

⟨Original article⟩

## Examination of the hearts and blood vascular system of *Eptatretus okinoseanus* using computed tomography images, diagnostic sonography, and histology

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**Summary** The heart is an organ that pumps blood by creating positive and negative pressures at a regular rhythm. Vertebrates improved the pump design by creating cardiac chambers, while accessory pumps that are present in many species, such as hagfish, remained redundant. However, the process by which the blood circulatory system develops in vertebrates, including the development of the heart, largely remains unknown. The Atlantic hagfish *Myxine glutinosa* has five accessory hearts, the functions of which remain unclear. Despite these findings, it is still an open question as to whether the blood circulatory system of *M. glutinosa* represents the ancestral condition of vertebrates. In this study, we examined the hearts and blood circulatory system of the hagfish *Eptatretus okinoseanus*.

We examined the structure of the hearts in *E. okinoseanus* using computed tomography images and diagnostic sonography and identified the three features of those hearts. First, *E. okinoseanus* has four hearts: two cardinal hearts, one branchial heart, and one portal heart. Second, we observed vascular blood circulations, the sinus, and two types of blood vessels (the dorsal aorta, anterior cardinal veins). Third, the hearts pump by creating positive and negative pressure at a regular rhythm. Histological analyses revealed many empty spaces in the heart tissues of all types. Those hearts pump blood in and out of the whole body in a similar manner as a sponge absorbs and discharges water. The cardinal hearts showed primitive characteristics similar to the skeletal muscle. The branchial heart was the main operator, while the cardinal and portal hearts were assisting because the pumping ability of the branchial heart was much greater than that of the other hearts, the cardinal and portal hearts.

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In the heart evolution of *E. okinoseanus*, four hearts were derived from the amphioxus. The two cardinal hearts in both sides of the brain may be primitive because their cell structure resembled that of the skeletal muscle from which it may have evolved.

**Key words:** *Eptatretus okinoseanus*, hagfish, heart, blood vascular system

## 1. Introduction

The heart is an organ that pumps blood by creating positive and negative pressures at a regular rhythm. One of the mysteries of the vertebrate heart concerns its early evolution. A possible vertebrate ancestral blood circulatory system can be found in the amphioxus a stem taxon of extant vertebrates<sup>1</sup>. Unlike most vertebrates, amphioxus do not have a completely closed blood circulation system and no distinct heart, and circulation is powered by smooth muscle peristaltic vessels<sup>2</sup>. Vertebrates improved the pump design by creating cardiac chambers and maintained redundant accessory pumps that are present in many species, including the hagfish (Myxiniiformes)<sup>3</sup>. Alternatively, they generate blood flow by rhythmical contractions of blood vessels. However, the process by which blood circulatory system develops in vertebrates, including the development of the heart, is still largely unsolved.

The blood circulatory system of the hagfish has been extensively studied as a key group for clarifying the early evolution of the vertebrate heart. This is because the hagfish together with the lamprey (Petromyzoniformes) is phylogenetically located between the amphioxus and all jawed vertebrates (Gnathostomata)<sup>4</sup>. As in other vertebrates, the Atlantic hagfish, *Myxine glutinosa*, has a closed blood circulation system. However, one of its unique features is the presence of five accessory hearts<sup>5</sup>. These accessory hearts are distributed in several places, including the cranial, trunk, and caudal regions. These accessory hearts can be distinguished from a “true” heart (which is occasionally called a branchial heart in *M. glutinosa*) because they lack the heart-specific cardiac muscle seen in other vertebrates.

The function of these accessory hearts is unclear, although it is likely that they have a role in assisting the “true” heart. The arterial blood pressure of *M. glutinosa* is extremely low, ranging from 3 to 8 mm Hg, the lowest among all vertebrates<sup>5</sup>. Such low arterial blood pressure may cause problems for efficient blood circulation. The accessory hearts of *M. glutinosa* may be important to increase venous blood pressure and help the blood returning to the “true” heart.

