Studies on Sexual Reproduction in the Colonial Volvocales (Chlorophyta), with Special Reference to Phylogenetic Relationships within the Order

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Introduction

The colonial Volvocales (Chlorophyta) includes three families, the Volvocaceae, the Astrephomenaceae and the Spondylomoraceae. These families are delineated by differences in the mode of colony formation and by the presence or absence of a gelatinous matrix encompassing the colonies (Bold and Wynne 1978, Starr 1980). All of them are coenobic, inhabitat fresh-water and undergo autocolony formation in asexual reproduction. Their life cycles are thought to be haplobiontic, with meiosis occurring at zygote germination (Stein 1958b, Coleman 1959, Starr 1975, Bold and Wynne 1978).

Recent advancement in the methods of unialgal cultures has contributed to our knowledge of the sexuality of the colonial Volvocales, especially in physiological and genetic, development, and in biosystematic studies (Starr 1970a, Wiese 1976, Coleman 1979, Kochert 1982). However, there remain many unresolved facets in the sexual reproduction of these algae, even at light microscope level, with particular regard to conjugation between gametes and zygote germination. Therefore, phylogenetic relationships within the colonial Volvocales have been discussed only on the basis of vegetative morphology (Crow 1918, Fritsh 1929 1935, Hoops and Floyd 1982ab 1983, Hoops 1984, Greuel and Floyd 1985).

The present series of studies were undertaken to elucidate the morphological details of sexual reproduction in the colonial

Volvocales at light microscope level, and to evaluate phylogenetic relationships within these algae based mainly on characteristics of sexual reproduction. Culture experiments performed here involve the use of new, or newly modified, methods for culturing the algae and for inducing both sexual reproduction and zygote germination. Details of the sexual reproduction of seven genera of the three families of the colonial Volvocales at light microscope level are described here, with particular attention to conjugation between gametes and zygote germination. Phylogenetic relationships within the order are outlined as demonstrated by the characteristics of sexual reproduction as well as by the other morphological features.

Materials and Methods

I. Materials and culture methods

The water or soil samples used in this study are listed in Table 1. Clones were established by the pipette-washing method (Pringsheim 1946) directly from water samples or from Petri dishes (90 X 20 mm) in which small amount of dried soil sample (ca. 0.5g) had been rewetted with distilled water. For isolating colonies of Volvulina, Astrephomene or Pyrobotrys, a boiled pea (Glycine max) was added to the Petri dish when rewetted. Cultures were grown in screw-cap tubes (15 X 180 mm, Fujimoto Rika, Tokyo) containing 12 ml of the growth medium (Table 2). Except for Pyrobotrys, these screw-cap tubes were maintained in an incubator, under controlled culture conditions (Table 2). Illumination was provided by cool-white fluorescent lamps.

For establishing Pyrobotrys cultures, an anaerobic method was used. After inoculating with the fresh growth medium, the screw-cap tube (15 X 180 mm) was capped somewhat loosely and enclosed within a double-cap tube (24 X 200 mm) (Figs. 1-3). Then the air in the screw-cap tube was exchanged for nitrogen gas using a vacuum pump and an injector through the double cap. This double-cap tube was maintained at 20°C, under 14:10 LD and 7000 lux (Table 2). One to two weeks after the inoculation of a single colony, abundant colonies of Pyrobotrys were usually detected by using a stereomicroscope. Stock cultures were

maintained at 20°C, under 14:10 LD and 1000-2000 lux, and were transferred monthly. A five-bacteriological medium, as described by Ichimura and Watanabe (1977), was used to test for the presence of bacteria in the Pyrobotrys cultures. Two weeks after inoculating the strains into this medium, which was maintained aerobically at 20°C (14:10 LD) or 35°C (darkness), they were examined microscopically. No bacterial growth was detected.

II. Induction of sexual reproduction and zygote germination

Gonium pectorale and Pandorina: Actively growing three- to six-day-old cultures were condensed to 1.5 ml and the condensed cultures of two complementary mating types were mixed in a watch glass (60 mm) supported on a glass triangle in Petri dishes (90 X 20 mm). About 5 ml of distilled water was added to the bottom of the Petri dishes to minimize water evaporation from the mixture. These Petri dishes were placed at 20°C, under 14:10 LD and 4000 lux (Table 2). To induce zygote germination, about one-week-old zygotes were pipetted into the growth medium solidified with 0.6 % agar in Petri dishes. These cultures were kept in the dark for about one month, after which the zygotes were transferred to the new liquid growth medium and placed under the usual illumination.

Gonium sociale: Sexual reproduction was induced as follows: a 4-day-old culture, which had been inoculated with 1 ml of actively growing culture, was condensed to 0.2-0.3 ml by centrifugation. Then, 1.5 ml of the mating medium (Table 2) was added to the condensed culture in a watch glass (60 mm) supported

by a glass triangle in Petri dishes (90 X 20 mm). About 5 ml of distilled water was added to the bottom of the Petri dish to minimize water evaporation from the watch glass. These Petri dishes were placed under the normal culture conditions (Table 2). To induce zygote germination, one- to two-week-old zygotes were pipetted into the growth medium lacking sodium acetate and solidified with 0.6 % agar in Petri dishes. These cultures were first put in darkness at 4°C for three months and then in darkness at 20°C for another three months, after which the zygotes were pipetted into the liquid growth medium and grown at 20°C, under 14:10 LD and 4000 lux.

Volvulina and Astrephomene: Mating reaction was induced by the methods described for Gonium pectorale and Pandorina. To induce zygote germination, two- to 10-day-old zygotes were pipetted into the growth medium solidified with 0.6 % agar in Petri dishes. These cultures were put in darkness for more than three months. Then, the zygotes were transferred to the liquid medium in a double-cap tube (18 X 150 mm), in which nitrogen gas was substituted for air using a vacuum pump and an injector. This double-cap tube was enclosed in red cellophane paper and placed under the usual illuminated culture conditions (Table 2).

Eudorina elegans var. elegans: Sexual reproduction was induced by adding 3 ml of mating medium (Table 2) to mixtures of four-day-old male and female cultures, which had been condensed from 12 ml to 0.5 by centrifugation, in a watch glass (60 mm) supported by a glass triangle in Petri dishes (90 X 20 mm). About 5 ml of distilled water was added to the bottom of the

Petri dish to minimize water evaporation from the mixture. The methods for zygote germination were the same as for Volvulina and Astrephomene.

E. elegans var. synoica: Sexual colonies developed spontaneously under the usual culture conditions (Table 2), several days after inoculating vegetative colonies into the fresh growth medium. Aplanozygotes were subsequently formed by conjugation between male and female gametes within sexual colonies. The zygotes turned reddish brown in color after about one week. These mature zygotes were pipetted into the growth medium solidified with 0.6 % agar in Petri dishes. These cultures were kept in darkness for about one month, after which the zygotes were transferred to the liquid medium and grown under the illuminated conditions (Table 2).

Volvox carteri: Sexual, male and female colonies were induced separately in a screw-cup tube: 1 ml of an actively growing culture of male or female strain and 0.5 ml of the "sexual inducer" (the fluid of male cultures in which abundant sperm packets had been formed) were inoculated into 12 ml of the new growth medium. Sexual colonies developed two or three days after the induction. Then, female colonies and mature male colonies (sperm packets) were mixed for fertilization and production of zygotes. To induce zygote germination, about one-week-old reddish brown zygotes were pipetted into the new growth medium in a watch glass (60 mm) supported by a glass triangle in Petri dishes (90 X 20 mm). About 5 ml of distilled water was added to the bottom of the Petri dishes to minimize water evaporation from the watch glass.

Pyrobotrys: Sexual reproduction was induced as follows: 1ml of an actively growing culture of P. elegans or P. casinoensis (homothallic strains) was inoculated into 12 ml of the mating medium (Table 2), in which nitrogen gas was substituted for air as described above in the culture methods for Pyrobotrys. (This exchange of the air for nitrogen gas was conducted for each of the following media after the inoculation.) Gametogenesis and fusion of gametes occurred within two to six days. For heterothallic strains of P. casinoensis, actively growing cultures (1 ml) of the two complementary mating types were inoculated into 12 ml of the mating medium, in which a small amount (1.0 g/l) of dried soil, from which the alga was isolated, was added. This culture was grown at 25°C, under 16:8 LD and 10000 lux.

For P. stellata, the two- to four-day-old culture in the mating medium was condensed to 0.2 ml by centrifugation and this pellet was inoculated into 12 ml of the fresh growth medium (Table 2) from which the two amino acids were eliminated. Sexual reproduction commenced within two days. To induce sexual reproduction of P. squarrosa, 1 ml of the four-day-old culture in the 1:1 mixture of the growth and mating media was inoculated into 12 ml of the growth medium. Gametogenesis and gametic union occurred in the early light cycle after four days.

For maturation of zygotes of the four species, 1-2 ml of the culture containing young plano- or aplanozygotes was inoculated into 12 ml of the fresh growth or mating medium. To induce zygote germination of heterothallic strains of P. casinoensis,

four-day-old zygotes, which had been inoculated into the fresh mating medium for maturation, were pipetted into 12 ml of the growth medium (Table 2) from which the two amino acids and sodium acetate were eliminated. These cultures were put in darkness aerobically at 4°C for more than three months, after which the zygotes were pipetted into the new growth medium and grown under the usual anaerobic, illuminated culture conditions (Table 2).

III. Methods of observation

Light microscopy was carried out using a Nikon LUR-Ke microscope equipped with phase optics. The extracellular, gelatinous matrix or envelope was observed by staining with methylene blue or haematoxylin. The staining methods developed by Rosowski and Hoshaw (1970) were used for detecting pyrenoids.

Observations and Discussion

I. Volvocaceae

The family Volvocaceae contains the largest number of genera among the three families of the colonial Volvocales. The colonies of all the members in this family were embedded in a gelatinous matrix. Except for Gonium and Basichlamys, the colonies are essentially radially spherical in shape and undergo inversion during colony formation and have an eight-celled embryo in which daughter cells are arranged in cruciate form (Smith 1950 1955, Bold and Wynne 1978). The genera in the Volvocaceae are delineated by differences in colonial morphology and sexual reproduction. Gametic union in this family is either isogamous, anisogamous or oogamous and the modes of sexual reproduction are heterogamous.

A. Genus Gonium Müller

The genus Gonium is characterized by having plate-like colonies and was originally established by Müller in 1773, based on a 16-celled species, Gonium pectorale. This species is most frequently encountered among the members of this genus and has a world-wide distribution. It was described under the names Pectoralia hebracia Bory (1824), Gonium helveticum Perty (1852) and Gonium helveticum (Perty) Diesing (1866) (Warming 1876, Bütschli 1884).

Pascher (1927) described a 16-celled species, Gonium formosum, based on planktonic material collected in Wisconsin, U.S.A. A 32-celled species was described by Prescott in 1942 as G. discoideum, and was collected in Louisiana, U.S.A. Pocock (1955), in her study of the genus Gonium in North America, established two new species, eight-celled G. octonarium and 16- or 32-celled G. multicoccum. In 1959, Pringsheim described G. quadratum, whose colonies have eight cells arranged cruciately. Recently, Watanabe (1977) established G. viridistellatum based on the cultured material isolated from soil collected in Okinawa Prefecture, Japan. This species is characterized by having eight-celled colonies in which the constitutive cells are arranged radiosymmetrically.

Two four-celled species of Gonium have been established. One of the two is Gonium sociale, which Dujardin (1841) originally described under the name Cryptomonas (subgen. Tetrabaena) socialis. Fromentel (1874) raised the subgenus

Tetrabaena to the genus level and proposed Tetrabaena dujardinii based on the type of Cryptomonas socialis Dujardin (1841). Warming (1876) was the first to refer this species to the genus Gonium and made a new combination, Gonium sociale (Dujardin) Warming. In 1904, Scherffel described a four-celled species, Gonium sacculiferum. However, Skuja (1956) considered this species to be separated from Gonium, based on the differences in colonial organization between G. sacculiferum Scherffel and the other species of Gonium. Therefore, he erected a new genus for this species and made a combination, Basichlamys sacculifera (Scherffel) Skuja. Some phycologists recognize this combination (e.g. Ettl 1983, Nozaki 1986) while others use the name Gonium sacculifera (e.g. Stein 1959, Bold and Wynne 1978, Starr 1978).

