

Culture studies of *Dictyosphaeria* (Chlorophyceae, Siphonocladales)

I. Life history and morphogenesis of *Dictyosphaeria cavernosa*^{1,2)}

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The life history of the marine green alga *Dictyosphaeria cavernosa* (FORSSK.) BOERGESEN from the southwestern part of Japan was studied by laboratory culture experiments and field observation. Plants from the field kept in sterilized sea water produced reproductive swarms after about one month in culture under the following conditions: 23°-25°C, 1000-2500 lux, and a 14-16hr L/10-8hr D cycle.

Three kinds of plants were collected in the field; those producing quadriflagellate zoospores, those producing biflagellate male gametes, and those producing biflagellate female gametes. No pronounced morphological differences were detected among these plants. However, two types of growth form could be distinguished; a smooth form and a rough form. The former consisted of uniformly sized cells which were smaller than those of the rough form, and the surface of the plant body was rather smooth. This type of plant always produced biflagellate male or female gametes. The rough form consisted of various sizes of cells, and its surface was rather rough. This type produced quadriflagellate zoospores or biflagellate male or female gametes. Both male and female gametes germinated parthenogenetically and developed into the smooth type of plant. Zoospores and zygotes developed into the rough type of plant. Germlings derived from male parthenogametes produced male gametes; those from female parthenogametes produced female gametes. Germlings from zoospores produced male or female gametes, while those from zygotes produced zoospores. The alga studied has an alternation of isomorphic generations. Cell division of germlings was 'segregative' and 'juvenile reproduction' was observed in some young unicellular germlings.

Key Index Words: Chlorophyceae; *Dictyosphaeria cavernosa*; life history; morphogenesis; reproduction; Siphonocladales; Valoniaceae.

A number of morphological and systematic studies of the genus *Dictyosphaeria* (DECAISNE 1842; HARVEY 1858; AGARDH 1887; ASKENASY 1888; MURRAY 1892; HEYDRICH 1892; CROSBY 1903; WEBER VAN BOSSE

1905; BOERGESEN 1912, 1913, 1940, 1952; ARNOLDI 1913; YAMADA 1925, 1934; SETCHELL 1926; NASR 1944; EGEROD 1952; VALET 1966) have been conducted, but very little has been published regarding its reproduction and life history. BOERGESEN (1912, 1913) observed 'zoosporangia' in *D. van Bosseae*, and ARNOLDI (1913) presented figures of zoospores and their formation taken from fixed specimens of *D. cavernosa* and *D. versluysii*. However, it is impossible to determine whether they were truly zoosporangia and zoospores since the authors did not observe living material. OLTMANN

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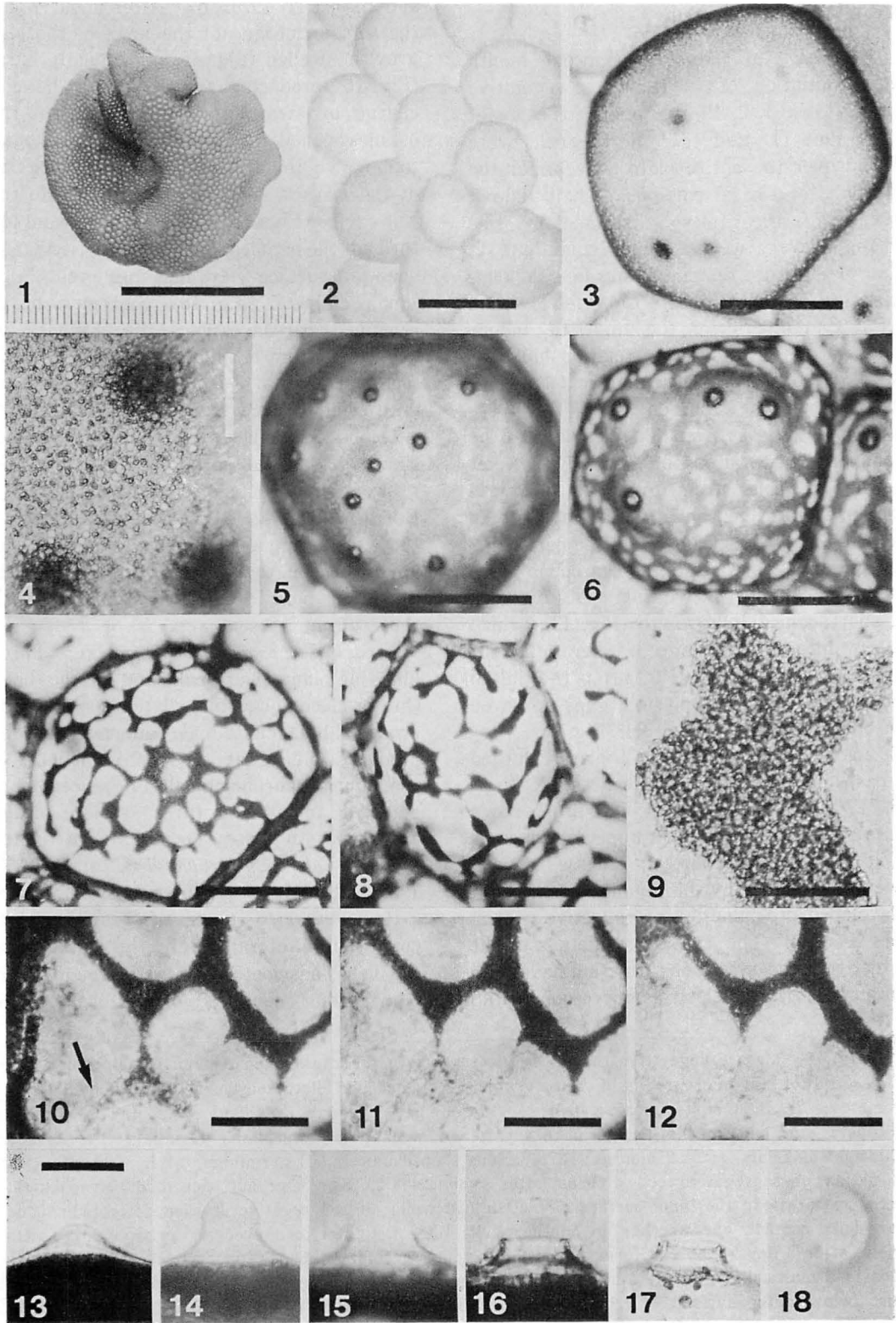
(1922), PRINTZ (1927) and FRITSCH (1935) referred to the reproduction of the genus without providing detailed information. Although EGEROD (1952) noted that both biflagellate and quadriflagellate swimmers had been reported, she mentioned neither the source of the information nor the characteristics of the swimmers. ENOMOTO and HIROSE (1972b) reported the formation of quadriflagellate swimmers in *D. cavernosa*. HORI and ENOMOTO (1978a, b) carried out electron microscope observations on cytoplasmic cleavage and nuclear division during swimmer formation in the same species. However, the complete sequence of development and life history have not been previously recorded for any species of *Dictyosphaeria*. Our research concerns the life history and development of three species of *Dictyosphaeria* in laboratory culture. The present paper presents the reproduction, morphogenesis and life history of *Dictyosphaeria cavernosa*.

