Korean J. Zool. 39:198-206, 1996

Adhesive Membranes of Oocyte in Four Loaches (Pisces: Cobitidae) of Korea

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Adhesive membranes in the oocytes of four loach fishes of Korea were investigated by light and electron microscopes to clarify the structural characteristics among the species. In the vitellogenic stage the adhesive membranes of oocytes could be classified into two forms as follows: 1) fence-shaped form of *Nemacheilus toni* and 2) non-adhesive membranous form of *Misgurnus anguillicaudatus*, *M. Mizolepis*, and *Lefua costata*. The adhesive membranes of oocytes in the loach fishes showed a species specificity with reference to their habitats and spawning properties.

KEY WORDS: Adhesive Membrane, Zona radiata, Oocyte

The loaches of the family Cobitidae are benthic small fishes inhabiting the freshwaters of the Europe, Asia and North Africa. The family Cobitidae of Korea contains 5 genera and 14 species, of which 5 species are endemic to Korea (Kim and Kang, 1993). In spite of their species diversity, Korean loaches have attracted little attention due to their small size and lack of market, and their usual habitat in the small streams. Because the phylogenetic classification is not possible without extending knowledge of the species level, it is neccessary to know better characters for species morphology. Occasionally, the morphological aspects of egg envelope have been used for taxonomic purpose in oviparous teleosts (Riehl, 1980; Johnson and Werner, 1986; Britz *et al.*, 1995). Adhesive membranes were reported in relation to their substrate spawning conditions in some fish species (Blaxter, 1969; Laale, 1980; Riehl and Greven, 1990, 1993). Kim and Park (1995) recently reported the four forms of the adhesive membrane in the genus *Cobitis* from Korea.

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It is purpose of this paper to comparatively investigate the adhesive membranes of 3 genera with 4 species except the genus *Cobitis* in the family Cobitidae of Korea and to discuss the differences of their forms.

Materials and Methods

Specimens investigated in present study were used the following fishes collected from several streams of the South Korea in spawning season from 1992 to 1994: *Nemacheilus toni* (Dybowski), *Misgurgus anguillicaudatus* (Cantor), *M. mizolepis* Günther, and *Lefua* costata (Kessler). For the light microscopy, their ovaries fixed with 10% formalin were isolated and embedded in paraffin following treatment of a series of graded alcohols, sectioned 5 μm and 7 μm, and stained with hematoxylin-eosin for histological examinations. Developmental stage of oocyte followed basis defined by Nagahama (1983). The adhesive membranes were investigated at the late yolk granules which yolk granules are gradually dispersed into entire

cytoplasm of oocyte. The number and size of the adhesive membranes were also measured using micrometer within 10 μm. For the transmission electron microscopy, adult females were anaesthetized with MS222. Their ovaries were excised and prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. Postfixation was performed in 1% osmium tetroxide in the same buffer. After dehydration in a graded alcohol series, specimens were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with JEOL-1200EX transmission electron microscope. For the scanning electron microscopy, their ovaries were prefixed and postfixed in same way of those of TEM. The samples were dehydrated in a graded alcohol series and dried to critical point in CO_2. The dried samples were coated with gold-palladium and observed with JEOL JSM-T330A scanning electron microscope.

Results

Developmental process of zona radiata and appearance of adhesive membrane

Oogenesis of the family Cobitidae commonly occurs by proliferation of oogonia on the ovarian lamella (Plate 1. A). After these proliferation, oogonia become early oocyte which consists of chromatin-nucleolus and perinucleolus (Plate 1. B, C). In perinucleolus stage, follicle cells are present, but do not completely circumscribe individual oocytes, and the zona radiata was not distinct (Plate 1. C). At the stage of yolk vesicle which first appears as the important constituents of yolk materials, oocytes are surrounded by a single layer of flattened follicle cells, and the zona radiata surrounding oocyte becomes more distinct (Plate 1. D, E). As vitellogenesis proceeds, most of oocyte cytoplasm become occupied by many dense yolk granules (late yolk granules), which are fused with each other to form yolk mass (Plate 1. E). In this period follicle cells and zona radiata could be distinguished from outer structure of oocyte.

