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Structure and Histochemistry of Skin of Mud Loach, *Misgurnus anguillicaudatus* (Pisces, Cobitidae), from Korea

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The structure of skin of a mud loach *Misgurnus anguillicaudatus* was described in relation with their histochemical nature from four regions of the skin. The epidermis has a strongly thick layer of two glandular cells, consisting of a elongate mucous cell and club cell, and a thin layer of superficial layer. The secretion of the elongated mucous cell was acid mucopolysaccharides in nature but the club cell did not give any histochemical reaction. A well defined lymphatic system, comprising small lymphocytes was present in the stratum germinativum layer of the epidermis. A pit organ of a pear-shaped structure was present below the epithelial cells and lie directly on the basement membrane. The organ has blood vessels serving the sense organs of the epidermis. There was a definite area showing acid mucopolysaccharides in the stratum laxum layer of the dermis. Small scales are present deep in the dermis except the top of the head. A great number of blood capillaries were found just under basement membrane. These structural features of skin in *M. anguillicaudatus* seem to be closely related with cutaneous respiration using air.

Key words : Skin, elongate mucous cell, club cell, acid mucopolysaccharides, *Misgurnus anguillicaudatus*

Introduction

The mud loach *Misgurnus anguillicaudatus* inhabits muddy swamps, ponds, and ricefields which are subjected to periodic drying. The fishes which live in habitats are able to constantly cover the oxygen demanded and can perform aquatic respiration with their gills. However, some fishes inhabit warm and stagnant reservoirs or environment undergoing periodic drought causing a reduction of dissolved oxygen. To adapt to these environments those fishes have developed additional respiratory apparatus to receive or hold air in the following organs; the intestine (Cobitidae), the skin (Cobitidae, Anguillidae, Nototeridae, Gobiidae), the branchial chambers (Anabatidae, Osphromenidae, Channidae), the swim bladder (Dipnoi), the labyrinthine organ (Anabas), and other (Liem, 1967; Johansen, 1970; Niva *et al.*, 1981; Munshi and Hughes, 1986; Moitra *et al.*, 1989; Itazawa and Hanyu, 1991; Ishimatsu *et al.*, 1998). These air breathing fishes are bimodal breathers-they are air breathing and water breathing. It has been known that the ratio between aerial and aquatic respiration depends on the oxygen and the carbon dioxide content of the water, the less oxygen in the water, the greater the percentage of aerial respiration (Liem, 1967; Ishimatsu *et al.*, 1979; Ishimatsu *et al.*, 1998).

The skin of air breathing fishes has certain features as a thicker epidermis due to a large glandular cell (*Misgurnus*, *Monopterus*), intraepithelial capillaries (*Periophthalmus*), and a well developed vascularization (*Heteropneustes*) and these structures may be related with the amphibious habit of the fishes (Jakuboski, 1958; Liem, 1967; Johansen, 1970; Mittal and Munshi, 1971; Mittal and Banerjee 1974; Whitear, 1986). The family Cobitidae has been known as air breathing fishes, cutaneous respiration, but the studied

species was just restricted to *Misgurnus fossilis* (Jakubowski, 1958). Moreover, the reports of *M. fossilis* showed the lack of a histochemical nature and a detailed structure in the skin. Therefore the purpose of this study was to get information on structure and histochemical nature of the skin, and discuss cutaneous respiration in *M. anguillicaudatus*.

Material and Methods

The observed 10 specimens were collected from muddy swamps or ponds and ranged from 75.5 mm to 125.2 mm in standard length. The specimens were fixed in 10% neutral formaldehyde. The skin fragments were taken from four regions; the top of the head, the dorsal region, the lateral region, and the abdominal region.

These fragments were dehydrated through a standard ethanol series to 100%, cleared in xylene and then embedded in wax (Paraplast, Oxford). Blocks were sectioned at 5 μm. Sections were deparaffinized and stained with Harris's hematoxylin, Ehrlich's hematoxylin, iron alum hematoxylin and counter-stained with eosin. Various histochemical tests such as alcian blue (AB, pH 1.0), giemsa blue, methylene blue, combined alcian blue-periodic acid schiff (Combined AB-PAS), Masson's trichrome for demonstration of lymphocytes, carbohydrates, and connective tissues.