Materials and Methods

Thalli of *Dictyosphaeria cavernosa* were collected from the intertidal zone at Ushuku, Ayamaru-misaki (Amami Island), Ooyama, Komesu and Gushichan (Okinawa Island), in the southwestern part of Japan. The collections were carried out from March to June of 1976-1980. Material was kept at 15°C and brought to the laboratory. After being rinsed with filtered and autoclaved sea water, each plant was placed in a separate glass vessel containing 100-150 ml of sterilized sea water. No nutrients were added. The medium was changed every 3-5 days. The plants were kept under four different conditions: (1) 16: 8 hr, 25°C; (2) 16: 8 hr, 30°C; (3) 14: 10 hr, 23°C; and (4)

14: 10 hr, 18°C. All culture vessels were exposed to a light intensity of 1000-2500 lux. Swimmers were discharged about one month after initiation of cultures. A quantity of swimmers was obtained using their positive phototactic response. After washing by micropipette, zoospores were transferred to glass tubes containing 15 ml of Provasoli's ES medium (prepared according to MCLACHLAN 1973). The tubes were kept under the dark condition for 1-3hr, while the zoospores scattered evenly and adhered to the inner surface of the tubes. The sex of the gametes was determined by crossing tests. Gametes were then washed and mixed in a watch glass. After 10 min, the resulting zygotes were transferred to glass blocks (20×20×2 mm) and were placed under dark condition for 30-60 min. The zygotes attached themselves to the substratum within 15-30 min, whereas unconjugated gametes continued swimming. The blocks were then rinsed with running sea water to remove the unconjugated gametes. The blocks with zygotes, which were detected by the presence of two stigmata, were transferred to glass vessels (60×90 mm) containing 150 ml of the same medium. Male and female gametes were separately transferred into tubes for the study of parthenogenesis. After the germlings grew to 0.3-0.5 mm, they were isolated with a pipette and placed in the tubes. When they reached 2-3 mm in size, each was transferred to a separate glass vessel containing 150 ml of medium. The culture of germlings was carried out under conditions (1) and (3). The medium was changed every 2 weeks. Cultures were not axenic, but they were strictly unialgal. For light microscopy, swimmers were fixed in 4% sea water glutaraldehyde.

Fig. 1. Mature vegetative thallus of *Dictyosphaeria cavernosa* from Okinawa Island, smooth form. Figs. 2-18. Light micrographs of *D. cavernosa*. 2. Vegetative cells; 3. Dark spots in fertile cell; 4. Dark spots; 5. Cytoplasmic gaps in fertile cell; 6. Enlarging of cytoplasmic gaps; 7. Cytoplasmic network; 8. Irregularly shaped protoplasmic masses; 9. Swimmers in a mass; 10-12. Swimmers (arrow) being liberated from a mass; 13. A short conical outgrowth of the cell wall; 14. Liberation tube; 15. Swollen apex of a liberation tube; 16. Burst apex; 17. Open ostiole for swimmer liberation, side view; 18. An ostiole, surface view. Scale: (Fig. 1)=20 mm, (Fig. 2)=1 mm, (Figs. 3, 5-8)=500 μm, (Fig. 4)=100 μm, (Figs. 9, 13-18)=50 μm, (Figs. 10-12)=200 μm.