The adhesive membranes firstly appear in yolk vesicles, but were not distinctive. But these adhesive membranes distinctively formed in outer zona radiata at late yolk granules. These adhesive membranes and zona radiata increased in size as oocyte grows.

Adhesive membranes

The adhesive membranes observed in present study could be classified into two forms : fence-shaped and non-adhesive membranous form (Plate 2). The adhesive membrane of each species and their characteristics are as follows.

Nemacheilus toni (Dybowski)

The adhesive membrane of this species showed unique fence-shaped feature that is distributed the outer zona radiata, and these fences are stained with hematoxylin. This form was included only *Nemacheilus toni* among the family Cobitidae. The fences of *N. toni* are 4-5 in number and 5.0-6.0 μm in length. The thickness of the zona radiata was measured about 4.0-5.0 μm (Plate 2. A, B). In TEM observations, the zona radiata consisted of three layers (Plate 2. C). The zona radiata externa is outermost layer and a site for attachment of adhesive membrane. The adhesive membrane seems to consist of compound chemicals. The zona radiata interna is beneath zona radiata externa, and the zona radiata subinterna consists of several layers showing different electron density. Microvilli and pore canal exist in the zona radiata, and the microvilli penetrated zona radiata through pore canals (Plate 2. C). Structure of egg surface in ovulated oocytes show hillock-like adhesive membrane (Plate 2. D)

Misgurnus anguillicaudatus (Cantor) and M. mizolepis Günther

Unlike almost species of the family Cobitidae, these two species have no the adhesive membrane on the outer zona radiata (Plate 2. E, F). The zona radiata of *M. anguillicaudatus* was distinctive, and was about 2.0-3.0 μm thick. And the thickness of the zona radiata of *M. mizolepis* was about 2.5-3.5 μm. Although the above two species have no adhesive membranes in the light microscopic observations, the fine structure of their zona radiata, particularly *M. anguillicaudatus*, showed a feature that numerous bundle of microvilli are

