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A Scanning Electron Microscopic Study on the Glochidial Encystment of a Freshwater Clam, *Anodonta arcaeformis* on the Host Fish, *Carassius auratus*

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ABSTRACT

A scanning electron microscopic study on the glochidium and glochidial encystment of *Anodonta arcaeformis* on the host fish *Carassius auratus* was conducted. The shape of the glochidium was apparently subtriangular and its average size was 270 μm x 260 μm x 145 μm. The glochidial shell valves were of the same size, kept together by a ligament that is 50.4 μm in length and 5.5 μm in width. Each of the glochidial shell valve had a long hook studded with many spines on the superior face. A large area of at the apex of the valve surrounding the base of the hook was provided with numerous small spines which became progressively smaller toward the periphery of the area. The glochidial shell valve consisted of two layers. The mantle cells line the glochidial shell valves and some of hair cells were observed. A larval thread was 2.3 μm in diameter. In the artificial infection of the glochidia to one of the natural hosts, *Carassius auratus*, it took about three to four hours to encyst the glochidia with epithelial cells of the fish fins. The encystment method was the cell migration from the neighboring epithelial cells.

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INTRODUCTION

Most unionoidean bivalves are larviparous, discharging glochidia from brooding sites in the marsupial demibranchs, and the mature larvae of them are temporary, obligatory parasites on the fins and/or gills of various freshwater fishes or a few species of externally-gilled aquatic amphibians. The larvae usually parasitize host fish for a period of 10-30 days (Mansur and Silva, 1999). Metamorphosis of the glochidial larvae depends on blood composition and immune responses of the host fish (Reculing, 1919; Arey, 1932; Kirk and Layzer, 1997). Recently Lima *et al.* (2006) achieved artificial cultivation of glochidia of the freshwater mussel *Anodonta cygnea* and observed the ultrastructure of early developmental stages by scanning electron microscopy, from the glochidial to the juvenile stage.

Anodonta arcaeformis is one of the representative species among the Korean freshwater bivalves. This study was conducted to find out the external ultra structures of the glochidia and to observe the successive stages of glochidial encystment following the glochidial attachment to the host fish.

MATERIALS AND METHODS

Several adult individuals of *Anodonta arcaeformis* (Unionoidea, Anodontidae) and of a freshwater species *Carassius auratus* (Cyprinidae) were collected from a stream in Taean County, Chungcheongnam Province, Korea. Each species was kept separately in aquaria in the laboratory until this experiment. In the experiment a gravid mussel was selected out of those kept in the aquarium and was dissected in a small quantity of dechlorinated tap water to cut the marsupia loose. The glochidia were shaken out into the finger bowls and collected for the experiments.

The glochidia were cleaned several times with saline solution and anaesthetized with menthol for SEM observation of the glochidium. Fully relaxed and well opened glochidia were washed again and fixed with 2.5% glutaraldehyde solution for 2 hours. The whole