Results and Discussion

Maturation of plants: About one month after initiation of the cultures, vegetative thalli (Figs. 1, 2, 19) became fertile under conditions (1) and (3). Fertile cells were found over the entire plant, except for the rhizoidal and basal portions. Thalli did not become fertile unless the medium was changed every week. Under conditions (2) and (4), plants remained sterile. A temperature of 18°C appears to be too low and a 30°C temperature too high to induce maturation. No distinct relationship was observed between maturation in laboratory cultures and the lunar day.

Swarmer formation: The first sign of the incipient maturation of thalli was their change in color from green to yellowish green. Subsequently, several dark green spots (Fig. 3), resulting from the partial aggregation of cytoplasm, appeared in each cell (Fig. 4). Just above each spot protruded a short conical outgrowth (Fig. 13), which later differentiated into a liberation tube (Fig. 14). Forty-eight hr after the initial change in color, cytoplasmic gaps appeared in the protoplast of each cell (Fig. 5). After 52 hr, the gaps in the protoplast enlarged to assume a brownish yellow network (Figs. 6, 7). After 60 hr, the network broke down into irregularly shaped protoplasmic masses (Fig. 8). After 65 hr, the masses, which were surrounded by thin hyaline membranes, developed into numerous reproductive swarmers (Fig. 9). The cytological details of the process of the swarmer formation have been observed with the electron microscope (HORI

and ENOMOTO 1978a, b). After about 72 hr, the thin membrane of the apex of the outgrowth swelled (Fig. 15) and finally burst (Fig. 16), producing an ostiole for the discharge of swarmers (Fig. 17). When the ostioles opened, the swarmers suddenly began to move vigorously in the peripheral regions of the masses and were liberated into the cell cavity (Figs. 10-12), and then swam out through the ostioles, which were circular in shape in surface view and measured 25-30 μm in diameter (Fig. 18). Swarmers never remained near the ostioles. The discharge of swarmers from a single reproductive organ took place within 3-5 min and that from an entire plant lasted from 1-3 hr. Discharge was observed only during the light period. Except for basal and rhizoidal cells, all cells of a plant became fertile simultaneously. BOERGESEN (1912, 1913) showed figures of the zoosporangia in *D. van Bosseae*. The appearance of the network and shape of the liberation pore are nearly identical with those of the present alga.

Kinds of reproductive swarmers: Three kinds of plants were collected in the field. One produced quadriflagellate zoospores, one produced biflagellate male gametes, and one produced biflagellate female gametes. No pronounced morphological differences were detected among the three. These results are similar to those reported for a related species, *Valonia macrophysa* (CHIHARA 1953, 1959), which is also known to be dioecious.

However, two types of growth forms could be differentiated in the plants from the field; a smooth form and a rough form. The former consisted of uniformly sized

Fig. 19. Mature vegetative thallus of *Dictyosphaeria cavernosa* from Amami Island, rough form. Figs. 20-34. Light micrographs of *D. cavernosa*. 20. Quadriflagellate zoospores; 21. Biflagellate male gametes; 22. Biflagellate female gametes; 23. Conjugated gametes; 24. Settled zygote; 25. Spherical zoospore germling forming a germ tube, after 4 weeks; 26. Unicellular germling with secondary rhizoidal filaments, after 2 months; 27. Various shaped unicellular germlings, after 3 months; 28. Initiation of segregative cell division, after 3 months; 29. 8 hr after initiation of division, daughter cells scatter along the inner surface of mother cell wall; 30. Segregative division, after 15 hr, daughter cells contact one another in mother cell; 31. Daughter cells become polygonal in surface view, after 3 days; 32. Pyriform multicellular young germling; 33. Secondary segregative cell division in a multicellular plant, 4 months after germination; 34. Daughter cells distributed peripherally just below outer surface of cell wall. Scale: (Fig. 19)=10 mm, (Figs. 20-23)=20 μm , (Fig. 24)=10 μm , (Fig. 25)=100 μm , (Figs. 26, 28-34)=1 mm, (Fig. 27)=3 mm.