Gonium pectorale Müller

The general features of sexual reproduction in Gonium pectorale Müller have been observed in cultures by Schreiber (1925), Stein (1958b) and Kusumoto et al. (1978). Nevertheless, some details on the sexual reproduction of this alga seem to have been left for more careful studies. I have observed the presence and role of cytoplasmic protrusions in conjugating gametes of G. pectorale under the phase contrast microscope.

Vegetative morphology. Colonies were flattened and contained eight or 16 cells embedded in gelatinous cellular sheaths and arranged in a single layer, measuring up to 65 um in width. A 16-celled colony had 12 peripheral and four central cells, forming a slightly curved square or rhomboid (Pl.

I A). An eight-celled colony formed a group of cells arranged in four zigzag rows of two each (Pl. I B). The cells were nearly spherical or angular in shape, measuring up to 18 μm in diameter, and had two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast. The chloroplast contained a single basal pyrenoid, but sometimes two or three ones observed.

Asexual reproduction. The general features of asexual reproduction were essentially the same as previously reported by Hartmann (1924), Stein (1958b) and Kusumoto et al. (1978). Each of eight or 16 vegetative cells divided three or four times successively to form an eight- or 16-celled daughter colony within the gelatinous sheath (Pl. I C). Second and third divisions were nearly parallel and perpendicular to the first division, forming a group of eight cells arranged in two rows four each (arrow, Pl. I C). The fourth division was parallel to the first division. Sometimes the divisions occurred only twice and a four-celled colony was formed (Pl. I D).

However, it is on the mode of flagellar elongation in the daughter colonies that my observations differed from the previous reports. Just after the successive divisions, each cell of the daughter colony grew only a single flagellum. As a result, the newly formed daughter colony had uniflagellate cells (P. I D, E). Later, before or after colony liberation, these cells began to grow the second flagellum (arrow, Pl. I F). The two flagella became equal in length within a half-day after the daughter colony formation.

Flagellar elongation of this type has not been previously reported in Gonium pectorale. The figures of Hartmann (1924 fig. C) indicate that the cells of a newly formed daughter colony within the parental cellular sheath have two flagella of equal length. I have examined six strains of G. pectorale which were sent from the Culture Collection of Algae at the University of Texas at Austin (UTEX 13, 805, 806, 826, 827 and 2075; Starr 1978). The same mode of flagellar elongation as in my strains has been observed in UTEX 827 and 2075 strains. But it was not determined in the other four UTEX strains. These strains produced no normal 16-celled colonies at my laboratory, probably because of modification or mutation during long-term preservation at the Collection.

Sexual reproduction. The strains isolated in this study were heterothallic, and mating reaction occurred in the early light cycle, one to three days after mixing colonies of two complementary mating types. Colony clumping, which is the first sign of a mating reaction in Pandorina (Coleman 1959, Rayburn and Starr 1974), Volvulina (Stein 1958a, Carefoot 1966) and Astrephomene (Stein 1958a, Brooks 1966), was not observed. A colony dissociated into individual ovoid to ellipsoidal cells surrounded by a gelatinous sheath (Pl. II A). These cells then cast off their sheaths (Pl. II B, C), and spherical, naked gametes were formed (Pl. II D). They were 5-12 μm in diameter and had the same organelles as vegetative cells, except for a slender cytoplasmic protrusion (arrow, Pl. II D), which was up to 5 μm long and was located at the base of the flagella. This transparent structure could be detected only by phase contrast

microscopy.

The gametes soon aggregated in a clump, with their flagellar tips sticking together, forming a single center (arrow, Pl. II E). Meanwhile, two of these gametes joined the tips of their protrusions (Pl. II F, G), the lengths of which were sometimes the same and other times not, forming a cytoplasmic bridge between the two gametes (Pl. II H). This bridge became shortened, allowing the anterior regions of the two protoplasts to approach each other, and the gametes fused (Pl. II I). Plasmogamy proceeded from lateral to posterior portions (Pl. II J). A quadriflagellate zygote was formed in the clumping group and then separated from the clump (Pl. II K). This motile zygote contained two stigmata and two pyrenoids, and entered a dormant period within the light cycle. Many of the zygotes gradually aggregated loosely and lost their flagella. Each zygote then secreted a smooth cell wall, around which a gelatinous wall was formed during the subsequent dark cycle. Two days after the mating reaction, the zygote had cast off its gelatinous wall, which remained attached to the surface of the former zygote (arrow head, Pl. III A). The zygote became reddish brown in color after about one week (Pl. III A), and measured 10-18 μm in diameter.

The zygotes began to germinate one or two days after they were transferred from the dark treatment in the agar medium to the liquid medium under the usual illumination. Initially the protoplast in the zygote underwent two equal divisions (Pl. III B, C), probably meiotic, to form four daughter cells. Part of

the zygote wall protruded and swelled, forming an ellipsoidal shape (Pl. III C). The four cells gradually squeezed out into the protruding wall (Pl. III D). Meanwhile, four biflagellate gone cells which were joined in a colony (germ colony) (Pl. III F) were released, leaving the empty zygote wall behind (Pl. III E). Each gone cell in the germ colony subsequently divided into a gone colony, as in asexual reproduction.

Starr (1962) reported a "protoplasmic protrusion" in the anterior region of the gamete in Volvulina pringsheimii Starr. He observed that plasmogamy was initiated by the union of the tips of the protrusions of the two gametes, and said that this structure was one of the diagnostic attributes by which V. pringsheimii may be distinguished from V. steinii Playfair. Later, however, Carefoot (1966) observed a very similar structure in the gamete of V. steinii and named it a "mating papilla". Mating papillae have been observed in the related isogamous genera Pandorina (present data) and Astrephomene (Brooks 1966, present data). The mating papillae observed in these algae are, in general, not as long as cytoplasmic protrusions of Gonium pectorale (Pl. II D, F), which also can be called mating papillae on the basis of their function.

Gonium sociale (Dujardin) Warming

The general features of sexuality in the four-celled species Gonium sociale (Dujardin) Warming have been studied in cultures by Starr (1955) and Stein (1959). Although they described conjugation between gametes, it seems have not been

determined whether the gametes of this alga bear mating papillae, which initiate plasmogamy, as do those of G. pectorale Müller (present data). Furthermore, zygote germination has not been previously observed in G. sociale.

The sexual reproduction of G. sociale was studied with particular regard to these two points. The presence and role of cytoplasmic protrusions in conjugating gametes and the details of zygote germination were observed.

Vegetative morphology. Colonies were square and contained four cells embedded in gelatinous cellular sheaths (Pl. IV A), measuring up to 32 μm in diameter. The cells were ovoid in shape, measuring up to 16 μm long, and had two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast with a single basal pyrenoid. The cells were connected to one another by protuberances of the sheaths.

Asexual reproduction. Each cell of the colony divided twice to form four-celled embryo within each gelatinous sheath Pl. IV B). Each daughter cell of the embryo then grew two flagella equally (Pl. IV C) and the daughter colony moved out from its parental sheath.

Sexual reproduction. The strains used in this study were homothallic, and sexual reproduction began near the onset of the light cycle, two or three days after adding the mating medium to the colonies.

Colony clumping, which is the first sign of mating reaction in Pandorina (Coleman 1959, Rayburn and Starr 1974), Volvulina (Stein 1958a, Carefoot 1966) and Astrephomene (Stein 1958a,

Brooks 1966), was not observed. Before the mating reaction, colonies had usually dissociated into individual ovoid to ellipsoidal cells surrounded by gelatinous sheaths (Pl. IV D), which were clearly recognized in their anterior portions (arrow heads, Pl. IV D). In the mating reaction, these cells gradually cast off their sheaths (Pl. IV E) as their flagellar tips stuck to those of other cells. As a result, spherical, naked gametes were formed (Pl. IV F, G). Sometimes the gametes were liberated from undissociated intact colonies. The gametes were 6-10 μm in diameter and had the same organelles as the vegetative cells. However, some of them bore a slender cytoplasmic protrusion at the base of the flagella (Pl. IV G). This transparent protrusion was up to 6 μm long and could be detected only by phase contrast microscopy.

Gametic union occurred one to two hours after the onset of the light cycle. Several to many gametes formed a clumping group, with their flagellar tips sticking together at the two opposite ends with regard to the protoplasts (Pl. IV H): one flagellum of each aggregating gamete reached to one end, while the other reached to the opposite end. Meanwhile, two of the gametes paired and plasmogamy took place. These two gametes sometimes were the same size, but only one of the pair bore an anterior cytoplasmic protrusion (Pl. V A). The presence or absence of this protrusion in these two gametes was independent of the relative size of the two protoplasts. When the gametes differed in size, either the larger or smaller gamete bore the protrusion. The tip of the protrusion from one gamete became more slender and connected with the region near the flagellar

base of the other gamete, forming a cytoplasmic bridge between the two gametes (Pl. V B). This bridge became shortened, allowing the anterior regions of the two protoplasts to approach each other (Pl. V C), and the gametes fused (Pl. V D). Plasmogamy proceeded from lateral to posterior portions (Pl. V E). A quadriflagellate zygote was formed in the clumping group and then separated from the clump. This motile zygote contained two stigmata and two pyrenoids (Pl. V F), and entered a dormant period after swimming for four to six hours. Many of the zygotes gradually aggregated loosely and lost their flagella. Each zygote then secreted a broad gelatinous wall in the late stage of the light cycle (Pl. V G). During the subsequent dark cycle, the zygote formed a cell wall ornamented with reticulation between the gelatinous wall and the surface of the protoplast (P. V H). This reticulate-walled zygote gradually accumulated green granules, which turned reddish brown in color after about one week, to form mature zygote (Pl. V I, J). It measured 16-22 μm in diameter.

The zygotes began to germinate two or three days after they were transferred from the dark treatment to the usual illumination in the liquid medium. Initially the zygote turned somewhat greenish in color. The protoplast in the zygote underwent two divisions (Pl. VI A, B), probably meiotic, to form four daughter cells. Part of the reticulate wall ruptured allowing the inner wall in this region to protrude and swell, forming an ellipsoidal shape (Pl. VI C). The four cells gradually separated from one another within this wall (Pl. VI C,

D). The tip of the protruding wall ruptured and four biflagellate protoplasts were released separately, leaving the empty zygote wall behind (Pl. VI E-G). These four gone cells had the same form and swam actively with their flagella. Each cell was ellipsoidal or elongate-ovoid in shape, but more or less asymmetrical in side view (Fig. 4b), measured 11-18 µm long (Fig. 4; Pl. VI H).

The liberated gone cell gradually altered its form as it swam. As its granules disappeared, a basal pyrenoid in its cup-shaped chloroplast, a stigma in its lateral middle portion and two contractile vacuoles near the base of the flagella could be seen. The cell became somewhat spherical and symmetrical in shape and secreted a gelatinous envelope which was detected in the anterior region of the cell.

About 12 hours after liberation, the gone cell began to divide to form a gone colony. Before cell divisions, the gone cell lost its stigma and pyrenoid, and its flagella became detached (Pl. VI I). The protoplast divided twice within its gelatinous envelope (Pl. VI J, K) to form a four-celled colony as in asexual reproduction. A stigma appeared in each cell, and each cup-shaped chloroplast developed a basal pyrenoid as the gone colony grew, giving the typical shape of Gonium sociale (Pl. VI L).