Results

The integument of the *M. anguillicaudatus* could be divided into three principal layers, the epidermis, the dermis and the subcutis (Figs. 1, 2).

Epidermis

The epidermis can be divided into three layers -the stratum germinativum, the middle layer and the outermost layer (Table 1). The average thickness of the epidermis was thickest at the top of the head (approximately 255 μm), and the thinnest in the dorsal region (approximately 169 μm). And the lateral region was 193 μm and abdominal region 178 μm (Table 1).

Table 1. Mean thickness of four skin regions in *Misgurnus anguillicaudatus*

Region \ Thickness	Epidermis (μm)	Dermis (μm)	Total skin (μm)
Dorsal region	169	110	279
Lateral region	193	127	320
Abdominal region	178	83	261
Top of head	255	33	288

Outermost layer

The superficial layer of the epidermis of *M. anguillicaudatus* composed of rather flattened cells arranged in 1 to 3 rows of cell and was thin. A few elongated unicellular mucous glands were expanded from the middle layer of the epidermis, sometimes, a few lymphocytes were present (Figs. 1, 2).

Middle layer

The middle layer had two glandular cells, elongate mucous cell and club cell. In between these skin glands the elongated spindle shaped epidermal cells were found vertically. The cell boundary of these cells was usually not clear as it greatly expanded or stretched out due to the collateral pressure of these glands (Figs. 1, 2A, 2B)

The elongate mucous gland had a basal, spherical or oval nucleus with a thin rim of slightly basophilic cytoplasm, pushed at the periphery of the cell due to the heavy accumulation of its basophilic secretions. Each cell had a large elongated spherical body and a short narrow neck. Its secretory matter was highly vacuolated and basophilic. The secretions had a blue color reaction with Combined AB-PAS reaction (Fig. 2C), blue with alcian blue (Fig. 2D), and faint blue with Masson's trichrome stain (Fig. 2B). The secretions gave a deep pink color with the giemsa reaction. Their nucleus were pink or red color with Combined AB-PAS reaction and Masson's trichrome stain. The elongate mucous glands varied in their size and distribution. These were unicellular of various dimensions reaching from 64~75 μm (in dorsal, lateral and abdominal region) to 200 μm (in the top of head in height). The elongate mucous gland had less than club cells except for the top of head in size (Fig. 2B). The mucous cells were extended to the superficial layer of epidermis in the dorsal, lateral, abdominal region, and top of the head (Figs. 1, 2B). In particular, the elongate mucous cells of the top of head were rather enlarged toward stratum germinativum and then they occupied two thirds of epidermis. Therefore the thickness of the epidermis in this region was thickest (Table 1, Fig. 1B).

