Korean J. Ichthyol. 9(1), 121~129, 1997

韓魚誌, 9(1), 1997

Egg Membrane in Five Cobitid Species of *Cobitis* (Pisces : Cobitidae)

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The five species of genus *Cobitis* 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 two form as follows: 1) granular form of *Cobitis lutheri*, *C. striata*, *C. sinensis*, and *C. sinensis-longicorpus*, 2) villous form of *C. melanoleuca*. Although *C. lutheri*, *C. striata*, *C. sinensis*, and *C. sinensis-longicorpus* possessed the same granular 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 *Cobitis* showed a species specificity closely related to their habitats and spawning properties.

Introduction

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* was 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 reclassified 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. Therefore the genus *Cobitis* in Korea is distributed into five species, *C. lutheri*, *C. striata*, *C. sinensis*, and *C. melanoleuda*. Because it had revealed that *C. granoei* and *C. melanoleuca* were conspecific, Nalbant (1993) reclassified *C. granoei* to the name *melanoleuca*, according to priority. In the Nakdong River drainage of Korea, *Cobitis sinensis - longicorpus* originated from *C. sinensis* and *I. longicorpus* was reported in reference to the morphological features, karyotype with diploid and triploidy and the histological features of the gonad (Kim and Lee, 1990, 1995 ; Kim and Park, 1993).

Because the phylogenetic classification is not possible without extending knowledge of the

This research was supported by a grant from the Basic Research Institute Program, Ministry of Education, Korea, 1995, Project no. BSRI 4426.

species level, 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 the 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 *Cobitis* and discusses the significance of their systematic position and habitats.

Materialsand Methods

Females of 5 cobitid fishes, *C. lutheri*, *C. striata*, *Cobitis sinensis*, and *C. melanoleuca* 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 0.1 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 - 1200 EX 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 CO2. The dried samples were coated with gold - palladium and observed with JEOL JSM - T 330A scanning electron microscope.

In the present study the distinction of late yolk granule stages in oocyte differentiation was observed 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 *Cobitis* consisted of three zone (Plate 1, A1, A2). The outermost zone of egg, zone 1 (Z1), has a site for attachment of adhesive membranes. 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, A1, A2). 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.

C. lutheri

C. lutheri have adhesive membrane of granule - shaped feature that is distributed the outer zona ratidata (Plate 1, A). The granules measured 1.0㎛ in diameter and uniformly dis-