In Gonium pectorale (present data), each of the two conjugating gametes bears a slender cytoplasmic protrusion (mating papilla) at the base of the flagella (Fig. 6; Pl. II F) and plasmogamy is initiated by the union of the tips of the two

protrusions (Pl. II F-H). In contrast, only one of the two conjugating gametes of G. sociale bore an anterior cytoplasmic protrusion (Fig. 5; Pl. V A) and the plasmogamy was initiated by the union between the tip of the protrusion and the anterior region of the other gamete which did not bear such a protrusion.

The germinating zygote of G. pectorale gives rise to four biflagellate gone cells joined in a colony (germ colony) (Pl. III F) (Schreiber 1925, Stein 1958b, Kusumoto et al. 1978). The same situation has been recently observed in G. multicoccum Pocock (Saito 1984) and G. viridistellatum Watanabe (unpublished data). However, the four gone cells were liberated separately from a germinating zygote of G. sociale (Pl. VI D-G).

Based on these differences in sexual reproduction between G. sociale and G. pectorale (the type species of Gonium), it seems natural to place G. sociale in a genus different from Gonium as well as from the other genera of the Volvocaceae, since both gametic union and zygote germination in G. sociale are unique. Therefore a new combination is proposed as follows.

Tetrabaena socialis (Dujardin) Nozaki comb. nov.

Basionym: Cryptomonas socialis Dujardin, Hist. nat. Infs.: 333, pl. 5, fig. 1, 1841.

Synonyms: Tetrabaena dujardinii Fromentel, Etudes Microsoaires: 214, 1874.

Gonium sociale (Dujardin) Warming, Bot. Tidsskrift ser. 3, **1**: 82, pl. 1, figs. 1-19, 1876

Tetragonium lacustre W. et G. S. West, J. Roy. Microsc. Soc. London **1896**: 160, pl. 2, figs.

1-13, 1896.

B. Genus *Pandorina* Bory

The genus *Pandorina* was established by Bory (1824) in his classification of the colonial flagellates, with a single species, *P. morum*. Since then, this genus has been frequently confused with the genus *Eudorina* Ehrenberg (1838) because the original description of *Pandorina* was too incomplete and inaccurate to differentiate between the two genera.

The complete life cycle of *P. morum* Bory was first described by Pringsheim (1870), who presented the first clear comparison of *Pandorina* and *Eudorina*. *Pandorina* has colonies in which cells are arranged compactly, while colonies of *Eudorina* have spherical cells arranged in the periphery of the gelatinous matrix.

Korshikov (1923a) described *P. charkowiensis* from Russia. This species has 32-celled colonies with multipyrenoid cells. Chodat (1931) established two 16-celled species, *P. smithii* and *P. minodii*. In 1932, Tiffany described *P. protuberans* based on planktonic material collected in Lake Erie, U.S.A. However, these four species were not established using studies of cultured material.

Schreiber (1925) and Coleman (1959) studied *P. morum* using laboratory cultures and showed its heterothallic and isogamous sexual reproduction. In 1974, Rayburn and Starr described *P. unicocca* based on cultured material. This species has 32-celled colonies, with a *Eudorina*-like appearance, but shows isogamous sexual reproduction. According to Rayburn and Starr (1974), the genus *Pandorina* is distinguished from *Eudorina* by having isogamous sexual reproduction.

Pandorina morum Bory

Sexual reproduction in P. morum Bory has been observed in cultures by Schreiber (1925) and Coleman (1959). However, it was not determined whether the isogametes of this species bear mating papillae, which initiates the plasmogamy, as do those of the related isogamous genera Volvulina (Starr 1962, Carefoot 1966), Gonium (present data) and Astrephomene (Brooks 1966, present data). The details of sexual reproduction in Pandorina morum are described here, with particular regard to the presence and role of mating papillae in conjugating gametes and the mode of gone colony formation.

Vegetative morphology. Colonies were ellipsoidal to spherical in shape and contained 16 or 8 cells compactly arranged in a gelatinous matrix (Fig. 7; Pl. VII A), measuring up to 53 µm in diameter. The cells were keystone-shaped to ovoid and had two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast with a single basal pyrenoid (Fig. 7), measuring up to 20 µm in surface diameter.

Asexual reproduction. Each cell of the colony underwent daughter colony formation equally. Prior to cell division, the constitutive cells, whose size were about 15 µm in surface diameter, became separated from one another and became spherical in shape within the swollen gelatinous matrix. As cell divisions progressed, the gelatinous matrix became more

swollen, revealing its internal structure (Pl. VII B), which formed a keystone-shaped space for each parental cell (16 spaces in case of a 16-celled parental colony). The gelatinous matrix of a 16-celled parental colony at last attained about 130 um in length.

Each parental cell embedded within this keystone-shaped space underwent colony formation. It usually divided four times successively to form a 16-celled plakea, which inverted to become a spheroidal daughter colony. Each cell of the daughter colony soon began to grow two flagella of equal length (Fig. 8). The stigma remained in one of the daughter cells up to this stage. When elongation of the new flagella was nearly completed, a new gelatinous matrix was secreted and a new stigma appeared in each cell of the daughter colony. The colony swam slowly within the keystone-shaped space of the gelatinous matrix and subsequently swam away from its parent. The newly formed daughter colony measured 16-18 μm in length.

Although Coleman (1959) did not report the existence of a keystone-shaped space within which each daughter colony develops (Pl. VII B), this space was clearly recognized in the same strains used by her: In 50-3 (UTEX 788; Starr 1978), In 50-11(UTEX 789), IN-Bl II-9 (UTEX 856) and Cal-68-8 (UTEX 880). This disagreement of the two observations may be caused by a difference in the methods of observation. In the present study, the materials were stained with methylene blue and observed by phase contrast microscopy. It is considered that this keystone-shaped space in asexual reproduction results from the direct

swelling of the gelatinous matrix which tightly surrounds the keystone-shaped cell in the vegetative phase (Fig. 7; Pl. VII A). A similar structure in the gelatinous matrix of Pandorina morum has been recently reported by Fulton (1978) using an electron microscope. Furthermore, it is noteworthy that in the 19th century, Pringsheim (1870) had already reported such spaces, within which daughter colonies develop, observing his natural collection of P. morum.

Sexual reproduction. Mating reaction occurred during the earlier light cycle, at least one dark cycle after the mixing of the colonies of the complementary mating types. The first step of the mating reaction was colony clumping, in which several to many colonies aggregated, with their flagella attached to one another (Pl. VII C). In this stage, the shape of the colony did not change at first for about two hours, but afterwards became swollen and developed gametes. As the gelatinous matrix of the colony swelled, the constitutive cells became separated from one another and were released from the matrix (Pl. VII D). These liberated naked cells functioned as gametes. They measured 5-10 μm in diameter and had the same organelles as the vegetative cells except that they bore a cytoplasmic papilla at the base of the flagella (Fig. 9; Pl. VII F). These papillae were varied in length, up to 3 μm long.

The gametes soon aggregated in a clump with their flagellar tips sticking together, forming a single center (Fig. 9; Pl. VII E). Gametic union occurred in this gamete clumping. Two of the gametes connected the tips of their anterior papillae, forming a cytoplasmic bridge between the gametes (Pl. VII G). This bridge

soon became shortened, allowing the anterior regions of the two protoplasts to approach each other, and the gametes fused. Plasmogamy proceeded from lateral to posterior portions (Fig. 9). A quadriflagellate zygote was formed in the clumping group and then separated from the clump (Pl. VII H). This motile zygote was spherical in shape and had two pyrenoids and two stigmata, measuring 8-15 μm in diameter. After swimming, a large number of zygotes aggregated and settled down with their flagella disintegrated, then entered a dormant period (Pl. VII I). The mating reaction mentioned above was completed during one light cycle. The zygote then lost its stigmata and secreted a heavy cell wall during the subsequent dark cycle (Pl. VII J). It turned reddish brown in color after about one week (Pl. VII K). This mature zygote measured 10-20 μm in diameter and its internal structure could not be seen because of an accumulation of reddish brown granules in it.

The zygote began to germinate one to two days after transfer from the agar medium in the dark to the liquid medium under the usual illuminated conditions. Part of the zygote wall protruded and the zygote became pyriform or ellipsoidal in shape (Pl. VIII A). Meanwhile, this protruding wall ruptured and a biflagellate gone cell was released, leaving its empty wall behind (Pl. VIII B, C). The gone cell was pyriform to ellipsoidal in shape just after liberation, but soon became spherical and swam slowly with its two flagella (Pl. VIII D). It was reddish brown in color because of persistence of the granules from the former zygote.

Before cell division, the gone cell secreted a gelatinous

envelope (Pl. VIII E) through which the two flagella projected. The gone colony was formed within this spherical envelope, which became swollen as the colony formation progressed. It became 20-40 μm in diameter. The gone cell within the envelope divided successively three or four times to from an eight- or 16-celled plakea (Pl. VIII F-H), which inverted and developed new flagella. A new colony (gone colony) was formed within the gelatinous envelope of the former gone cell (Pl. VIII I). During cell divisions, the gelatinous envelope containing the developing embryo swam by means of the two flagella which were retained intact from the original gone cell. The reddish brown granules from the original zygote remained in each cell of the gone colony, but gradually vanished to form a green colony after liberation from the envelope, giving the typical shape of Pandorina morum. The zygote germination and gone colony formation described above was completed during one light cycle.

During the mating reaction, there were some cells and plakeas which escaped from the colonial envelope, settled down on the bottom of the container and initiated colony formation. Such a colony was four-, eight- or 16-celled and often participated in the mating reaction. This phenomenon occurred only during mating.

The existence of mating papillae of gametes has not been previously reported in the genus Pandorina. However, the papillae were detected in P. morum and were followed in the conjugating gametes (Pl. VII F, G) using a phase contrast microscope. Although Coleman (1959) documented zygote germination and gone colony formation in this species using

laboratory cultures, the persistence of the two flagella provided by the original gone cell during gone colony formation and the gelatinous envelope within which the gone colony develops were not mentioned. However, Pringsheim (1870) and Korshikov (1923b) observed such a gelatinous envelope in their natural collections.

Pandorina unicocca Rayburn et Starr

The general features of sexual reproduction in Pandorina unicocca Rayburn et Starr have been observed by Rayburn and Starr (1974) when they described this species. However, it seems that they did not describe in detail conjugation between gametes and gone colony formation. Details of sexual reproduction in P. unicocca are described here, with particular reference to these two points.

Vegetative morphology. Colonies were ellipsoidal in shape, measured up to 90 μm long, and contained 32 or 16 cells loosely arranged to form a hollow sphere in a gelatinous matrix (Fig. 10, 11; Pl. IX A, B). The cells were ovoid to spherical in shape and had two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast with a single basal pyrenoid (Figs. 10, 11), measuring up to 18 μm in diameter.

Asexual reproduction. Cell divisions usually occurred when the size of the cells attained about 20 μm in diameter. Each cell of the colony underwent daughter colony formation, but did not always divide synchronously. Occasionally some of the cells lagged (Pl. IX C).

As cell divisions progressed, the gelatinous matrix of the parental colony became more swollen, and a transparent membrane became separated from the surface of the developing embryo (Pl. IX C). This transparent membrane was spherical in shape and reached 32 μm in diameter.

Each daughter colony was formed in this vesicle. Usually four or five longitudinal divisions occurred successively to form a 16- or 32-celled plakea and a spheroidal colony was formed after inversion (Pl IX D). During the inversion each daughter cell began to grew two flagella, one of which became much longer than the other when the new gelatinous matrix was secreted in the daughter colony. Consequently the longer flagellum seemed to project from the new matrix, while the shorter one was embedded in it. Therefore, the newly formed daughter colony apparently had uniflagellate cells (Pl. IX E) and swam within the transparent vesicle in the parental confluent gelatinous envelope (Fig. 12), which measured about 300 μm long, for a 32-celled parental colony. Up to this stage, the stigma of the parental cell remained in one of the daughter cells of the developing embryo. New stigmata, however, soon appeared in the individual cells, after the daughter colony was released from the parent. The newly formed daughter colonies measured 23-26 um long. It took several hours from the first cell division to the release of the daughter colony. A single basal pyrenoid subsequently developed and the two flagella became equal in length in each individual cell.

