

# Unpaired Ultimobranchial Glands of the African Lungfish, *Protopterus dolloi*

Youko Shinohara-Ohtani<sup>1</sup> and Yuichi Sasayama<sup>2\*</sup>

<sup>1</sup>Sagami Chemical Research Center, Nishi-ohnuma 4-4-1, Sagamihara, Kanagawa 229-0005, Japan

<sup>2</sup>Noto Marine Laboratory, Faculty of Science, Kanazawa University, Uchiura, Ishikawa 927-0553, Japan

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**ABSTRACT**—The ultimobranchial glands of juvenile African lungfish (*Protopterus dolloi*) (14 individuals; total body length 25–205 mm) were immunohistochemically examined. In individuals larger than 36 mm, one ultimobranchial gland was close to the left afferent branchial arteries. The topography of the ultimobranchial gland was similar to that of salamanders and sharks, but not to teleosts. With body growth, the ultimobranchial gland was vascularized and the parenchymal cells were gradually immunostained with anti-calcitonin antibody. In all individuals examined, the ultimobranchial gland existed only on the left side of the pharynx. These observations are discussed from a phylogenetic viewpoint.

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## INTRODUCTION

Lungfish are members of the Osteichthyes. This fish is a key animal to understand the evolution of vertebrates, because it is near the ancestor that evolved teleosts and tetrapods. Therefore, lungfish have been discussed so far from various characters of their morphology (Jarvik, 1955; Cloutier and Ahlberg, 1996). Furthermore, recently, the primary structure of insulin of the African lungfish (Conlon *et al.*, 1997) and the neurohypophysial hormone genes of the Australian lungfish (Hyodo *et al.*, 1997) have been clarified, and these hormones are now known to be more similar to those of tetrapods than to teleosts. Therefore, lungfish are becoming more and more important in the study of the evolution of the vertebrates.

The ultimobranchial gland is an endocrine organ that secretes the bone-mineralizing hormone calcitonin. As this hormone is thought to have an important role in calcium homeostasis, all jawed vertebrates have ultimobranchial glands or have cells originating from this gland. The morphology and topography or development of the ultimobranchial glands have been examined so far in various non-mammalian vertebrates (Table 1). Three types of ultimobranchial gland exist: (1) one pair of the ultimobranchial glands on both sides of the pharynx; (2) unpaired glands on only the left side; and (3) one gland found near the heart and esophagus. However, no reports exist so far about the ultimobranchial glands of the lungfish and coelacanth. Only one figure drawn by Kerr (1910) is known, in which the position of the ultimobranchial gland is shown on the left side of the pharynx as a postbranchial body

in an African lungfish (no description of the species), without an explanation in the text.

This study was made using juvenile African lungfish (*Protopterus dolloi*) to 1) describe the ultimobranchial glands using immunohistochemistry, 2) compare the form of the glands with other vertebrates, and 3) discuss the paired or unpaired development of the ultimobranchial glands from the viewpoint of phylogeny.

## MATERIALS AND METHODS

Lungfish were obtained from a commercial source. Their initial body size was about 25 mm in total length. They were fed with frozen larvae of *Chironomus* or porcine liver three times a week, and were kept at 25°C under dim light. Fourteen individuals were used in this study, and their total body length was 25, 36, 38, 40, 40, 43, 72, 81, 110, 117, 155, 185, 195 and 205 mm. Individuals of 25–43 mm could swim actively, although external gills were still conspicuous. With growth, however, they became inactive. In smaller individuals, the anterior part of the body was cut off, and a notch was made in the pharynx. This anterior part was then fixed in Bouin's solution or in Bouin's solution without acetic acid in a shaker for 5 hr. In larger individuals, only the portion surrounding the pharynx was dissected out, and fixed similarly. Tissues were dehydrated, were embedded in paraffin, and were sectioned serially at 8 µm. The sections were stained by the labeled streptavidin biotin method (Giorno, 1984) using anti-salmon calcitonin antiserum, or with hematoxylin and eosin. The anti-calcitonin antiserum raised in rabbits by injections of synthetic salmon calcitonin I-BSA conjugate was used in this study. The specificity of the immunoreaction was checked by using normal rabbit serum in place of the primary antiserum, and by using primary antiserum inactivated by adding excess synthetic salmon calcitonin.

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\* Corresponding author: Tel. +81-768-74-1151;  
FAX. +81-768-74-1644.