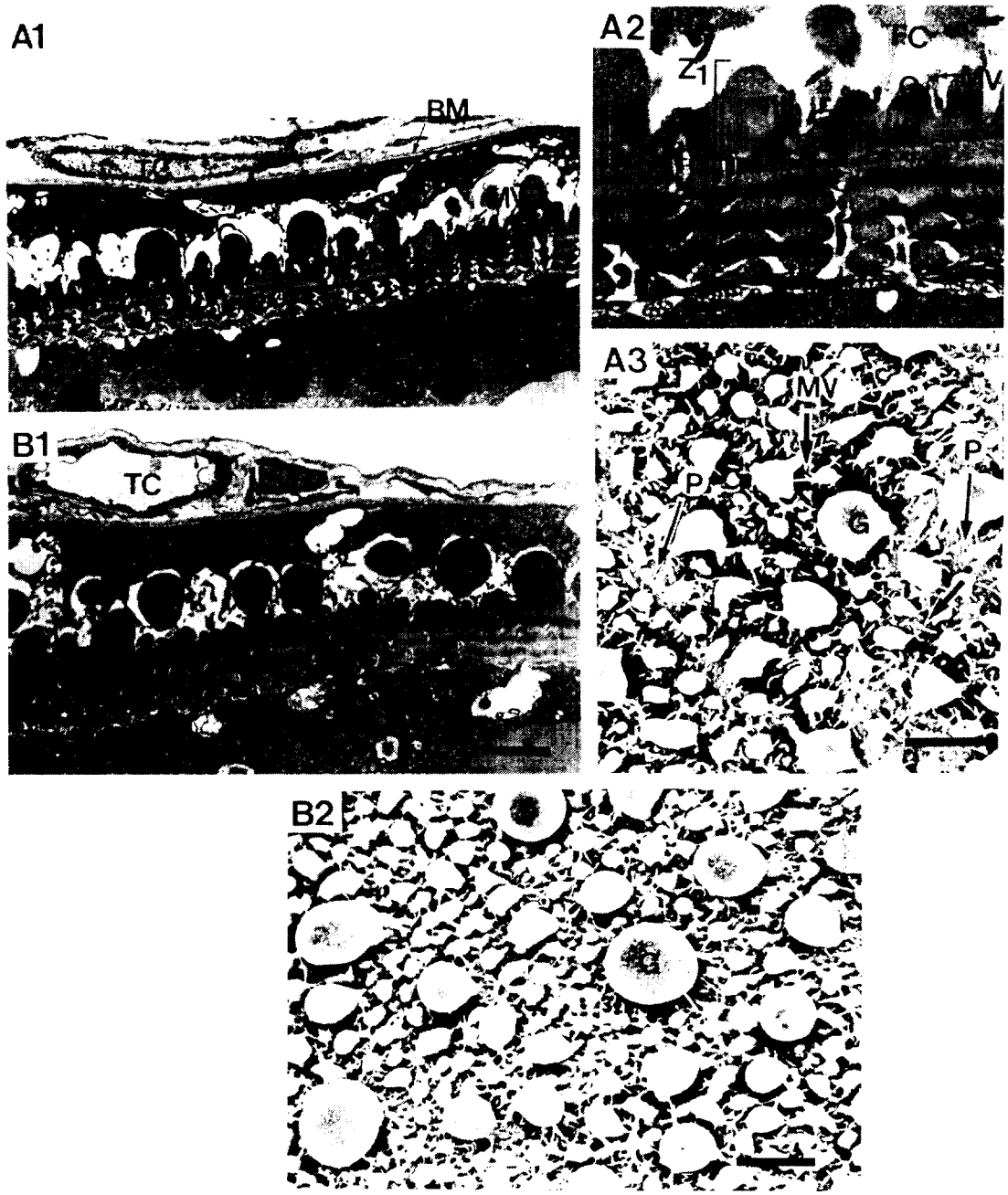

Plate 1. Transmission electron micrographs (TEM) and scanning electron micrographs (SEM) of various adhesive membranes on the outer zona radiata of five loaches of Korea. A. TEM (A1 and A2) and SEM (A3) of *C. lutheri*. BM. base membrane ; FC, follicle cell ; G, granular form ; GC, granulosa cell ; MV, microvillus ; OO, ooplasm ; P, pore canal ; TC, thecal cell ; Z1, Z2, Z3, zona radiata (ZR)1, zona radiata 2, zona radiata 3 ; 1, 2, 3, 4, layers of electron density. B. TEM (B1) and SEM (B2) of C. striata. G, granular form ; GC, granulosa cell ; TC, thecal cell ; ZR, zona.radiata. Bar=2 ㎛(A1, A2, B1), 5㎛(A3, B2).

tributed on the Z1 of the zona radiata at a density of 7~8 number/10㎛. The zona radiata consisted of three zone, and Z3 consisted of five layers, showing different electron densities. The zona radiata was about 2.0~3.0㎛ thick (Plate 1, A1, A2). Pore canals and microvilli from oocyte distributed throughout the zona radiata (Plate 1, A2). In SEM, the zona radiata contained numerous pore canals and microvilli, and granular projections, adhesive membrane (Plate 1, A3).

C. striata

In *C. striata*, the external surface of the zona radiata possessed a number of uniformly granules, 1.5~2.0㎛ in diameter, at a density of 5~6 numbers/10㎛ (Plate 1, B). The zona radiata, about 3.0~4.0㎛ thick, consisted of three zone, of which Z3 composed of four layers showing different electron densities (Plate 1, B1). The zona radiata contained numerous pore canals and microvilli, and granular projections. In SEM, the outer surface of zona radiata was covered with numerous granulestwined vines (Plate 1, B2).