My observation on asexual reproduction in P. unicocca was different from that of Rayburn and Starr (1974) with regard to

the parental gelatinous matrix. I have observed transparent vesicles, within which each daughter colony develops (Fig. 12; Pl. IX C, D), and this structure is thought to have surrounded the parental cell tightly in the vegetative phase. Though Rayburn and Starr (1974) did not report such a vesicle in asexual reproduction, it was clearly observed in the same strains they studied [103 (UTEX 2127; Starr 1978), 104 (UTEX 2128), 105 (UTEX 2031) and 106 (UTEX 2032)]. This disagreement between the two observations might be caused by a difference in the methods of observation. The materials in the present observation were stained with methylene blue and observed by phase contrast microscopy. Such a vesicle, within which each daughter colony is formed in the parental confluent gelatinous envelope, has been reported in several species of the related genera Eudorina and Pleodorina; i.e. E. elegans Ehrenberg (Hartmann 1924, Iyengar 1933), E. indica Iyengar (Doraiswami 1940), P. illinoisensis Kofoid (Merton 1908) and P. californica Shaw (Chatton 1911). However, in another species of Pandorina, P. morum (present data), each daughter colony developed in a keystone-shaped space formed in the gelatinous matrix of the parental colony (Fig. 8; Pl. VII B), and was not formed in a vesicle in the parental gelatinous matrix.

It is considered that the difference in the parental gelatinous matrix in asexual reproduction between P. unicocca and P. morum is derived from a structural difference in the matrix of the vegetative colony. From my observation of these two species, this difference was also revealed when the colonies

were swollen and constitutive cells became separated from one another under unfavorable culture conditions. The gelatinous matrix of the colony is said to maintain the colonial arrangement of cells in P. morum (Fulton 1978). Therefore, the difference of the gelatinous matrix may reflect a morphological difference between the two species (Rayburn and Starr 1974) (Figs. 7, 10, 11; Pl. VII A, Pl. IX A, B), namely, a difference in the degree of the separation of the constitutive cells of the colony.

Furthermore, the mode of flagellar elongation in daughter colony formation in P. unicocca is similar to that of Eudorina-Pleodorina (Goldstein 1964): unequal flagellar elongation causing newly formed daughter colonies to appear to have uniflagellate cells (Fig. 12). In Pandorina morum, however, each cell of the newly formed daughter colony grew two equal flagella (Fig. 8).

Based on these two characteristics in asexual reproduction, the structure of the parental gelatinous matrix and the mode of flagellar elongation, it may be postulated that Pandorina unicocca is more closely related to the genus Eudorina, rather than to P. morum, although Pandorina and Eudorina have been delineated by differences in gametic differentiation in sexual reproduction (Smith 1930, Thompson 1954, Rayburn and Starr 1974).

Sexual reproduction. The strains observed in this study were heterothallic and the mating reaction occurred soon after mixing of the colonies of two complementary mating types.

The mode of the mating reaction was essentially the same as that of Pandorina morum Bory as previously described. Colony clumping (Pl. IX F) and gamete release (Pl. IX G) occurred

and naked, spherical biflagellate gametes were formed. The gamete bore a cytoplasmic protrusion, the mating papilla, near the base of the flagella (Pl. IX H). Plasmogamy was initiated by the union of the anterior mating papillae of the two gametes during colony clumping (Pl. IX I) and proceeded laterally from anterior to posterior portions. A quadriflagellate zygote was formed (Pl. IX J) and then separated from the clump. Many of the zygotes subsequently aggregated (Pl. IX K) and entered a dormant period, with their flagella disintegrated (Pl. IX L). In the earliest case, a clump of these aplanozygotes was formed within one hour after the mixing of the colonies.

The aplanozygotes then lost their stigmata, secreted a heavy cell wall during the following day (Pl. X A), and became reddish brown in color after about one week (Pl. X B). These mature zygotes measured 10-20 μm in diameter.

The zygotes began to germinate within a day after transference from the darkness to the usual illumination. Part of the zygote protruded into a thin-walled protuberance. The reddish brown protoplast grew two flagella as it squeezed out into the protuberance (Fig. 13; Pl. X C), in which two or three hyaline bodies were observed. These bodies are considered to be the degenerate products of meiotic division.

The thin-walled protuberance ruptured and the reddish brown gone cell was released (Fig. 14; Pl. X D), leaving the empty zygote wall behind (Pl. X F). The gone cell was somewhat ellipsoidal or oval in shape and was surrounded by a gelatinous envelope through which the two flagella projected (Fig. 15; Pl. X

E). This envelope was spherical in shape and became more swollen as gone colony formation progressed. It measured from 15 to 45 μm in diameter.

After swimming for several hours, the gone colony within the envelope began cell division to form a gone colony (Figs. 16-21; Pl X G-L). Three, four or five successive divisions occurred to form an eight-, 16- or 32-celled plakea (Figs. 16-19; Pl. X G-J), which inverted to become a spheroidal colony (Fig. 26; Pl. X K). This gone colony then grew new flagella and secreted a gelatinous matrix (Fig. 21; Pl. X L). It took about five hours from the first cell division to this stage.

The two flagella provided by the gone cell remained to stick to one of the daughter cells of the peripheral region of the plakea during cell division (Fig. 16-19). They were, however, detached from the cell in the late stage of inversion and the gelatinous envelope containing the gone colony came to a halt (Figs. 20, 21). The reddish brown granules from the zygote still remained in each cell of the newly formed gone colony, but vanished gradually after liberation from the envelope. Consequently a green colony was formed within a day. Each cell of the colony had a stigma, a cup-shaped chloroplast with a single basal pyrenoid and two equal flagella. The gone colony just after formation generally measured about 15 μm long, in case of an eight-celled colony, and about 25 μm, in case of a 32-celled colony.

The presence and role of the mating papillae (Pl. IX H) in the conjugating isogametes of Pandorina unicocca as in P. morum (Pl. VII F, G) has been clarified here, although Rayburn and

Starr (1974) did not mention such features in their studies. Furthermore the two flagella provided by the gone cell during gone colony formation have been followed. The gone colony developed within the envelope as it swam by means of the two flagella which were retained, intact, from the original gone cell (Figs. 15-21).

C. Genus Volvulina Playfair

Playfair (1915) described the genus Volvulina based on the species V. steinii. He distinguished this alga from Pandorina and Eudorina on the basis of its loose investing membranes of the cells. Pascher (1927) and Printz (1927) both regarded V. steinii Playfair as a probable species of Eudorina. Korshikov (1938a) observed its asexual reproduction and plano- and aplanozygotes and suggested that it was very similar to Pandorina. However, Pocock (1953) resolved their doubts about the genus Volvulina through re-examination of the preserved type material and a detailed cultural study. She characterized V. steinii as having a spheroidal colony with 16 lenticular to hemispherical cells whose chloroplasts normally lack pyrenoids.

The second species was described by Skvortzow in 1957 as V. playfairiana, but his description is insufficient to know whether it is a good species or not. Starr (1962) considered this species to be immature forms of V. steinii. Stein (1958a) and Carefoot (1966) studied the morphology and sexuality of V. steinii using laboratory cultures. They observed that the vegetative cells of this alga lacked pyrenoids, while pyrenoids developed in the zygotes.

Starr (1962) established a third species, V. pringsheimii based on cultured material isolated from pond water collected in Texas, U.S.A. According to him, the presence of pyrenoids in the vegetative cells in V. pringsheimii Starr is the most definitive of the characters which distinguish V. pringsheimii from V. steinii.

Volvulina steinii Playfair

The general features of sexual reproduction in Volvulina steinii have been observed in unialgal cultures by Stein (1958a) and Carefoot (1966). However, some details of sexual reproduction in this alga seem to have been left for more careful studies, especially regarding zygote germination and gone colony formation. Sexual reproduction in Japanese strains of V. steinii is described in detail here, with particular regard to this point. In addition, some details of vegetative morphology are also presented.

Vegetative morphology. Colonies were ellipsoidal to spherical in shape and usually contained 16 cells arranged in four whorls round the periphery of the gelatinous matrix (Figs. 22, 23; Pl. XI AB), measuring up to 65 μm long. The cells were lenticular to hemispherical in shape, measuring up to 20 μm in surface diameter. Each cell was separately embedded in an individual sheath (a keystone-shaped space) formed in the gelatinous matrix of the colony. The structure of the gelatinous matrix could be clearly observed in material stained with haematoxylin (Pl. XI C). The 16 keystone-shaped spaces surrounded a small hollow in the center of the colony (Pl. XI D).

Each cell contained a massive cup-shaped chloroplast which was somewhat striated on the surface, and had two equal flagella. Two to ten or more contractile vacuoles were scattered on the anterior surface of the cell (Figs. 22, 23; Pl. XI A). Each of

the four cells in the most anterior whorl had a single stigma. In some colonies, another stigma was observed in one of the posterior cells (Fig. 23).

The chloroplast did not show pyrenoids in the younger age of the culture (Fig. 23; Pl. XI A). Three days after the inoculation of the culture, however, a single pyrenoid, or sometimes two or three, appeared in each chloroplast of the cells. This pyrenoid was located in the brim of the cup-shaped chloroplast (Fig. 22; Pl. XI B). Using the staining methods of Rosowski and Hoshaw (1970), the pyrenoid could be clearly detected two days after the inoculation, but could not be observed in one-day-old culture. Colonies growing in soil-water-pea medium (Starr 1964) began to show an observable pyrenoid in each chloroplast of the cells four days after the inoculation.

The present observation agreed, to some extent, with that of the culture studies on Volvulina steinii by Pocock (1953), Stein (1958a) and Carefoot (1966), except for the presence or absence of pyrenoids in the chloroplasts of the vegetative cells. Although these three authors did not mention the formation of pyrenoids in the vegetative cells, it was clearly detected, not only in the Japanese strains, but also in the three strains which Carefoot (1966) investigated [FA-4 (UTEX 1525, Starr 1978), SC-22 (UTEX 1527) and C2-13 (UTEX 1531)]. All the strains developed pyrenoids in the brim of the cup-shaped chloroplast as the culture aged, though his three strains began to show observable pyrenoids later in the Japanese strains. The

pyrenoid of his strains appeared in four to seven-old cultures growing in the synthetic medium (Table 2) or in five- to 13-day-old cultures grown in the soil-water-pea medium. A similar type of pyrenoid development in the vegetative cells of V. steinii was reported by Korshikov (1938a) in his natural collection. It is considered, therefore, that the species V. steinii has the potential of forming pyrenoids in the brim of the cup-shaped chloroplasts in the vegetative cells under some culture or growth conditions. This potential seems likely to be related to the previous observations of Stein (1958a) and Carefoot (1966) that zygotes of this alga develop pyrenoids.

Asexual reproduction. Each cell of the colony formed a daughter colony. Cell division usually occurred when the cell reached about 20 μm in surface diameter (Pl. XI F). Four successive divisions formed a 16-celled plakea, and a spheroidal colony was formed after inversion.

During colony formation, the two flagella remained stuck to one or two of the daughter cells until the spherical colony was formed. Many contractile vacuoles were shared out among the daughter cells. The stigma, when present, remained attached to one of the daughter cells and ultimately ended up in one of the cells in the three posterior whorls of the daughter colony. The pyrenoid of the chloroplast, when present, was seen for a time in one of the daughter cells, but gradually became indistinct.

In the late stage of the inversion, each daughter cell began to grew two equal flagella. A 16-celled compact colony, measuring 16-20 μm long, was formed in each keystone-shaped space of the parental gelatinous matrix (Fig. 24; Pl. XI F). The

daughter colony then swam away from the parental gelatinous matrix. Stigmata then developed in the anterior four cells as the colony became bigger, giving the typical shape of Volvulina. It took three to four hours from the first cell division to the release of the daughter colony.