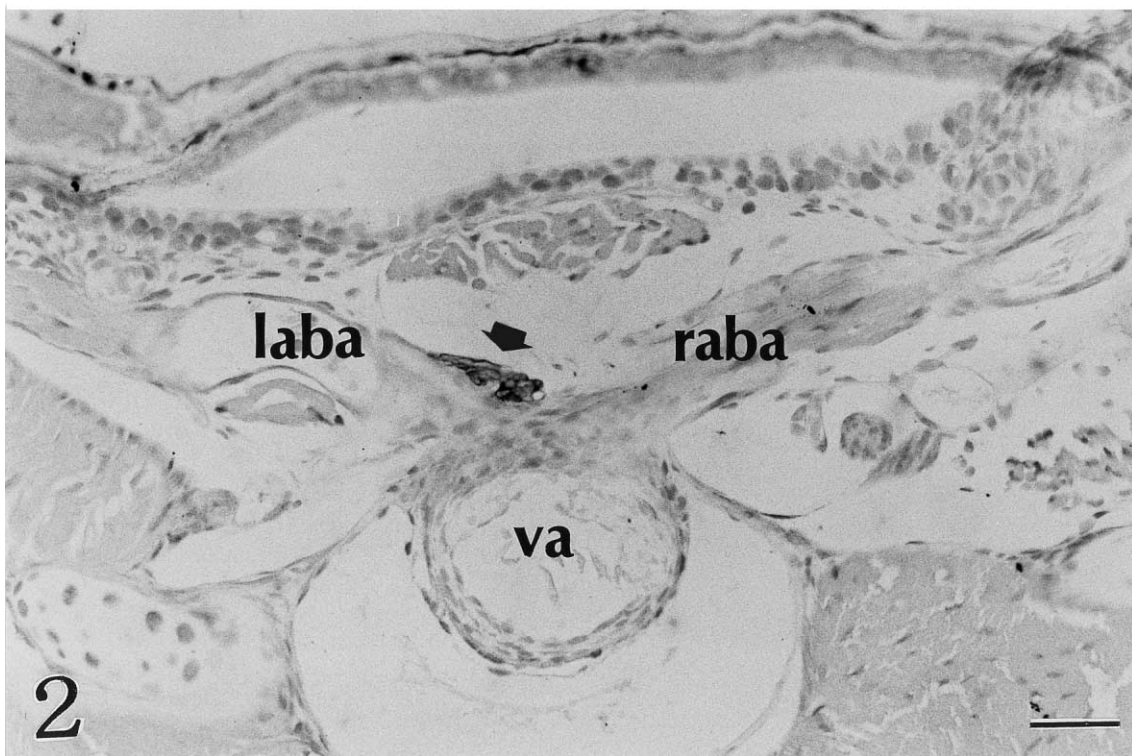
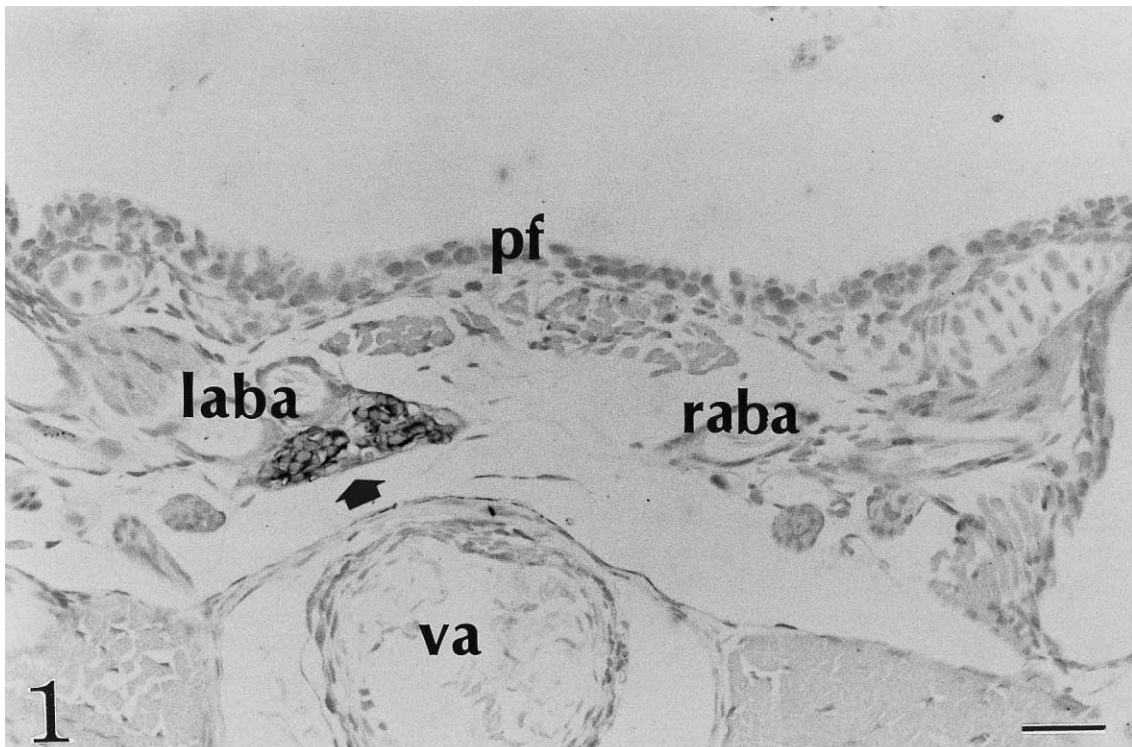
**Table 1.** The status of the ultimobranchial glands, showing the development in juveniles and/or the condition in adults

