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Plant Rake and Algal Pouch of the Larvae in the Tropical Ascidian *Diplosoma similis*: An Adaptation for Vertical Transmission of Photosynthetic Symbionts *Prochloron* sp.

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ABSTRACT—The embryos of *Diplosoma similis* are brooded within the thick walled tunic of the colony in isolation from the symbiotic algae *Prochloron* sp., which are in the cloacal cavity of the parent colony. Prior to the spawning, the plant rake, a tassel-like structure, protrudes from the postero-dorsal end of the larval trunk and extends into the cloacal cavity. The algal cells in the cloacal cavity adhere to the plant rake. When the larvae are spawned, the trunk tunic extends posteriorly and forms a pouch entirely covering the plant rake. The algal cells are packed in the pouch (algal pouch) enveloping the basal part of the tail. The cell density of the algae in the pouch is much higher than that in the colony, suggesting that the plant rake functions for gathering and concentrating the symbionts into the algal pouch. In the course of metamorphosis, the algal pouch expands and turns into the cloacal cavity of the young colony. The high density of algal cells in the pouch would ensure that the young colony possesses the symbiotic algae of appropriate cell density in the cloacal cavity, and the colony can sufficiently receive benefits from the symbionts just after the settlement.

INTRODUCTION

Photosynthetic symbiosis is known in various metazoans, such as sponges, cnidarians, flat worms, bivalves, nudibranchs, and ascidians. Among them, some species acquire the symbionts from the environment (horizontal transmission) and the others receive the symbionts from their parent (vertical transmission). The mode of symbiont transmission varies from species to species.

Prochloron sp. is a kind of prokaryotic algae with the same chlorophyll pigments, chlorophyll (chl.) *a* and *b*, as those found in the chloroplasts of green algae and higher plants (Lewin, 1976). It occurs in coral reef areas, almost exclusively as a symbiont of some colonial ascidians of the family Didemnidae (Tunicata, Chordata) (Lewin and Cheng, 1989). The *Prochloron* cells (PrCs) are invariably contained within the cloacal cavity or the colony surface, except for *Lissoclinum punctatum* Kott, 1977, in which nearly half of the PrCs occurs intracellularly in the tunic (Hirose *et al*., 1996, 1998).

In didemnid ascidians, colonies brood their embryos and spawn tadpole larvae, and the larvae always carry PrCs. There are various differences in the larval structure for holding the symbionts among genera or species (Eldredge, 1967; Kott, 1977, 1980, 1982). The fact that there is a diversity of modes of vertical transmission led Kott (1980) to suggest that the modes of transmission had independently evolved several times. Therefore, the didemnid-*Prochloron* symbiosis is an attractive system for comparative investigation of the mechanism of transference of the symbionts and will provide a better understanding of the evolution and adaptation of larval structures for the vertical transmission.

In many of the *Prochloron* bearing didemnids, the spawned larvae carry the PrCs by attaching them to the tunic surface of the larval trunk. These larvae develop various structures for holding the symbionts. Eldredge (1967) reported that the larva of *Diplosoma virens* (Hartmeyer, 1909) develops an algal pouch around the basal part of the tail and the packed algal cells are distributed in this pouch. Kott (1980, 1981, 1982) reported that the larvae or embryos of *Diplosoma midori* (Tokioka, 1954), *D. multipapillata* Kott, 1980, *D. similis* (Sluiter, 1909), and *D. virens*, have a tassel-like organ, *rastrum* or plant rake, an outgrowth of the larval tunic to which algal cells adhere. The plant rake is taken in the incipient cloacal cavity at the spawning of the larvae (Kott, 1981). These descriptions indicate that the embryos or early larvae form the plant rake, and then the algal pouch replaces the plant rake as a symbiont carrier structure in these *Diplosoma* species. However, the detailed histological study has not been done on this dramatic change of the larval structures.

In this article, I describe the morphology of the embryos, larvae, and metamorphosing larvae of *D. similis* by means of a combination of histology and scanning electron microscopy. This description leads to speculation about the evolution of

