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Adhesive Membranes of Egg in Five Cobitid Species of *Iksookimia* (Pisces: Cobitidae)

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The five species of genus *Iksookimia* from Korea were investigated by electron microscopes to clarify the adhesive membranes on zona radiata. In the late vitellogenic stage the adhesive membranes could be classified into three form as follows: 1) villous form of *Iksookimia koreensis*, *I. pumila*, and *I. hugowolfeldi*, 2) cotton-shaped form of *I. longicorpus*, and 3) vine-shaped form of *I. choii*. And although *I. koreensis*, *I pumila*, and *I. hugowolfeldi* possessed the same villous form, it was evident that fine structure of the zona radiata varied according to species. The adhesive membranes and fine structures of zona radiata in *Iksookimia* showed a species specificity related to closely their habitats and spawning properties.

KEY WORDS: Adhesive Membranes, Zona Radiata, *Iksookimia*, Oocyte

The loaches of family Cobitidae are benthic small fishes inhabiting the freshwater of the Europe, Asia and North Africa. The family Cobitidae of Korea contained 5 genera and 14 species. Among them, the genus *Cobitis* composed of 9 species, of which 5 species are endemic to Korea (Kim and Kang, 1993). Recently, Nalbant (1993, 1994) erected the genus *Iksookimia* separating it from *Cobitis* on the basis of the shape of pectoral fin ray in male and their color patterns on the body sides. And Nalbant transfered 4 Korean cobitid fishes, *Cobitis koreensis*, *C. longicorpus*, *C. choii*, and *C. pumila*, to the genus *Iksookimia*. Also, Nalbant (1993) described a new species, *Iksookimia hugowolfeldi*, representing a cobitid fish which is distributed only in the Yongsan River of Korea.

Korean cobitid fishes show such a species diversity, but there is occasionally taxonomic confusion due to their diverse color patterns. Because the phylogenetic classification is not possible without extending knowledge of the species level, Therefore, it is necessary to know better characters for species morphology. In many taxonomic groups, the adhesive membranes on the surface of the zona radiata are found and used for taxonomic purpose (Riehl, 1980; Johnson and Werner, 1986; Hirai, 1993; Britz *et al.*, 1995). The adhesive membranes were reported in relation to substrate-spawning conditions in many fishes (Blaxter, 1969; Laale, 1980; Riehl and Greven, 1990, 1993). Also, Kim and Park (1995, 1996) recently reported the several forms of the adhesive membranes in some genera of Cobitidae from Korea.

This paper describes the adhesive membranes and fine structures of zona radiata for the five species of *Iksookimia* and discusses the significance of such to their systematic position and habitats.

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Materials and Methods

Females of 5 cobitid fishes, *Iksookimia koreensis*, *I. pumila*, *I. hugowolfeldi*, *I. longicorpus*, and *I. choii*, were used and collected from several streams of the South Korea in spawning season from 1992 to 1995.

For the transmission electron microscopy (TEM), adult gravid females were anaesthetized with MS222. Their ovaries were excised and prefixed in 2.5% glutaraldehyde in 0.1M 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 (SEM), 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.

In the present study the distinction of late yolk granule stages in oocyte differentiation was based on previously reported histological criteria (Kim and Park, 1995, 1996).

Results

In TEM observations of the late yolk graule stage, the zona radiata of *Iksookimia* consisted of three zone (Plate 2. A). The outermost zone of egg, zone 1 (Z1), has a site for attachment of adhesive membranes. The beneath Z1, zone 2 (Z2) has less electron-dense substance. The innermost zone, zone 3 (Z3) consists of several layers, heterogeneous zone, showing different electron densities by species. Microvilli and pore canals exist in Z2 and Z3, and the microvilli projected from oocyte toward the follicle cell through pore canals (Plate 1, 2). The thickness (Z2+Z3) of zona radiata also varied according to species. The microvilli and pore canals were easily observed in all surface of zona radiata.

The adhesive membranes distributed throughout the zona radiata and were easily distinguished from microvilli of zona radiata. The adhesive membranes of each species and their characteristics are as follows (Plate 1, 2).