<i>Squalus acanthias</i>	One pair develops, but right side degenerates	Camp, 1917
<i>Dasyatis akajei</i>	One pair present	Sasayama and Oguro, 1992
<i>Protopterus</i>	In the figure left side only, no description in the text	Kerr, 1910
<i>Protopterus dolloi</i>	Only left side	in this study
<i>Anguilla japonica</i>	One gland on the pericardium and esophagus	Otani <i>et al.</i> , 1975
<i>Onchorhynchus keta</i>	One pair develops	Sasayama <i>et al.</i> , 1989
<i>Carassius</i>	One pair develops, but unpaired in adults	Giacomini, 1912
<i>Carassius auratus</i>	One gland on the esophagus	Sasayama and Oguro, 1992
<i>Oryzias latipes</i>	One gland on the Cuvierian duct	Sasayama <i>et al.</i> , 1995
<i>Hypogonophis rostratus</i>	One pair develops	Marcus, 1908
<i>Ichthyophis glutinosus</i>	One pair develops, but maximum size in the larvae	Klump and Eggert, 1935
<i>Typhlonectes compressicada</i>	No glands in adults	Sasayama, unpublished data
<i>Hynobius nigrescens</i>	Only left side develops	Sasayama <i>et al.</i> , 1986
<i>Onychodactylus japonicus</i>	Only left side present	Oguro <i>et al.</i> , 1983
<i>Amblystoma</i>	Only left side present	Baldwin, 1918
<i>Amblystoma opacum</i>	Only left side present	Uhlenhuth and McGowan, 1924
<i>Triturus v. viridescens</i>	Only left side present	Steinitz and Stone, 1954
<i>Cynops pyrrhogaster</i>	Only left side present	Oguro <i>et al.</i> , 1983
<i>Amphiuma</i>	One pair develops	Wilder, 1929
<i>Necturus</i>	One pair develops	Wilder, 1929
<i>Xenopus laevis</i>	One pair develops, but degenerates	Sterba, 1950
<i>Xenopus laevis</i>	Degenerated in adults	Sehe, 1960
<i>Bufo</i> , <i>Hyla</i> , <i>Pelobates</i>	One pair present	Boschwitz, 1960
<i>Pseudacris nigrata</i>	One pair present	Robertson and Swartz, 1964
<i>Rana esculenta</i>	One pair develops	Maurer, 1888
<i>Rana fusca</i>	One pair develops	Watzka, 1933
<i>Rana japonica japonica</i>	One pair develops	Sasayama <i>et al.</i> , 1976
<i>Chrysemys picta</i>	One pair, but right side degenerative	Clark, 1971
<i>Pseudemys scripta</i>	One pair, but right side degenerative	Clark, 1971
<i>Crocodilus porosus</i>	One pair develops, but right side degenerates	Hammar, 1937
<i>Lacertidae</i>	Only left side present	Peters, 1941
<i>Takydromus tachidromoides</i>	Only left side present	Sasayama and Oguro, 1992
<i>Elaphe quadrivirgata</i>	One pair present	Yoshihara <i>et al.</i> , 1979
<i>Elaphe conspicillata</i>	One pair present	Yoshihara <i>et al.</i> , 1979
<i>Rhabdophis tigrinus tigrinus</i>	One pair present	Yoshihara <i>et al.</i> , 1979
<i>Agkistrodon halys</i>	One pair present	Yoshihara <i>et al.</i> , 1979
<i>Laticauda semifasciata</i>	One pair present	Yoshihara <i>et al.</i> , 1979
<i>Gallus</i>	One pair develops	Bolk <i>et al.</i> , 1937
<i>Sturnus vulgaris</i>	Scattered in both sides	Clark, 1983

## RESULTS

In an individual of 25 mm, the smallest among the fishes examined, the ultimobranchial gland had not differentiated. However, in an individual of 36 mm, the ultimobranchial gland was found only on the left side of the pharynx. In the lungfish, the arterial trunk from the heart becomes five pairs of afferent branchial arteries to both sides of the jaw. The ultimobranchial gland was present adhering to the last two afferent branchial arteries of the left side (Fig. 1). The ultimobranchial gland was a thick sheet-like tissue with a lumen in the central part. The cytoplasm of every parenchymal cell was little and the number of calcitonin-immunoreactive cells were few. The end of the gland reached almost to the branching point to the right

