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Habitats and Air Uptake Based on Analysis of Skin Structure of Two Korean Bullheads, *Pseudobagrus brevicorpus* and *P. koreanus* (Pisces; Bagridae)

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Abstract: The skin of the Korean bullheads, *Pseudobagrus brevicorpus* and *P. koreanus*, is composed of epidermis, dermis and subcutis. The epidermis has three layers, the outermost layer, middle layer and stratum germinativum. The epidermis consists of two types of gland cells, an unicellular mucous cell of sulfomucin and a large club cell having sometimes two nuclei. The epidermis has numerous intraepithelial blood vessels in *P. brevicorpus* but not at all in *P. koreanus*. Lymphatic spaces containing lymphocytes are well developed in mainly the stratum germinativum. The dermis lacks scales and consists mostly ofbundles of coarse collagen fibers. The collagen bundles are arranged in parallel to each other in the dorsum and lateral region toward the dorsum, but vertically at intervals in the abdomen and lateral region toward the abdomen. Considering this unique skin structure, the two species are likely to exercise cutaneous respiration as a dual respiratory system to overcome hypoxic conditions which frequently occurs in their habitats.

Key words: Korean bullheads, *Pseudobagrus brevicorpus, P. koreanus,* skin, cutaneous respiration

The Korean endemic bullheads, *Pseudobagrus brevicorpus* and *P. koreanus*, are demersal freshwater fishes that live in large rocky or stony bottoms of the upper or middle stream (Park and Kim, 2002). The water level in such a stream varies considerably with season. During the dry season when water levels are low, the water flow is reduced and many areas become hypoxic, that is, the water contains little dissolved oxygen. Even under such harsh conditions, however, life is maintained. Among the Korean silurid fishes, *Liobagrus mediadiposalis* is an air-breathing fish and can utilize oxygen in the air, whose content is much higher, in order to overcome the hypoxic condition in the water (Park et al., 2003). *P. brevicorpus* and *P. koreanus* show very similar habitat to *L. mediaposalis*. Air-breathing behavior occurs during prolonged seasonal exposure to dry mud or swamp, during brief standings by receding waters, and during volitional emergence (Jakubowski, 1958; Johansen, 1970; Mittal and Munshi, 1971; Mittal et al., 1980; Graham, 1997). Through habitat-based ecological observation and histological study of *P. brevicorpus* and *P. koreanus,* whether they can uptake air through skin and whether they can be designated as indicator species can be determined.

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MATERIALS AND METHODS

Eight adults of *Pseudobagrus brevicorpus* (52. 7 to 69. 4 mm SL) were collected in the Nakdong River, Korea, May, 2006, and ten adults of *P. koreanus* (195.7 to 215.3 mm SL) in Gosan stream, Korea, June, 2006. The specimens were fixed in 10% neutral buffered formaldehyde, and subsequently skin fragments, about 5×5 mm^2, were taken from three regions: dorsum, lateral region and abdomen.

For histological examination, the skin fragments were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). We deparaffinized 5 μm sections and stained them with Harris hematoxylin, Ehrlich hematoxylin, counter-stained with eosin, and Masson trichrome stain for general histology.

We stained for polysaccharides with alcian blue (AB) at pH 1.0 and 2.5, and periodic acid-Schiff (PAS). We used the PAS technique in combination with AB (pH 2.5), and

AB (pH 2.5)-PAS, to distinguish between neutral and acidic mucins. We stained for acid mucin by metachromatic reaction with toluidine blue. We used high iron diamine (HID) with AB to assess the nature of the acid mucin. The above staining techniques followed the methods described by Humason (1972). For photography and skin evaluation, we used Carl Zeiss vision (LE REL. 4.4, Germany).

For scanning electron microscopic observations of the skin, three specimens were fixed in each stage of larval development for 24 h under 4°C in cacodylate-buffered 2.5% glutaraldehyde, dehydrated by a graded series of ethanol, dried to a critical point with liquid CO_2. The dried materials were coated with gold by ion sputtering and then examined under a Hitach S-450 scanning electron microscope.