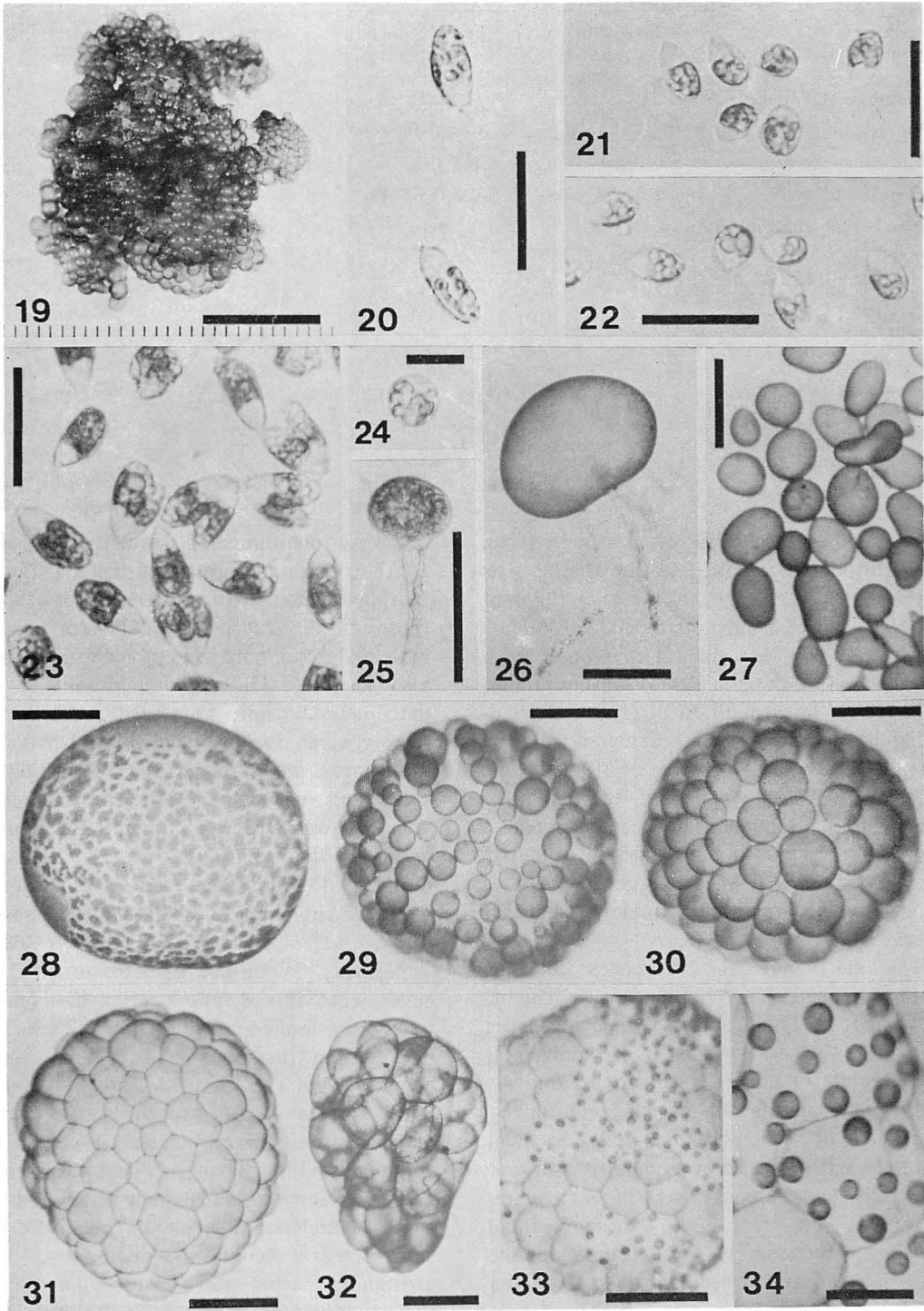


Table 1. Comparison of characteristics of reproductive swarmers.

Swarmer characteristic	Male gamete	Female gamete	Zoospore	Planozygote
Number of flagella	2	2	4	4
Length of flagella	12.0-14.0-16.0 μ m	12.0-14.0-16.0 μ m	17.0-18.0-19.0 μ m	12.0-14.0-16.0 μ m
Length of body	8.0-9.0-9.5 μ m	8.0-9.0-9.5 μ m	14.5-15.5-17.0 μ m	14.0-15.0-16.0 μ m
Breadth of body	5.0-5.5-6.0 μ m	5.0-5.5-6.0 μ m	6.0-7.5-8.0 μ m	6.5-7.0-8.0 μ m
Number of chloroplast platelets	4-5	4-5	6-8	8-10
Number of stigmata	1	1	1	2
Number of posterior granules	2-3	2-3	2-3	4-6
Papillum	+	+	+	+
Phototaxis	+	+	+	?
Color of condensed suspension	yellow	yellowish-green	yellow	yellow

cells, smaller than those of the rough form, and the entire surface of the thallus was rather smooth (Fig. 1). This type of plant always produced biflagellate male or female gametes. The cell size of the rough form varied, and its surface was rather rough (Fig. 19). The rough form produced quadriflagellate zoospores or biflagellate male or female gametes.

Among 91 plants collected at random in the field, 18 individuals produced zoospores, 29 produced male gametes and 44 produced female gametes. This suggests that all three kinds of plants commonly occur in the field.

The morphological differences detected among swarmers are summarized in Tab. 1.