Despite these findings, it is still an open question as to whether the blood circulatory system of *M. glutinosa* represents the ancestral condition of vertebrates. This is mainly because our current knowledge of the blood circulatory systems of lower vertebrates is quite limited. The present study described the blood circulatory system of the hagfish, *Eptatretus okinoseanus*. The genus *Eptatretus* is the only member of the Eptatretiformes, a sister taxon of the Myxiniiformes. Our data should be informative for clarifying the phylogenetic distribution of the unique blood circulatory system found in *Myxine* sp.

## 2. Materials and methods

### 2.1. Materials

Three *E. okinoseanus* specimens were examined, which were collected from the East China Sea off Okinawa Island (26°28.946'N, 127°41.207'E), at a depth between 550 and 600 m. All specimens were female. The total lengths (TL) and weights of each specimen were 73 cm TL and 990 g, 80 cm TL and 1100 g, and 89 cm TL and 1200 g, respectively.

### 2.2. Computed tomography (CT)

CT images were obtained at the Okinawa Churaumi Aquarium using a SOMATOM Spirit CT scanner (SIEMES Medical) at an X-ray setting of

130 kV and 50–80 mA. X-ray intensities were measured using an RLS detector with two channels spaced at 5-mm intervals. The scanner gathered projection images that were then reconstructed into 1.25-mm slices. The CT scan slices were processed using OsiriX software (version 2. 7. 5, 32 bit; Osirix Foundation, Geneva), which enabled a three-dimensional reconstruction of the hearts. An Iopamiron 300 (Bayer Co., Ltd., Japan) was used as the contrast medium of the CT image. During CT image capture, the specimens were anesthetized with 0.2 mL alfaxan (Meiji seika, Co., Ltd., Japan).

### 2.3. Histology

All excised tissue specimens were fixed with formalin, embedded in paraffin, and sliced with a microtome to a thickness of 3  $\mu\text{m}$ . After stretching and removing the paraffin, the specimens were stained with hematoxylin and eosin (HE)<sup>6</sup> or phosphotungstic acid–hematoxylin (PTAH)<sup>7</sup>.

### 2.4. Diagnostic sonography

Ultrasonographic imaging was performed on a living *E. okinoseanus* at the Okinawa Churaumi Aquarium using the sonography diagnostic imaging system FAZONE M (Fujifilm Co., Ltd., Japan). The sonography transducer was placed on the body surface, and the experiment was conducted in seawater maintained at ca. 4°C.

### 2.5. Terminology

Anatomical terminology was described according to Kardong<sup>8</sup>.

## 3. Results

One branchial heart and two accessory hearts (cardinal and portal hearts) were observed (Figs. 1A–1D). The cardinal hearts were a paired organ located in both sides of the brain (Fig. 1A). The branchial heart was located between the gills and the liver and consists of four chambers, the sinus venosus, atrium, ventricle, and ventral aorta (Fig. 1B). The portal heart was a small, round-shaped organ located on the surface of the intestine (Fig.

1C).

Figure 2 shows CT images of *E. okinoseanus*. The upper figure shows the head area consisting of the two cardinal hearts, the sinus, and two types of blood vessels (the dorsal aorta and anterior-cardinal-veins). Blood flow from the sinus to the two cardinal hearts via the dorsal aorta was observed. The lower figure shows the branchial and anterior body cavities consisting of the branchial and portal hearts and three blood vessels (anterior cardinal veins). A portal heart in one of the three anterior cardinal veins was observed.

Figure 3 shows a photomicrograph of heart tissues stained with HE. The original objective magnification was  $\times 40$ . There were many empty spaces (white spaces among the cells) in the three types of heart tissues (Fig. 3A, cardinal hearts; Fig. 3B, branchial heart; Fig. 3C, portal heart). These empty spaces suggested that the hearts push blood in and out of the whole body in a similar manner as a sponge absorbs and discharges water.