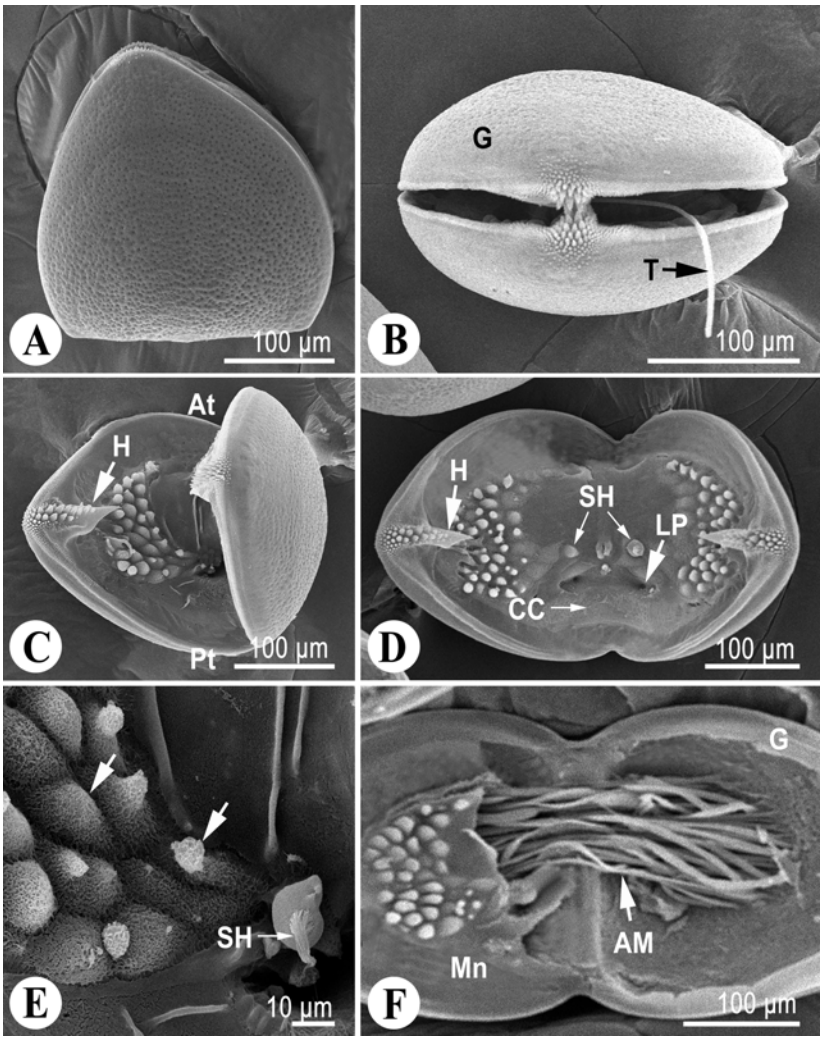

Fig. 1. External shapes of the glochidium. **A**: A glochidium with the its valves closed. **B**: Apex of the glochidium showing hooks and larval thread (T). **C**: A glochidium partly open its valves. **D**: A glochidium widely open its valves. It shows hooks (H), sensory hair cells (SH), lateral pit (LP), and posterior ciliary organ (CC). **E**: Large mantle cells (arrowed) and sensory hair cell (SH) in the mantle. **F**: A glochidium showing the adductor muscle bundles (AM) under the mantle layer (Mn) artificially removed in part.

glochidia fixed were dehydrated in a series of graded concentrations of alcohol and amyl acetate mixture and dried with critical point dryer. To observe the shell structures, some of the glochidia were put into 10% KOH overnight to remove the flesh part of the glochidia and washed several times with redistilled water. The cleaned shells were air dried. The specimens chemically dehydrated or air dried were coated 200 nm in thickness with gold sputter for SEM observation.

