. **(F, G)** Embryo at late-somite stage (10 hours post-fertilization) injected with FITC-labeled mock morpholino-oligo at one-cell stage. **(F)** and **(G)** are bright-field and fluorescent (filter for FITC) microscopy, respectively. Scale bars in **(E–G)**, 0.5 mm.

quality is essential for rearing early larvae of most marine fish (Shields, 2001). The survival ratio at metamorphosis from hatching was 60–75% in this study. The small-scale culture system designed here, using one 18-L tank for rotifers and 5-L beakers for the larvae (Fig. 1D) enabled nearly 2000 larvae to grow to completion of metamorphosis. Fertilized eggs can now be collected from spawning tanks at NRIA, Mie, Japan (Uji et al., 2013). We are now trying to establish a culture system for parental sole that would supply fertilized eggs throughout the year by control of light and temperature conditions. Rearing experiments in the laboratory using the culture system designed here would make the bamboo sole an ideal model for use in experimental biology research.

Development of asymmetry

Bamboo sole larvae complete metamorphosis in a shorter period of time (21–23 days) compared with those of the Japanese flounder (35 days; Watanabe et al., 2008). Eye migration and pigmentation of the ocular side complete in 8–10 days. Such rapid larval and metamorphic developments are clearly advantageous for developmental biology research. Five types of external morphologic abnormalities were noted in this study, characterized by incomplete anterior extension of the dorsal fin and by abnormal eye location and ocular-side pigmentation. In total, 50% of juveniles exhibited one or more external abnormalities that could be attributed to metamorphic developmental anomalies. None of the anomalies observed here were observed among adult bamboo sole collected from the Seto Inland Sea for brood stock. In addition, larvae from the same brood stock that were reared at lower density, about one per 50 ml, in a larger tank (100 L) did not show such high ratio of abnormal development, even though rotifers reared in similar conditions were given. It is therefore suggested that the high ratio of anomalies observed here could be correlated with high-density culture in a small beaker.

Except for incomplete anterior extension of the dorsal fin, which is specific to sole, other malformations observed here, including abnormal eye location and pseudoalbinism of the ocular side, appear often commonly in the Pleuronectiformes when cultured in tanks (Aritaki et al., 2004; Aritaki and Tagawa, 2012). Because the mechanisms underlying these anomalies are unclear, identification of the causative factors using bamboo sole larvae would enhance current understanding of the development of asymmetry in the Pleuronectiformes. The bamboo sole can therefore be considered as a good model, in place of Japanese flounder, for investigating the developmental control systems governing eye migration and lateralized pigmentation in the skin.

Developmental loss of the pectoral fin

Absence of pectoral fins is a unique morphologic characteristic observed in the bamboo sole, *H. japonica*. The order Pleuronectiformes consists of two suborders, Psettodoidei and Pleuronectoidei. The latter is composed of three major groups (Fig. 1A): first, Citharoides; second, Soleidae and Cynoglossidae, both including so-called soles; and third, Scopthalmidae, Paralichthyidae and Pleuronectidae, including turbot and Japanese flounder. The bamboo sole belongs to the Family Soleidae, in which, however the Senegal sole, *Solea senegalensis*, retains its pectoral fins. The tongue sole, *Cynoglossus semilaevis* (Family Cynoglossidae) lacks pectoral fins. A genome database for *C. semilaevis* is available at NCBI (Map Viewer; https://www.ncbi.nlm.nih.gov/mapview/). Unlike soles, other members of the Pleuronectiformes have pectoral fins, as shown here for Japanese flounder (Fig. 5K). Thus, within the Pleuronectiformes, it is thought that the lack of pectoral fins is unique to some members of the Soleidae and Cynoglossidae.

It is well known that pelvic fin loss or reduction sometimes occurs in teleosts, such as pufferfish, including fugu, *Takifugu rubripes*, and stickleback, *Pungitius pungitius* (Shapiro et al., 2006; Tanaka et al., 2005). Both fugu and stickleback retain pectoral fins. Contrary to these species, the bamboo sole lacks pectoral fins but retains pelvic fins. In most moray eels, both pectoral and pelvic fins have been lost (Bohlke et al., 1989), analogous to limb loss in snakes. Thus, pectoral fin loss seems to have occurred independently in teleosts, but loss of the pectoral fins while retaining the pelvic fins is unique to soles.