Figure 4 shows a photomicrograph of heart tissues stained with HE or PTAH. The original objective magnification was  $\times 1000$ . Cross-striation was found in three types of heart tissues. The cardinal heart tissues were thick and straight, while the branchial and portal heart tissues were thin and branching. Cardinal hearts appeared similar skeletal muscle tissues, suggesting that this heart evolved from the skeletal muscle.

Figure 5 shows successive heart images using diagnostic sonography. In the upper panel, the probe of the sonograph was placed on three points (A, B, or C) which corresponded with the three heart types. The second (A), third (B), and fourth panels (C) from the top showed the cardinal hearts, branchial heart, and portal heart, respectively. These data showed the time direction from left to right and show three phases: the expression phase at the start, the compression phase, and the expression after compression. The heart rates of the three heart types were measured from the time between the expansion phase to the compression phase. The volume of the three heart types was calculated as an ellipsoid volume ( $4\pi \times \text{length} \times \text{width} \times \text{high}/3$ ) when *E.*

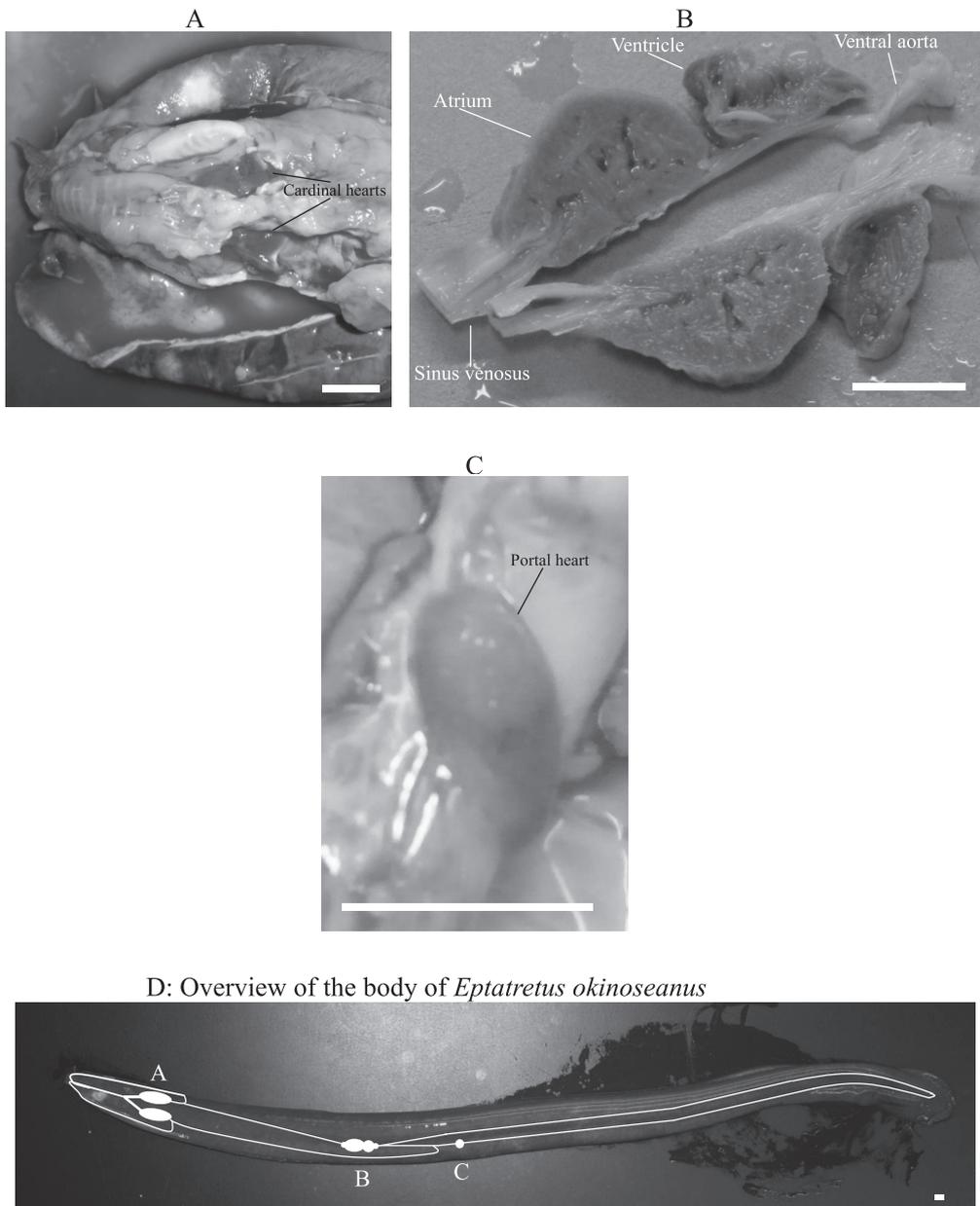