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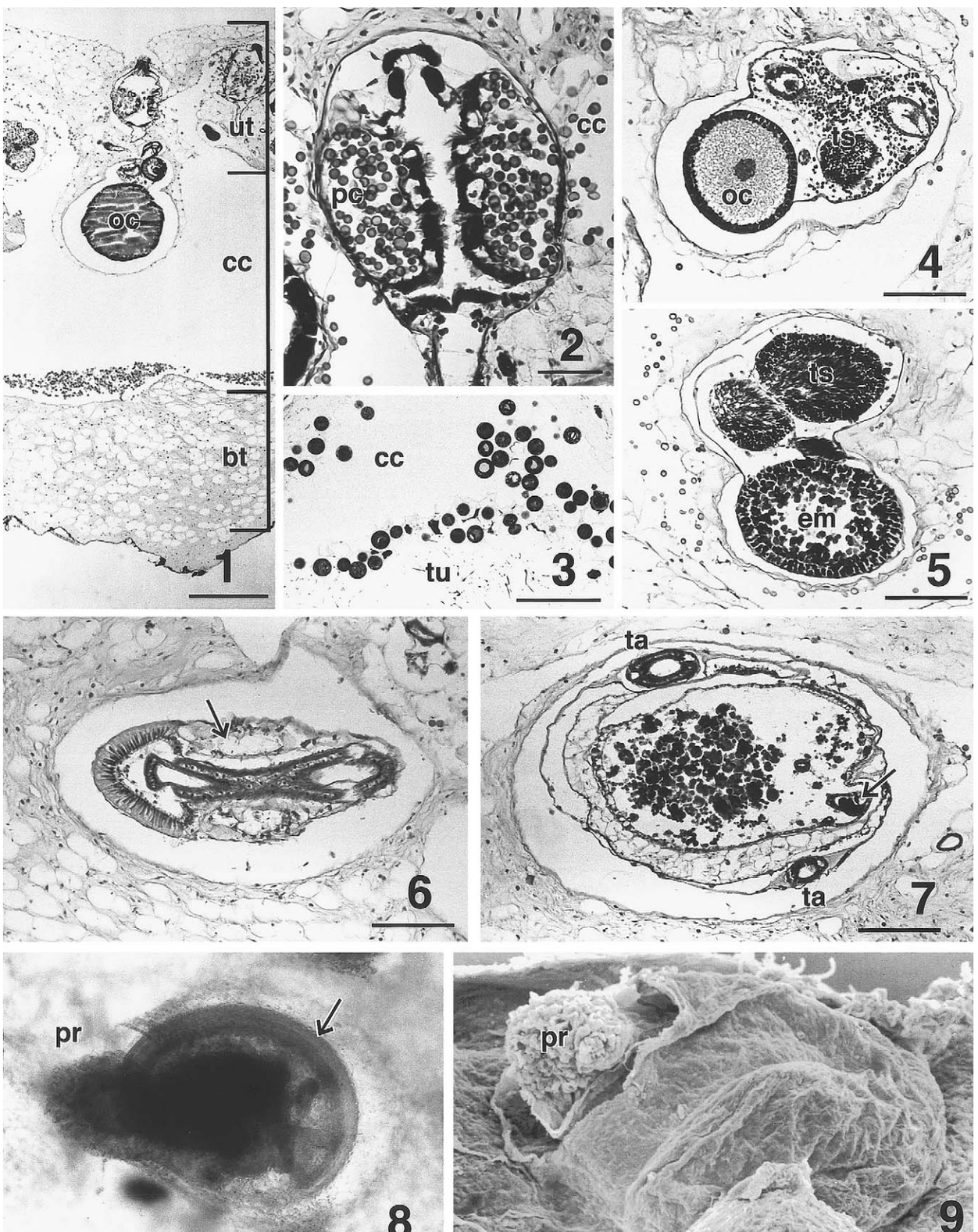