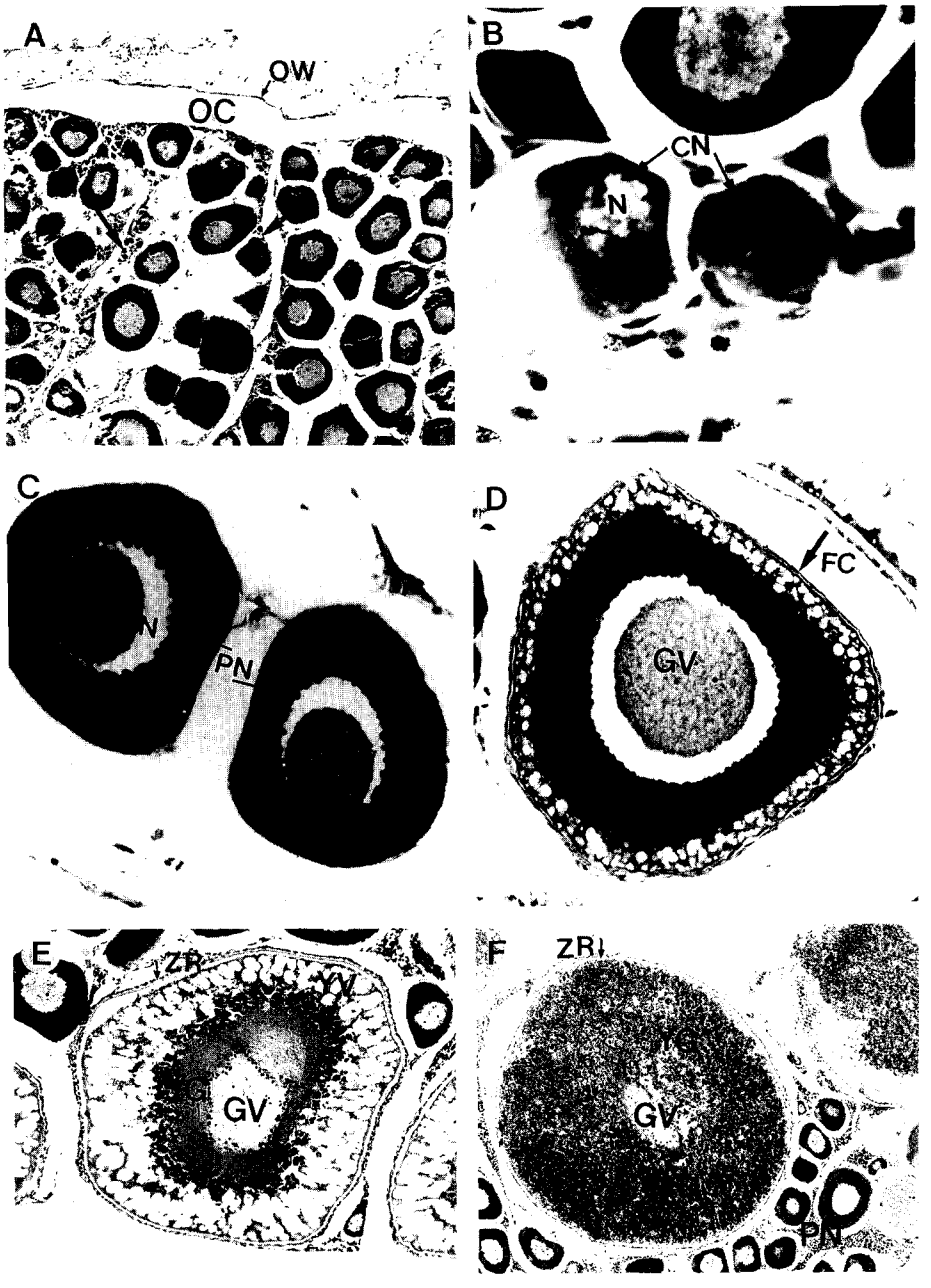

Plate 1. Developmental stage of ova in *Misgurnus anguillicaudatus* from Korea. A. Developmental stage of oocyte proliferate on the ovarian lamella (arrows) of ovary surrounded by ovarian wall (OW). OC; ovarian cavity. × 100. B. Chromatin-nucleolus stage (CN, undergo proliferation by mitotic division. × 1200. C. In peri-nucleolus stage, multiple nucleoli become located around the periphery of nucleus (N), nucleus and oocyte increase in size. PN; peri-nucleolus. × 400. D. Flattened follicle cell (FC) become visible in the yolk vesicle (YV) stage which is first yolk materials. × 200. E. In early yolk granule stage, cytoplasm become occupied by yolk granule (YG). GV; germinal vesicle, YV; yolk vesicle, ZR, zona radiata. × 200. F. In late yolk granule, most of cytoplasm become occupied by many dense yolk granules (YG) fused with each other to form yolk mass. In this period, zona radiata (ZR) become more distinctive than early yolk granule. GV; germinal vesicle, PN; peri-nucleolus stage. × 80.