Fig. 1. Anatomy of *Eptatretus okinoseanus*.

A: Cardinal hearts. B: Branchial heart. Tissue samples were fixed in 10% buffered formalin and cut horizontally. C: Portal heart. D: Body overview. The presumed circulation is showed by a white line, and the hearts are shown as white bulges. Scale bar: 1 cm.

*okinoseanus* was anatomized. The flow rate was calculated as the heart volume multiplied by the heart rate.

Table 1 shows the ability (heart rate and flow rate) and the heart volume of the three heart types. The heart volumes were 0.0926, 0.289, and 0.0250 cm<sup>3</sup> for the cardinal hearts, branchial heart, and portal heart, respectively. The heart rates were 1.08,

1.20, and 0.339 beats/s for the cardinal hearts, branchial heart, and portal heart, respectively.

#### 4. Discussion

In addition to the “true” brachial heart in *E. okinoseanus*, we also found three accessory hearts (i.e., a pair of cardinal hearts and a single portal

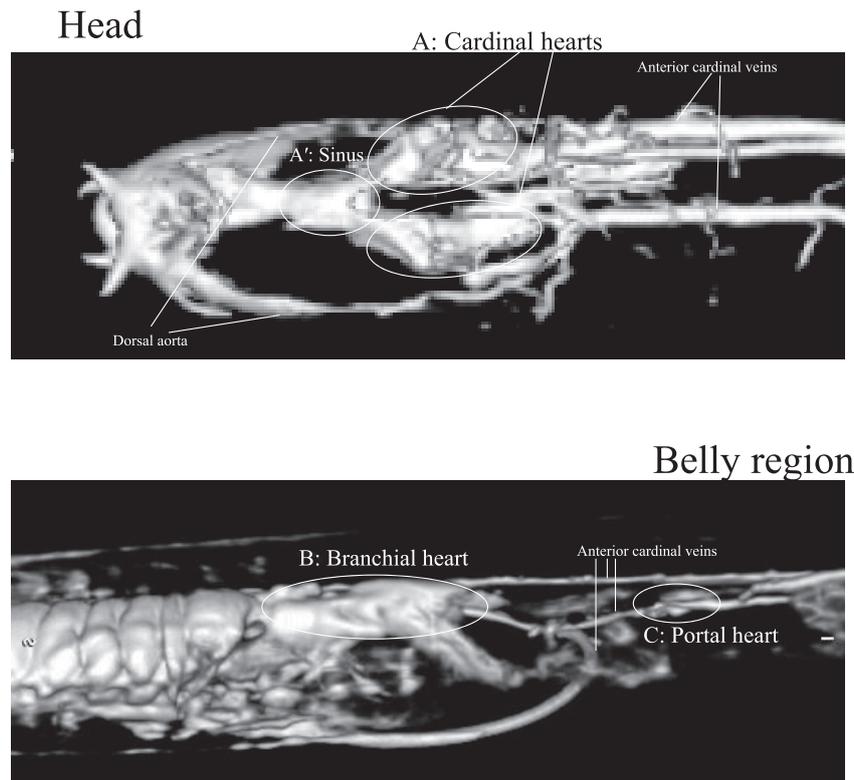


Fig. 2. CT scan data of *Eptatretus okinoseanus*. A: Cardinal hearts. B: Branchial heart. C: Portal heart. The CT images were expressed using Osirix software. A viewer and a color look-up table set 3D volume rendering and VR muscle-bones, respectively.