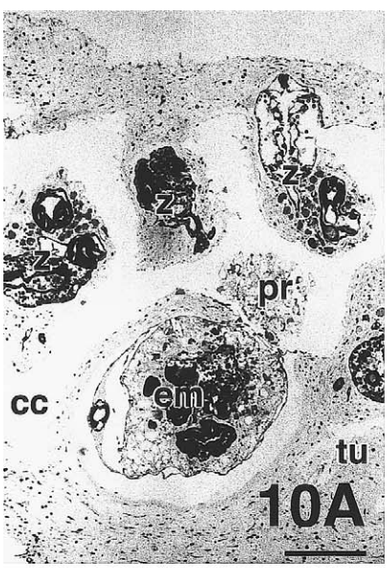

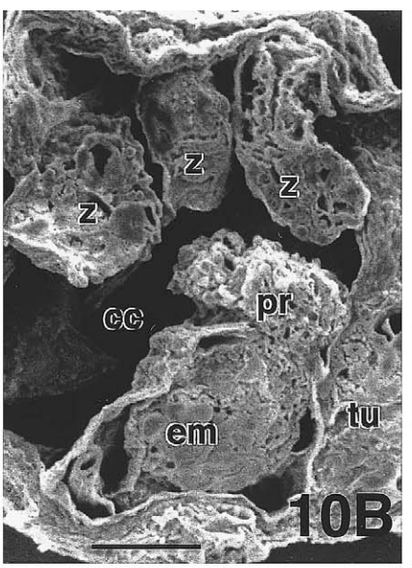

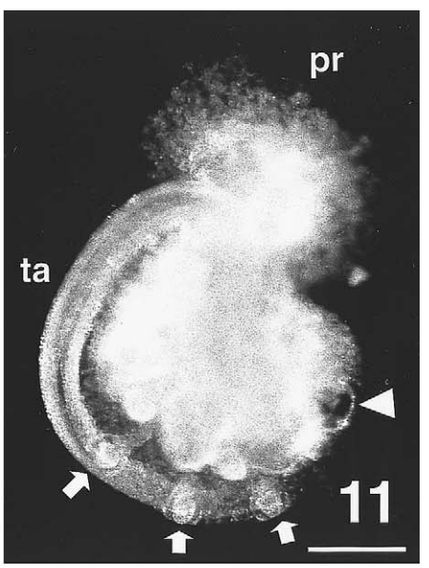

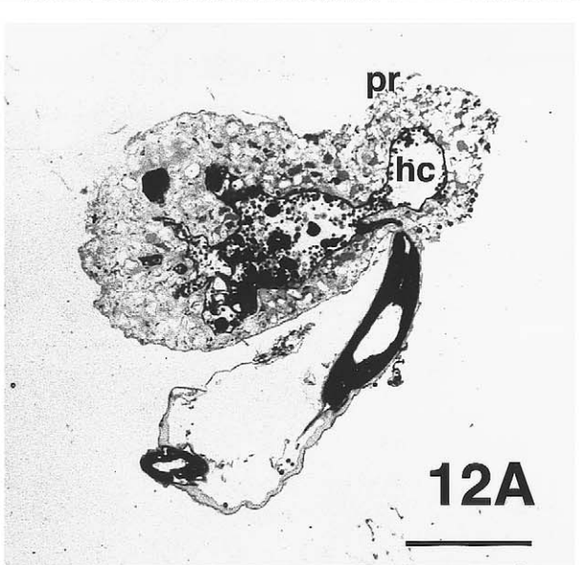

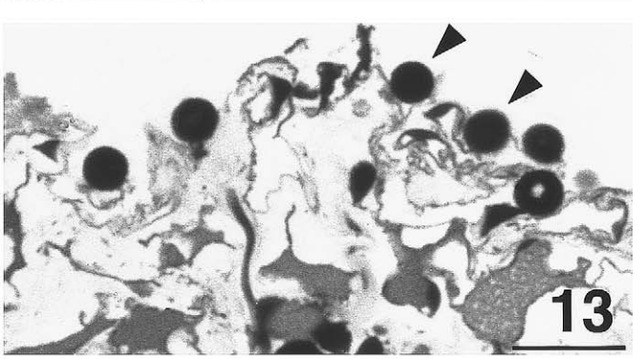

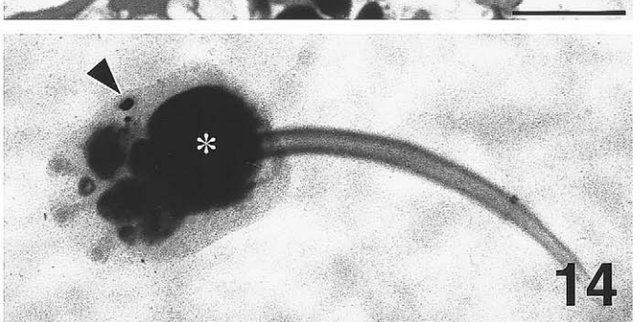

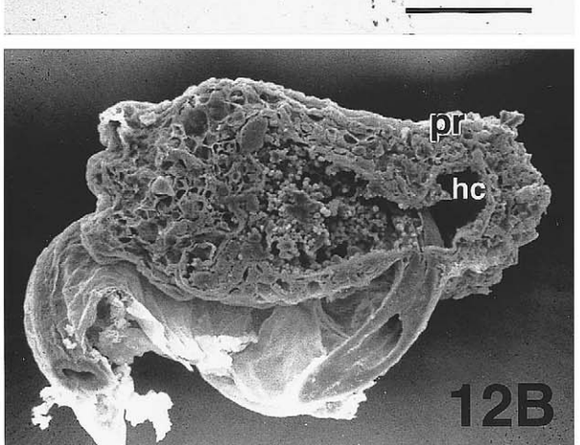

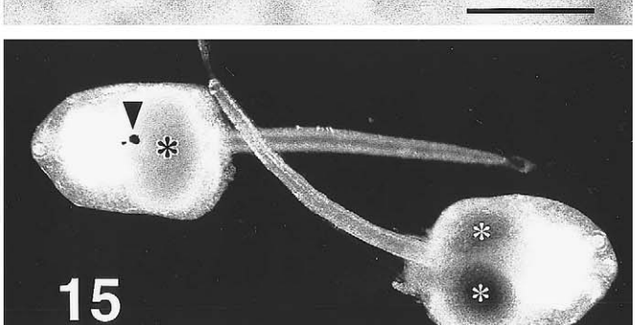

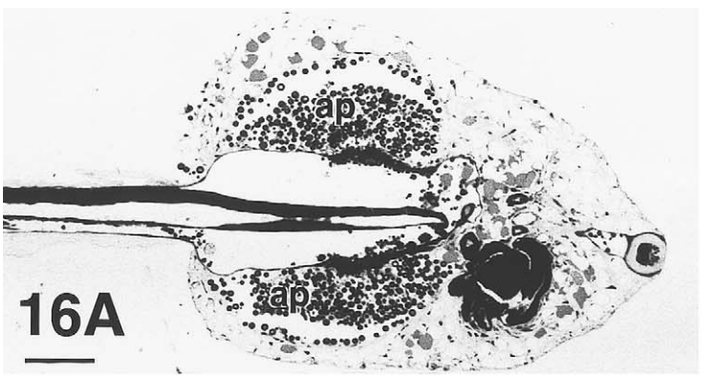

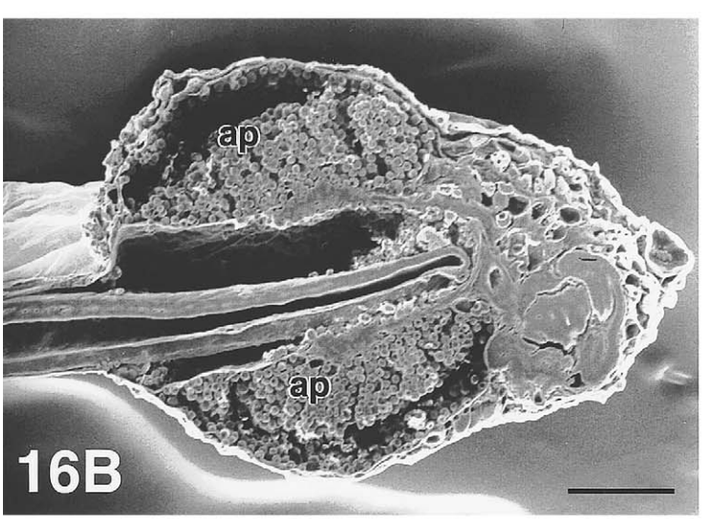

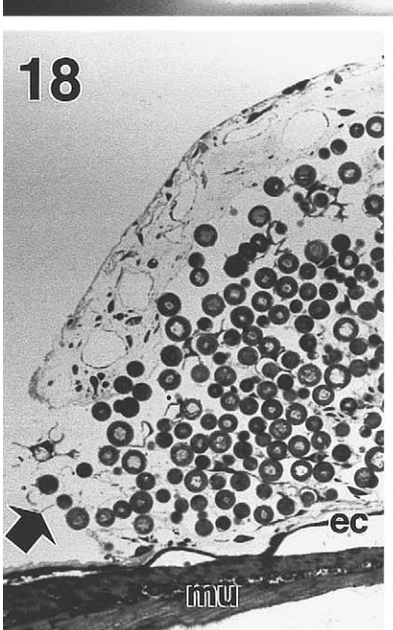

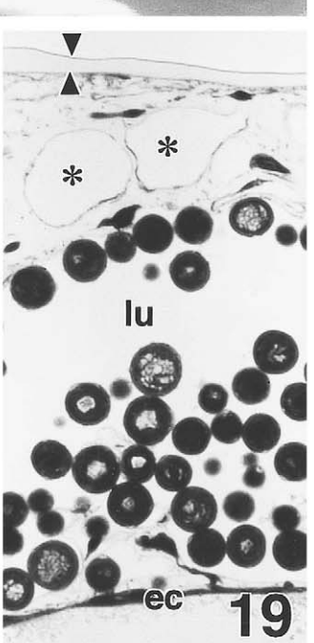

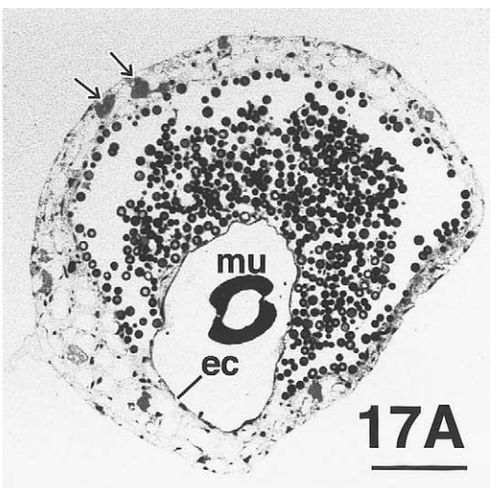

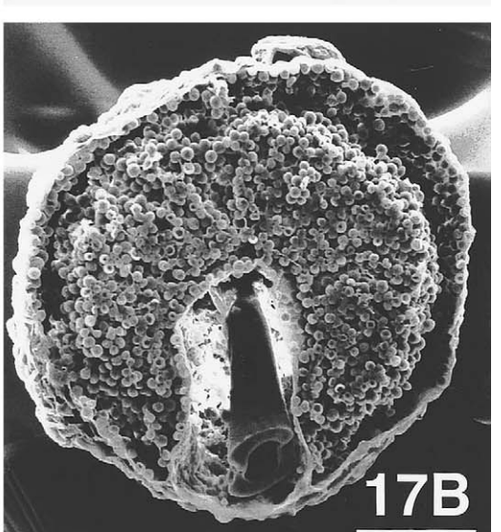

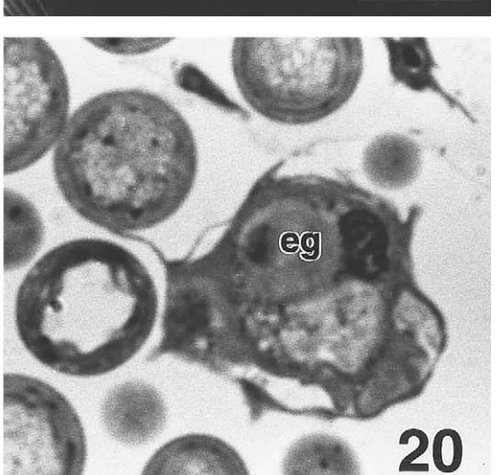

vertical transmission of symbiotic algae in this species.

MATERIALS AND METHODS

Animals

Colonies of *Diplosoma similis* are common on branches of dead coral and green algae (*Dictyosphaeria* spp.) in the vicinity of the Hawaii Institute of Marine Biology (HIMB), Coconut Island, Hawaii. The colonies are gelatinous sheets of 1–2 mm thick and uniformly green due to the symbiotic algae *Prochloron* sp. In the colonies exposed to the sunlight, the tunic contained white