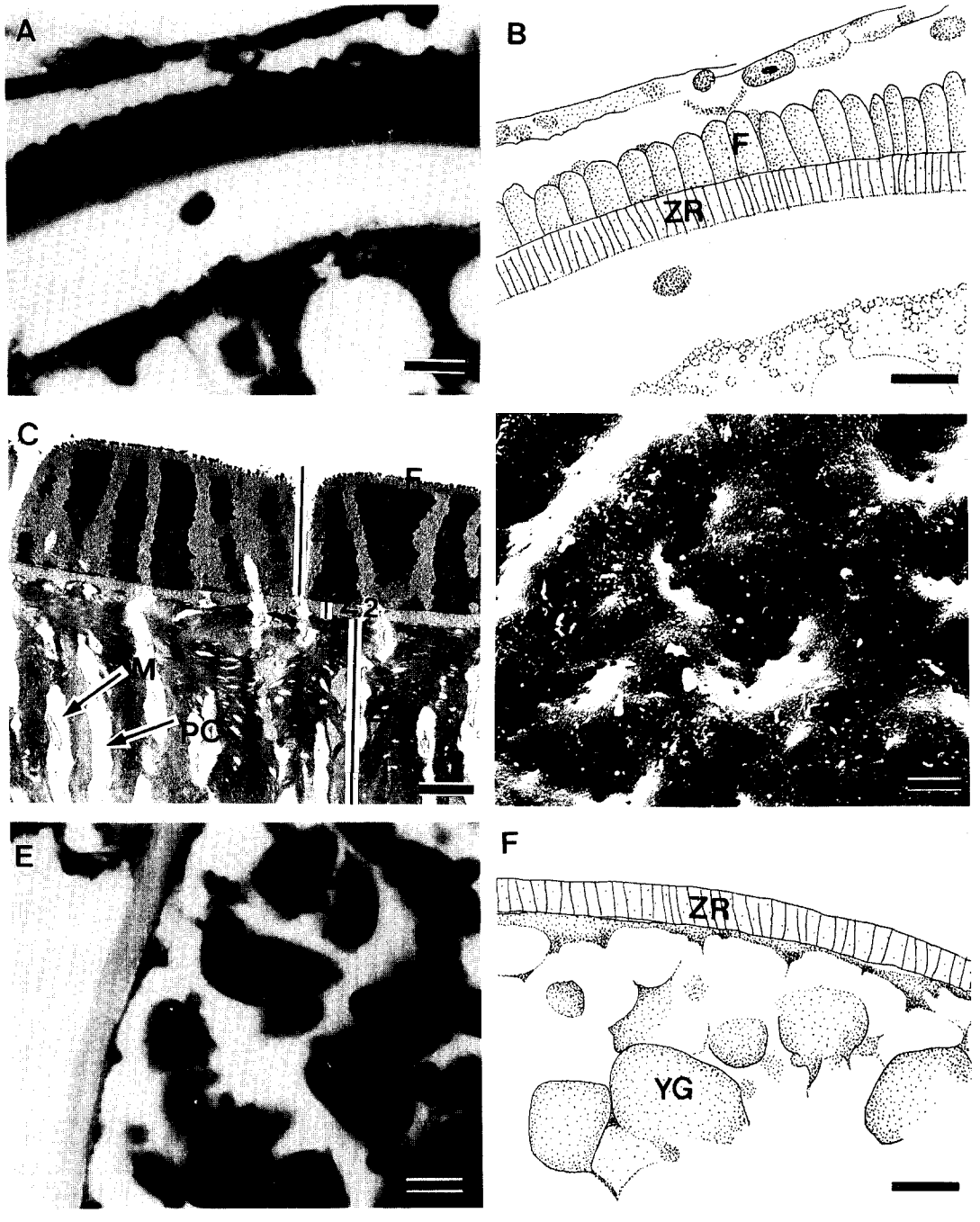

Plate 2. Photographs of various adhesive membranes on the outer zona radiata of four loaches of Korea. A-D. Fence-shaped form of adhesive membrane of *Nemacheilus toni*. A. Light micrograph. Bar=10 μm, B. Schematic diagram of LM (left); F, fence; ZR, zona radiata, Bar=10 μm, C. Transmission electron micrograph: F, fence; M, microvillus; PC, pore canal; Z_1, zona radiata externa; Z_2, zona radiata interna; Z_3, zona radiata subinterna. Bar=2 μm, D. Scanning electron micrograph. Bar=5 μm. E-F. Non-adhesive membranous form of *Misgurnus anguillicaudatus*. E. Light micrograph. Bar=10 μm, F. Schematic diagram of LM (left); YG, yolk granule. ZR, zona radiata. Bar=10 μm.