Below the elongate mucous cell other glandular

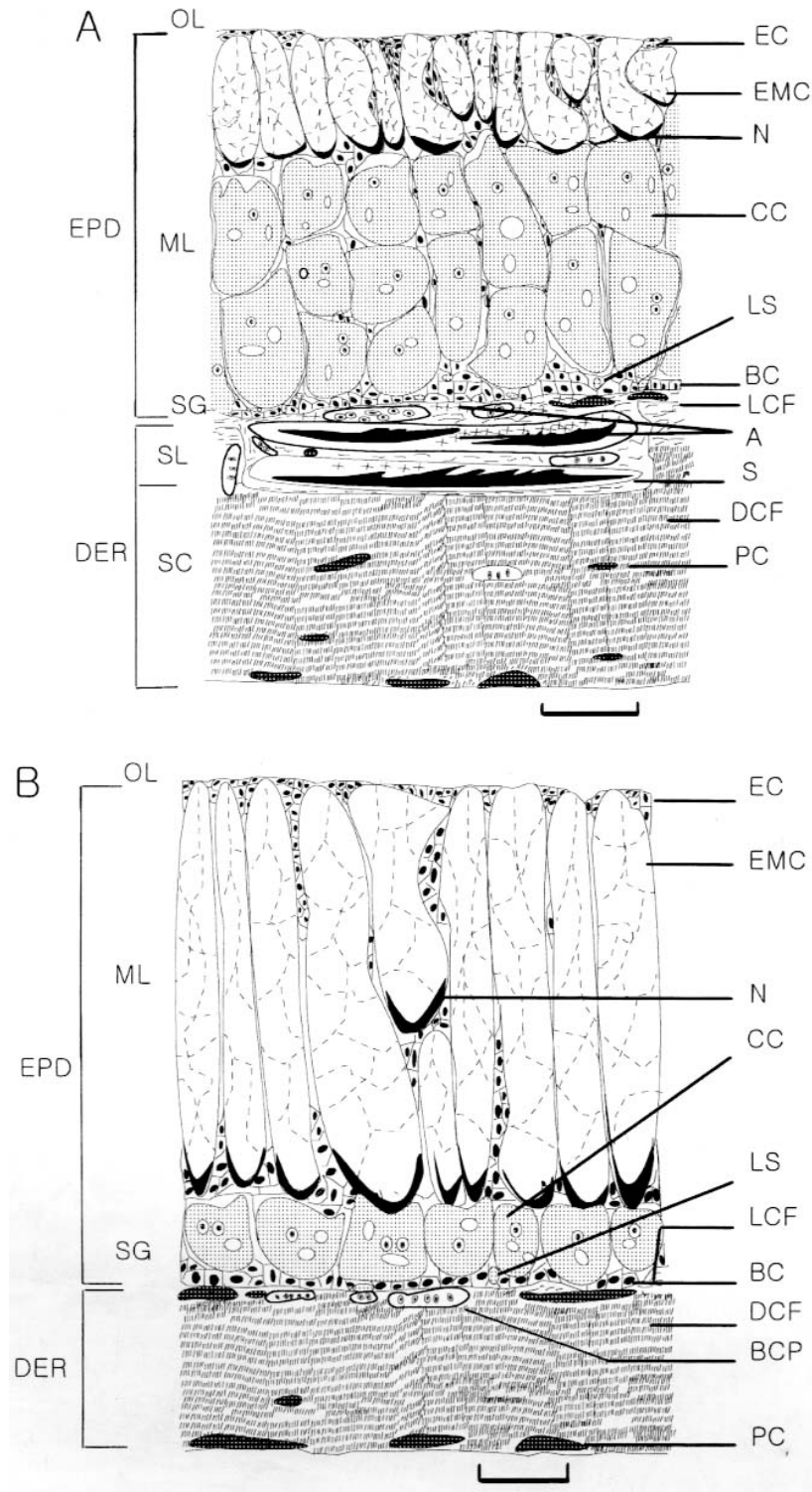
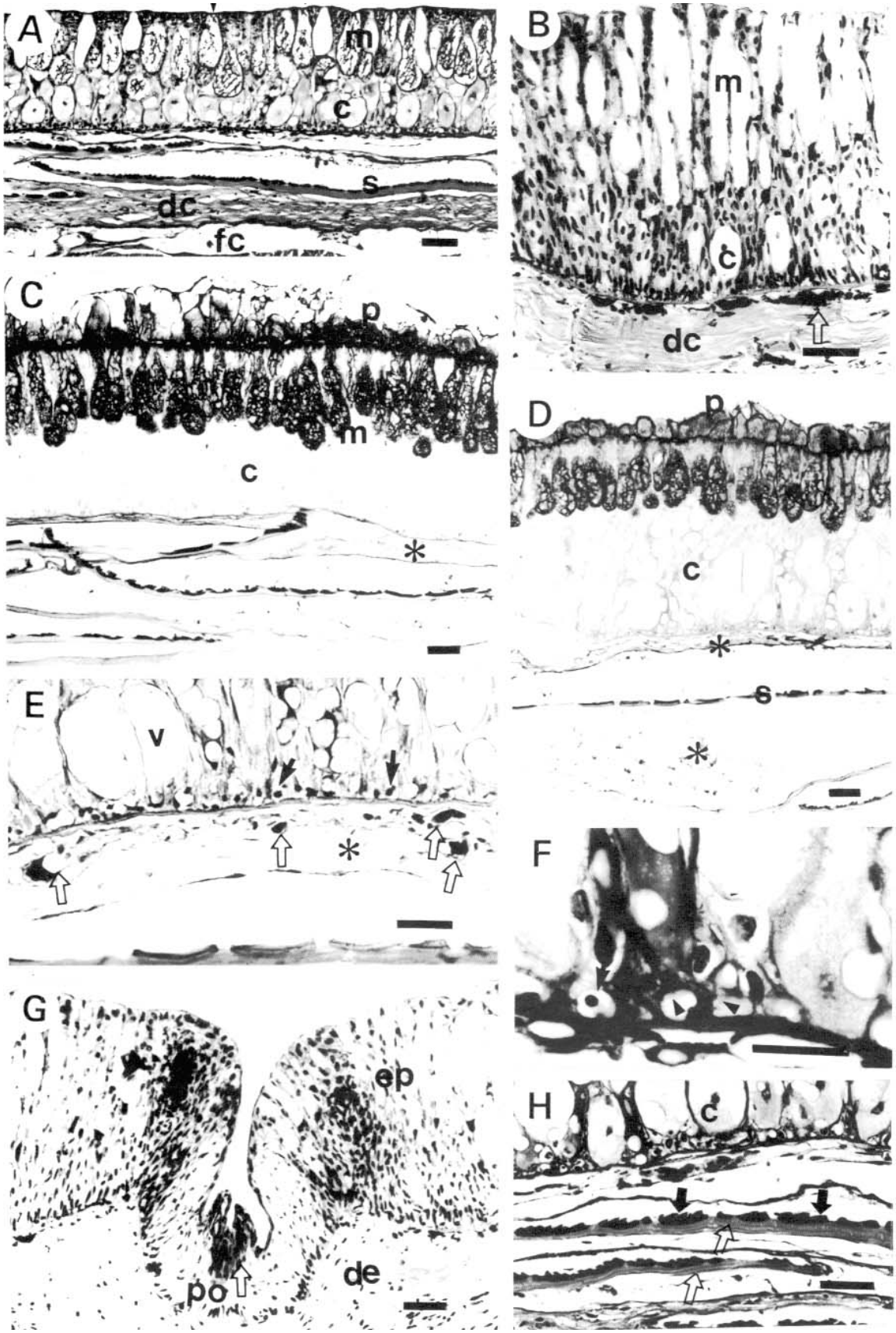