I. koreensis

I. koreensis have adhesive membrane of villus-shaped feature that is distributed the outer zona ratidata (Plate 1. A, B). The villi measured 1.0-1.5 μm in length and uniformly distributed on the Z1 of the zona radiata at a density of 3-4/10 μm in number. The zona radiata consisted of three zone, and Z3 consisted of six layers, showing different electron densities. The zona radiata was about 2.0-2.5 μm thick. Pore canals and microvilli from oocyte distributed throughout the zona radiata. In SEM, the zona radiata contained numerous pore canals and microvilli, and villous projections, adhesive membrane.

I. pumila

In *I. pumila*, the external surface of the zona radiata possessed a number of uniformly distributed villi, 1.0-1.5 μm in length, at a density of 3-4 numbers/10 μm (Plate 1. C, D). The zona radiata, about 2.5-3.0 μm thick, consisted of three zone, of which Z3 composed of nine layers showing different electron densities.

I. choii

I. choii showed distinctive feature like twined ivies or vines, and was the longest among the adhesive membranes of *Iksookimia* (Plate 1. E, F). The vines measured 3.0-4.5 μm in length and distributed on the Z1 of the zona radiata at a density of 4-5 numbers/10 μm. The zona radiata was about 5.0-6.0 μm thick. The zona radiata consisted of three zone, and Z3 consisted of four layers. Pore canals and microvilli from oocyte exist in Z2 and Z3 of zona radiata (Plate 1. E). In SEM, the outer surface of zona radiata was covered with numerous twined vines (Plate 1. F).

I. longicorpus

This species showed cotton-like structure, thicker and longer characteristics than villi of villous form (Plate 2. A, B). Such a adhesive