The asexual reproduction of Volvulina steinii is essentially the same as that of Pandorina morum Bory, but different from that of P. unicocca Rayburn et Starr and Eudorina (see the previous description), with regard to the structure of the parental gelatinous matrix and the mode of new flagellar elongation. It may be suggested, therefore, that there appears to be a close phylogenetic relationship between Volvulina steinii and Pandorina morum.

Sexual reproduction. The first step in the mating reaction was colony clumping (Pl. XI G). As the colonies were clumping, all the cells of the colony were released from the gelatinous matrix (Fig. 25; Pl. XI H, I). These cells functioned as gametes. The gametes resembled vegetative cells, except for their spherical shape and a cytoplasmic mating papilla at the base of the flagella (Fig. 26; Pl. XI J).

The gametes soon aggregated in a clump, with their flagellar tips sticking together, forming a single center (Fig. 26; Pl. XI K). Gametic union occurred in this clumping. Two of the gametes, whose sizes might be either the same or not, joined their papillae (Pl. XII A) and the anterior regions of the two protoplasts fused. Plasmogamy proceeded from lateral to posterior portions (Fig. 26; Pl. XII B). A quadriflagellate

zygote was formed and separated from the clump (Pl. XII C). The zygote was spherical in shape and had two stigmata and two pyrenoids (Fig. 27). It took about one hour from the beginning of colony clumping until this stage. Motile zygotes soon settled down, shortened their flagella and entered a dormant period (Fig. 28; Pl. XII D). They secreted a cell wall during the following day (Fig. 29; Pl. XII E) and became reddish brown in color after about one week (Fig. 30; Pl. XII F). Mature zygotes measured 9-21 μm in diameter.

Zygotes under illuminated and anaerobic conditions usually began to germinate within a day after transfer from the dark treatment. Initially, part of the zygote wall distended into a thin-walled protuberance, into which the reddish brown content grew two flagella (Figs. 31, 32). In this space, hyaline bodies, probably meiotic products, were observed (Fig. 32; Pl. XII G). The thin-walled protuberance subsequently ruptured and a biflagellate gone cell escaped, leaving the empty wall behind (Fig. 33; Pl. XII H). The gone cell was spherical in shape (Fig. 34; Pl. XII I) and secreted a gelatinous envelope around itself (Fig. 35). About two hours after release, the gone cell divided within the envelope to form a gone colony, as in asexual reproduction (Pl. XII J-L). Throughout the cell divisions, the two flagella provided by the original gone cell remained functional and attached to the surface of the gelatinous envelope. The gelatinous envelope containing the developing embryo swam with these two flagella until the late inversion stage (Figs. 36-39). A four-, eight- or 16-celled gone colony was formed within the gelatinous envelope (Fig. 40; Pl. XII L).

The reddish brown granules recognized in the zygote remained in the gone colony, but disappeared gradually after liberation from the envelope common to the original gone cell, giving the typical shape of V. steinii.

The only observation on zygote germination for the genus Volvulina was reported by Carefoot (1966) on V. steinii, and it differs from the present observation. Carefoot (1966) said that during zygote germination, the zygote wall disintegrated, and did not remain as an empty hull. Furthermore, he did not mention the gelatinous envelope in which gone colony develops, nor did he comment on the fate of the two flagella provided by the gone cell during the gone colony formation. These differences might be caused by the method of induction of zygote germination. According to Carefoot (1966), 2.5-3-hour-old mating materials were put in darkness for 9-10 days, and these dark-treated materials were not placed under anaerobic conditions. In contrast, this study used 2-10-day-old zygotes and placed them under anaerobic conditions after dark treatment of more than three months.

D. Genus Eudorina Ehrenberg

The genus Eudorina was erected by Ehrenberg in 1832 and included a single species, E. elegans, which has a world-wide distribution. E. echidna was described by Swirenko (1926). However, this species is considered to be a pathological form of E. elegans Ehrenberg (Huber-Pestalozzi 1961), though it was recognized by Pascher (1927) and Hu et al. (1980). Smith (1930) created three species of the genus, E. unicocca, E. plusicocca and E. carteri. Korshikov (1938b) established E. cylindrica from Russia, and Prescott (1955) described E. interconnexa, based on plankton sample collected in the Panama Canal Zone.

Goldstein (1964) studied the comparative morphology and sexuality of this genus based on 73 clones isolated from 44 natural populations under controlled laboratory conditions. He included the genus Pleodorina Shaw (1894) in Eudorina because the presence of sexual compatibility among three species of Eudorina and Pleodorina illinoisensis Kofoid, as well as the transformation from somatic cells to reproductive cells in P. californica Shaw under some experimental conditions. However, some phycologists do not recognize this taxonomic treatment on Pleodorina (e.g. Bold and Wynne 1978, Ettl 1983). Goldstein (1964) characterized E. elegans as having multipyrenoid vegetative cells and synonymized E. plusicocca Smith and E. carteri Smith with E. elegans. He classified E. elegans into one dioecious [var. elegans] and two monoecious varieties [var. synoica Goldstein and var. carteri (Smith) Goldstein].

Eudorina elegans Ehrenberg var. elegans

Sexual reproduction in Eudorina elegans Ehrenberg was studied in culture by Schreiber (1925), Goldstein (1964) and Kusumoto et al. (1976). However, none of these investigators documented conjugation between male and female gametes. Although Pocock (1937) and Iyengar (1937) reported their observations of conjugation in this species, their results conflict with regard to the initial site of plasmogamy in the male gamete. No observations of the details of gone colony formation have been reported for E. elegans. In this paper, the details of sexual reproduction in E. elegans var. elegans are described, with particular regard to these two points.

Vegetative morphology. Colonies were ellipsoidal and contained 32 or 16 cells, loosely arranged to form a hollow sphere in a gelatinous matrix, measuring up to 90 μm long (Pl. XIII A). The cells were nearly spherical in shape, measured up to 18 μm in diameter, with two equal flagella, a stigma, two large contractile vacuoles at the base of the flagella, and a cup-shaped chloroplast with several pyrenoids.

Asexual reproduction. Each cell of the colony divided four or five times to form a 16- or 32-celled plakea, which inverted. A spheroidal colony developed within a transparent vesicle in the parental gelatinous matrix (Pl. XIII B). After inversion, each cell of the daughter colony grew two flagella, one of which became much shorter than the other when the new gelatinous matrix

was secreted. Therefore, newly formed colonies have apparently uniflagellate cells. These two flagella, however, became equal in length after the colony was released from the parent.

Sexual reproduction. The strains used in this study were heterothallic, and sexual reproduction occurred one to three days after mixing male and female colonies within mating medium (Table 2).

Male colonies usually produced sperm packets early in the light cycle. Each cell of the colony formed a plakea, which inverted. Sperm packets developed within transparent vesicles in the confluent gelatinous envelope of the male colony (Pl. XIII C) and then swam freely from the parental colony. The packets were hemispherical, with 16 or 32 biflagellate spindle-shaped cells, and measured 13-20 μm in diameter. (Pl. XIII D, E). Simultaneously with or after sperm packet formation, the female colony became immobile and swollen. The cells of the colony separated from each other and gradually cast off their transparent vesicles inside the confluent gelatinous envelope (Pl. XIII H). The mature spherical female gamete retained its flagella in the gelatinous envelope and measured 14-20 μm in diameter.

The sperm packets swam to the mature female colonies (Pl. XIII F) and dissociated into individual male gametes. They surrounded the female colonies and swelled, and the now spherical male gametes separated from one another (Pl. XIII G). Soon they reverted to the spindle shape. Each cell measured 10-15 μm long, and contained a stigma, two contractile vacuoles and two flagella of equal length (Fig. 41). The shape of the cell varied as it

swam.

The male gamete bore a slender cytoplasmic protrusion up to 7 μm long at the base of the flagella (Fig. 41; Pl. XIII I, J). This transparent structure could only be detected by phase contrast microscopy.

The free swimming male gametes penetrated the gelatinous matrix of the female colony (Pl. XIII G). After a male gamete passed through the envelope, it swam slowly around the female gamete, landed on the lateral anterior portion and oriented its anterior end towards the flagellar base of the female cell (Fig. 42; Pl. XIV A). The anterior end of the male gamete then entered the anterior region of the female gamete and plasmogamy proceeded laterally and posteriorly (Figs. 43-45; Pl. XIV B, C).

The spherical quadriflagellate zygote contained one or two stigmata, two short flagella belonging to the male gamete, and two long ones from the female gamete (Fig. 46; Pl. XIV D). It entered a dormant period either while still in the female colony or after leaving it. Its flagella disintegrated, a smooth cell wall was secreted within a day (Pl. XIV E) and green granules accumulated and then turned reddish brown in color after about one week (Pl. IV F).

During the dark treatment, the zygotes turned grayish brown in color. They began to germinate two or three days after they were transferred to light. Initially, part of the outer cell wall ruptured, allowing the inner wall in this region to protrude and swell conspicuously (Pl. XV A). The grayish brown protoplast or gone cell grew two equal flagella as it squeezed out into the

protuberance (Pl. XV B, C). One or two hyaline bodies, probably meiotic products, were also observed (Pl. XV A, D). The thin-walled protuberance then ruptured and the biflagellate gone cell moved out, leaving its empty wall behind (Pl. XV D, E). The gone cell was spherical in shape and swam slowly (Fig. 47; Pl. XV F). Before cell division, the gone cell secreted a gelatinous envelope, through which the two flagella projected (Fig. 48). The gone colony was formed within this envelope, which became swollen as colony formation progressed. It became 40-50 μm in diameter.

Several hours after liberation, the gone cell divided four or five times successively, forming a plakea (Fig. 49-53; Pl. XV G, H), which inverted and formed new flagella. A 16- or 32-celled colony was formed within the gelatinous envelope of the former gone cell (Fig. 54; Pl. XV I). Throughout cell divisions, both of the two flagella of the original gone cell remained functional and remained attached to the surface of the envelope (Figs. 49-53). The attachment site was connected to one of the inner daughter cells by a transparent strand. In the eight-, 16- and 32-celled stages, it was connected to a cell located in the corner of the square plakea (Figs. 51-53). The gelatinous envelope containing the developing embryo swam with these two flagella until the late inversion stage (Figs. 49-53).

The cells of the gone colony appeared uniflagellate just after formation (Fig. 54) because one of the two flagella was much shorter than the other. The shorter flagellum was embedded in the new matrix of the colony, whereas the longer one penetrated it. They became equal in length after the colony was

released. The grayish brown granules in the zygote remained in the gone colony (Fig. 54), but became inconspicuous as the colony grew, giving the typical shape of E. elegans.

Two investigators have observed conjugation between male and female gametes in Eudorina elegans and made conflicting observations. Pocock (1937) stated that the anterior end of the male gamete entered the female gamete. Iyengar (1937) described in detail the fact that the posterior end of the female gamete entered the female cell. Here, it was observed that fusion was initiated by the entrance of the anterior end (including the flagellar base) of the male gamete into the anterior region of the female gamete, with fusion proceeding laterally and posteriorly (Figs. 42-46; Pl. XIV A-C).

These observations suggest that the slender cytoplasmic protrusion at the base of the flagella of the male gamete (Fig. 41; Pl. XIII I, J) might be a mating structure. However, it could not be determined if the structure actually participated in the initial fusion, because conjugation occurred in the gelatinous matrix of the female colony (Figs. 42-46) and protrusion could not be clearly observed by either bright field or phase contrast microscopy. To my knowledge, this structure has not been previously reported for male gametes of Eudorina.