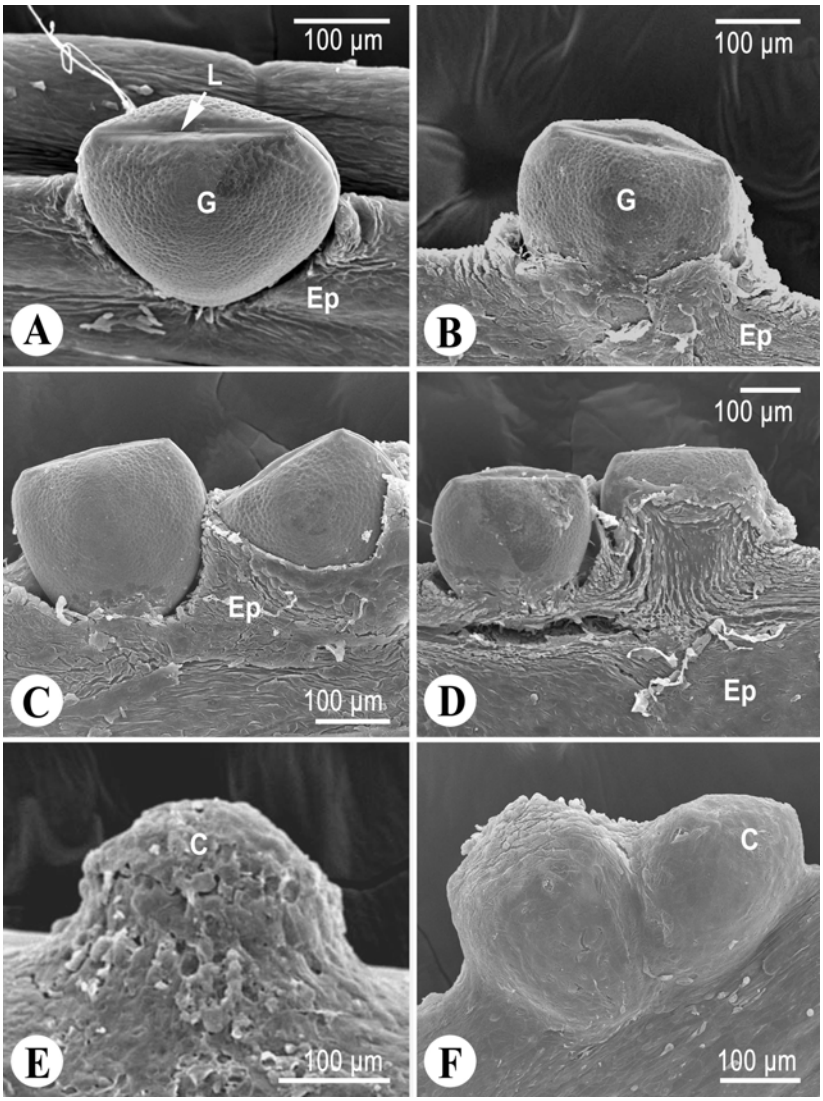

Fig. 2. Glochidial encystment process. **A**: Thirty minutes after attachment. G, glochidium; L, ligament; Ep, epithelium of the host fish fin. **B**: One hour after attachment. The epithelial cells (Ep) are moving toward the attached glochidium (G) to cover it. **C**: Two hours after attachment. **D**: Two and half hours after attachment. Epithelial cells of the gill epithelium (Ep) are showing active pattern of migration **E**: Four hours after attachment. The epithelial cells of the gill roughly covered the glochidium to form a cyst (C). **F**: Five days after attachment. The glochidial cysts (C) are completely formed and the epithelial cells looked stable with several cell layers.

Many of glochidia collected were transferred to the bottom of a fish globe containing 15 cm deep dechlorinated tap water at room temperature. The host fishes (*Carassius auratus*) from the aquarium were allowed to swim around in the fish globe which contained lots of glochidia at the bottom. Some time after the fishes were introduced, the glochidia moved up one by one to the fish by snapping their valves. The fish infected with about 5 to 10 glochidia within five minutes were transferred to a small fish globe for further observations and experiments. The fish infected with the glochidia were sacrificed for observations of successive stages of encystment at an interval of 30 minutes from the time of infection up to 5 hours after the infection. After that, the fish were sacrificed at an interval of 24 hours. The fish fins infected with the glochidia were cut out and fixed with 2.5 % glutaraldehyde for 2 hours, dehydrated in a series of graded concentrations of alcohol and amyl acetate mixture, and dried with critical point dryer. The specimens dehydrated and dried with critical point dryer were coated 200 nm in thickness with gold sputter for SEM observation.

All of the specimens dried chemically or by air were observed with a SEM (JSM-5410).

RESULTS

1. Morphology of the Glochidium

The shape of the glochidium is apparently subtriangular and its average size is 270 μm x 260 μm x 145 μm. The glochidial shell valves are of the same size, kept together by a ligament that is 50.4 μm in length and 5.5 μm in width (Fig. 1A, C). Each of the glochidial shell valve has a long hook studded with many spines on the superior face (Fig. 1A, C-F). A large area of at the apex of the valve surrounding the base of the hook is provided with numerous small spines which become progressively smaller towards the periphery of the area (Fig. 1D). There are numerous niches scattered all over the surface. The glochidial shell valve consists of two layers. The mantle cells line the glochidial shell valves and some of hair cells are observed (Fig. 1A, B). A larval thread is 2.3 μm in diameter (Fig. 1B).

2. Glochidial Encystment

In the artificial infection of the glochidia to one of the natural hosts, *Carassius auratus,* it took about three to four hours to encyst the glochidia with epithelial cells of the fish fins (Fig. 2A-F). During the period of early encystment, the epithelial cells of fins of the host fish actively migrated toward the attached glochidium and began to cover it (Fig. 3A-F). At this stage, connections between the epithelial cells became loose and the shapes of the epithelial cells changed into various shapes according to their situations (Fig. 3B-E). The walls of the cysts thickened with several layers of epithelial cells migrated after four to five days after the glochidial infection (Fig. 2F).