RESULTS

General habitat

Pseudobagrus brevicorpus prefers ecological conditions of 30 to 50 cm/sec in water velocity, pebbly bottoms or small crevices overlapped with small and large pebbles of 5 to 20 cm in diameter and 50 to 120 cm in water depth. In this habitat, *Coreoperca herzi, Liobagrus mediadiposalis, Odontobutis platycephala, Niwaella multifasciata,* and *Koreocobitis naktongensis* coexist. The habitats of *P. koreanus* were similar to *P. brevicorpus.* While the habitats of *P. brevicorpus* and *P. koreanus* are usually filled with water throughout year, they sometimes undergo a dramatic change in water level because the substrata consisting mostly of pebbles or rocks are exposed to air during drought season. Consequently only a little water remains under rocks and pebbles.

General feature of the skin

The skin of the two Korean bullhead fishes could be divided into three principal layers, the epidermis, the dermis, and the subcutis (Fig. 1A; Fig. 3A).

Epidermis

The epidermis is composed of three layers, the outermost layer, middle layer and stratum germinativum (Fig. 1A; Fig. 3B). Average thickness of the epidermis in three regions (dorsum, lateral region and abdomen) is 167. 4 μm to 312. 6 μm in *P. koreanus* and 153. 4 μm to 274. 6 μm in *P. brevicorpus*. Among the three regions, the abdomen is the thickest, and the dorsum is the thinnest (Table 1).

The thin outermost layer consists of several types of epidermal cells including polygonal cells and a few flattened cells which are arranged in 3 to 12 rows, and mucous gland cells (Fig. 1A; Fig. 3C). Most mucous cells are spherical or some flask-shaped structures, but some in the abdomen have a large spherical body and a short narrow neck (Figs. 1A to 1F; Fig. 2). The nucleus is basal, spherical or oval with a thin rim of slightly basophilic cytoplasm. The size of mucous cells varied, ranging from 12.2 μm to 16.5 μm in height in *P. koreanus* and 13.6 μm to 16.0 μm in *P. brevicorpus.* The mucous cells in both species give a deep red color in PAS reaction were diastase resistant, γ-metachromasia with toluidine blue, blue with AB at pH 1.0 and 2.5 (Figs. 1B to 1F, Table 2).

The middle layer is the thickest and composed mainly of voluminous club cells, a few flask or spherical mucous cells and a few lymphocytes (Fig. 1A; Figs. 3B and 3C). In between these skin glands the elongated spindle-shaped epidermal cells are found. The cell boundaries of these cells are usually not clear as they are greatly expanded or stretched out due to the collateral pressure of these glands. The club cells arranged in 2 to 11 layers are oval or spherical in shape (Figs. 1A to 1F; Fig. 2; Figs. 3C and 3D) and they are more numerous than the spindle-shaped epidermal cells. They are usually uninucleate, with an oval nucleus, but are sometimes binucleate with two nuclei very close to each other. Their cytoplasms are finely granular or more or less homogeneous in nature and invariably show some shrinkage due to fixation. Some have a few vacuoles in their cytoplasm. Each club cells are varied in height ranging from average of 58.2 μm to 80.0 μm in *P. koreanus* and 48.1 μm to 58.1 μm in *P. brevicorpus*. In both species, the region occupied by larger club cells is thicker-the abdomen consisting of numerous large club cells has the thickest middle layer, average 80.0 μm in *P. koreanus* and 58.1 μm in *P. brevicorpus*. The thickness of the skin as well as the middle layer is greatly affected by their club cell's features. The club cell's nuclei stain blue and their cytoplasms are stained light pink with hematoxylin/eosin staining. However, they do not have histochemical reaction for mucosubstances as in the mucous cell (Figs. 1B to 1F). Interestingly, for *P. brevicorpus*, the middle layer is characterized by the presence of fine blood capillaries accompanied by dermal collagen (Figs. 2A to 2D). Vascular capillary networks are scattered in the mid-epidermis. However, there are no such blood capillaries in *P. koreanus.*