pigment cells, and some part of the tunic was bluish probably due to structural color. The colonies, together with the substratum, were transferred to plastic cups (100 mL) or to an outdoor tank with running seawater.

Each colony spawned a number of larvae during the day. The spawned larvae were either immediately fixed or were transferred to plastic dishes filled with seawater so that they could metamorphose and settle.

Immature larvae were dissected from colonies which had been fixed with glutaraldehyde and preserved in 50% ethanol.

Microscopy

Unfixed colonies were hand-sliced with razor blades into 1–0.5 mm thick vertical sections. Unfixed larvae and these colony sections were mounted with seawater and observed under a light microscope. Prior to the observation, some colonies and larvae were vitally stained with 0.01% neutral red diluted with seawater to distinguish the acidic components.

Colonies and larvae were fixed in 2.5% glutaraldehyde containing 0.45 M sucrose and 0.1 M sodium cacodylate (pH 7.4); additional colonies were fixed in 10% formalin-seawater. Some of the fixed colonies were dehydrated through a butanol series and embedded in paraffin. Six μm thick sections were prepared and stained with Delafield's hematoxylin and eosin. The specimens fixed with glutaraldehyde were rinsed with 0.45 M sucrose-0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide-0.1 M sodium cacodylate. They were dehydrated through an ethanol series, and embedded in low-viscosity epoxy resin. Some larvae were embedded in styrene resin. The sections of 1 μm thick were stained with 1% toluidine blue for light microscopy. For scanning electron microscopy (SEM), the dehydrated specimens were immersed in hexamethyldisilazane (HMDS; 30 min, 2 times). They were air-dried and sputter coated with gold-palladium, and examined in a Hitachi S-570 scanning electron microscope at 10–20 kV. To examine the internal structures in SEM, the specimens embedded in styrene resin were sectioned until the desired structures were exposed, and then the resin was removed from the specimens in acetone (1 hr, 2 times). Then the specimens were immersed in HMDS and processed as mentioned above.