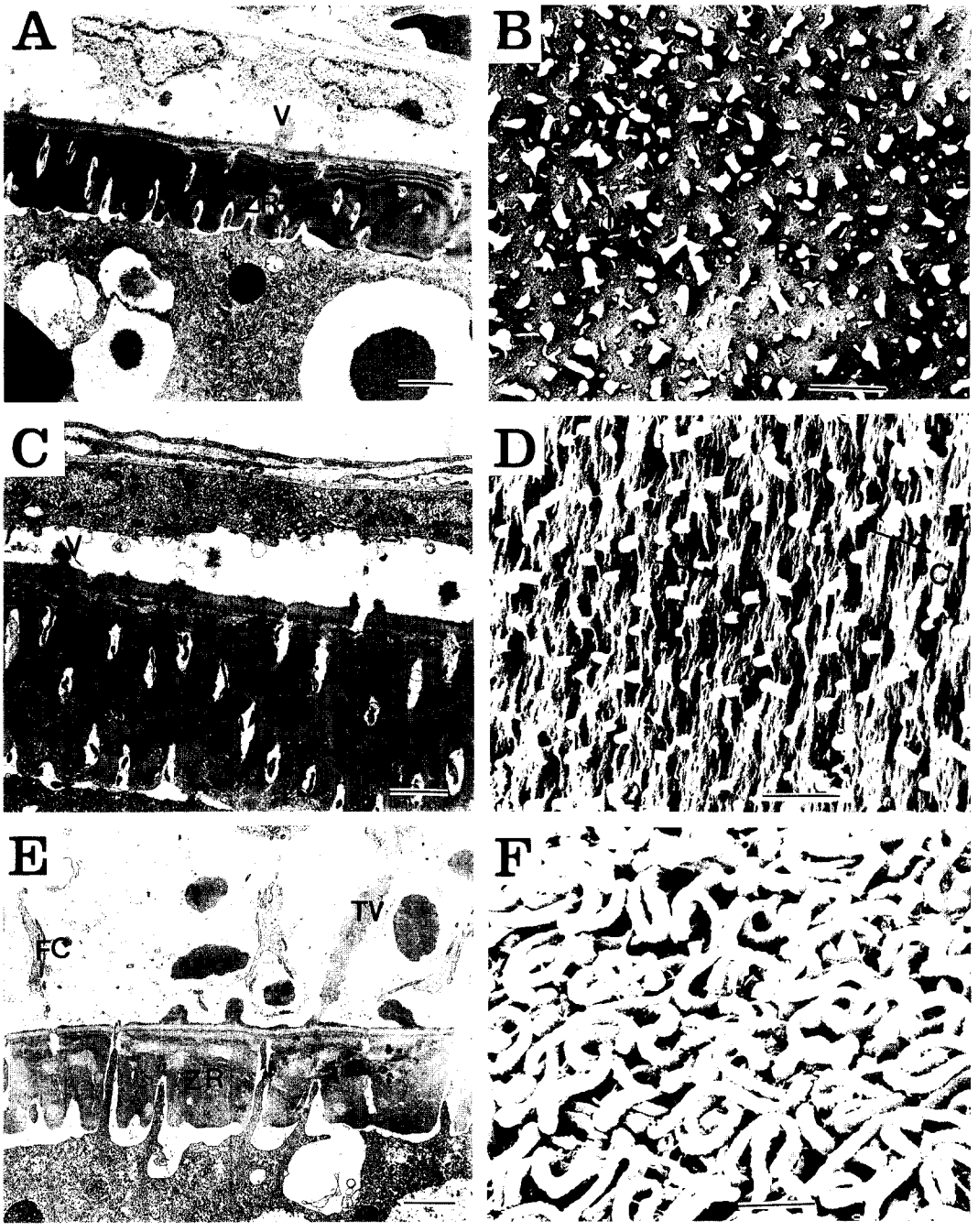

Plate 1. Transmission electron micrographs (TEM) and scanning electron micrographs (SEM) of various adhesive membranes on the outer zona radiata of five loaches of Korea. A-B. TEM (A) and SEM (B) of *I. koreensis*. MV, microvillus; PC, pore canal; V, villous; ZR, zona radiata. Bar=2 μm, 5 μm. C-D. TEM (C) and SEM (D) of *I. pumila*. MV, microvillus; PC, pore canal; V, villous; ZR, zona radiata. Bar=0.9 μm, 5 μm. E-F. TEM (E) and SEM (F) of *I. choii*. FC, follicle cell, TV, twined vine. Bar=2 μm, 5 μm.