Fig. 1. A diagrammatic representation of the dorsal skin (A) and the top of head (B) in *Misgurnus anguillicaudatus* showing its structural organization. A, definite area; BC, basal epithelial cell; BCP, blood capillary; CC, club cell; DCF, dense collagen fibers; DER, dermis; EC, superficial epithelial cell; EMC, elongated unicellular mucous cell; EPD, epidermis; LCF, loose collagen fibers; LS, lymphocytes; ML, middle layer; N, nucleus; OL, outermost layer; PC, pigment cell; S, scale; SC, stratum compactum; SG, stratum germinativum; SL, stratum laxum. Bar = 40 μm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cell, club cells, were found, and they were oval or spherical in shape (Figs. 1, 2). They were usually uninucleate, having an oval nucleus, sometimes binucleate having two nuclei very close to each other. The cytoplasm was finely granular or more or less homogeneous in nature and invariably showed some shrinkage due to fixations. Each club cell, showed various dimensions, was approximately 30~75 μm in height. With hamatoxylin/eosin stained preparations, the nuclei of club cells showed deep blue and the cytoplasm was stained light pink (Fig. 2A). In the Combined PAS-AS reaction, alcian blue, and giemsa stain they did not give any histochemical test for acid mucopolysaccharides (Figs. 2C, 2D). With Masson's trichrome stain preparations they were negative (Fig. 2B). The club cells were more numerous than elongate mucous cell (Fig. 1A). A large number of club cells were invariably found in epidermis in either 1 to 2 layers in dorsal and abdominal region or 2 to 3 layers in lateral region, but just one layer in top of head. Some of the club cells had a few vacuoles in their cytoplasm (Figs. 1, 2E).