The stratum germinativum consists of a single layer of cuboidal cells or a few columnar cells. Each basal cell has a prominent, lightly stained, centrally placed spherical or oval nucleus, and the cytoplasm is homogeneous (Fig. 1A) Between the basal cells, small, oval or round lymphatic spaces are present (Fig. 1A). They contain one to three small lymphocytes with deeply stained nuclei and small amounts of faintly stained cytoplasm. The lymphocytes are seen in the middle layer and sometimes in the outermost layer of the epidermis, and are PAS positive and AB positive, and they turn green in Masson trichrome.

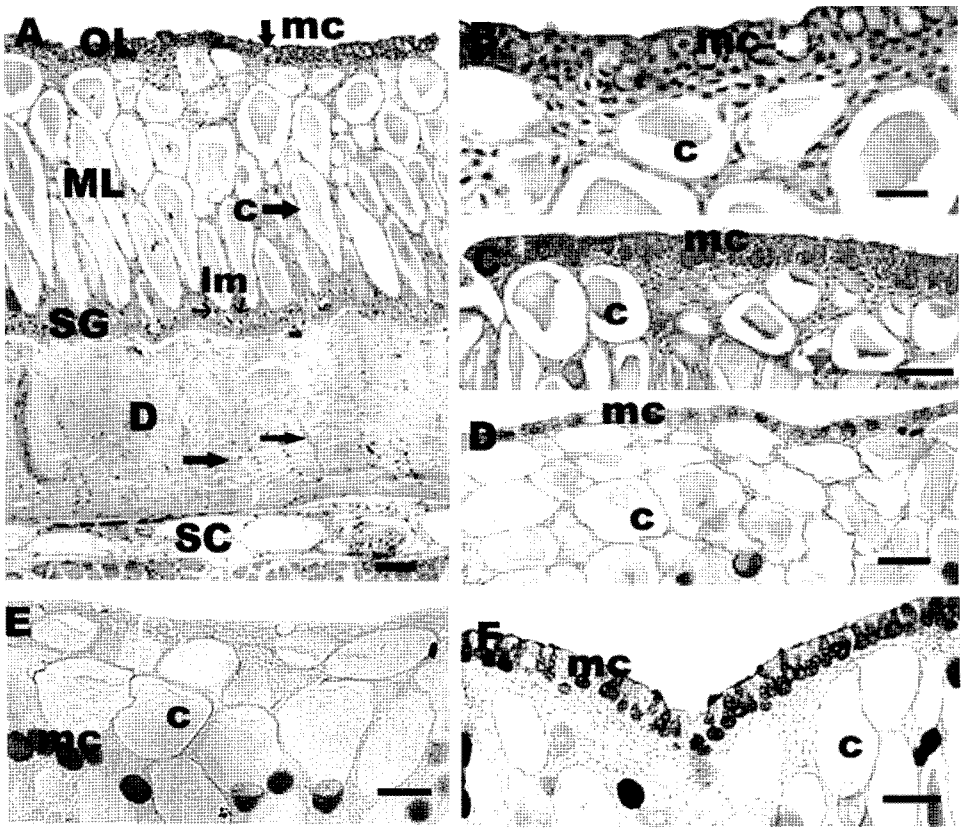

Fig. 1. Transverse sections of the skin of *Pseudobagrus brevicorpus* (A to C) and *P. koreanus* (D to F). A, The dorsal skin with haematoxylin and eosin. The skin is composed of epidermis including outermost layer (OL), middle layer (ML), and stratum germinativum (SG), dermis (D) and subcutis (SC). The epidermis has epidermal glands of mucous cell (mc) and club cell (c), and there are numerous lymphocyte (lm) at the stratum germinativum; Some collagen bundles are vertically arranged at intervals in the dermis (arrows). Bar = 50 μm; B, The dorsum with haematoxylin and eosin The mucous cells (ms) and club cells (c) are seen. Bar = 20 μm; C, The abdomen with Toluidine blue. The mucous cells (mc) are positive and the club cells (c) negative. Bar = 20 μm; D, The dorsum with AB (pH 1.0). The mucous cells (mc) situated at the outermost layer are positive. Note that the club cells negative. Bar = 20 μm; E, The dorsum with PAS staining. The mucous cells (mc) scattered at the middle layer of the epidermis are positive. No club cells (c) show any reaction. Bar = 20 μm; F. Lateral skin with HID stanining. The mucous cells (mc) of the outermost layer are positive but the club cells (c) are negative. Bar = 20 μm