heart). The heart is an organ that pumps blood by creating positive and negative pressures at a regular rhythm. According to this definition, accessory hearts can be categorized as a heart in a broad sense. The pumping ability of the branchial heart (1.25 L/h) was much greater than that of accessory hearts (0.358 L/h and 0.0305 L/h in cardinal and portal hearts, respectively) (Table 1). This indicates that the branchial heart is likely to be the main driver of the blood circulatory system. The photomicrographs showed there were many empty spaces in the three accessory hearts (Fig. 3A–3C) that structurally resembled sponges. In humans, 64% of whole body blood in the veins and venules are maintained as blood reservoirs. The hearts of *E. okinoseanus* may not be able to stock much blood because the veins and venules are narrow. Those hearts may pump blood in and out of the whole body in a similar manner as a sponge absorbs and discharges water. The two cardinal hearts near the brain in *E.*

*okinoseanus* may be primitive hearts because their cell structure appeared similar to that of the skeletal muscle (Fig. 4).

The present study showed that the blood circulatory system of *E. okinoseanus* is similar to that of *M. glutinosa* having accessory hearts. According to some molecular-based phylogenetic studies, the Eptateretiformes and Myxiniformes form a monophyletic group<sup>9,10</sup>. The presence of accessory hearts in *E. okinoseanus* indicates that this is not a specific feature of *M. glutinosa*, but is probably shared in the Eptateretiformes and Myxiniformes clade. On the other hand, the number of accessory hearts varies among these taxa, and *M. glutinosa* has two more accessory (caudal) hearts than *E. okinoseanus*. Although the number of accessory hearts could have changed through their evolutionary history, this is beyond the scope of this study.

It is still unclear whether the presence of accessory hearts is the ancestral condition of vertebrates,