C. sinensis

C. sinensis have granular adhesive membrane on the outer zona radiata (Plate 2, C). The granules measured 1.3~3.0㎛ in diameter and distributed random on the Z1 of the zona radiata at a density of 2~3 numbers/10㎛ (Plate 2, C). The zona radiata was about 2.5~3.0㎛ thick. The zona radiata consisted of three zone, and Z3 consisted of four or five layers.

C. sinensis - longicorus

This species have adhesive membrane of granule - shaped feature which is distributed the outer zona ratidata (Plate 2, D). The granules measured 1.0~1.7㎛ in diameter and uniformly distributed on the Z1 of the zona radiata at a density of 3~5/10㎛ in number. The zona radiata consisted of three zone, and Z3 consisted of six layers showing different electron densities. The zona radiata was about 3.5~4.5㎛ thick. Pore canals and microvilli from oocyte distributed throughout the zona radiata (Plate 2, D).

C. melanoleuca

C. melanoleuca have adhesive membrane of villus - shaped feature, thinner and longer characteristics than granules of granular form (Plate 2, E). The villi measured 1.5~2.0㎛ in length and distributed on the Z1 of the zona radiata at a density of 9~11 numbers/10㎛ (Plate 2, E1). The zona radiata was about 5.5~6.0㎛ thick. The zona radiata consisted of three zone, and Z3 consisted of eigth layers (Plate 2, E1). Pore canals and microvilli from oocyte exist in Z2 and Z3 of zona radiata. In SEM, the outer surface of zona radiata was covered with numerous villi (Plate 2, E2).

Discussion

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). 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