Zoospores: The teardrop-shaped zoospores (Figs. 20, 47) possessed a small papilla and four flagella of equal length at the anterior end. Each contained a parietal chloroplast composed of 6-8 polygonal flat discoidal platelets with 1 or no pyrenoid. It was not clear whether the basal parts of each platelet were connected. The zoospores had 2-3 conspicuous small white granules at the posterior end and a reddish yellow stigma near the lateral side. The discharged zoospores swam energetically for 1-3hr, showing a positive phototactic response.

Gametes: The gametes (Figs. 21, 22, 48,

49) were distinguished from the zoospores by their size and number of flagella. Other morphological features were identical to those of the zoospores. The response of both male and female gametes to light was also positive. The sex of male and female gametes could only be determined by cross testing, since no morphological or behavioral differences were recognized. However, a condensed suspension of the male gametes was yellow, whereas that of the female gametes was yellowish green. Gametes discharged from the same reproductive organ or thallus did not conjugate with each other.

Zygotes: When the gametes of different sexes were mixed, aggregation of gametes took place immediately. After 2-3 min, the aggregation dispersed and 5-10 min later about 60% of the gametes had conjugated. Their fusion began anteriorly, then gradually progressed laterally until the cells fused completely (Figs. 23, 50). The planozygotes had two stigmata, four flagella and 8-10 chloroplast platelets. The planozygotes did not show a clearly recognizable phototaxis. Their swimming period was shorter than that of the unconjugated gametes. ARNOLDI (1913) presented figures of swarmers labeled 'Zoosporen', which measured 8-10 μ m in length. Judging from the length of the body, how-

ever, it seems likely that they were not zoospores but gametes.

Germination and development: Zygotes and zoospores began development immediately after settling on the substratum. No period of dormancy was observed. Unfused male and female gametes developed parthenogenetically. The mode of germination, the developmental sequence and the growth rate of zoospores were fundamentally identical with those of the zygotes and the parthenogametes. After shedding their flagella they rounded up (Figs. 24, 51) and formed a cell wall within 24 hr (Fig. 52). They increased their volume and developed into spherical bodies (Figs. 53-55). After 3 weeks, each spherical body produced a germ tube (Figs. 25, 56), which developed into a primary rhizoidal filament (Figs. 57-60). The cell contents remained within the original body. During the following month, the germlings produced several secondary rhizoidal filaments (Fig. 26). Such unicellular rhizoidal filaments formed from the lowermost end of young plants were also observed by BOERGESEN (1912). After two months germlings had grown to about 2.0-2.5 mm in diameter, and had various shapes (Fig. 27).

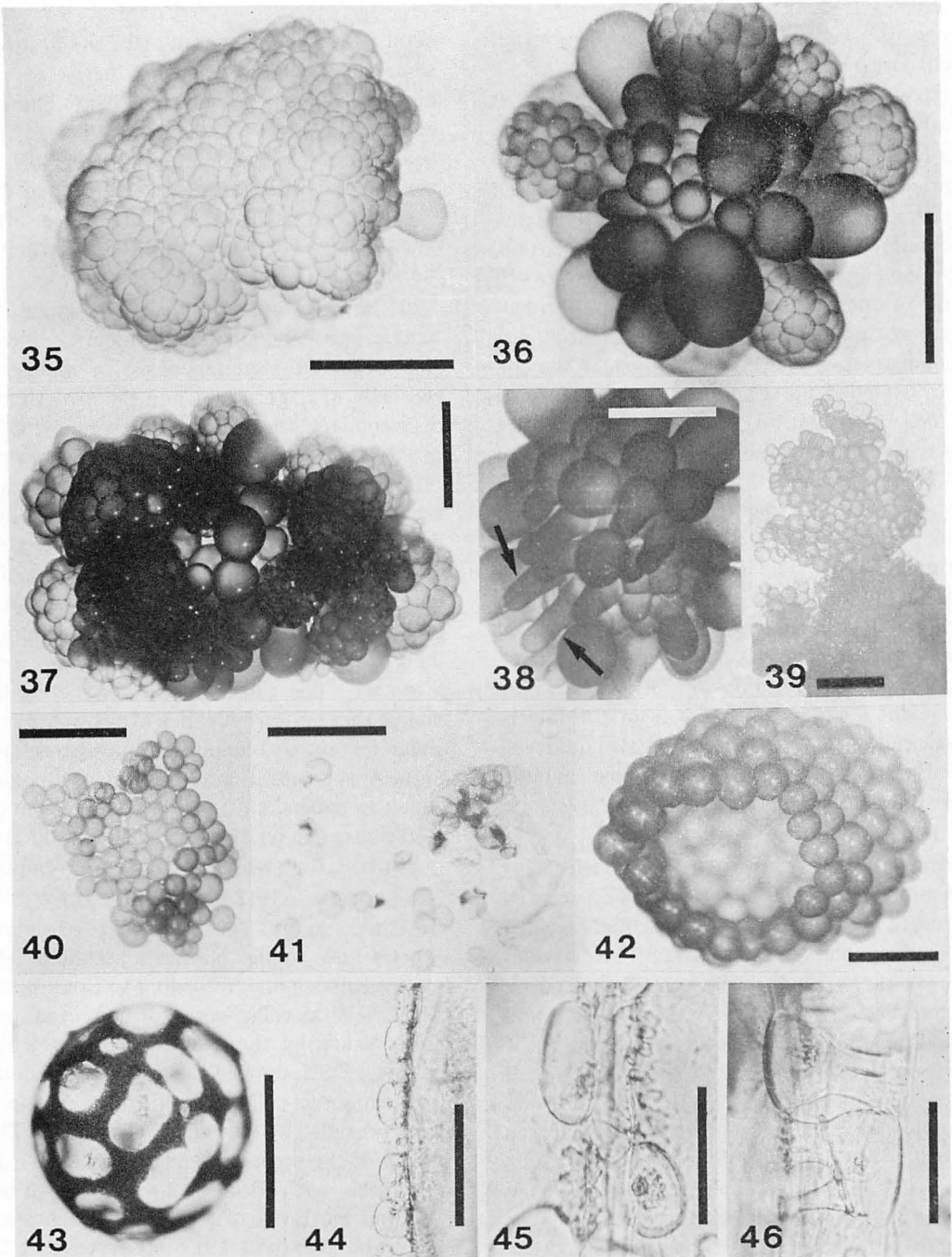
Morphogenesis: About three months after germination, the first cell division occurred in unicellular germlings which had reached 3-5 mm in diameter (Fig. 28). The cell division is of the segregative type described by BOERGESEN (1912, 1913), ARNOLDI (1913) and EGEROD (1952). The complete process of division will be reported in greater detail in a subsequent article. Briefly, division usually began at midnight (Fig. 28) and was finished by morning (Fig. 29). About 8 hr after initiation, many spherical protoplasmic bodies had been formed in a single layer along the entire inner surface of the mother cell wall (Fig. 29). They formed a cell wall and increased their volume rapidly, thus becoming daughter cells. After 15 hr, the cells came into contact with one another (Figs. 30, 32). After three days, the cells matured becoming polygonal in surface view (Fig. 31), producing a hollow, monostromatic plant body essentially identical to the adult