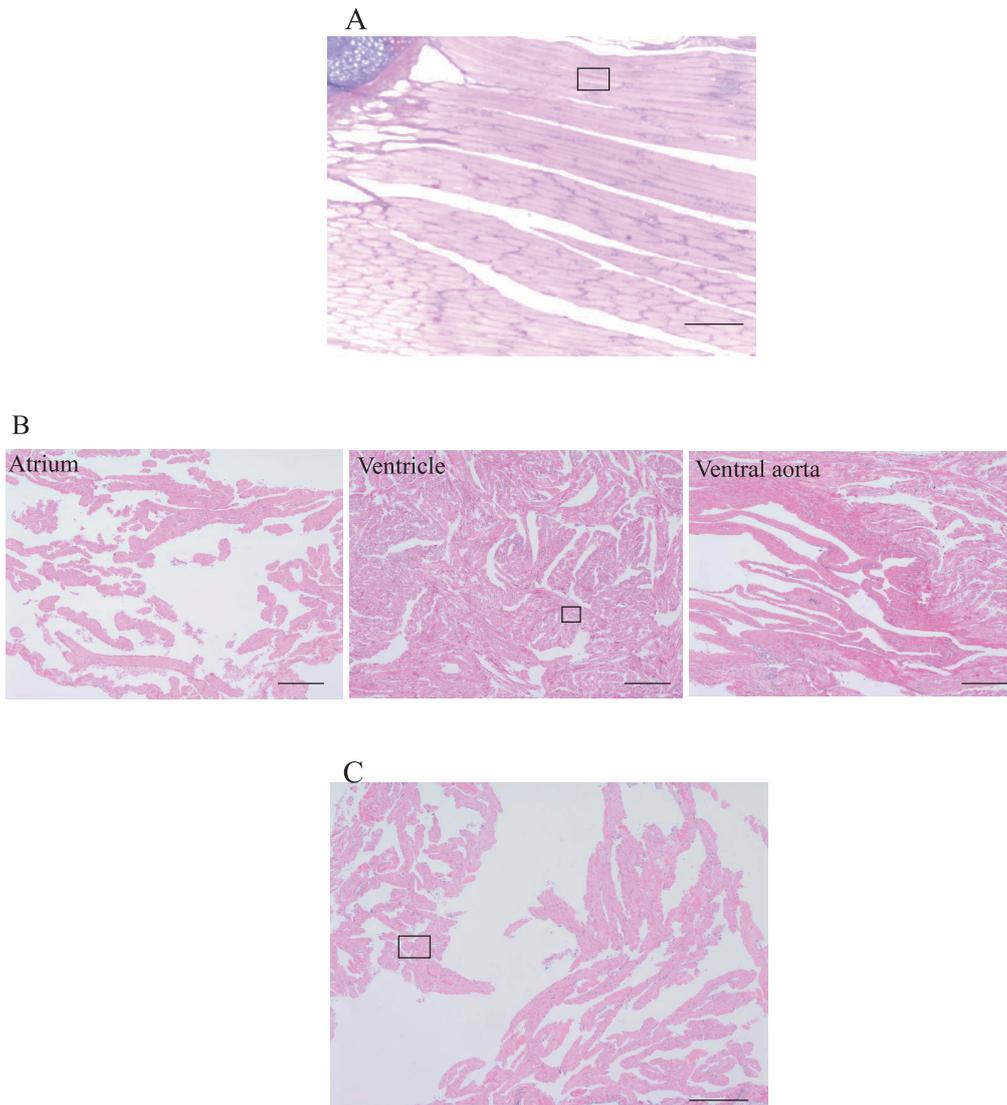


Fig. 3. A photomicrograph showing heart tissues stained with hematoxylin and eosin. The original objective magnification was  $\times 40$ . Scale bar: 0.5 mm. Fig. 4 shows higher magnification of the squares in Fig. 3.  
A: cardinal hearts. B: branchial heart. C: portal heart

but it is noteworthy that the muscular structure of *E. okinoseanus* cardinal hearts possibly represents a primitive condition of the vertebrate heart. Although cardinal hearts have the ability to pump like a “true” heart, their histology more closely resembles the skeletal muscle than the “true” heart muscle. In general, the vertebrate heart is composed of a cardiac muscle characterized by highly branching muscle fiber bundles. In contrast, accessory heart muscles lack branching and more closely resemble skeletal muscles that surround blood vessels in vertebrates. It is widely accepted that the vertebrate heart is derived

from blood vessels<sup>2</sup>. We can hypothesize that the heart of primitive vertebrates was composed of skeletal muscles, as seen in the cardinal hearts of *E. okinoseanus* and *M. glutinosa*.

We have studied lactate dehydrogenase (L-Lactate: NAD oxidoreductase, EC 1.1.1.27) A and B subunits (LD-A, B) in hagfishes, including *E. okinoseanus*, since 1997<sup>11-19</sup>. Vertebrates, including hagfishes, mainly have two subunits: LD-A rich skeletal muscle and B rich heart muscle<sup>20, 21</sup>. In our studies, two LD-Bs contained in six different hearts were found (data not shown), and the LD-A and -B