Area measurement in the sections

The density of *Prochloron* cell was measured in the tissues of colonies, larvae and metamorphosing larvae. A rectangular area was randomly selected in each of six independent sections (1 μm thick) of epoxy embedded specimens. The proportion of the areas occupied by PrCs or cavities in the selected area was measured by digital images on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

Colony and Embryos

In *Diplosoma similis*, each zooid was separately embedded in a tunic and shares a cloacal cavity with adjacent zoo-

Fig. 1. Cross section of the colony of *D. similis* (paraffin section stained with hematoxylin and eosin). The colony consists of three layers; upper tunic (ut), cloacal cavity (cc), and basal tunic (bt). oc, oocyte. Scale bar=250 μm.
Fig. 2. An enlargement of the pharynx of the zooid (paraffin section). PrCs are distributed in peribranchial cavity (pc) and cloacal cavity (cc). Scale bar=50 μm.
Fig. 3. An enlargement of the cloacal cavity (cc) and the cavity wall of the tunic (tu) (resin section). Many PrCs adhere to the complicated surface of the cavity wall. Scale bar=50 μm.
Fig. 4. An enlargement of the gonad containing an oocyte (oc) and testis (ts) (paraffin section). Scale bar=100 μm.
Fig. 5. A blastula embryo (em) and testis (ts) in the gonad (paraffin section). Scale bar=100 μm.
Fig. 6. A tail-bud embryo in the basal tunic (paraffin section). Bladder tunic cells (arrowed) are distributed in the tunic of the embryo. Scale bar=100 μm.
Fig. 7. A tailed embryo in the basal tunic (paraffin section). Arrow, pigment granule of the sensory vesicle; ta, cross section of the tail. Scale bar=100 μm.
Fig. 8. An immature larva in the basal tunic protrudes a plant rake (pr) into the cloacal cavity. Arrow indicates the tail encircling the larval trunk. Scale bar=0.5 mm.
Fig. 9. A plant rake (pr) sticking out from the opening of the tunic wall encasing the immature larva (SEM). Scale bar=200 μm.
Fig. 10. A pair image of resin section (A) and SEM (B) of the cross section of the colony containing an immature larva (em) with a plant rake (pr). cc, cloacal cavity; tu, tunic; z, zooid. Scale bar=200 μm.
Fig. 11. An immature larva dug out from the basal tunic. Arrows, adhesive apparatus; arrowhead, sensory vesicle; pr, plant rake; ta, tail. Scale bar=200 μm.
Fig. 12. A pair image of resin section (A) and SEM (B) of semi-frontal section of the immature larva with a plant rake (pr). hc, hemocoelic chamber. Scale bar=200 μm.
Fig. 13. An enlargement of the part of the plant rake. PrCs (arrowheads) adhere on the complicated surface of the plant rake. Scale bar=20 μm
Figs. 14–15. The spawned larvae. Lateral (Fig. 14), dorsal (upper larva in Fig 15), and ventral (lower larva in Fig 15) view of the spawned larvae. Arrowhead, granules of sensory vesicle; asterisk, algal pouch. Scale bar=200 μm.
Fig. 16. A pair image of resin section (A) and SEM (B) of the semi-frontal section of the spawned larva. ap, algal pouch. Scale bar=100 μm.
Fig. 17. A pair image of resin section (A) and SEM (B) of the cross section of the posterior part of the larval trunk. Vacuoles of some bladder tunic cells are stained with toluidine blue (arrows). ec, tail ectoderm; mu, tail muscle. Scale bar=100 μm.
Fig. 18. Postero-dorsal part of the algal pouch (resin section). Arrow indicates the opening of the pouch. ec, tail ectoderm; mu, tail muscle. Scale bar=50 μm.
Fig. 19. Enlargement of the posterior part of the algal pouch (resin section). Asterisks, the vacuoles of the bladder tunic cells; arrowheads, larval tunic layer; ec, tail ectoderm; lu, lumen of the algal pouch. Scale bar=20 μm.
Fig. 20. A phagocytes in the algal pouch (resin section). The phagocyte is engulfing some PrCs (eg). Scale bar=10 μm.

ids. In the vertical cross section, the colony was divided into three layers; upper tunic, cloacal cavity, and basal tunic (Fig. 1). In the tunic, there were many cells with large vacuoles. The vacuoles were stained with neutral red, suggesting they contain acidic fluid. The color of pH test paper indicated that the pH of exudative fluid from the squashed colony was about 1.4. Each zooid was separately embedded in the upper tunic and a gonad was found at the posterior end of the zooid.

PrCs were found in peribranchial and cloacal cavities of the colonies (Fig. 2), but not in the other parts of the colonies or on the colony surface. The PrCs were not uniformly distributed in the cavities; many PrCs could be seen adhering to the wall in this area, and some were almost embedded in the tunic (Fig. 3). Algae were not observed in the gonad (Fig. 4). It seems that early embryogenesis proceeds in each zooid (Fig. 5), and then, the embryos shift out of the zooid to the basal tunic and start to be brooded there. The tunic wall separates the embryos from the cloacal cavity in which many PrCs were distributed. Therefore, no PrCs were associated with the embryos (Figs. 6, 7).