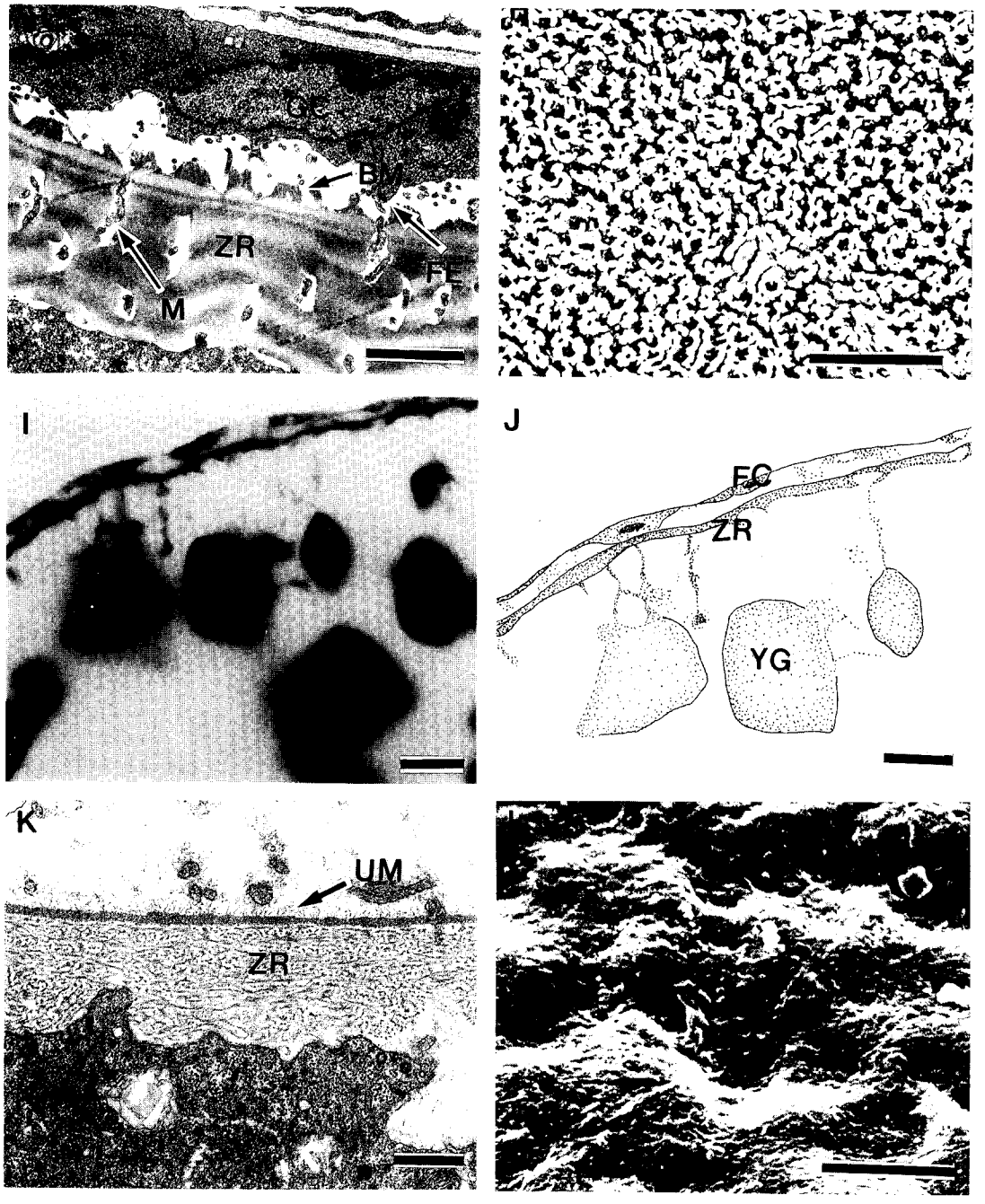

Plate 3. Photographs of various adhesive membranes on the outer zona radiata of four loaches of Korea. G-H. Non-adhesive membranous form of *Misgurnus anguillicaudatus*: G. Transmission electron micrograph. BM, bundle of microvilli; FE, follicular epithelium; GC, granulosa cell; M, microvillus; ZR, zona radiata. Bar=2 μm, H. Scanning electron micrograph. Bar=5 μm. I-L. Non-adhesive menbranous form of *Lefua costata*: I. Light micrograph. Bar=10 μm, J. Schematic diagram of LM (left). FC, follicle cell; YG, yolk granule. ZR, zona radiata. Bar=10 μm, K. Transmission electron micrograph; UM, very short microvilli; ZR, zona radiata. Bar=0.5 μm, L. Scanning electron micrograph. Bar=5 μm.

attached to the zona radiata externa (Plate 3. G). The zona radiata subinterna consisted of five layers showing different electron density. The microvilli and pore canal exist in the zona radiata and the microvilli penetrated zona radiata through pore canals (Plate 3. G). The egg surface have numerous canal-like structures (Plate 3. H).