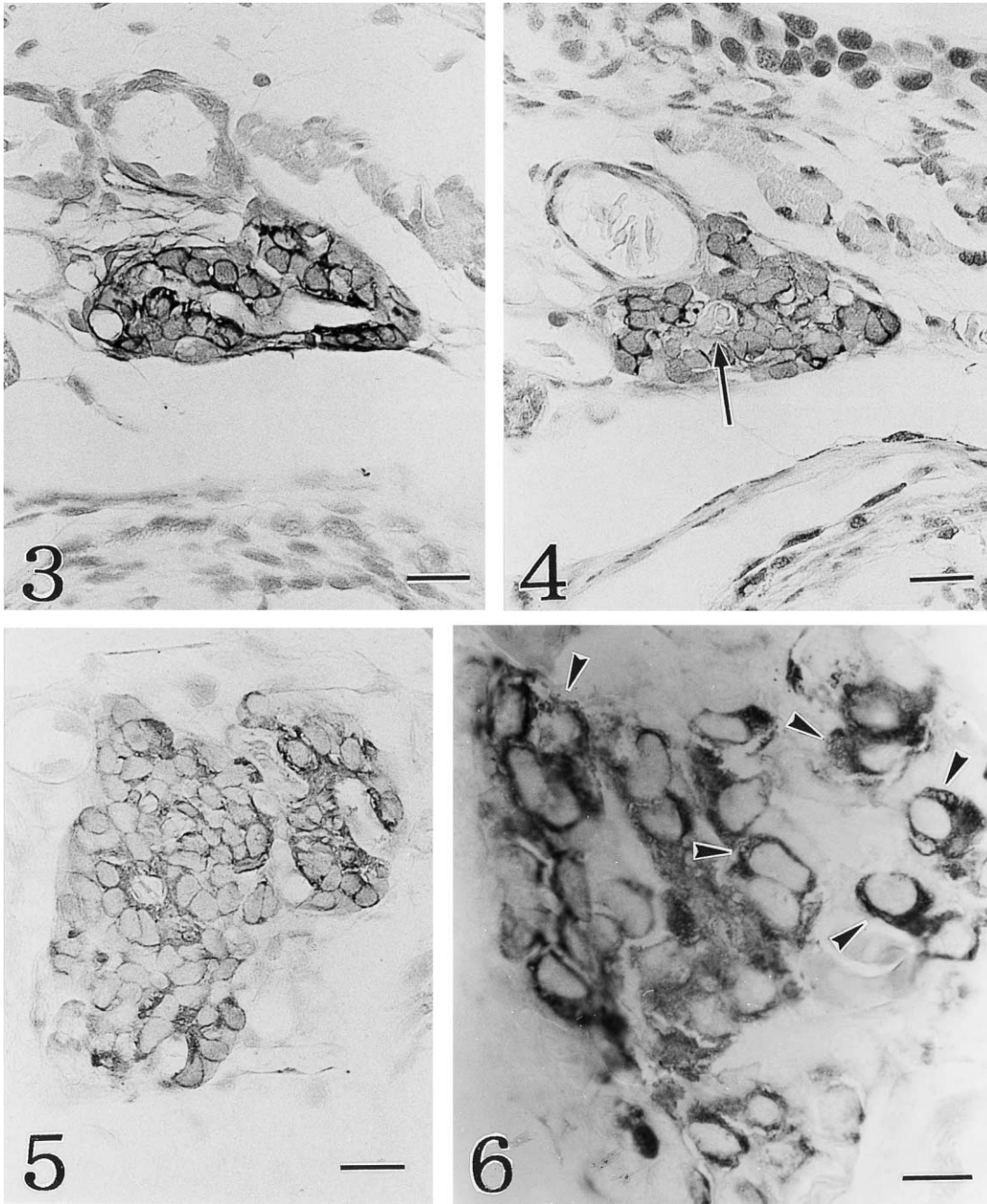
and left of the fifth afferent branchial artery (Fig. 2). However, at this stage, near the right afferent arteries no ultimobranchial gland nor calcitonin-immunoreactive cells were found. In this specimen and a 38 mm individual, a lumen was in the gland (Fig. 3). In a 40 mm individual, the parenchyma was well vascularized (Fig. 4). In an individual of 43 mm, although one part of the tissue still adhered to the left afferent branchial artery, the ultimobranchial gland had begun to separate from the artery (Fig. 5). In a 81 mm individual, the calcitonin-immunoreactive cells of the gland had bubble-like particles in the cytoplasm, which might suggest active metabolism of the cells (Fig. 6). In an individual of 117 mm, the ultimobranchial gland was independent from, but close to, the afferent branchial artery as a thick and compact body (Fig. 7). The calcitonin-im-



**Fig. 1.** Ultimobranchial gland (arrow) of a 36 mm individual. Immunostained section. This gland contains a lumen in the central part. Note that the ultimobranchial gland does not exist on the right side. laba, left afferent branchial artery; pf, pharyngeal floor; raba, right afferent branchial artery; va, ventral aorta. These abbreviated words are used also in following figures. Bar = 70  $\mu$ m.