plant. About one month after the first division, the secondary division occurred also during the dark period (Fig. 33). The secondary daughter cells were formed just below the exposed outer surface of the mother cell wall, and were arranged in a single layer (Fig. 34). Therefore, even though they enlarged, the thalli remained monostromatic saccate structures.

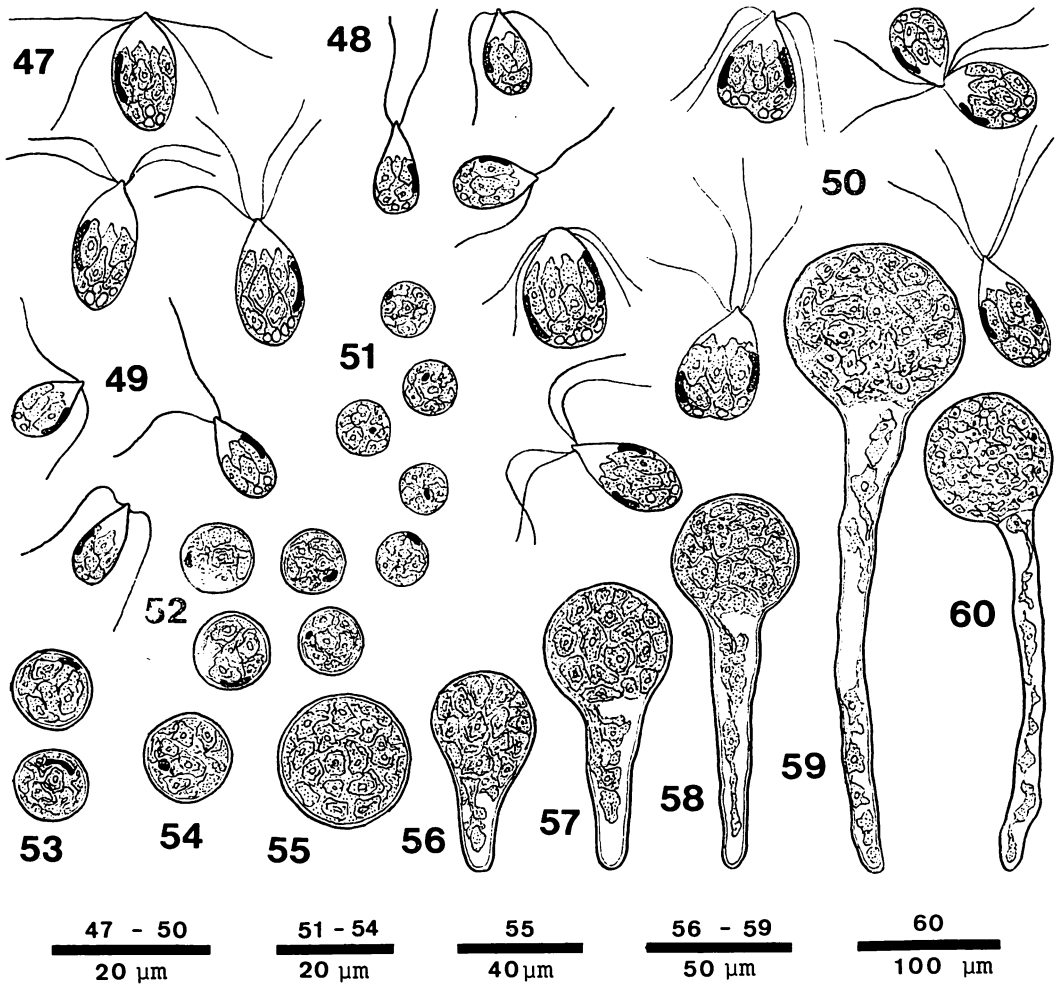
In the germlings derived from male and female parthenogametes, secondary cell division occurred simultaneously in almost all the cells of a germling and the growth rate of secondary daughter cells was synchronized. These germling, therefore, developed into the smooth form (Fig. 35). In contrast, the secondary cell division in the germlings derived from zoospores and zygotes did not take place simultaneously in all cells of a germling. It occurred only in scattered group of cells (Fig. 33). These germlings therefore developed into the rough form (Fig. 36). The causal factor for the formation of these different types of growth forms under laboratory conditions may be related to the synchronization of secondary division which is dependent, not on environmental conditions, but on the kind of reproductive swarmer from which the plant developed.