Prior to the spawning, a tassel-like structure protruded from the postero-dorsal part of the trunk of the immature larvae and it was extended into the cloacal cavity of the parent colonies (Figs. 8–10). This structure has been described as a *rastrum* or a plant rake in several *Diplosoma* species (Kott, 1980, 1982). The PrCs in the cloacal cavity adhered to the plant rake. At this stage, the trunk and the plant rake were about 0.5 mm and 0.3 mm long, respectively (Fig. 11). The plant rake was mainly composed of the tunic in which many tunic cells were distributed, and the postero-dorsal part of the posterior hemocoelic chamber protruded into the plant rake (Fig. 12). The surface of the plant rake was folded and held PrCs (Fig. 13).

When the larvae get out of the tunic and move into the cloacal cavity, the plant rakes seem to be taken into the larval trunk. The larval trunk gets longer during this process, and the tunic of the larval trunk evidently grows posteriorly and forms a pouch that envelops the plant rake. The pouch, named the algal pouch (Eldredge, 1967), surrounded the basal part of the tail and was packed with PrCs.

Larva

The larvae that have just spawned were about 0.8 mm long excluding the tails. The algal pouch occupied the posterior half of the trunk and it looked green due to large number of PrCs it contained (Fig. 14). The algal pouch was a cavity that almost encircles the basal part of the tail but not the ventral side (Fig. 15). In the algal pouch, the ectoderm of the tail swelled (Fig. 16). In cross section, the algal pouch was horseshoe-shaped (Fig. 17). The ventral half of the algal pouch was partitioned into right and left chambers by the tunic and the ectoderm of the tail. The algal pouch had an opening at the postero-dorsal end of the trunk (arrowed in Fig. 18). The wall of the pouch consisted of tunic, and it was not lined with epidermis. The inner surface of the pouch wall had a complicated surface like that of cloacal wall in the colony. Although some of the PrCs were partly embedded in the wall, the PrCs never invaded the tunic through the tunic cuticle. Many vacuolated tunic cells were distributed in the tunic of the larval trunk (Figs. 16–19). This type of tunic cells has been described as “bladder (tunic) cells” in some ascidians, and some of them are known to contain acidic fluid (cf. Stoecker, 1980). In *D. similis*, the vacuoles of these cells were vitally stained with neutral red, and thus, they evidently contain acidic fluid. In the posterior part of the wall of the algal pouch, there was a thin tunic layer that overlays the tunic of the larval trunk (arrowheads in Fig. 19). This outer tunic layer was a larval tunic that will be thrown off during the metamorphosis. In addition to the bladder tunic cells, amoeboid-shaped tunic cells were distributed in the tunic and some of them migrated from the tunic into the cavity of the algal pouch. They often engulfed the PrCs and disintegrated them in their phagosomes (Fig. 20).

Larvae initially attached themselves to the substratum with the adhesive apparatus, and the epidermal ampullae subsequently extend and spread to hold the substratum. The tail was withdrawn and the algal pouch was expanded to become the cloacal cavity of the young colony. The opening of the algal pouch turned into a cloacal aperture.

Cell density of the *Prochloron* sp.

Cell density of *Prochloron* sp. in the algal pouch of the larva appeared to be higher than that in cloacal cavity in the colonies (Figs. 3, 18). The comparison of the amount of PrCs per unit area in the photomicrographs clearly demonstrated this tendency (Table 1); the *Prochloron* density in the algal pouch was more than 2.5 times higher than that in the cloacal cavity of the colonies. In the metamorphosing larvae, because the algal pouch increases in volume as it transforms into the cloacal cavity, the density of PrCs was intermediate between the densities of PrCs in the larvae and in the colonies.

Table 1. Area occupation of PrCs in histological sections of algal pouch or cloacal cavity.

specimens	Average (%)	SD	examined area of the cavity ($\times 10^4$ μm^2)
larva (algal pouch)	59	5.7	6.5
metamorphosing larva	40	5.0	4.4
colony (cloacal cavity)	22	8.0	6.4