**Fig. 2.** Caudal end of the ultimobranchial gland (arrow) of Fig. 1, which reaches the branching point of the left and right branchial arteries. Bar = 70  $\mu$ m.





**Fig. 3.** Ultimobranchial gland with a lumen in a 38 mm individual. Immunostained section. Bar = 40  $\mu$ m.

**Fig. 4.** Vascularized ultimobranchial gland in a 40 mm individual. Immunostained section. The arrow shows a blood capillary with red blood cells. Bar = 40  $\mu$ m.

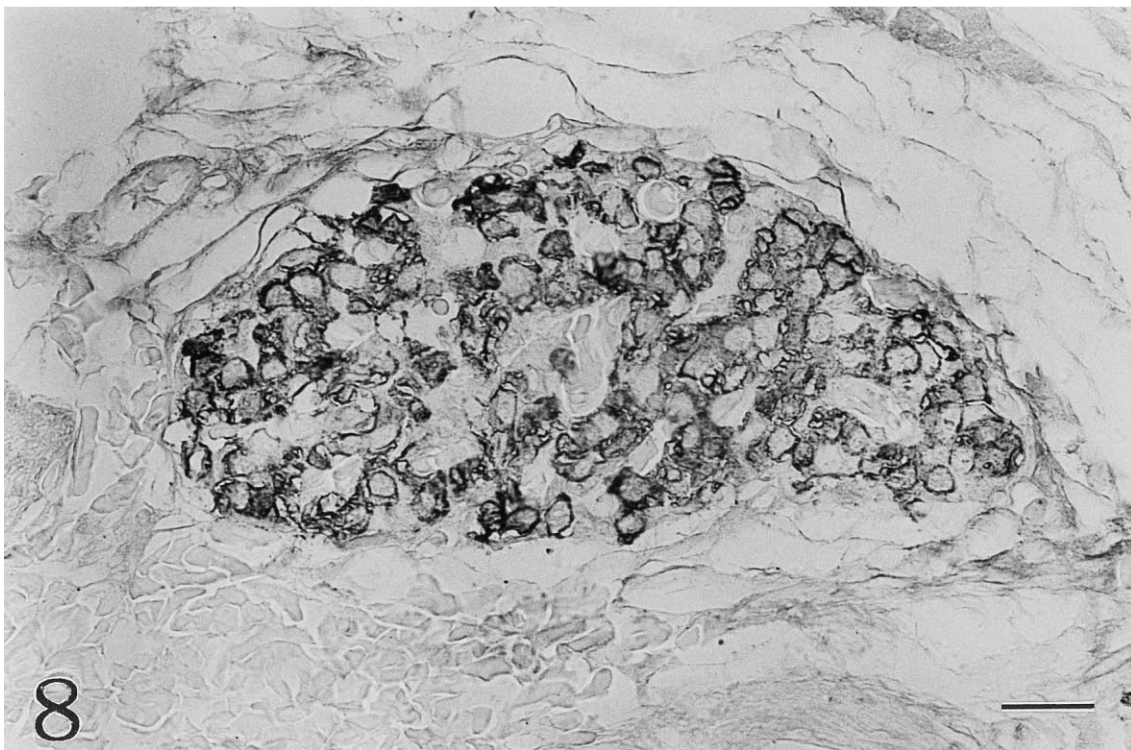
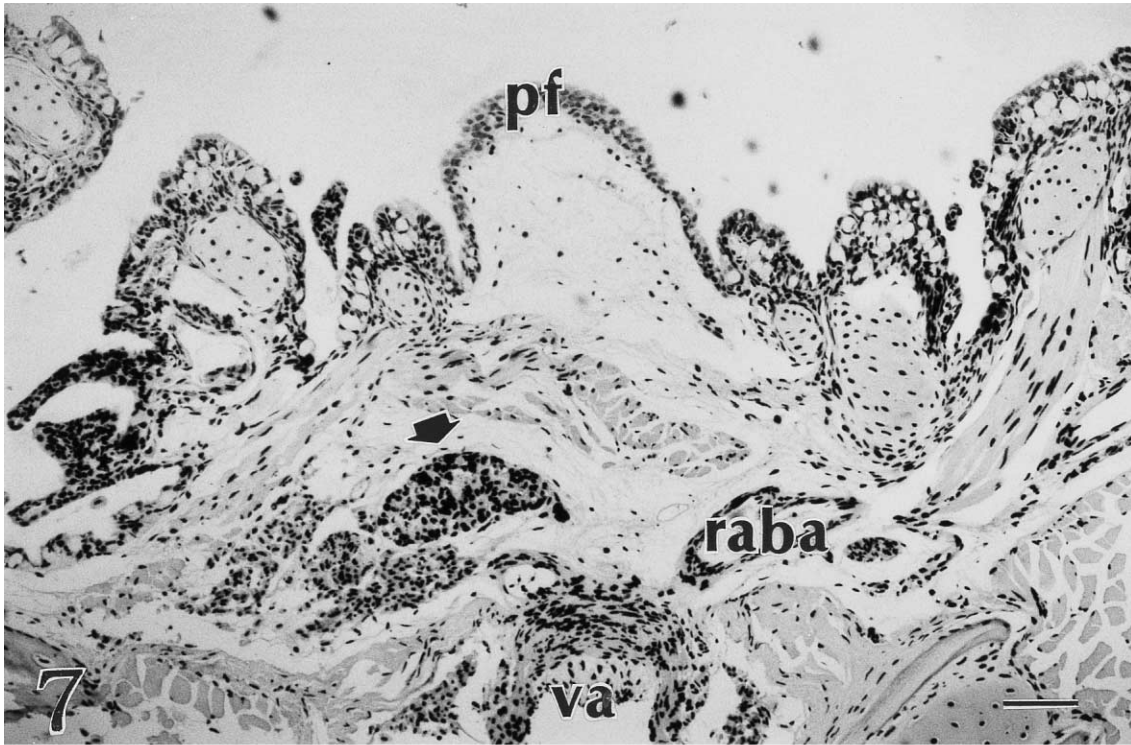
**Fig. 5.** Ultimobranchial gland in a 43 mm individual, which began to be independent from the labia. Immunostained section. Bar = 40  $\mu$ m.