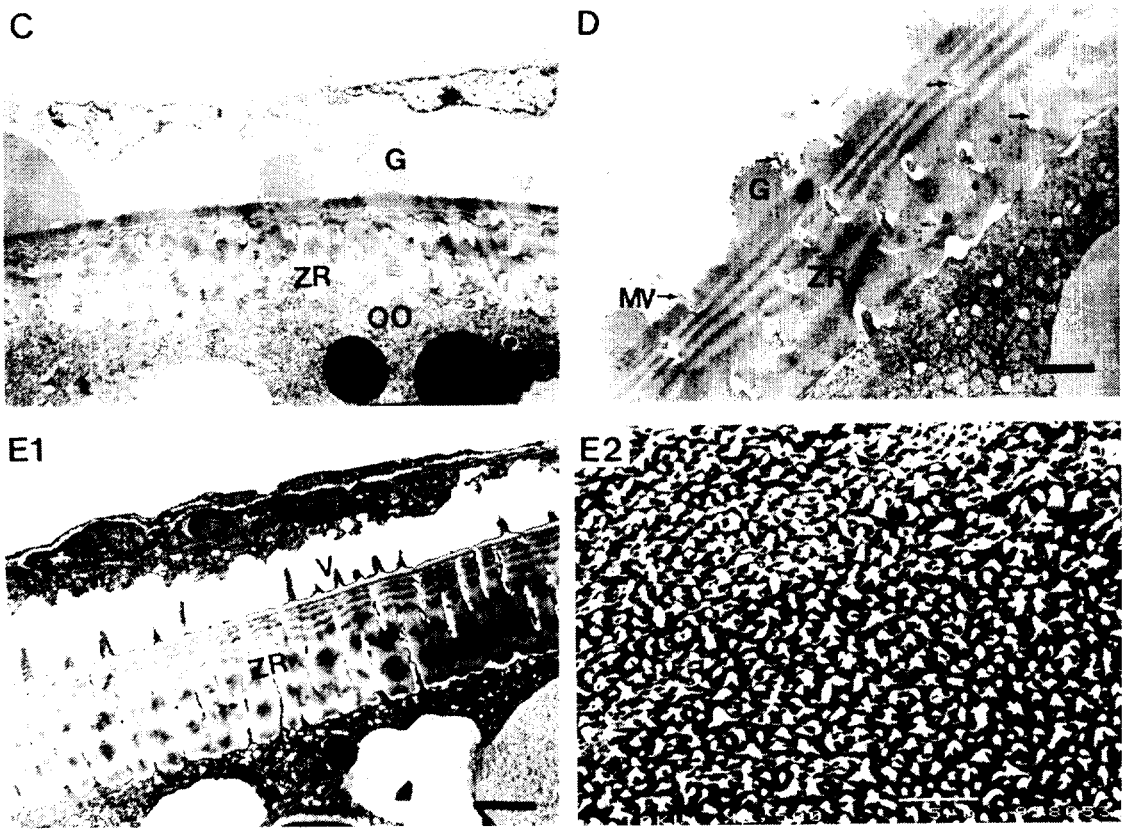

Plate 2. Transmission electron micrographs (TEM) and scanning electron micrographs (SEM) of various adhesive membranes on the outer zona radiata of five loaches of Korea. C. TEM of *C. sinensis*. G, granular form ; OO, ooplasm ; ZR, zona radiata. Bar=2 ㎛. D. TEM of *C. sinensis - longicorpus*. G ; granular form ; MV, microvilli. OO, ooplasm ; ZR, zona radiata. Bar=2㎛. E. TEM (E1) and SEM (E2) of of *C. melanoleuca*. ZR, zona radiata ; V, villous form. Bar=2㎛(C, D, E1), 5㎛(E2).

(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).

By SEM and TEM observations of the adhesive membranes for the five species of *Cobitis* in the family Cobitidae, it was found that two forms of adhesive membranes are attached to the outer zona radiata during the late yolk granule stage : 1) granular form, 2) villous form. The granular form appeared in *C. lutheri, C. striata, C. sinensis,* and *C. sinensis - longicorpus* But these species were distinctly different from each other in the size or number of granules as well as the thickness and electron density of the zona radiata. Especially *C. sinensis - longicorpus* has been known as a hybrid origin between *Cobitis sinensis* and *Iksookimia longicorpus*(Kim and Lee, 1990, 1995 ; Kim and Park, 1993). On the one hand, *I. longicorous* showed cotton - like form that it is quite

a different from that of *C. sinensis* in adhesive membrane (Park and Kim, in pressing). But the adhesive membrane of *C. sinensis - longicorpus* showed not a intermediate form between *C. sinensis* and *I. longicorpus,* but granular form like that of *C. sinensis.* Of course, *C. sinensis - longicorpus* in fine structures was different from *C. sinensis* with the same form in the adhesive membrane.. Also, *C. melanoleuca* have unique villous form of adhesive membrane, differing from granular form of three species.

The 5 cobitid fishes inhabit nearly sandy bottom. According to authors (Ivankov and Kurdyayeva, 1973 ; Hirai, 1993), it had been reporeted that the morphological character of the primary membrane (zona radiata) indicated adaptation to spawning and egg development, and that structure of the zona radiata are closely related to environmental factors and systematic relationships. Therefore, it seems that the structure of adhesive membrane in *Cobitis* may be deeply related to the their habitats with species specificity.

The zona radiata of *Cobitis* consisted of three zone (Z1, Z2, Z3). The Z3, the innermost zone, consists of several layers which show different electron densities by species. Microvilli and pores were 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 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 (L nning and Hagstr m, 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.

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). Therefore, the fine structures on the adhesive

membranes of *Cobitis* could be used as a taxonomic good character for the species identification related to their habitats.

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기름종개속 *Cobitis*(어강, 미꾸리과) 어류 5종의 난막

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기름종개속 *Cobitis* 어류 5종의 부착막의 미세구조(adhesive membrane)를 조사하기 위하여 전자현미경으로 성숙란을 조사한 결과, 난황형성시기인 난황구 후기의 zona radiata에는 다음과 같은 2가지 형태의 부착막 구조가 존재하였다. *Cobitis lutheri, C. striata, C. sinensis* 그리고 *C. sinensis -longicorpus*는 과립형(granular form)을 보였으며 *C. melanoleuca*는 융모형(villous form)을 보였다. 그러나 *C. lutheri*와 *C. striata* 그리고 *C. sinensis, C. sinensis -longicorpus*는 같은 과립형의 부착막구조를 가지고 있으나 이들 과립의 크기와 분포 수 그리고 난막의 미세구조에서 종간에 뚜렷한 차이를 보여주었다. 이러한 기름종개속 어류의 난막과 부착막 구조는 그들의 서식처와 산란습성에 관련된 종의 특이성을 보여 주고 있어서 분류학적으로 주목되었다.