Although zygote germination in Eudorina was reported by Schreiber (1925) and Goldstein (1964) for E. elegans and Palik (1955) and Waters (1960) for E. illinoisensis (Kofoid) Pascher, none of these authors completely described the fate of the gelatinous envelope and the two persistent flagella of the

original gone cell during gone colony formation. This paper presents observations on the presence of the gelatinous envelope surrounding the gone colony (Fig. 54; Pl. XV I). The two flagella persisted until the late inversion stage (Figs. 47-53).

Eudorina elegans Ehrenberg var. synoica Goldstein

Although zygote germination in Eudorina elegans has been observed in the dioecious variety, E. elegans var. elegans (Schreiber 1925, Goldstein 1964), the zygotes of the monoecious variety of this species have not previously been induced to germinate. This paper describes the zygote germination in a monoecious variety, E. elegans var. synoica Goldstein.

Vegetative morphology. Colonies were ellipsoidal in shape and contained 32 or 16 cells loosely arranged to form a hollow sphere in a gelatinous matrix (Pl. XVI A, B), measuring up to 120 µm long. The cells were nearly spherical in shape, measuring up to 20 µm in diameter, and had two equal flagella, a stigma, two large contractile vacuoles at the base of the flagella, and a cup-shaped chloroplast with several pyrenoids.

Asexual reproduction. The mode of asexual reproduction was essentially the same as that of E. elegans var. elegans, described above. A spheroidal daughter colony with apparently uniflagellate cells developed within a transparent vesicle in the parental gelatinous matrix (Pl. XVI C).

Sexual reproduction. Sexual colonies were monoecious. Several cells situated at random in the colony developed into sperm packets while the remaining cells served as female gametes

(Pl. XVI D). Conjugation between male and female gametes occurred within the sexual colony to form zygotes.

The zygotes began to germinate within a day after they were transferred from the agar surface in the dark to the liquid medium under the usual illuminated conditions. Initially, part of the outer wall ruptured. The inner wall in this region then protruded and swelled, forming an ellipsoidal shape (Pl. XVI E, G).

Meanwhile, the protoplast within the zygote frequently underwent a transverse division (Pl. XVI H). As a result, two approximately equal-sized protoplasts were formed in the zygote wall (Pl. XVI H). Subsequently, the two protoplasts separated from each other within the expanding wall (Pl. XVI I, J). A hyaline body, probably a meiotic product, could be observed (arrow head, Pl. XVI I, J). The tip of the wall then ruptured and two biflagellate gone cells were released separately, leaving the empty zygote wall behind (Pl. XVI K, L). Each of these two gone cells had the potential to develop into a gone colony.

However, relatively small zygotes occasionally did not undergo a transverse division when they germinated. In the germinating, ellipsoidal zygote, a small hyaline body budded off from the protoplast within the protrudent wall (arrow head, Pl. XVI E, F). The protoplast then grew two equal flagella as it squeezed out into the protruding wall (Pl XVI F). The tip of this wall subsequently ruptured and a single biflagellate gone cell moved out, leaving its empty wall behind.

Irrespective of these two types of zygote germination, the

liberated gone cells had the same form, and developed into gone colonies as described above for E. elegans var. elegans (Figs. 47-54). The gone cell was nearly spherical in shape, 12-18 µm in diameter, and contained reddish brown granules from the former zygote. Before cell division, the cell secreted a gelatinous envelope from which the two flagella projected. The cell subsequently divided successively to form a gone colony as in asexual reproduction within the gelatinous envelope. During colony formation, the envelope containing the developing embryo moved by means of the two flagella which were retained intact from the original gone cell.

Although the germinating zygote of E. elegans var. elegans gives rise to a single gone cell (Schreiber 1925, Goldstein 1964, present data) (Pl. XV A-D), that of E. elegans var. synoica frequently produced two equal biflagellate gone cells (Pl. XVI G-K). A similar situation has been reported by Waters (1960) using heterothallic and dioecious strains of E. illinoisensis (Kofoid) Pascher, in which two viable gone cells are most frequently produced in the germinating zygote. Therefore, the number of viable gone cells from a germinating zygote does not indicate with whether the colonies of Eudorina are dioecious or monoecious.

E. Genus Volvox Linné

Leeuwenhoek (1700) was the first to recognize and describe Volvox, using his newly invented microscope. However, the generic name Volvox was established by Linné (1758) with the type species, V. globator. The present-day conception of V. globator is based on the descriptions of Ehrenberg (1832 1838).

The only valid species described before 1900 are V. globator Linné, V. aureus Ehrenberg, V. carteri Stein and V. tertius Meyer. Since 1900, new species have been described by Powers (1907 1908), West (1910 1918), Playfair (1914 1915 1918), Smith (1920), Shaw (1922c), Rich and Pocock (1933), Pocock (1933a) and Korshikov (1939).

Shaw also described five genera that he considered closely related to, but distinct from, Volvox. These genera are Besseyosphaera (1916), Campbellosphaera (1919), Merrillosphaera (1922a), Copelandosphaera (1922b) and Janetosphaera (1922c). Printz (1927) made each of Shaw's genera a section of Volvox and established the section Euvolvox [=Volvox] to include the species referable to Volvox as delimited by Shaw.

In his comparative study in the genus Volvox, Smith (1944) retained four of the sections recognized by Printz (1927) but rejected two (Campbellosphaera and Besseyosphaera). He recognized 18 species of Volvox and classified them into four sections based on the differences in the gelatinous matrix of the colony, the shape of the somatic cells and the cytoplasmic strands connecting the cells in the colony.

Recently Starr (1970b) described V. pocockiae based on

cultured material isolated from soil collected in a shallow pond in Mexico. This species is the second species in the section of Janetosphaera, and characterized by having dwarf male colonies.

Volvox carteri Stein f. kawasakiensis Nozaki f. nov.

Volvox carteri Stein belongs to the section Merrillosphaera, in which the cells of the colonies are not connected by cytoplasmic strands (Smith 1944). This species is characterized by having special females and dwarf males in sexual reproduction, and a unique embryogenesis, in which the reproductive cells of the next generation are produced by unequal cytokinesis (Shaw 1922a, Metzner 1945, Kochert 1968, Starr 1969).

Since Stein (1878) described V. carteri, five infraspecific taxa in this species have been established, namely, var. typica Shaw (1922a), var. manilana Shaw (1922a), var. weismannia (Powers) Shaw (1922a), f. nagariensis Iyengar (1933) and var. hazenii Metzner (1945). These taxa are delineated by differences in asexual colonies as well as in sexual, male and female colonies.

Recently, strains of V. carteri have been isolated from soils collected in Kawasaki-shi, Kanagawa Prefecture, Japan. These strains showed some morphological differences from those of the taxa within V. carteri with particular regard to the male colonies, and are considered to be a new taxon. Using these strains, it was possible to observe actual fertilization between sperm and egg in detail for the first time in the oogamous genus Volvox.

The morphology, sexual reproduction and taxonomy of V. carteri f. kawasakiensis Nozaki f. nov. are described here.

Asexual colonies. The mature asexual colonies of V. carteri f. kawasakiensis were nearly spherical in shape (Pl. XVII A-C), contained 500-3000 (usually 1000-2000) cells embedded in individual sheaths in the periphery of the gelatinous matrix (Pl. XVII D-F) and colonies measured up to 750 μm long. The somatic cells were nearly spherical in shape, measured up to 10 μm in diameter and had two equal flagella, two contractile vacuoles and a cup-shaped chloroplast with a single pyrenoid (Pl. XVII D, E). The gonidia were located in the posterior half to two thirds of the colony (Pl. XVII A-C) and their number was usually 8-14, but increased up to 30 (Pl. XVII C) when the colonies were inoculated repeatedly (at intervals of 2-3 days) into the new growth medium. The gonidia were spherical in shape, measuring up to 78 μm in diameter (Pl. XVII G).

This organism is very similar to V. carteri f. nagariensis with regard to the number of gonidia (Table 3). Iyengar (1933) noted that the number in f. nagariensis collected in India is usually 15 but sometimes as many as 21. Starr (1969) reported that the number in f. nagariensis from Japan is generally up to 16. On the other hand, Iyengar (1933) reported that the colony of f. nagariensis measures up to 1003 μm in diameter and comprises 8000 cells. Starr (1969) estimated the colony cell number of Japanese strains of the same form as near 5000. However, I have measured the diameter of the colonies of the same material (UTEX 1885 and 1886; Starr 1969 1978) and found it to be up to 1100 μm. Colonies of V. carteri f. kawasakiensis, however, measured up to

750 μm long and comprised up to 3000 cells. Therefore, f. kawasakiensis can be distinguished from f. nagariensis by its smaller size and the smaller colony cell number in asexual colonies.

Male colonies. The induced cultures produced two types of male colonies with regard to the presence or absence of somatic cells. The total number of somatic cells and androgonidia (sperm packets) was 16, 32, 64, 128 or 256. If the number was 16 or 32, the male colony was composed of only androgonidia or sperm packets (Pl. XVIII B, C, N). In 128- or 256-celled male colonies, there were both small somatic cells and large androgonidia (sperm packets) (Pl. XVIII H-J). The ratio of somatic cells to androgonidia was nearly 1:1. However, 64-celled male colonies exhibited both of these types, some with only androgonidia (sperm packets) (Pl. XVIII E-G) while others with a 1:1 ratio of somatic cells to androgonidia (Pl. XVIII D). The mature male colonies were ovoid to spherical and measured 70-200 μm long (depending upon the colony cell number).

Male colonies composed of only androgonidia or sperm packets (without somatic cells) have not been previously reported in V. carteri, but V. (sect. Merrillosphaera) spermatosphaera Powers (1908) and V. (sect. Janetosphaera) pocockiae Starr (1970b) produce only such male colonies. Although the asexual colonies of V. spermatosphaera and V. carteri are similar morphologically (Smith 1944), V. spermatosphaera has no special females (Starr 1970b) and the gonidia of the next generation are not produced by unequal cytokinesis (Starr personal

communication).

The other type of male colony (with a 1:1 ratio of somatic cells to androgonidia) in V. carteri f. kawasakiensis has been reported for f. nagariensis by Starr (1969). I have observed male colonies of the same form (UTEX 1886) and obtained the same results as Starr (1969) that f. nagariensis produces only male colonies with a 1:1 ratio of somatic cells to androgonidia. Therefore, production of male colonies composed of only androgonidia (without somatic cells) is the most definitive character which distinguishes V. carteri f. kawasakiensis from the other infraspecific taxa of V. carteri. Starr (1969) states that the ratio of somatic cells to androgonidia in male colonies may well serve as taxonomic features separating varieties and/or forms within V. carteri.

Female colonies. Colonies were nearly spherical to ovoid in shape and had nearly the same colony cell number as the asexual colonies (Pl. XIX A, B). They contained small somatic cells and large reproductive cells (eggs) which were scattered among the somatic cells except for the anterior region of the colonies (Pl. XIX A-C). The number of eggs ranged from 42 to 81. These eggs were smaller than the gonodia in asexual colonies (Pl. XIX C).

The number of eggs in female colonies of V. carteri f. kawasakiensis is the largest within V. carteri (Table 3). Next to this form, f. nagariensis has the largest egg number. Starr (1969) reported the number of eggs in female colonies of f. nagariensis ranges from 20 to 64, but the usual number is 35-45. I have observed female colonies of the same strain (UTEX 1885) and estimated the egg number 24-42 under the present culture

conditions.

Development of the colonies. The developmental processes of asexual and female colonies in V. carteri f. kawasakiensis were essentially the same as reported by Starr (1969) for f. nagariensis. During plakea formation, large cells (reproductive initials) and small ones (somatic initials) were produced by unequal cytokinesis (Pl. XVII H-J). Subsequent divisions formed a hollow, spherical plakea from which protruded the large gonodia or eggs of the next generation. An anterior phialopore was surrounded by four lips (Pl. XVII K, Pl. XIX D). Inversion began with opening of the lips (Pl. XVII L, Pl. XIX E) and proceeded to form a spheroidal colony in which the gonidia or eggs were situated inside the colony (Pl. XVII M-O, Pl. XIX F, G).