Stratum germinativum

This layer was arranged in 1 to 3 rows of cells consisting of germinative cells and lymphocytes. The germinative cells were cuboidal and provided with a prominent centrally placed oval or spherical nucleus. In between these cuboidal cells were found small oval or round lymphatic space (Fig. 1). One or two small lymphocytes had deeply stained nuclei, surrounded by small mounts of faintly stained cytoplasm were invariably found inside these space (Figs. 2E, 2F). In Masson's trichrome stained preparations, the nuclei of these lymphocytes took a deep red color and the cytoplasm was stained light red or pink (Fig. 2E). With the methylene blue and the giemsa stain they showed no clear reaction. The lymphocytes penetrated the middle layer, very often reaching up to the outer cell layers of the epidermis.

The pit organs, single or in groups of two to three were distributed on the surface of the skin (Fig. 2G). Each pit organ is a pear-shaped structure, sunk below the epithelial cells and lie directly on the basement membrane. In the Intraepithelial layer there were blood vessels serving the sense organs of epidermis.

Dermis

The dermis was separated by a thin basement membrane (Fig. 1). The dermis consisted of relatively thin outer loose connective tissue layer, stratum laxum, and a thin inner compact layer, stratum compactum (Figs. 1, 2)

Stratum laxum

This layer was distinguished by the presence of a thin scale, which is lodged in pockets in the connective tissues (Figs. 1A, 2). A loose connective tissue layer lying below the basement membrane was richly supplied with blood capillaries, which was connected with blood vessels of the stratum compactum and subcutis by capillaries running between the scale pockets. The loose connective tissue of the stratum laxum was mainly composed of a thin collagen bundle, stained green in Masson's trichrome stained preparations. A layer of sparsely distributed pigment cells was found below the basement membranes, and also in the connective tissue pockets on the underside of the scales (Figs. 1, 2A-D).

The scales of *M. anguillicaudatus* consisted of

Fig. 2. Structure and histochemistry of skin of Misgurnus anguillicaudatus. (A) Cross section of dorsal skin showing the structure of the epidermis and dermis. The epidermal cells are marked by arrow head (Ehrlich's hematoxylin); (B) Cross section of the top of head showing absence of scale, enlargement of the elongated mucous cell occupying a great area of epidermis, and small club cells. Pigment cells (arrow) are founded just under basement layer (Masson's trichrome stain); (C) A view of the dorsal skin showing Combined AB-PAS reaction positive elongated mucous glands and its productions, and a definite area (*) just under basement membrane. The club cells were negative in Combined AB-PAS reaction; (D) A view of the ventral skin showing histochemical reaction with alcian blue (AB); (E) A view of the ventral skin showing lymphocytes (solid arrows) and a great number of blood capillaries (arrows) with Masson's trichrome stain; (F) A view of lymphatic spaces (small arrow heads) and lymphocytes (large arrow) of dorsal skin (Harris's hematoxylin); (G) A pit organ of the top of head showing a pear-shaped structure. Blood capillaries (marked by arrow) were present in intraepithelial layer (Masson's trichrome stain); (H) The structure of scale consisting of the upper bony layer (solid arrows) and the inner fibrillary plate (arrows) (Ehrlich's hematoxylin). (**Abbreviations**: c, club cell; dc, dense collagen fibers; de, dermis; ep. epidermis; fc; fatty cell; m, elongate mucous cell; p, productions of mucous gland; po, pit organ; s, scales; v, vacuoles). Bar = 25 μm.