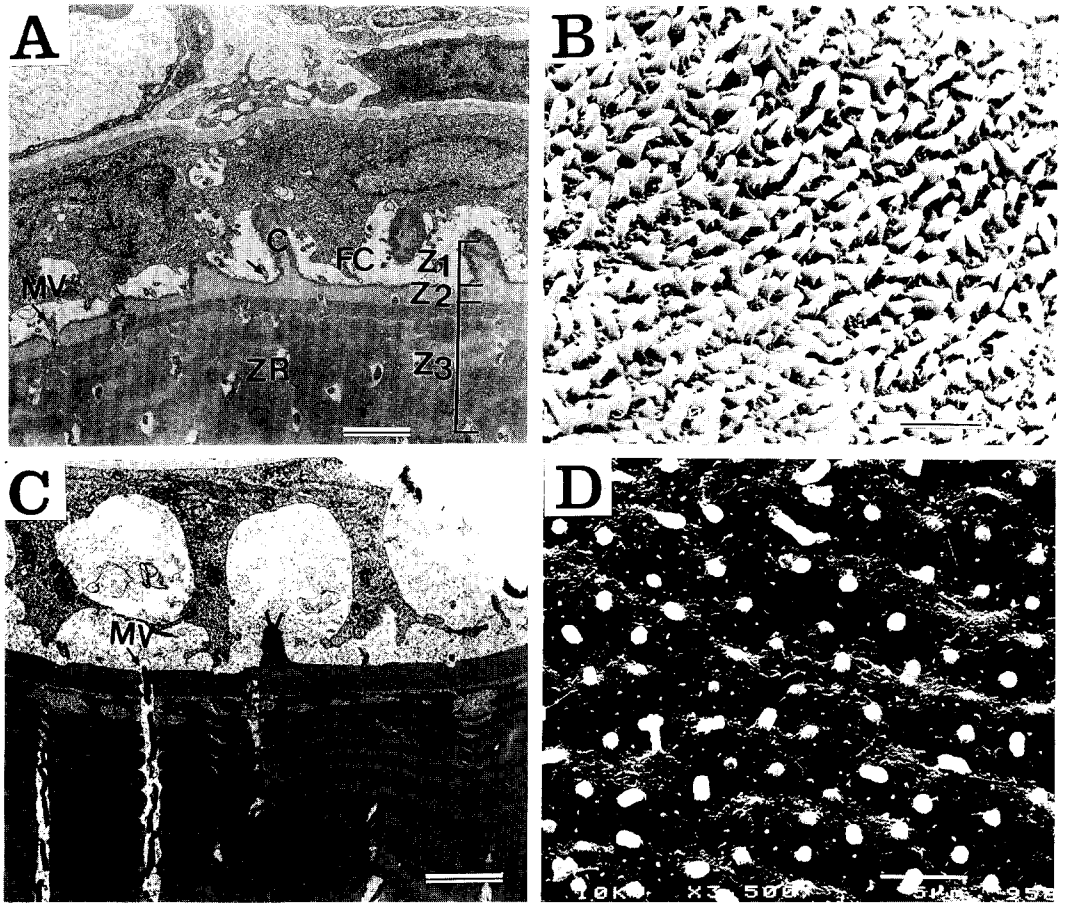

Plate 2. Transmission electron micrographs (TEM) and scanning electron micrographs (SEM) of various adhesive membranes on the outer zona radiata of five loaches of Korea. A-B. TEM (A) and SEM (B) of *I. longicorpus*. C, cotton-like form; FC, follicle cell; MV, microvillus; ZR, zona radiata; Z_1, zona radiata 1; Z_2, zona radiata 2; Z_3, zona radiata 3. Bar=2 μm, 5 μm. C-D. TEM (C) and SEM (D) of *I. hugowolfeldi*. MV, microvillus; ZR, zona radiata. Bar=2 μm, 5 μm.

strucutres were attached to the Z1 of zona radiata of *I. longicorpus*. The cottons measured 2.0-2.5 μm in length and densely distributed at a density of 10-15/10 μm in number. The thickness of zona radiata measured 5.0-7.0 μm, and Z3 showed six layers. The pore canals and microvilli were easily observed in TEM. In SEM, the surface of the zona radiata was ornamented with cotton-shaped form (Plate 2, B).

I. hugowolfeldi

In *I. hugowolfeldi*, the Z1 of the zona radiata have numerous villi, about 1.0-1.7 μm in length at a density of 2-4 μm numbers/10 μm (Plate 2. C, D). The thickness of the zona radiata was about 2.7-3.2 μm. The Z3 composed of very much thirteen layers, showing different electron densities (Plate 2. C). The Z2 and Z3 possessed pore canals and microvilli from oocyte.

Discussion

The structural and morphological differences of zona radiata have been reported in other Korean cobitid fishes (Kim and Park, 1995, 1996). The differences of zona radiata structure in teleosts were species specific (Lönning and Hagström,

1975; Riehl and Greven, 1993). Ivankov and Kurdyayeva (1973) hypothesized that the morphological character of the primary membrane (zona radiata) indicated adaptation to spawning and egg development. Hirai (1993) reported that structure of the zona radiata are closely related to environmental factors and systematic relationships.

By SEM and TEM observations of the adhesive membranes for the five species of *Iksookimia* in the family Cobitidae, it was found that three forms of adhesive membranes are attached to the outer zona radiata during the late yolk granule stage: 1) villous form, 2) cotton-shaped form, 3) and vine-shaped form. The villous form appeared in *I. koreensis*, *I. pumila*, and *I. hugowolfeldi*. In the villous form, three species seemed to be similar with each other in the size or number of villi, but it was recognized that the thickness and electron density of the zona radiata varied by species. In the thickness of zona radiata, *I. koreensis* was about 2.0-2.5 μm, *I. pumila* was about 2.5-3.0 μm and *I hugowolfeldi* was about 2.7-3.2 μm. *I. koreensis* and *I. pumila* could be discerned by such a difference of the thickness of zona radiata. On the other hand, Z3 of zona radiata, innermost zone, composed of heterogeneous layers, layer of different electron density, according to species. The Z3 consisted of six layers in *I. koreensis*, nine layers in *I. pumila*, and thirteen layers in *I. longicorpus*. *I. koreensis* and *I. pumila* could be easily classified due to their morphological aspects, whereas *I. hugowolfeldi* and *I. longicorpus* were not so. However, there was clear difference between *I. hugowolfeldi* and *I. longicorpus* in the adhesive membranes. *I. hugowolfeldi* has cotton-shaped form differing from villous form of *I. longicorpus* in the adhesive membrane, and thicker zona radiata, 5.0-7.0 μm thick, and Z3 of zona radiata consisted of six layers, much less layers than that of *I. longicorpus* with thirteen layers. Therefore, two cobitid species could be classified by the differences of adhesive membranes and fine structures of zona radiata. *I choii* showed unique vine-shaped form, twined ivies or vines, and the vine was the longest among the adhesive membranes of *Iksookimia*. Microvilli and pore canals of *Iksookimia* distributed on the Z2 and Z3 of zona radiata, and the microvilli projected from oocyte toward the follicle cell. The pore canals distributed throughout the zona radiata contribute to the transportation of nutrients from the follicle cell to the developing egg body (Hurley and Fisher, 1966; Nagahama, 1983; Groot and Alderdice, 1985).