**Fig. 6.** Ultimobranchial gland in a 81 mm individual. Immunostained section. Note that the cytoplasm of some calcitonin-immunoreactive cells includes bubble-like particles (arrowheads). Bar = 20  $\mu$ m.

munoreactive cells were arranged reticularly (Fig. 8), and had many bubble-like particles in the cytoplasm. In a 185 mm individual, a small mass of calcitonin-immunoreactive cells existed on the left side in addition to an authentic ultimobranchial gland (Fig. 9a, b). This small tissue seems to be so-called para-ultimobranchial gland (Fig. 9c).

In all lungfish examined, ultimobranchial glands and cal-

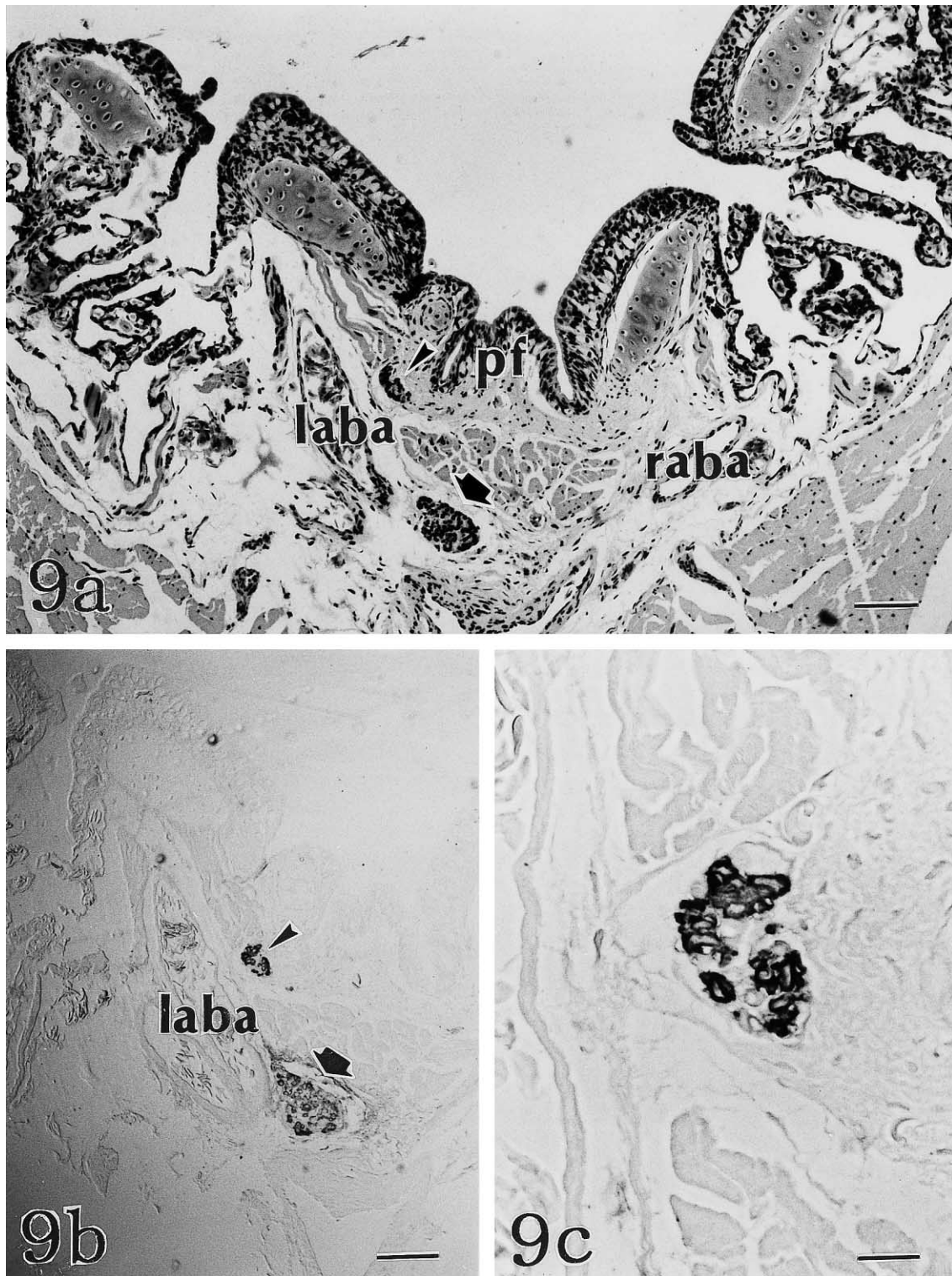
citonin-immunoreactive cells could not be found on the right side of the pharynx. Therefore, in this species, in individuals at least larger than 36 mm fish, we may conclude that the ultimobranchial gland is present only on the left side of the pharynx. Figure 10 shows the position of the gland schematically, with comparisons to that of the shark, eel and salamander.



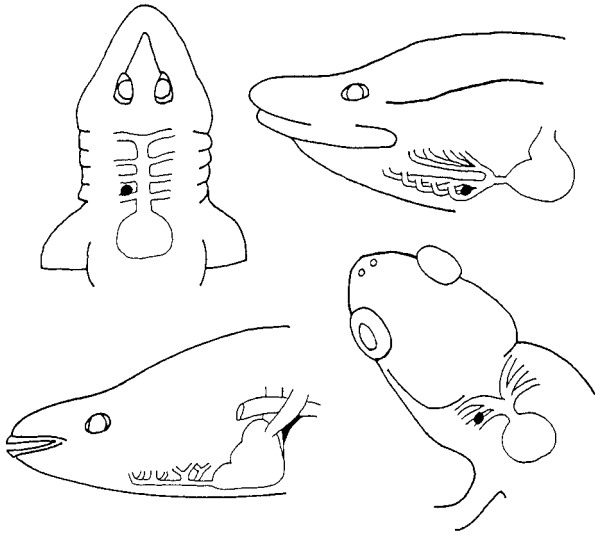