DISCUSSION

In *Diplosoma similis*, the larvae seem to collect the photosynthetic symbionts from the cloacal cavity of the parent colony using a special organ, the plant rake. No algal cells were observed in eggs and early embryos. Embryogenesis initially proceeds in the gonad and then in the basal tunic. In this species, fertilization probably occurs in the gonad and the embryos subsequently move into the tunic, as reported in *Diplosoma listerianum* (Burighel *et al*., 1987; Burighel and Martinucci, 1994). The embryos are separated from PrCs by the tunic, until the immature larvae extend the plant rake (or

rastrum) into the cloacal cavity through an opening of the tunic wall. The plant rake is composed of larval tunic and the posterior part of the hemocoelic chamber protrudes into the plant rake. The surface of the plant rake is highly folded and PrCs in the cloacal cavity appear to be caught on the surface of the plant rake (Fig. 13). Although Kott (1981) suggested that the PrCs become entangled with the plant rake that brush them off the cloacal wall as the larvae are released from the tunic into the cloacal cavity, the plant rake has already been replaced by the algal pouch in the larvae in the cloacal cavity. Therefore, the many of the PrCs may be collected in earlier stage when the plant rake extended through an opening of the tunic wall (Figs. 8–10).

When the larvae hatch out of the brooding chamber in the basal tunic, the plant rake has disappeared and the symbionts are contained in the larva's algal pouch which occupies the posterior half of the larval trunk. Because the total length of the trunk and plant rake of the immature larvae is almost the same as the length of the trunk of the spawned larvae, the tunic of the larval trunk evidently overgrows the plant rake and forms the algal pouch. The bulge of the hemocoelic chamber associated with the plant rake is not found in the spawned larvae; this bulge is probably retracted during the algal pouch formation. Kott (1980) described that the plant rake is pulled into the presumptive cloacal cavity as the tail is withdrawn into the hemocoelic chamber in *Diplosoma virens*. However, because I did not observe the plant rake in spawned larvae whose tails are not withdrawn at all, I concluded that algal pouch formation occurs within the parent colony in *D. similis*.

The plant rake and the algal pouch are supposed to be a special adaptation for collecting and holding a large amount of PrCs. To date, these organs have been found only in the species of the genus *Diplosoma* (Kott, 1980). In other *Prochloron* bearing didemnids, the larvae usually hold PrCs by attaching them on the surface of the trunk tunic. In *D. similis*, the PrCs are densely packed in the algal pouch, and the density is much higher (>2.5 times) than that in the cloacal cavity of the colonies (Table 1). This high density of the PrCs in the algal pouch can not be explained by a simple diffusion of the PrCs into the algal pouch and suggests that the plant rake both collects and concentrates the PrCs. Since the algal pouch (or presumptive cloacal cavity) expands its volume during metamorphosis, the density of the PrCs gradually decreases. The *Prochloron* density in the young colony would be same as or slightly higher than that of the parent colony. The young colony, therefore, has sufficient amount of the PrCs as compared to its parent colony, and the colony can sufficiently receive (nutritional?) benefit from the symbionts just after the metamorphosis.

The larvae should be large enough to carry sufficient amount of PrCs, but large larvae would be easily found and preyed on by sight feeders, such as fishes. For instance, Olson and McPherson (1987) reported that 87% of the spawned larvae were consumed by fish predation in a *Prochloron* bearing didemnid *Lissoclinum patella* (Gottschaldt, 1898). Whereas the larvae of *D. similis* may also suffer intensive predation, the larval trunk is armed with the bladder tunic cells that are abundantly distributed throughout the tunic of the larval trunk. The vital staining with neutral red revealed that the cells contain acidic fluid in their vacuoles and these would presumably lessen the risk of predation. In *D. similis*, even the tail-bud stage embryo possesses a thick tunic in which bladder tunic cells are distributed (Fig. 6).

Although all PrCs are distributed outside the tunic in *D. similis*, some of the PrCs in the algal pouch are endocytized by free amoeboid cells that may go out of the tunic (Fig. 20). Whereas endocytosis of PrCs by the host cells has been reported in the peribranchial cavity or in the branchial basket of adult *Lissoclinum voeltskowi* (Michaelsen, 1920) (Cox, 1983) and in the tunic of adult *L. punctatum* (Hirose *et al*., 1996), this is the first report of the intracellular distribution of PrCs in larvae. Because some of the engulfed PrCs are degenerative, they will be digested subsequently as suggested for *L. voeltskowi*. By contrast, the PrCs engulfed by the tunic cells appears to be healthy in *L. punctatum*, and, thus, intracellular symbiosis may be stably established as "tunic phycocytes" in this species. In *D. similis*, the amoeboid cells carrying PrCs are unlikely to change into tunic phycocytes or some intracellular symbiosis systems, since I could not find intracellular distribution of PrCs in the parent colonies.

The present study revealed the process of vertical transmission of the symbiotic algae in *D. similis*. The larvae pay costs to develop special structures, the plant rake and algal pouch, for collecting and holding a large amount of PrCs. This suggests that the PrCs provide great benefit for the host, and they are regarded as obligate symbionts of the *Prochloron* bearing didemnids.

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