Dermis

In both species, the dermis is the thickest in the abdomen and their average thickness ranges from 80.9 μm to 218.8 μm in *P. koreanus* and from 74.9 μm to 102.4 μm in *P. brevicorpus* (Table 1).

The dermis lacks scales and consists mostly of bundles of coarse collagenous fibers arranged compactly in several layers (Fig. 1A). The collagen bundles are arranged in parallel to each other in the dorsum and lateral region toward the dorsum, but vertically at intervals in the abdomen and lateral region toward the abdomen (Fig. 1A). This layer is weakly PAS positive and become deep green in Masson trichrome-stained preparations for collagen. Pigment cells and blood capillaries are present just beneath the basement membrane or sometimes randomly in this layer. The pigment cells are more abundant in the dorsum and lateral region.

Subcutis

This layer includes the nerves and numerous blood vessels

Table 1. A Relative thickness of three skin regions of *Pseudobagrus koreanus* and *P. brevicorpus*

Region \ Species	*P. koreanus* (n = 10)				*P. brevicorpus* (n = 8)			
	Epidermis (μm)		Dermis (μm)		Epidermis (μm)		Dermis (μm)	
	Mean	Min-Max	Mean	Min-Max	Mean	Min-Max	Mean	Min-Max
Dorsum	167.4	150.6-180.6	80.9	66.9-94.9	153.4	136.3-179.4	74.9	71.1-85.8
Lateral region	203.1	190.4-226.0	135.5	125.0-139.3	185.4	168.2-205.3	86.0	68.4-96.3
Abdomen	312.6	259.0-354.6	218.8	207.7-236.2	274.6	243.0-310.4	102.4	83.8-132.9

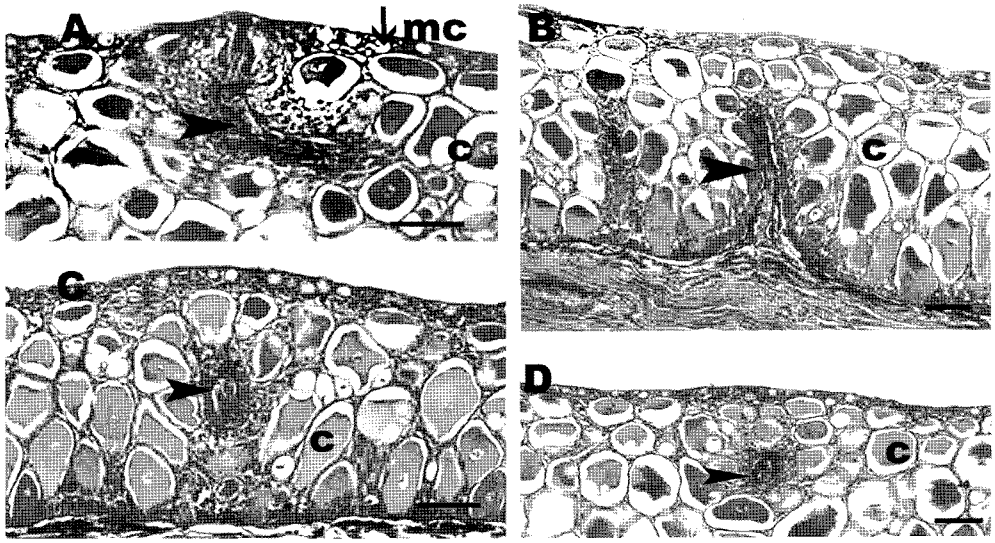

Fig. 2. Transverse sections of the skin of *Pseudobagrus brevicorpus* with haematoxylin and eosin. Lots of intra-epidermal blood vessels (arrows) are presention all three regions of the epidermis. A, dorsum; B, lateral region; C, abdomen; D, abdomen. *Abbreviations*; mc, mucous cell; c, club cell. All bars indicate 50 μm.