**Fig. 7.** Ultimobranchial gland in a 117 mm individual. Hematoxylin and eosin staining. The arrow shows the ultimobranchial gland. Note that no ultimobranchial gland is on the right side. Bar = 200  $\mu$ m.

**Fig. 8.** Ultimobranchial gland in a 117 mm individual. Adjacent section of Fig. 7 stained immunohistochemically, showing magnification of the ultimobranchial gland. Note that calcitonin-immunoreactive cells are arranged reticularly and have many bubble-like particles as in Fig. 6. Bar = 40  $\mu$ m.





**Fig. 9.** (a) Ultimobranchial gland with a para-ultimobranchial tissue in a 185 mm individual. Hematoxylin and eosin staining. The arrow shows the ultimobranchial gland; the arrowhead shows the para-ultimobranchial tissue. Note that no ultimobranchial gland is on the right side. Bar = 200  $\mu$ m. (b) Adjacent section of (a) stained immunohistochemically, showing ultimobranchial gland (arrow) and a para-ultimobranchial tissue (arrowhead). Bar = 200  $\mu$ m. (c) Magnification of a para-ultimobranchial tissue. Immunostained section. Bar = 30  $\mu$ m.



**Fig. 10.** A schematic drawing of the position (not the size or shape) of the ultimobranchial glands in four species of vertebrates. Top left, shark; top right, lungfish (side view); lower left, eel (side view); lower right, salamander.