The eggs are largely divided into buoyant and demersal egg in teleosts, and most stream fishes have demersal eggs with adhesive stickiness (Lagler *et al.*, 1977). The demersal eggs of teleosts are comprised of three functional types: non-adhesive, adhesive and twine eggs (Mito, 1979). The contact mechanisms of adhesive eggs according to species. Some species of Osmeridae and *Plecoglossus altivelis* have an adhesive membrane covering the animal hemispheres of egg (Kanoh, 1952; Honma and Tamura, 1962). Eggs attaching to substrate by thread or filaments on the chorion have been described in *Oryzias latipes*, and some species of Cyprinodontiformes (Tsukahara, 1971; Hart *et al.*, 1984; Riehl and Greven, 1993) and Cobitidae (Kim and Park, 1995, 1996). In addition, various patterns of adhesive membranes were reported : wart-like appendage in some of Pleuronichthidae (Mito, 1963), hexagonal pattern in *Cynolebias melanotaenia* and *C. ladigesi* (Worum and Sheldon, 1976), lamellar structure of Pleuronectinae (Hirai, 1993), and equidistant ridges of Perciformes (Britz *et al.*, 1995).

Korean benthic loaches inhabited different habitats, such as pebble, sand, or mud bottom by species (Kim and Son, 1984; Kim and Lee, 1987; Kim and Kang, 1993). Especially, *I. choii* lived under sand, differing from other cobitid fishes which mostly inhabited on pebble bottoms. The cobitid fishes of villous form mainly inhabited on the pebble bottoms of the middle and lower streams with slow current, whereas *I. longicorpus* of cotton-shaped form inhabited pebbly bottoms of the middle and upper streams, or rapid current. Therefore, there can be suggested that the forms of adhesive membrane may be deeply related to the their habitats with species specificity.

Filament-like adhesive membranes 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 covering material of the egg membrane in adhesive eggs 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). Koya *et al.* (1995) reported that the down-like layer formed on the Z1, outermost zone of vitelline envelope of *Hexagrammos octogrammus*, functions as an adhesive membranes.

The adhesive membrane and structure of the zona radiata in teleosts is well documented, and their morphological aspects have occasionally been used for taxonomic purposes (Laale, 1980; Groot and Alderdice, 1985.; Kjesbu and Kryvi, 1989; Hirai, 1993; Riehl and Greven, 1993; Britz *et al.*, 1995; Koya *et al.*, 1995). The present observation indicates that the adhesive membranes of *Iksookimia* could be used as a taxonomic good character for the species identification related to their habitats.

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참종개속(*Iksookimia*) 어류 5종의 난 부착막 구조

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Iksookimia(신칭, 참종개속) 어류 5종의 부착막 구조(adhesive membrane)를 조사하기 위하여 전자현미경으로 성숙란을 조사한 결과, 난황형성시기인 난황구 후기의 zona radiata에는 다음과 같은 3가지 형태의 부착막이 구별되었다. *Iksookimia koreensis*, *I. pumila* 그리고 *I. hugowolfedi*는 융모형(villous form)을 보였으며, *I. longicorpus*는 원추모양의 솜 형태(cotton form)을 보여주었으며, *I. choii*는 덩쿨모양(vine form)의 독특한 부착막 구조를 가지고 있었다. *I. koreensis*와 *I. pumila*, 그리고 *I. hugowolfeldi*는 같은 융모형의 부착막구조를 가지고 있으나 난막의 미세구조는 종간에 뚜렷한 차이를 보여주었다. 또한 참종개속 어류의 난막과 부착막 구조는 그들의 서식처와 산란습성에 관련된 종의 특이성을 보여 주고 있어서 분류학적으로 주목되었다.