two distinct layers-the upper bony layer and the inner fibrillary plate (Fig. 2H). However the top of the head had no scale. The bony layer carried concentrically arranged ridges of striae on its free surface while the lower fibrillary plate was mainly composed of parallel collagen fibers. The upper bony layers of the scale was basophilic in nature, and appeared blue in alcian blue and a deep green color in Masson's trichrome stained preparations. The lower fibrillary plate was eosinophilic, weakly Combined AB-PAS positive, alcian blue negative. With the Masson's trichrome stain preparations, it showed a green color.

A definite area was present between the basement membrane and the scales. This material looked blue with Combined AB-PAS reaction, blue in alcian blue, pink with giemsa stained preparations, and was negative in Masson's trichrome stain preparations (Figs. 2C-2E). Also, these materials were present in some of free space in which scales are lodged (Fig. 2D).

Stratum compactum

This was a relatively thick layer and was composed of coarse, compactly arranged collagen fiber bundles in several layers. A few collagen fiber bundles ran vertically at intervals. This layer took a deep green color in Masson's trichrome stained preparations for collagen. Between them there was a few pigment cells and blood capillaries.

Subcutis

This was a boundary tissue that binds the stratum compactum with the underlying muscle bundles. In routine hematoxylin and eosin preparations this layer invariably shows numerous spaces which are occupied by fat cells (Fig. 2A). The main branches of the nerves and blood vessels was found in this layer.

Thickness of skin

In the top of the head the thickness of the epidermis was thickest, about 255 μm, due to more enlargement of mucous cell in the top of the head, but the dermis was thinnest (about 33 μm), because of the absence of the scale causing great reduction of its thickness (Table 1). On the one hand, the dorsal, the lateral, and the abdominal region was similar to each other in their thickness. The thickness of skin was thickest in the lateral region (about 320 μm), and thinnest in abdominal region (about 261 μm).

Discussion

The capacity of cutaneous respiration depends on the structure of the skin and the degree of its vascularization based on their habitats. The structure of skin of *M. anguillicaudatus* was characterized by a thick epithelial layer having large two glandular cells, a thin superficial layer consisting of 1 to 3 rows of cells, the localization of small scales inside the dermis, a well defined lymphatic system, a definite area of acid mucopolysaccharides, and a large number of blood capillaries just under the basement membrane. These features of the skin of *M. anguillicaudatus* may be closely related with respiration using skin.

The thickness of epidermis in *M. anguillicaudatus* was thicker, approximately 169 to 255 μm (Table 1), as in those of other known air breathing fishes. In the thickness of the epidermis *M. fossilis* was 182~397 μm (Jakubowski, 1958), *Heteropneustes fossilis* 98 μm, *Mastacembelus pancalus* 44 μm, *Amphipnous cuchia* 119 μm (Mittal and Munshi, 1971), and *Monopterus albus* 75 μm (Liem, 1967). The thickness of the epidermis results from the strong development of the glandular cells forming its thickest layer. In the dorsal region, the thickness of the epidermis was 169 μm because of mucous cells (about 64 to 75 μm in height) occupying half of epidermis. The thickness of epidermis in the top of head was 255 μm due to great enlargement of mucous cells (about 200 μm in height) occupying two thirds of the epidermis. The size and abundance of mucous cells plays an important role in supporting and maintaining the normal relationship of the cutaneous respiration. The content of the mucous cell was acid mucopolysaccharides, but the club cell was not mucopolysaccharides. Both skin glands contained a lot of water, and oxygen may easily penetrate them towards the deeper layers of the skin (Jakubowski, 1958; Mittal and Munshi, 1971). The Letterer (1959) and Rogers (1961) have reported that mucus substances had great ability to bind a large amount of water. Hora (1934) and Mittal *et al.* (1980) has shown that mucus has a remarkable power of precipitating mud held in suspension in water and that the mucus secreted by the skin in air breathing fishes may also be used in keeping the skin clear for respiration.