## DISCUSSION

The developmental processes of the ultimobranchial glands of this lungfish are closely similar to those of the salamander (*Hynobius nigrescens*) (Sasayama *et al.*, 1986), and are also in part similar to the salmon (*Onchorhynchus keta*) (Sasayama *et al.*, 1989) and to the frog (*Rana japonica japonica*) (Sasayama *et al.*, 1976). In the salamander, at the stage in which yolk granules remain in the cytoplasm, the ultimobranchial gland arises as a protrusion of the pharyngeal floor only on the left side in the direction of the heart. With body growth, calcitonin-immunoreactive cells gradually increase in number in the parenchyma of the gland. However, from the right side of the pharyngeal floor, nothing arises. In the salmon and frog, the ultimobranchial glands are formed bilaterally in the pharynx. Each anlage on both sides assumes a follicular structure from the early stages, which remains for life, and is different from the lungfish. Also in the salmon, calcitonin-immunoreactive cells in the parenchyma increase in number with body growth. Therefore, the fact is interesting that lungfish already include the prototype development of the salmon and amphibia.

The position of the ultimobranchial glands of lungfish is closely similar to that of the salamander (Sasayama *et al.*, 1986) and the spiny shark (*Squalus acanthias*) (Yamada *et al.*, 1991), but not to teleosts (see Fig. 10). In the lungfish, the ultimobranchial gland is on the left side at the basal portion of the fifth afferent branchial artery. In the metamorphosed salamander, the afferent branchial arteries are transformed as three pairs of aortic arches. The ultimobranchial gland is close to those arches, and only on the left side. Also in the adult spiny shark, the ultimobranchial gland is on the left side near the last afferent branchial artery. However, in most teleosts, the position of the ultimobranchial gland is not related to the

afferent branchial arteries, at least in the adults (Pang, 1971; Sasayama *et al.*, 1989, 1995). The ultimobranchial glands are on/in the transverse septum that separates the heart and the viscera. In some species, such as goldfish, the gland is on the ventral side of the esophagus (Sasayama and Oguro, 1992). In all jawed vertebrates examined so far, the ultimobranchial gland is formed by an invagination of the pharyngeal floor in the direction of the heart. In most vertebrates, the ultimobranchial glands are fundamentally located anterior to the heart. Although in adult frogs the ultimobranchial glands appear to be present just beneath the glottis (Robertson, 1971), the relative position is the same. Furthermore, in the stingray (*Dasyatis akajei*), the ultimobranchial glands are just above the heart (Sasayama and Oguro, 1992), which may be related to the extreme flattening of the body. Nevertheless, only in teleosts is the ultimobranchial gland located just posterior to the heart, including the position of the esophagus, although the reason is not clear.

On the other hand, even in the same animal group, paired and unpaired ultimobranchial glands are present (see Table 1). Also, in some animals, the ultimobranchial glands in the juveniles develop as paired glands, but in adults they are unpaired, because the right ultimobranchial gland degenerates to varying degrees. In addition, even if in juveniles the ultimobranchial glands develop bilaterally, in the adult stage both sides degenerate, as seen in apoda and *Xenopus*. These observations suggest that among vertebrates, the necessity of the ultimobranchial glands is different from species to species to varying degrees even among their life stages. In the lungfish examined in this study, calcitonin-immunoreactive cells were never found on the right side as a complete gland or even as scattered cells. In one individual, a small mass of calcitonin-immunoreactive cells was found on the same side as the ultimobranchial gland. This cell mass might have separated from the main gland during development, and then migrated there. Why the right ultimobranchial gland is not present in the lungfish is unknown. However, this may be related to a phenomenon that in the lungfish, lungs are formed at the dorsal position by the torsion of lung rudiments to the right side. In fact, in *Protopterus* the right lobe of the lung is relatively small as compared to the left at least during the early development (Kerr, 1910).

In cartilaginous fishes, which are one of the old animals, the ultimobranchial glands develop bilaterally, at least in the juveniles (Camp, 1917). The ultimobranchial glands may have originally developed bilaterally. However, when considering the data in Table 1, the tendency seems to be that in the same animal group, animals with one paired ultimobranchial glands are morphologically more specialized, compared to the animals with an unpaired gland: the ray is more specialized than the shark, the frog is more specialized than the salamander, and the snake is more specialized than other reptiles. Nevertheless, the form of the ultimobranchial glands remains in the prototype condition. The phylogenetical meaning of this phenomenon is not clear at present.

Although in the adult salmon ultimobranchial glands are



present bilaterally, most adult teleosts have an unpaired gland just behind the heart. This may be another specialization in which one pair of glands fuse with each other during development.

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