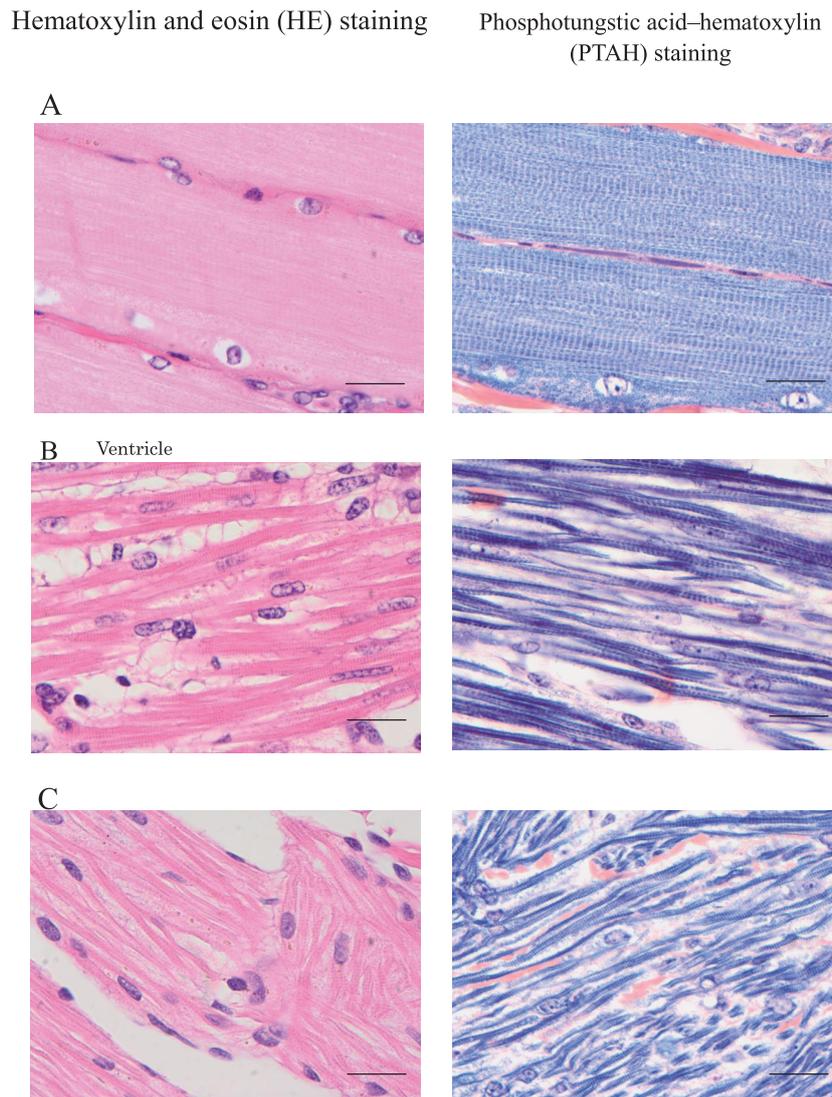


Fig. 4. A photomicrograph showing heart tissues stained with HE or PTAH. The original objective magnification was  $\times 1000$ . Scale bar: 20  $\mu\text{m}$ . This shows a higher magnification of squares in Fig. 3.  
A: cardinal hearts. B: branchial heart. C: portal heart

in vertebrates were of one type. Lampreys have only one LD. In the evolution from lamprey to hagfish, LD changed from LD to one LD-A and two LD-Bs. In the future, we will examine the ratio of the two LD-Bs in the three heart types, which may determine clear relationships between vertebrate heart evolution and those of LDs.

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we observed vascular blood circulation, the sinus, and two types of blood vessels (the dorsal aorta and anterior cardinal veins). Third, the hearts pump by creating positive and negative pressure at a regular rhythm. We examined heart histology to determine heart function. We observed many empty spaces in all heart tissue types, and hearts pump blood in and out of the whole body in a similar manner as a sponge absorbs and discharges water. The cardinal hearts showed primitive characteristics similar to the skeletal muscle. The branchial heart was the main operator, while the cardinal and portal hearts were