BOERGESEN (1912) and EGEROD (1952) presented figures of nonseptate primary vesicles and young plants septated by the first cell division. According to BOERGESEN (1912), several cells were formed in a primary vesicle by the first cell division, while EGEROD (1952) showed that nearly one hundred daughter cells were formed in a primary vesicle by the first division. The results of the present study (Figs. 29-31) do not agree with BOERGESEN's observation, but confirms EGEROD's report. BOERGESEN (1912) and ARNOLDI (1913) observed that 2-6 daughter cells were formed in a mother cell by the secondary division. This observation differs from the present findings, which note the formation of 7-16 daughter cells (Figs. 33, 34).

Six months after germination, the germlings had grown to about 30 mm in diameter (Fig. 37), and the basal cells of the germ-



Figs. 35-42. Cultured plants of *Dictyosphaeria cavernosa*. 35. Cultured germling, smooth form, derived from male parthenogamete, after 5 months; 36. Cultured germling from zoospore, rough form, after 5 months; 37. Cultured germling from zygote, rough form, after 6 months; 38. Basal cells of germling elongated and differentiated into rhizoidal cells (arrows); 39. Ruptured plant; 40. Isolated fragment; 41. Daughter cells artificially isolated from mother cell; 42. Cross section of thallus, showing hollow, monostromatic nature. Figs. 43-46. Light micrographs of *D. cavernosa*. 43. Unicellular germling becoming fertile; 44. Row of intercellular tenaculæ consisting of simple haptera; 45. Intercellular tenaculæ consisting of simple haptera; 46. Tenaculæ consisting of bifurcated haptera. Scale: (Figs. 35-37, 39, 41 and 42)=5 mm, (Fig. 38)=3 mm, (Fig. 40)=10 mm, (Fig. 43)=500 μ m, (Fig. 44)=100 μ m, (Figs. 45 and 46)=50 μ m.



Figs. 47-60. Line drawings of *Dictyosphaeria cavernosa*. 47. Quadriflagellate zoospores; 48. Biflagellate male gametes; 49. Biflagellate female gametes; 50. Planozygotes; 51. Settled zoospores, 18 hr after settling; 52. Settled zoospores with cell wall, after 24 hr; 53. Spherical body derived from zoospore, after 1 week; 54. Spherical body derived from zoospore, stigma still remaining, after 2 weeks; 55. Spherical body derived from zygote, after 3 weeks; 56. Germination of spherical body derived from zoospore, after 3 weeks. Figs. 57-59. Elongation of germ tube. 57. After 23 days; 58. After 25 days; 59. After 4 weeks; 60. Germling with primary rhizoidal filament, after 5 weeks.

ings had differentiated into long rhizoidal cells (Fig. 38). This agreed with the observations of BOERGENSEN (1912), ARNOLDI (1913) and EGEROD (1952). As mentioned above, the rhizoidal cells of the mature thallus of *D. cavernosa* are not derived from the primary rhizoidal filament but are formed by the elongation of basal cells. In contrast, in the allied species *Valonia macrophysa* (CHIHARA 1953, 1959), *V. ventricosa* (CHIHARA 1959), *Siphonocladus pusillus* (JÓNSSON 1957)

and *Boergesenia forbesii* (ENOMOTO and HIROSE 1972a), a settled reproductive cell immediately produces a germ tube which later develops directly into the rhizoidal cells.

Maturity of germlings: After 6-7 months, the germlings became fertile and liberated swimmers under conditions (1) and (3). Germlings from male parthenogametes produced biflagellate male gametes and those from female parthenogametes produced biflagellate

female gametes. Those from zoospores produced biflagellate male or female gametes respectively. Those from zygotes produced quadriflagellate zoospores. A few germlings derived from zoospores produced quadriflagellate zoospores. Germlings from these zoospores also produced quadriflagellate zoospores. This seems to be a form of 'accessory reproduction' by means of zoospores, as defined by IYENGAR (1951).