The development of male colonies in V. carteri f. kawasakiensis was somewhat different from that of f. nagariensis (Starr 1969). In induced male cultures of f. kawasakiensis, parental gonidia either divided equally until the last plakeal stage (16-, 32- or 64-celled plakea) (Pl. XVIII K), or only the last division was unequal to form a 64-, 128- or 256-celled plakea. In the former case, all the cells of the colonies became androgonidia after inversion (Pl. XVIII L-N). Therefore these colonies had no small somatic cells (Pl. XVIII E, N). In the latter case, nearly equal number of large and small cells were formed. The large one became androgonidia and the small ones developed into somatic cells (Pl. XVIII D, H). Thus the 1:1 ratio of somatic cells to androgonidia was formed. A similar situation has been reported by Starr (1969) for V. carteri f. nagariensis, which has only

male colonies with the 1:1 ratio of cell types. In both types of male colonies of V. carteri f. kawasakiensis, all the cells of the newly formed compact male colony grew two equal flagella. Therefore, even the male colonies without any somatic cells swam with the flagella of the androgonidia slowly within the vesicle of the parental gelatinous matrix, before sperm packet formation.

After secretion of a new gelatinous matrix and enlargement, in both types of male colonies, the androgonidia detached their flagella and divided successively to form sperm packets (Pl. XVIII I). The packets were composed of 64 or 128 biflagellate, elongate sperm, compactly arranged in one layer forming plates (Pl. XVIII B, C, F, G, J), measuring 15-23 μm in diameter. After sperm packet formation, the male colonies with biflagellate somatic cells swam out from their parent and then released sperm packets, while those without flagellate somatic cells (composed of only sperm packets) remained within the parent (Pl. XVIII A) when releasing the packets.

Sexual reproduction. After female colonies and sperm packets were mixed, the packet attached itself to the posterior portion of the female colony, with the flagellar side facing the female (Pl. XX A, B). Then, the packet swelled and dissociated into individual biflagellate sperm, which soon penetrated the female at the attached portion (Pl. XX C). Each sperm was spindle-shaped and measured 11-17 μm long. It contained a stigma and two contractile vacuoles in the anterior portion, and a cup-shaped chloroplast filling the posterior portion. Although the male gametes of Eudorina elegans Ehrenberg bore a tubular cytoplasmic protrusion (putative mating structure) at the base of

the flagella (Fig. 41; Pl. XIII I, J), such a structure was not identified in the sperm of Volvox carteri, even using phase contrast microscopy (Pl. XX D).

After penetration, the sperm swam rapidly within the female colony and came to rest on the lateral anterior portion of the egg. It oriented its anterior end (including the flagellar base) to the anterior pole of the egg. The sperm attached itself tightly to the surface of the egg, laterally and elongated its body (Fig. 55; Pl. XX E). As soon as the sperm retracted its posterior tail (Fig. 56; Pl. XX F), it rapidly entered the anterior pole of the egg from the anterior to the posterior end (Fig. 57, 58; Pl. XX G, H). The fate of the flagella of the sperm could not be traced after plasmogamy. Fertilization was observed about four hours after the mixing of female colonies and sperm packets.

After fertilization, the zygote secreted a thin cell wall during the subsequent day (Pl. XXI I, J). This wall became thick and reticulated after about four days, and the zygotes became reddish brown in color after about one week (Pl. XX K, L). The mature zygotes, measuring 27-38 μm in diameter, were released from the parental female as it disintegrated.

The zygotes began to germinate one or two days after they were transferred to new growth medium. Initially, part of the outer reticulated wall ruptured (Pl. XX M) allowing the inner thin wall in this region to protrude and swell conspicuously (Pl. XX N). The reddish brown protoplast, or gone cell, grew two equal flagella as it squeezed out into the protuberance, in which

one or hyaline bodies, probably meiotic products, were observed (Pl. XX N). The thin-walled protuberance then ruptured and the reddish brown biflagellate gone cell moved out, leaving its empty wall behind. The gone cell was nearly spherical in shape and secreted a gelatinous envelope through which two flagella projected. As this envelope swelled conspicuously, the protoplast within the envelope divided to form a gone colony, as in asexual reproduction (Pl. XX O). Until the inversion stage of colony formation, the envelope containing the developing embryo swam by means of the two flagella which were retained intact from the original gone cell (Pl. XX O). The newly formed gone colony was reddish brown in color because of the persistence of pigment from the original zygote. After liberation from the envelope, the colony gradually became green as it grew. The number of of gonodia in the gone colony was generally four (Pl. XX P).

Clonal cultures started from the individual gone colonies were either male or female. Of 31 gonal cultures analyzed, there were 17 female and 14 male cultures produced.

Although the persistence of both the gelatinous envelope within which the gone colony develops and the two flagella provided by the gone cell during gone colony formation have not been previously documented in V. carteri, a similar situation was reported for V. (sect. Volvox) rousseletii G. S. West (Pocock 1933b).

Taxonomic account. Based on the present study and data from relevant literature, the species V. carteri can be divided into six infraspecific taxa based on the differences in asexual and

sexual colonies (Table 3). Although infraspecific taxa in Volvox [Merrillosphaera] carteri have been established as either forms or varieties, the six taxa are seen to be the same taxonomic rank. Iyengar (1933) represents the infraspecific taxa of this species as forms and recent investigators have used his classification (e.g. Starr 1969 1978, Kochert 1975, Birchem and Kochert 1979). Therefore, the six taxa within V. carteri are postulated to be represented as follows:

Volvox carteri Stein f. carteri. Stein, Der Organisms der Infusionsthiere. 3 Abt. 1 Hälfte. Leipzig: 134, 1878.

Volvox globator Carter (non Linnė). Carter, Ann. & Mag. Nat. Hist. 3rd. Ser.3: s, pl. 1, figs. 1, 3, 4, 7, 8, 10, 1859.

Merrillosphaera carteri (Stein) Shaw var. typica Shaw, Philip. J. Sci. 21: 119, pls. 7, 8, 1922a.

Volvox carteri Stein f. weismannia (Powers) Iyengar, J. Linn. Soc. Bot. 49: 364, 1933.

Volvox weismannia Powers, Trans. Amer. Microsc. Soc. 28: 152, pl. 24, figs. 25, 27, pls. 25, 26, 1908.

Merrillosphaera carteri (Stein) Shaw var. weismannia (Powers) Shaw, Philip. J. Sci. 21: 121, 1922a.

Volvox carteri Stein var. weismannia (Powers) Iyengar in Smith, Trans. Amer. Microsc. Soc. 63: 291, 1944.

Volvox carteri Stein f. manilana (Shaw) Iyengar, J. Linn. Soc. Bot. 49: 364, 1933.

Merrillosphaera carteri (Stein) Shaw var. manilana Shaw,

Phillip. J. Sci. **21**: 120, pls. 1-6, 1922a.

Volvox carteri Stein f. nagariensis Iyengar, J. Linn. Soc. Bot. **49**: 371, figs. 7A, 8F, pl. 28, fig. 18, 1933.

Volvox carteri Stein var. nagariensis Iyengar in Smith, Trans. Amer. Microsc. Soc. **63**: 295, 1944.

Volvox carteri Stein f. hazenii (Metzner) Nozaki stat. nov.

Volvox carteri Stein var. hazenii Metzner, Bull. Torrey Bot. Club **72**: 129, figs. 1-119, 1945.

Volvox carteri Stein f. kawasakiensis Nozaki f. nov.

Diagnosis:

Volvox carteri Stein f. kawasakiensis Nozaki f. nov. (Pl. XVII-XX)

Colonia asexualis matura fere spherica aut ovia, usque ad 750 µm longa, ex 500-3000 (plerumque 1000-2000) cellulis composita, 8-30 (plerumque 8-14) gonodia continens. Colonia mascula ovia aut spherca, ex 16-256 cellulis composita, sine cellulis somaticis vel cum pariter cellulis somaticis et androgonidiis. Colonia feminea fere spherica auto ovia, 42-81 ova continens.

TYPE LOCALITY: Minamikase, Nakahara-ku, Kawasaki-shi, Kanagawa Prefecture, Japan. Soil samples were collected by the author in January 1984.

HOLOTYPE: Pl. XVII A.

II. Astrephomenaceae

The family Astrephomenaceae contains only a single genus Astrephomene. This genus was established by Pocock in 1953 and contains a single species, A. gubernaculifera. Superficially, Astrephomeme's 32-, 64- or 128-celled spheroidal colonies with a gelatinous matrix resemble those of the family Volvocaceae. However, Astrephomene does not undergo inversion during colony formation, a characteristic of the volvocacean algae. It was on this basis that Pocock (1953) erected the new family, Astrephomenaceae.

Stein (1958a) and Brooks (1966) studied morphology and sexuality of this alga using unialgal cultures and observed its sexual reproduction in detail. Cave and Pocock (1956) counted the chromosome number and Boorks (1972) studied the physiology of A. gubernaculifera Pocock.

Since Pocock (1953) described A. gubernaculifera, no other species has been erected in the Astrephomenaceae, although Brooks (1966) studied the sexual compatibility of A. gubernaculifera using, 26 strains isolated from soils collected in various localities of the U.S.A. and Mexico.

Recently, however, I isolated two morphologically distinct colonies of Astrephomene from soils collected in Kanagawa Prefecture, Japan (Table 1). The vegetative colony and reproduction of one is identified to A. gubernaculifera. However, the other is believed to be a new species. The morphology, asexual and sexual reproduction of the two species of Astrephomene are described here.

Astrephomene gubernaculifera Pocock

Vegetative morphology. Colonies were ellipsoidal to spherical in shape, contained 64 or 32 (rarely 128 or 16) cells embedded in gelatinous cellular sheaths in the periphery of the colony (Fig. 59; Pl. XXI A, B) and lacked a watery gelatinous matrix encompassing the whole colony (Pl. XXI G). The 64- or 32-celled colony contained four or two, respectively, small somatic cells at the posterior pole (Pl. XXI B-D). The cellular sheaths were pentagonal or hexagonal in front view and compactly adhered to one another to form a hollow colonial structure (Pl. XXI B, E, F; Fig. 59). Mature 64-celled colonies measured 90-150 µm long. The cells were nearly spherical in shape and had two equal flagella, a stigma, many small contractile vacuoles on the surface and a cup-shaped chloroplast (Fig. 60). The chloroplasts did not show pyrenoids in any age of the culture. The stigma was large in the anterior cells, but gradually diminished towards the posterior pole (Fig. 59; Pl. XXI A). Reproductive cells measured up to 18 µm in diameter and somatic cells up to 10 µm in diameter.

The vegetative morphology agreed well with that described by Pocock (1953).

Asexual reproduction. The general features of asexual reproduction were essentially the same as previously described by Pocock (1953), Stein (1958a) and Brooks (1966). Each cell, except the posterior somatic cells, divided successively into a

daughter colony without inversion (Pl. XXI I). The second and third divisions were parallel and perpendicular to the first division, forming a group of eight cells arranged in two rows of four each (arrow, Pl. XXI H). The group gradually became more convex towards the outside of the parental colony to form a spheroidal colony within the parental cellular sheath during further divisions (Pl. XXI I).

However, it is on the mode of flagellar elongation in a daughter colony that my observations differed from previous reports (Pocock 1953, Stein 1958a, Brooks 1966). Just after the successive divisions, each reproductive cell in the newly formed colony grew only one flagellum, while the posterior two or four somatic cells grew two equal flagella (Pl. XXI J). After colony liberation, each reproductive cell began to grow the second flagellum. The two flagella in the reproductive cell subsequently became equal in length.