Lefua costata (Kessler)

Lefua costata showed non-adhesive membranous form on the outer zona radiata such as two species of genus *Misgurnus* (Plate 3. I, J). However, the thickness and the features of the zona radiata of *L. costata* were obviously different from those of the genus *Misgurnus*. In the light microscopic observations, the thickness of zona radiata of *L. costata* was under 0.2 μm, and was the thinnest among the observed species. The thinnest zona radiata was more clearly observed in TEM observations (Plate 3. K). The zona radiata externa have numerous very short microvilli. The zona radiata interna and the zona radiata subinterna were the thinnest among the observed species. Their egg surfaces were covered with hay-like structures (Plate 3. L).

Discussion

The development and structure of oocyte in many fishes have been examined (Honma and Tamura, 1962; Gilkey, 1981; Nagahama, 1983; Hakima, 1984; Ntiba and Jaccarini, 1990; N'da and Deniel, 1993). However, those of the other loaches except the genus *Cobitis* (Kim and Park, 1993, 1995) were not still reported. In the present study, the zona radiata, membrane showing radial striations, become visible in the period of yolk vesicle, and as vitellogenesis proceeds they become more distinct and increased in size. The formation of the zona radiata and the growth of oocyte in the loaches well accorded with results reported in other fishes (Wourms, 1976; Wallace and Selman, 1981; Nagahama, 1983; Kjorsvik and Davenport, 1984; Groot and Alderdice, 1984; Kim and Park, 1995).

The adhesive membranes are attached to zona radiata of full-grown oocytes (Laale, 1980; Kjesbu and Kryvi, 1989; Riehl and Greven, 1993). Various forms of adhesive membrane have been known in many taxonomic groups. Honma and Tamura (1962) observed that *Plecoglossus altivelis* have a large number of fence-shaped adhesive membranes on the outer zona radiata of animal poles. And the filament-shaped adhesive membrane was observed on the outer zona radiata in *Oryzias latipes* and some species of Cyprinodontiformes (Tsukahara, 1971; Hart *et al.*, 1984; Riehl and Greven, 1993). Some species of *Silurus* and *Pungitius tymensis* are known adhesive membrane which are surrounded by a gelatinous mass (Kobayakawa, 1985). In addition, various patterns of adhesive membrane immediately before or after fertilization were reported by Laale (1980): hexagonal pattern in *Cynolebias ladogesi*, bead-like granules in *Clinocottus recalvus*, button-shaped appendage in *Apeltes quadracus*, wart-like appendage in some of Pleuronichthidae, and adhesive disc consisting of a bundle of threads or filaments in some *Gobius*. Koya *et al.* (1995) also reported that *Hexagrammos octogrammus* have adhesive material on the surface of the chorion.

Korean benthic loaches inhabited different habitats, such as pebble, sand, or mud bottom by the species (Kim and Son, 1984; Kim and Lee, 1988; Kim and Kang, 1993). In regard to adhesive membrane of genus *Cobitis*, Kim and Park (1995) reported that the loaches have four forms of adhesive membranes which are comprised of granular form, villous form, filamentous form, and saw-like form.