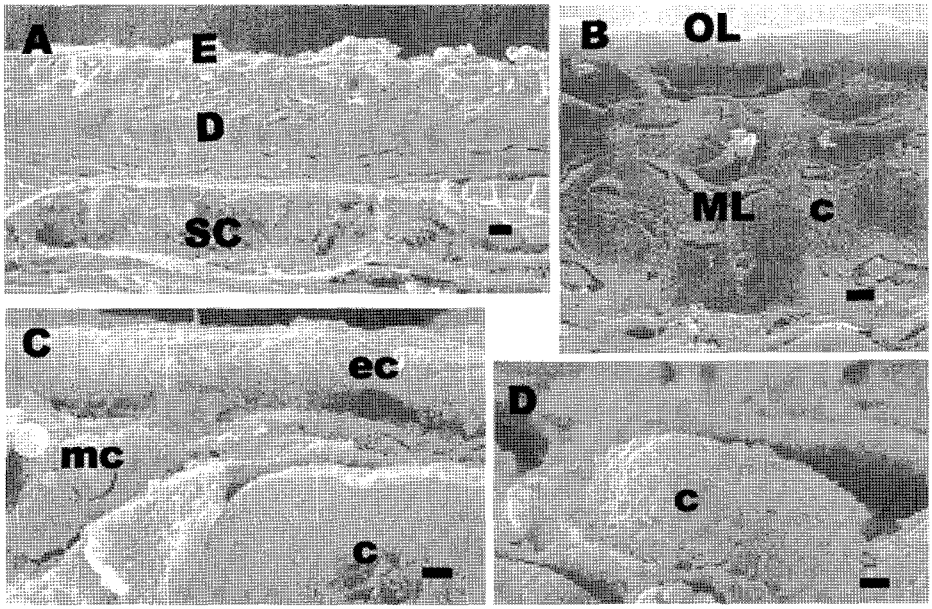

Fig. 3. Scanning electron micrograph of the dorsal skin of *Pseudobagrus brevicorpus*. A, The skin has epidemris (E), dermis (D) and subcutis (SC). Bar = 20 μm; B, The middle layer (ML) of the epidermis is thicker due to large club cells (c) than the outermost layer (OL). Bar = 50 μm; C, Epithelial cells (ec), mucous cell (mc), and club cell (c) are easily seen. Bar = 20 μm; D, Surface structure of the club cell (c). Bar = 20 μm.

under the dermis, and there are numerous empty spaces composed of fat cells in hematoxylin /eosin stained preparations (Fig. 1A).

DISCUSSION

The two Korean catfishes, *Pseudobagrus brevicorpus* and *P. koreanus*, are fresh water benthic fishes that prefer small streams with pebbly or rocky bottoms (Kim and Park, 2002). Through field observation, we confirmed that these habitats have the possibility to become dry during drought periods, causing shortage in dissolved oxygen for the fish. Among freshwater fishes, some catfishes (*Heteropneustes fossilis, H. microps* and *Liobargus mediadiposalis*) and some loaches (*Iksookimia koreensis* and two species of *Misgrunus*) have similar habitats as *P. brevicorpus* and *P. koreanus*. The hypoxic condition leads the fishes to respire by air through skin, which is termed cutaneous respiratory system (Graham, 1997; Park, 2002; Park et al., 2001 and 2003).