The stratum laxum layer of the dermis in *M.*

anguillicaudatus had a definite area showing acid mucopolysaccharides. This area did not penetrate the epidermis. This area was found just under the basement membrane or present in some of free space in which scales were lodged. The presence of mucopolysaccharides in the stratum laxum was correlated with the semiterrestrial ecological habits of fish (Mittal and Munshi, 1971). Rogers (1961) reported that 1 g of mucopolysaccharides could bind or release 200 g or 500 g of water. Thus it may be concluded that the presence of mucopolysaccharides in the stratum laxum is adaptive modification for cutaneous respiration of *M. anguillicaudatus*.

M. anguillicaudatus had a vascularization system consisting of a pit organ and lymphatic space in the epidermis. A pit organ of a pear-shaped structure was present below the epithelial cells and lies directly on the basement membrane. Jakubowski (1958) described the pit organ as looplike vessels serving the sense organs of the epidermis in *M. fossilis* and considered that the vascularization of looplike vessels may be proof of air breathing. A well-defined lymphatic system, comprising a series of lymph spaces containing small lymphocyte was present in the stratum germinativum layer of the epidermis. These lymphocytes penetrated intraepithelial layer. Such structures were not reported by Jakubowski (1958) in the mud loach, *M. fossilis*. The lymphatic system had functions of the supply of nutrition to the stratum germinativum for cell proliferation and to protect the epidermis from microorganisms or foreign proteins for protection of the epidermis (Mittal and Munshi, 1971).

M. anguillicaudatus had small scales embedded in the superficial layer of the dermis. The small scales, rudimentary scales or absence of scales was found in a burrowing and a mud-dwelling fish (Amphipnous, Monopterus) and it was considered as an adaptation to its peculiar mode of life (Liem, 1967; Mittal and Munshi, 1971; Whitear, 1986).

The blood capillaries of skin are very important to air breathing fishes. Tamura *et al.* (1976) reported that Periophthalmus cantonensis have intraepithelial capillaries. In *M. fossilis*, *Anguilla*, *Amphinous*, and *Monopterus* it was known that the blood vessels are present in the dermis (Jakubowski, 1958; Liem, 1967; Lethbridge and Potter, 1982). In this case, diffusion of oxygen took place readily across the mucous coat of the epithelium though the blood vessels were situated at the deep dermis (Mittal and Munshi, 1971; Perry and McDonald, 1993). The cutaneous respiration of *M. anguillicaudatus* may have occurred in the dermis because a large number of blood capillaries were close to each other just below the basement membrane.

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한국산 미꾸리 *Misgurnus anguillicaudatus* (Pisces, Cobitidae) 피부 구조 및 조직화학적 특징

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한국산 미꾸리 *Misgurnus anguillicaudatus*의 피부 구조 및 조직화학적 특징을 조사하기 위해 등, 측면, 복부, 머리부분의 피부를 조사하였다. 미꾸리의 epidermis는 elongated mucous cell과 club cell의 2개의 gland로 구성되었으며 두께는 아주 두꺼웠다. mucous cell은 acid mucopolysaccharides의 물질을 분비하였으나 club cell은 mucopolysaccharides에 어떠한 반응도 보이지 않았다. 표피의 기저층은 림파구를 가지는 lymphocytic space가 발달하였으며 기저막과 상피세포 사이에 배모양의 감각세포인 pit organ이 존재하였다. Dermis의 stratum laxum에는 acid mucopolysaccharides를 나타내는 부분(a definite area)이 존재하며, 이러한 물질은 비늘에도 분포하고 있다. 작은 비늘이 dermis속에 파 묻혀 있으나 머리부분에는 없다. 또한 많은 양의 모세혈관들이 기저막 바로 아래에 존재하였다. 피부의 이러한 특징들은 미꾸리가 공기를 이용하는 피부호흡과 밀접한 관계가 있다.