By light microscopical observations of the adhesive membranes of oocytes for four species of the except genus *Cobitis* in the family Cobitidae, it was found that two forms of adhesive membrane are attached to the outer zona radiata during the vitellogenesis: 1) fence-shaped form, and 2) non-adhesive membranous form. *Nemacheilus toni* with the fence-shaped form inhabits sandy and pebbly bottoms of rapids. However, non-adhesive menbranous form was found in the *Misgurnus anguillicaudatus*, *M. mizolepis*, and *Lefua costata*, which inhabit near stagnant habitat like muddy bottoms or swampy bottoms. In particular, the zona radiata of *L. costata* was characterized

by the thinnest thickness in the Korean loach fishes. By the difference of the thickness of the zona radiata, therefore, *L. costata* could be easily distinguished from the two species of the genus *Misgurnus* having non-adhesive membrane. The fence-shaped form was shown only in *N. toni* among the loach fishes. The zona radiata generally consisted of three layers: the zona radiata externa is outermost layer and a site for attachment of adhesive membrane, the zona radiata interna is beneath zona radiata externa, and the zona radiata subinterna consists of several layers showing different electron density. The zona radiata externa of *N. toni* have large chemical compound-like process. But in the genus *Misgurnus* and *Lefua* having the non-adhesive membrane their zona radiata externa have not a large process but a numerous bundle of microvilii or numerous very short microvilii. Particularly, the zona radiata interna and zona radiata subinterna of *L. costata* were very thin. And although genus *Misgurnus* and *Lefua* have the same non-adhesive membranous form, structure of egg surface showed evident difference that *M. anguillicaudatus* have a numerous canal-like structure, while *L. costata* was covered with hay-like structure. Therefore, there can be suggested that the forms of adhesive membranes may be deeply related to the their habitats with species specificity.

The functional aspects of these adhesive membrane have been known in many taxonomic groups. Mito (1979) reported that the demersal eggs of teleosts are comprised of three functional types: non-adhesive, adhesive and twine eggs. Also, Lagler *et al.* (1977) reported that fish eggs is largely divided into buoyant and demersal egg, and most stream fishes have demersal eggs with adhesive stickiness. Filament-like adhesive membrane have been known from egg of substrate-spawning teleosts (Blaxter, 1969; Kjesbu and Kryvi, 1989; Riehl and Greven, 1990, 1993). Also Laale (1980) suggested that the adhesive membrane consisted of several materials as mucus, mucine, and mucilage, or gelatin, and that various adhesive membranes have adhesive properties which enables the eggs to become attached to vegetation, submerged objects, and to one another. In the American smelt *Osmerus mordax*, the egg has a low stalk which is adhesive and becomes attached to the stony bottoms of streams in the spawning season (Lagler *et al.*, 1977). In the brook silverside *Labidesthes sicculus*, the egg has a single elongate filament that serves first for temporary flotation, and then for attachment (Lagler *et al.*, 1977). The fishes, having adhesive membrane, are most nonbuoyant eggs (Blaxter, 1969; Lagler *et al.*, 1977). In filamental structures, some differences in their size, distribution, and number were useful, and have been used for distinction of property of egg (Laale, 1980). Kim and Park (1995) suggested that in Korean loaches various forms of the adhesive membranes might be related to a species specificity with reference their habitats and spawning properties.

So, we believe that the adhesive membranes of oocytes in the loach fishes could be used as a taxonomic good character for the species or genus identification.

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(Accepted March 11, 1996)

한국산 미꾸리과 어류 4종의 난모세포의 부착막

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한국의 미꾸리과 Cobitidae 어류 3속 4종의 난모세포의 부착막(adhesive membrane)을 광학현미경과 전자현미경을 이용하여 조직학적으로 조사한 결과, 난황물질 형성시기인 난황구 후기의 zona radiata 외측에는 다음 2가지 형태의 부착막이 구별되었다. *Nemacheilus toni*는 울타리형(fence-shaped form)을 보여 주었으며, *Misgurnus anguillicaudatus*와 *M. mizolepis*, 그리고 *Lefua costata*는 무구조형(non-adhesive menbranous form)을 나타냈다. *Lefua costata*의 zona radiata의 두께는 미꾸리속의 2종보다 얇아 두 속은 쉽게 구별된다. 이상과 같이 기름종개과 어류의 알에서 나타나는 부착막 구조는 그들의 서식처와 산란습성에 관련된 종의 특이성을 보여 주고 있어서 분류학적으로 주목되었다.