We previously reported that fin muscles also degenerate during pectoral fin degeneration in the bamboo sole (Uji et al., 2013). Of further interest, we here found that the larval-type pectoral fin skeleton, consisting of coracoid and fin disk cartilages, forms during the early larval stage in the bamboo sole, after which both are completely lost during metamorphosis. In pelvic fin loss during development of fugu, the fin buds and cartilaginous skeleton do not form (Tanaka et al., 2005). Pectoral fin loss in the bamboo sole differs markedly from pelvic fin loss in fugu in that the larval-type fin skeleton forms and then degenerates during metamorphosis. It has been suggested that lateral positioning signals for pelvic fin bud formation are absent in fugu (Tanaka et al., 2005). In snakes, it has been proposed that forelimb loss is correlated with trunk elongation, where expansion of the Hox gene expression domain for thoracic identity leads to forelimb loss (Cohn and Tickle, 1999). However, it is conceivable that positioning signals for pectoral fin buds is at least retained in the bamboo sole, and the scenario for snake forelimb loss is not applicable to soles.

In teleosts, including zebrafish and Japanese flounder (refer Fig. 5J, K), fin disk cartilage is generally segmented into proximal radials that support the fin rays, by which the pectoral fin skeletal system changes from larval-style to adult-style (Dewit et al., 2011; Grandel and Schulte-Merker, 1998). In the bamboo sole, the fin disk cartilage is lost without forming proximal radials, and fin rays do not appear. Although larval pectoral fin development has been described in the zebrafish (Gibert et al., 2006; Yano et al., 2012), the developmental program that controls the transition from larval-style to adult-style pectoral fins remains largely unknown. In the bamboo sole, we speculate that while the positioning signal system and the system for larval pectoral fin outgrowth described below are retained, the developmental program for transition from larval-type to adult-type pectoral fin has been lost. We also suggest that degeneration of pectoral fins is likely accompanied by apoptosis. It may be possible to approach genes correlated with this developmental program, for example, by comparing transcriptomes of larval pectoral fin by RNA-Seq between the bamboo sole and Japanese flounder. The genome database of the tongue sole, also lacking pectoral fins, would also help the analyses.

From an ecological perspective, although all members of Pleuronectiformes with an asymmetric body are bottom fish, feeding behavior largely differs between soles and other families: soles consume benthic materials while moving slowly along the bottom; whereas most species such as Japanese flounder are fish-eaters that actively swim to hunt. From our observations, bamboo sole move slowly along the tank bottom by waving their dorsal and anal fins, and rarely swim spontaneously away from the bottom. Pectoral fins are unnecessary for such movements, so we suppose that pectoral fin loss in the sole co-evolved with this behavioral pattern, moving slowly by the power of dorsal and anal fins, only enabled by their flattened asymmetric body form. Because pectoral fins are essential for hunting zooplankton during the larval stage before establishing the flattened asymmetric body form, the soles may have evolved pectoral fin loss while retaining larval fin skeletal system.

Microinjection system for one-cell-stage embryos

The microinjection system designed in the present study for injection of bamboo sole one-cell-stage embryos and prolongation of the one-cell stage by cooling on ice allow injection into the cytoplasm during a window of up to 1 h. The survival ratio after microinjection was 50–60% just after injection and around 20% by the late-somite stage. Such a low rate of survival is presumably due to Na^+ influx from seawater through the damaged yolk membrane at injection. Addition of bovine serum albumin or polyethylene glycol into the seawater for embryonic culture did not improve the survival ratio (data not shown). Even though the survival ratio is low, injection into 100 cells per day is possible. This would hopefully facilitate gene knockout using CRISPR-Cas9 (Hsu et al., 2014) and injection of transgenes into embryos to produce transgenic bamboo sole.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

QC and MT performed culture of larvae and photographs. MM designed microinjection system. MK, YS and SN performed staging of larvae TS and SM designed culture system of adults and larvae. HY and TS wrote the manuscript.

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