DISCUSSION

It has been well known that there are two types of glochidia in the Unionidae. One is provided with stout hook on the ventral margin of the valves and the other is quite different shape and entirely hookless (Lefevre and Curtis 1910a, b). The mussels that belong to the genus *Anodonta* have glochidia provided with hooks. The structure of the glochidia of the *Anodonta* species has been described by Surber (1912, 1914), Arey (1924), Wood (1974a, b), Giusti *et al.* (1975) and Tompa (1979). Heard (1999) reviewed early discoveries and interpretations of the association of glochidia and host fish.

Wood (1974a) made a very detailed report on the light microscopic structure of the glochidium of *Anodonta cygnea.* Since ultramicroscopic studies on the glochidia of unionceans were performed by Giusti (1973) and Giusti *et al.* (1975), many researchers presented new features of the glochidia of various mussels. Jeong *et al.* (1992) reported anatomical and ultrastructural data on the glochidium of *Anodonta arcaeformis flavotincta.* Jupiter and Byrne (1997) reported light and scanning electron microscopy of the embryos and glochidia larva of the Australian freshwater bivalve *Hyridella depress.*

Recently, Araujo and Ramos (1998, 2002) described the glochidium of *Margaritifera auricularia.* Mansur and Da Silva (1999) described glochidia of five mussel

species from South America. This study focused on the fine external structures of the glochidium of *Anodonta arcaeformis* and on the process of the glochidial encystment. The shape of glochidium of *Anodonta arcaeformis* was apparently triangular and unequal lateral as described on the species that belong to the genus *Anodonta* by Brondiewicz (1968), Wood (1975) and Giusti *et al.* (1975).

The shape and the structure of the glochidium of *Anodonta arcaeformis* were similar to those of other species previously reported by many authors. The shape of the glochidium is apparently subtriangular and its average size was 270 μm x 260 μm x 145 μm. The glochidial shell valves were of the same size,