Table 2. A summary of the histochemical tests performed to show to the nature of the mucous cell of the epidermis in *Pseudobagrus koreansis* and *P. brevicorpus*

Techniques employed	Gland cells	
	Mucous cell	Club cell
Weigerts iron hematoxylin	±B	+++PN
Masson trichrome	+G	++G
Periodic acid-Schiff (PAS)	++R	-
Alcian blue (1.0)	±B	-
Alcian blue (2.5)	+ or ++B	-
Alcian blue/PAS	++B, BR, R	-
Toluidine blue	±γ-meta	ortho
High iron diamine	±N	-
High iron diamine/alcian blue	+BN	-

B, blue; BN, bluish black; BR, bluish red; G, green; N, black; R, red; γ-meta, γ-metachromasia; ortho, orthochromasia; +, increasing intensity of reaction; ±, fairly present.

The three catfishes and two loaches showed a unique skin structure, which is another character of cutaenous respiratory fishes; a more thick epidermis consisting of unicellular mucous cells of acid sulfomucins (some sialomucins) and club cells, the presence or absence of intra-epidermal blood vessels and no scale (Jakubowski, 1958; Mittal and Munshi, 1971; Whitear, 1980; Graham, 1997; Park et al., 2003). The skin of *P. brevicorpus* and *P. koreanus* also revealed close similarities to the above air breathing fishes.

Based on the similarities in habitats and skin structure, therefore, it is possible that *P. brevicorpus* and *P. koreanus* carry out cutaneous respiration to cope with extreme conditions. However, there is a difference regarding the intra-epidermal blood vessels between the two species. *P. brevicorpus* has numerous blood vessels over all three regions of the epidermis, but *P. koreanus* did not. The presence of the intra-epidermal blood vessel in *P. brevicorpus* may mean that the uptake of air can occur closer to surface than in deep dermis of *P. koreanus*. There is a high possibility that *P. brevicorpus* may frequently become hypoxic than in *P. koreanus* without the intra-epidermal blood vessels. This difference shows that the two species have unique microhabitats. Among the well-known aquatic air breathers, mentioned above, *L. mediadiposalis* has intra-epidermal blood vessels but others do not no ones (Jakubowski, 1958; Mittal and Munshi, 1971; Graham, 1997; Park, 2002; Park et al, 2001 and 2003).

The mucous cell in the epidermis was acidic sulfomucins in nature. Acidic sulphated mucopolysaccharides in mucous cells have also been reported in other cutaneous respiratory fishes: *Monopterus, Mastacembelus, Amphipnous, Misgurnus, Liobargus, Iksookimia* (Mittal and Munshi, 1971; Mittal and Banerjee, 1974; Mittal et al., 1980; Park and Kim, 1999 and 2001; Park et al., 2003). They function to hold a large amount of water or keep the skin clear for respiration (Rogers, 1961; Mittal et al., 1980). Their skins also have no scales, a known characters for air-breathing *M. albus* (Liem, 1967) and *L. mediadiposalis* (Park et al., 2003), which may be an adaptation for free locomotion with bent body (Mittal and Munshi, 1971).

A well-defined lymphatic space with small lymphocytes was present in the epidermis. This is known in air-breathing fishes such as *Heteropneustes, Mastacembelus, Amphipnous, Iksookimia, Misgurnus, Periopthalmus* (Mittal and Munshi, 1971; Park, 2002a and 2002b), and is thought to protect the epidermis (Mittal and Munshi, 1971).

Therefore, we conclude that *P. brevicorpus* and *P. koreanus* are closely related in that they can respire through their skins, based on ecological observations and histological features of skin required for cutaneous respiratory fishes. All environmental factors should be maintained in nature, without artificial developments or water pollutions which may cause destruction of water-ecology. They are endemic species of Korea and especially *P. brevicorpus* is a first-grade endangered wild fauna and flora designated by the Ministry of Environment. Therefore, they should be used as a biological indicator species for conditions in a watershed. More efforts are required in the future to ensure them as indicator species.

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