Although detailed cytological observations are not presented in this study, the results of culturing from generation to generation suggest that *Dictyosphaeria cavernosa* is heterothallic and that it has an alternation of isomorphic generations. As all three kinds of plants were obtained during the same season in the field and under the same conditions in culture, *D. cavernosa* seems to have no seasonal alternation of generations. This type of life history is also reported in a related species, *Valonia macrophysa* (CHIHARA 1953, 1959).

Vegetative propagation: Basal and rhizoidal cells did not become fertile. When they were removed from a plant and cultured separately in fresh medium they developed into normal monostromatic saccate plants. In laboratory culture, when adult plants were maintained in stagnant medium for a long time, they lost their organization and became convoluted monostromatic membranous plants (Fig. 39). If such a plant was separated into several fragments (Fig. 40) and cultured separately, each fragment developed into a normal new plant. This result supports EGEROD's (1952) observation that "by the addition of new segments and repeated fragmentation that thallus may continue to propagate vegetatively". When the daughter cells formed by the first division were artificially isolated from the mother cell (Fig. 41), they also developed into normal new plants. This confirms the opinion of BOERGENSEN (1912) that daughter cells "formed by the cell division, may occasionally become free and be able to grow" into new plants.

Extracellular protoplasts which were squeezed out of plants at least 3-5 mm in

diameter and placed in sea water (23°-25°C) in a watch glass produced many spherical bodies within 24 hr which also developed into normal new plants. Such a phenomenon has also been confirmed in other coenocytic green algae, such as *Bryopsis plumosa* (TATEWAKI and NAGATA 1970), *Boergesenia forbesii* (ENOMOTO and HIROSE 1972a), and *Derbesia tenuissima* (RIETEMA 1973).

Juvenile reproduction: When young unicellular germlings (1-2 mm diam.) derived from zoospores, zygotes or both sexes of parthenogametes were cultured at a high population density (500-600 germlings/28 cm²/150 ml medium), they neither grew nor divided. If these young unicellular germlings were then isolated and transferred into fresh medium, sometimes they became fertile within 5-10 days (Fig. 43). The process of maturation of unicellular germlings is essentially identical with that of multicellular normal adult plants. This seems to be an example of 'juvenile reproduction' (HIROSE 1954).

Systematic remarks on specific characters: Five morphological characteristics have been pointed out and discussed by several authors (WEBER VAN BOSSE 1905; BOERGENSEN 1912, 1913, 1940; ARNOLDI 1913; YAMADA 1925; SETCHELL 1926; EGEROD 1952; VALET 1966) as specific criteria for this genus: 1) the structure of plants (solid or hollow), 2) the presence or absence of spinulose trabeculae on the inner cell wall, 3) the shape of the spinulose trabeculae, 4) the shape of intercellular tenaculae, and 5) the cell size of mature plants. Each of these features was examined in our cultured germlings. In all cultures, multicellular germlings were always monostromatic and hollow (Fig. 42). No thalli remained solid throughout the entire developmental sequence. The hollow structure of the plant resulted from the distribution of daughter cells in the mother cell. No spinulose trabecula was observed in any germling cell. Usually, an intercellular tenaculum consisted of a simple hapteron, 35-50 μ m in diameter (Figs. 44, 45); however, bifurcated haptera (35-50 μ m in diam.) were sometimes observed (Fig. 46). The shape

of tenaculæ was not always uniform, but the dimensions of the trabeculæ were within a constant range. The cell size of mature plants varied from 600 to 4500 μm in diameter according to culture conditions, especially temperature, light intensity and density of plant population. It also varied with the kind of swarmer and with the portion of the plant. Therefore, the thallus structure, presence or absence of spinulose trabeculæ and size of intercellular tenaculæ appear to be effective specific criteria. In contrast, cell size of mature plants and the shape of haptera were not useful criteria in this study.

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榎本幸人・奥田一雄*: 緑藻キッコウグサの生活史と体形成について

奄美大島, 沖縄本島産のキッコウグサは 23°-25°C, 1000-2500 lux, 長日条件で成熟, 株を異にして 2 鞭毛雄性配偶子, 2 鞭毛雌性配偶子, 4 鞭毛遊走子を形成する。配偶子は雌雄同型。生殖細胞を形成するこの 3 種の藻体は基本的には同一構造であるが, 細部で 2 型, 即ち smooth form と rough form とが区別される。雌雄配偶子は単為発生し smooth form に発達, 配偶子を形成する。遊走子, 接合子は rough form に生長, 遊走子からの発芽体は配偶子を, 接合子からの発芽体は遊走子を形成する。本種は同型世代交代型の生活史をもつ。季節的な世代の交代はない。細胞質分裂は“segregative”である。単細胞段階の幼体で“juvenile reproduction”が観察された。(656-24 兵庫県津名郡淡路町岩屋, 神戸大学理学部臨海実験所; *051 北海道室蘭市母恋南町 1-13, 北海道大学理学部海藻研究施設)