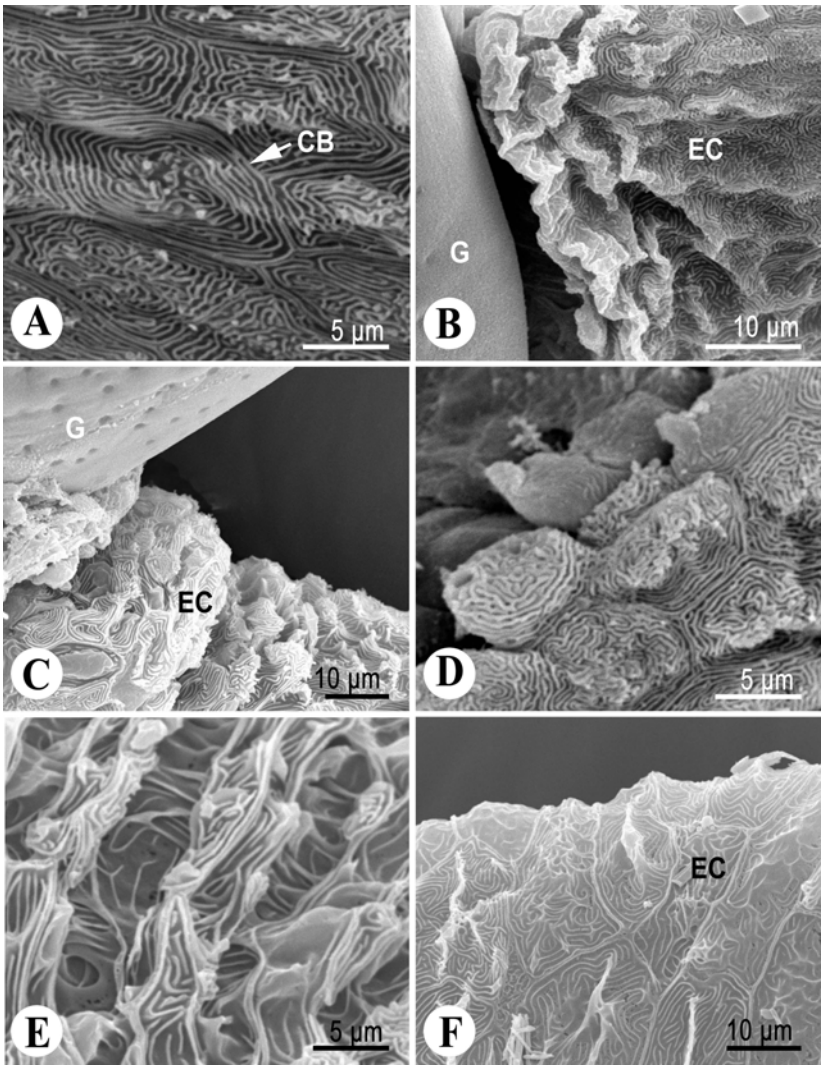

Fig. 3. Shape changing patterns of the gill epithelial cells after glochidial attachment. **A**: Normal epithelial cell surfaces of the host fish fin showing regularly arranged surface ridges and clear cell borders (CB). **B**-**D**: Epithelial cells actively migrating toward the attached glochidium(G) 30 minutes after attachment. **E**: The migrating epithelial cells of the fish fin showing very irregular shapes and arrangement of the epithelial cells. **F**: The epithelial cells (EC) of the fish fin near from a glochidial cyst. It shows almost normal pattern of cell shapes and arrangement, but they look still unstable even on the 5th day after attachment.

kept together by a ligament that was 50.4 μm in length and 5.5 μm in width. Each of the glochidial shell valve had a long hook studded with many spines on the superior face. A large area of at the apex of the valve surrounding the base of the hooks was provided with numerous small spines which became progressively smaller toward the periphery of the area. The mantle cells lined the glochidial shell valves and some hair cells existed. Jeong *et al.* (1992) reported the existence of microvilli on the mantle surface of *Anodonta arcaeformis* and mentioned that they may have a role in absorbing the nutrient during metamorphosis in the host animals.

Two types of hair cells were found in the mantle of the glochidium. One type of them was positioned solitarily at the central and subperipheral areas of the mantle and possessed several hairs per each. The second type possessed a bunch of hairs per each dome-like ciliated cell. These two types of hair cells presumably perceived mechanical and chemical stimuli as mentioned by Wood (1974). Another type of the hair cells with numerous hairs was located in the group in the posterior margin near the lateral pits. Mansur and Da Silva (1999) called this region posterior ciliary organ. This organ may concern with fluid current in the glochidial valves. A larval thread 2.3 μm in diameter was positioned in the middle of the mantle, ventral center to the hinge line of th shell valve, and seemed to be in a suitable position to maintain the balance of the glochidial body when pulled for attachment to its host.

According to Jeong *et al.* (1992) the larval thread was non-cellular and was not membrane bounded in *Anodonta arcaeformis flavotincta.* Wood (1974a) reported that the larval thread of *Anodonta cygnea* was a non cellular structure of a mucoid natures that gave a positive periodic acid Schiff reaction (PAS). On the processes of cyst formation, it took about three to four hours to encyst the glochidia with epithelial cells of the fins of a natural host fish *Carassius auratus.*

Young (1911), Lefevre and Curtis (1912) and some other earlier researchers stated that direct proliferation of the cells of the host tissue provided the material for the cyst that encloses the attached glochidium. In opposition to the opinions of the above researchers, Arey (1932a, b, c), Jeong (1989), and Jeong *et al.* (1992) asserted that the process of cyst formation is one of cell migration whereby neighboring host cells assemble and actively push forward over the invader until the wound is closed and the glochidium is covered. It may be taken into consideration that in case the time for encystment lasts only for several hours like in this study, the cells participating in the encystment can not be supplied enough by direct proliferation of the neighboring cells because of the long interval of the usual cell divisions. According to the experiment in this study, the epithelial cells of fins of the host fish actively migrated toward the attached glochidium and began to cover it during the period of early encystment. At this stage, connections between the epithelial cells became loose and the shapes of the epithelial cells changed into various shapes according to their situations. This shows a pattern of cell movement during morphogenesis, a cyst formation. The walls of the cysts thickened with several layers of epithelial cells migrated after four to five days after glochidial infection.

The glochidium of *Anodonta arcaeformis* possessed long hooks at the ventral apex of each valve. It took about 3 to 4 hours to cover the attached glochidium with one layer of the epithelial cells of the host fish fins. The encystment method was cell migration from the neighboring epithelium. This is a meaningful report showing the gradual processes of the glochidial encystment and clear views of the host's cell migration during the processes under the scanning electron microscope.

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