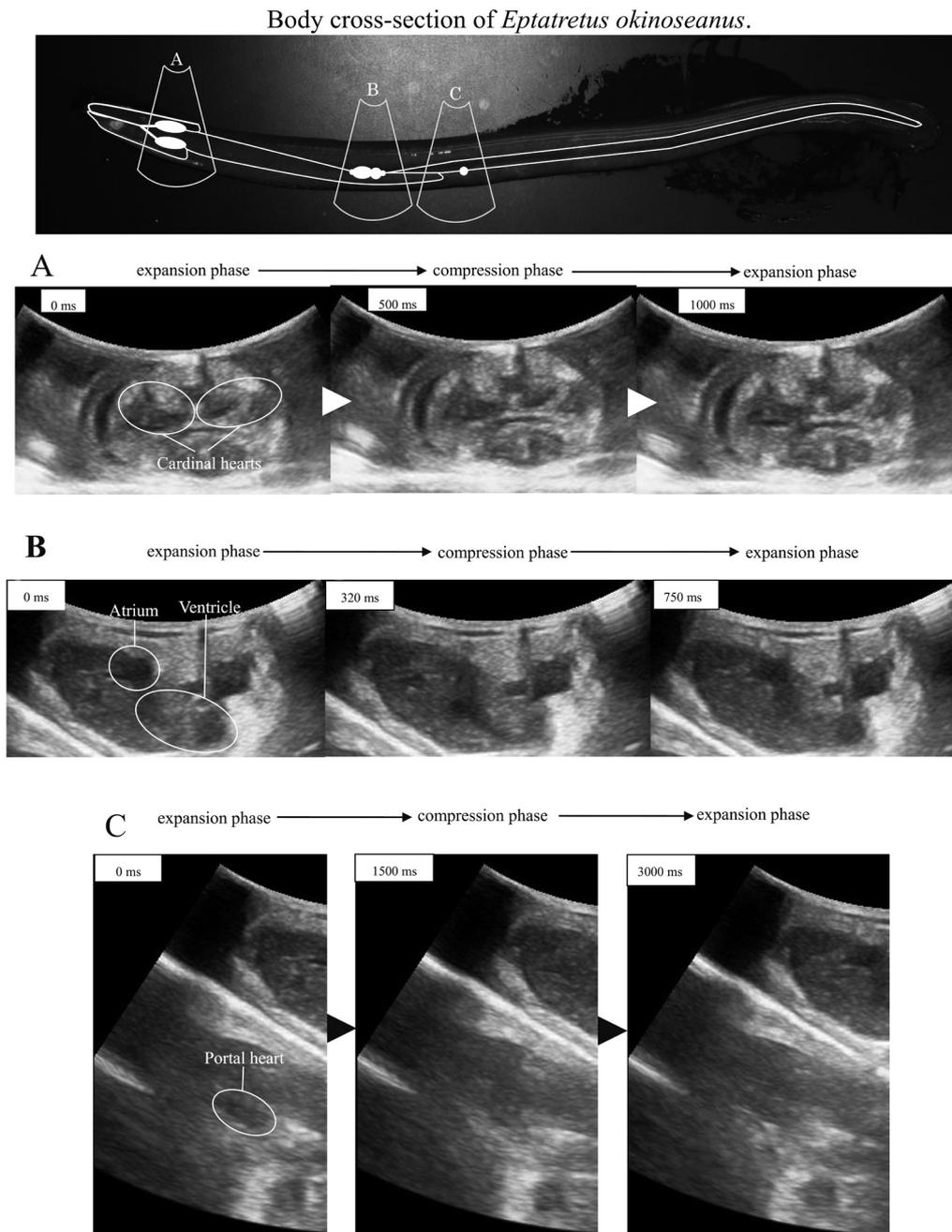


Fig. 5. Successive heart images using transthoracic echocardiography.  
A: cardinal hearts. B: branchial heart. C: portal heart

Table 1. The ability of three types of hearts in *E. okinoseanus*

	Volume (cm <sup>3</sup> )	Heart rate (beats/s)	Flow rate (L/h)
Cardinal hearts (two pieces)	0.0926	1.08	0.358
Branchial heart	0.289	1.20	1.25
Portal heart	0.0250	0.339	0.0305

assisting.

In heart evolution, the four hearts of *E. okinoseanus* were derived from the amphioxus. The two cardinal hearts in both sides of the brain may be primitive hearts because their cell structure resembled that of the skeletal muscle. Thus, the heart may have evolved from the skeletal muscle.

### Conflicts of interest

The authors declare no conflict of interests.

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