Sexual reproduction. Sexual reproduction was heterothallic and isogamous. During colony clumping or after the colony dissociated into individual cells, each reproductive cell escaped from its cellular sheath to become a biflagellate gamete. The gamete was spherical in shape and had the same organelles, except that it bore a mating papilla at the base of the flagella (Pl. XXI K). Plasmogamy was initiated by the union of the tips of the mating papillae of the two gametes during gamete clumping. Here many of the gametes aggregated in a clump with their flagellar tips ticking together, forming a single center. A quadriflagellate zygote was formed in the clumping group and then

separated from the clump (Pl. XXI L). The planozygote developed two, or sometimes more, pyrenoids and then settled down to become a smooth-walled aplanozygote, which turned reddish brown in color after about one week. The mature zygotes were spherical in shape and measured 12-20 μm in diameter (Pl. XXI M).

Under culture conditions for zygote germination, part of the zygote wall at first protruded (Pl. XXI N). The protruding wall then ruptured and a single biflagellate gone cell moved out, leaving the empty wall behind (Pl. XXI O). The gone cell was spherical in shape (Pl. XXI P) and secreted a gelatinous envelope through which the two flagella projected. Two to four hours after liberation, the gone cell settled down and divided into a gone colony within the envelope, as in asexual reproduction (P. XXI Q, R).

Although Brooks (1966) documented in detail gametic union and zygote germination of A. gubernaculifera, the gelatinous envelope within which the gone colony develops (Pl XXI Q, R) was not mentioned.

Astrephomene perforata Nozaki sp. nov.

Vegetative morphology. Colonies were ellipsoidal to spherical in shape and contained 64 or 32 (rarely 128 or 32) cells embedded in gelatinous cellular sheaths in the periphery of the colony (Fig. 61; Pl. XXII A-G). Irrespective of the colony cell number, 64 or 32, the colony contained two small somatic cells at the posterior pole (Fig. 61; Pl. XXII D). A watery gelatinous matrix encompassed the whole colony (Pl. XXII G). The

cellular sheaths were roughly circular in front view and were interconnected with neighboring cells to form a hollow colonial structure (Fig. 61; Pl. XXII A-C). The interconnecting sheaths formed small triangular, sometimes square, fenestrations between each cell (Fig. 61; Pl. XXII E, F). Mature 64-celled colonies were measured 110-200 μm long. The cells were lenticular to spherical in shape and had two equal flagella, many contractile vacuoles on the surface and a cup-shaped chloroplast (Fig. 62). The chloroplasts showed no pyrenoids in younger cultures (Pl. XX A, B, D). However, usually one, but sometimes two or three pyrenoids appeared in the brim of the cup-shaped chloroplast in three- to four-day-old cultures, when grown in the synthetic medium (Table 2) (Fig. 62; arrow, Pl. XXII C). Using the staining methods of Rosowski and Hoshaw (1970) the pyrenoids were clearly recognized in two-day-old cultures, but could not be detected in one-day-old cultured cells. The stigma was large in the anterior cells but gradually diminished towards the posterior pole (Fig. 61; Pl. XXII A). Reproductive cells measured up to 19 μm in surface diameter and somatic cells up to 10 μm in surface diameter.

Astrephomene perforata Nozaki differs from A. gubernaculifera in the shape of the cellular sheaths and the presence of pyrenoids in the vegetative cells. The cellular sheaths of A. gubernaculifera adhered compactly to one another and seemed to be pentagonal or hexagonal in front view because of their mutual compression (Fig. 59; Pl. XXI E, F). In contrast, the sheaths of A. perforata seemed to be roughly circular in

front view and were interconnected with the neighboring sheaths (Fig. 61; Pl. XXII E, F). The interconnecting sheaths showed small triangular or square fenestrations between cell. This difference could be clearly recognized in the materials stained with haematoxylin (Pl. XXI E, F, Pl. XXII E, F), irrespective of the age of the colony or culture conditions. The specific name perforata comes from these fenestrations formed by the interconnecting sheaths. In addition, colonies of A. perforata showed a watery gelatinous matrix surrounding the interconnecting sheaths (Pl. XXII G). It could be only recognized by ink preparation. However, as mentioned by Starr (1980), colonies of A. gubernaculifera never had such a matrix (Pl. XXI G).

The chloroplasts of the vegetative cells of A. gubernaculifera did not show pyrenoids in any age of the culture (Fig. 60; Pl. XXI A-D), as reported by Pocock (1953) and Stein (1958a). In A. perforata, on the other hand, one to three pyrenoids appeared in the brim of the cup-shaped chloroplast of each vegetative cell in older cultures (Fig. 62; Pl. XXII C).

The 64- or 32-celled of A. gubernaculifera contained four or two posterior somatic cells, respectively (Fig. 59; Pl. XXI C, D), while that of A. perforata had two somatic cells irrespective of the colony cell number, 64 or 32 (Fig. 61; Pl. XII D).

Asexual reproduction. Each cell, except the posterior somatic cells, divided successively to form a daughter colony without inversion (Pl. XX. I). The second and third divisions were parallel and perpendicular to the first division, forming a group of eight cells arranged in two rows of four each (arrow, Pl. XXII H). The group gradually became more convex towards the

outside of the parental colony to form a spheroidal colony within the parental gelatinous sheath during further divisions (Pl. XXII I). Just after the divisions, each reproductive cell in the newly formed colony grew only one flagellum, while the posterior two somatic cells grew two equal flagella (Pl. XXII J). After the daughter colony was released, each reproductive cell began to grow a second flagellum. The two flagella in the reproductive cells subsequently became equal in length.

Asexual reproduction in A. perforata was essentially the same as in A. gubernaculifera with regard to the mode of cell divisions and new flagellar elongation in both reproductive and somatic cells of daughter colonies.

Sexual reproduction. General features of sexual reproduction in A. perforata were essentially the same as those of A. gubernaculifera as described above.

Sexual reproduction was heterothallic and isogamous. During colony clumping (Pl. XXIII A) or after the dissociation of colonies into individual cells, each reproductive cell escaped from its cellular sheath (Pl. XXIII B) to become a biflagellate gamete. The gamete was spherical in shape and had the same organelles except that it bore a mating papilla at the base of the flagella (Pl. XXIII D). Many of the gametes soon formed the gamete clumping (Pl. XXIII C). Plasmogamy was initiated by the union of the mating papillae of the two gametes during gamete clumping, proceeding to form a planozygote. The planozygote was quadriflagellate and spherical in shape and had two or more pyrenoids. After swimming, several of the zygotes settled down

into a group of aplanozygotes (Pl. XXIII E), which turned reddish brown in color after about one week. These mature zygotes were smooth-walled, measuring 12-20 μm in diameter (Pl. XXIII F).

During zygote germination, a single biflagellate gone cell were released from the zygote wall (Pl. XXIII G-I). Two hyaline bodies, putative meiotic products, also could be seen (arrows, Pl. XXIII G, H). The gone cell was spherical in shape (Pl. XIII J), containing the reddish brown granules of the former zygote, and secreted a gelatinous envelope through which the two flagella projected. Two to four hours after liberation, the gone cell divided into a gone colony within the envelope, as in asexual reproduction (Pl. XXIII K, L). The two flagella provided by the original gone cell usually detached from the envelope just prior to the cell division, but sometimes remained attached to the envelope and functioned until the four- to 16-celled stage.

Diagnosis:

Astrephomene perforata Nozaki sp. nov. (Figs. 61, 62; Pls. XXII, XXIII)

Colonia ellipsoida aut spheric, ex 64 vel 32 cellulis composita, in vaginis cellulosis inclusis. Colonia 64 vel 32 cellularis 2 cellulas somaticas parvas continens. Vaginae cellulosae e fronte coloniae visu quasi circulares, vaginis propinquis interconnexae. Fenestrationes triangulares vel quadratae parvae inter quamque vaginam cellulosam formatae. Cellulae lenticulares auto sphericae. Chloroplastus poculiformis, in culturis junioribus nullas pyrenoides continens, in culturis vetustioribus in eius margine singularem pyrenoidem

formans. Reproductio non-sexualis coloniis filialibus effecta, ex omnibus cellulis, cellulis somaticis exceptis, sine inversione formatis. Reproductio sexualis isogametis effecta, ex omnibus cellulis reproductivis sine divisione liberatis formatis. Cum germinatione zygota zoosporam biflagellatam singularem generans.

TYPE LOCALITY: Nagae, Hayama-cho, Kanagawa Prefecture, Japan. Soil samples were collected by the author in December 1980.

HOLOTYPE: Figs. 61, 62.

III. Spondylomoraceae

The family Spondylomoraceae is based on Spondylomorum Ehrenberg (1848) as the type, but there are doubts as to the existence of this genus (Pringsheim 1960). The family is characterized by having coenobic colonies in which cells are arranged in tiers which are held together without the encompassing gelatinous matrix common to the families Volvocaceae and Astrephomenaceae (Bold and Wynne 1978, Starr 1980).

The genus Spondylomorum is characterized by having colonies with eight or 16 quadriflagellate cells. The four-celled colonies of Pascherina Silva (1959) [Pascheriella Korshikov (1928)] have biflagellate cells, the chloroplasts of which contain pyrenoids. The most encountered genus in this family is Pyrobotrys Arnoldi (1916). This alga has colonies composed of biflagellate cells whose chloroplasts lack pyrenoids.

Sexual reproduction in the Spondylomoraceae is known for Pascherina and Pyrobotrys, both genera exhibiting isogamy (Starr 1980). However, zygote germination in this family has not been observed.

Genus Pyrobotrys Arnoldi

The genus Pyrobotrys Arnoldi (1916) has a world-wide distribution but its occurrence is sporadic. This taxon also appears in the literature under the names Uva Playfair (1914) (Bourrelly 1962 1966, Fott 1967, 1971) or Chlamydobotrys Korshikov (1924) (Pascher 1927, Pringsheim 1960). Silva (1972), however, has resolved this nomenclatural confusion and used the name Pyrobotrys. Approximately 10 species have been described, mainly on the basis of differences in vegetative morphology (Huber-Pestalozzi 1961, Sarma and Shyam 1974).

Pringsheim (1960) established pure cultures of this alga collected in various habitats in Europe, South Africa and the United States, but he could not distinguish species because of the small morphological differences among strains, and the great morphological variability within one clone. Recently Sarma and Shyam (1974) observed P. acuminata Sarma et Shyam, and Hoops and Floyd (1982b) studied two species, P. stellata (Korshikov) Korshikov and P. elongata Korshikov. However, neither of them used pure or unialgal cultures of these organisms. Furthermore, the details of asexual and sexual reproduction based on pure cultured material have not been observed in Pyrobotrys.

The present study was undertaken to evaluate species concepts in Pyrobotrys and to observed its sexual reproduction in detail, and is based on the use of new pure culture methods (see Materials and Methods) and observations on 60 clones isolated from soils collected in various localities in Japan (Table 2). The morphology, asexual and sexual reproduction and taxonomy of the

four species of Pyrobotrys, P. stellata, P. squarrosa (Korshikov) Korshikov, P. casinoensis (Playfair) Silva and P. elegans (Behlau) Nozaki, comb. nov., are described here.

Pure cultures. Pringsheim (1960) established pure cultures of Chlamydobotrys [Pyrobotrys]; however, the change air conditions was not mentioned in his methods for culturing this alga. I have established pure cultures of four species of Pyrobotrys by new methods. Cultures were grown in a newly modified synthetic medium (Table 2) and placed under anaerobic conditions by exchanging the air for nitrogen gas. With the exception of P. casinoensis, anaerobic conditions were required for establishment of pure cultures.

Pyrobotrys is usually found in waters rich in organic matters (Bold and Wynne 1978, Starr 1980), where abundant bacteria grow and exhaust oxygen to alter the water to an anaerobic environment. Although various methods for pure or clonal cultures of algae have been described (e.g. Pringsheim 1946, Starr 1964 1978, Stein 1973, Nisizawa and Chihara 1979), little is known about the methods for establishing anaerobic pure cultures.

Using the pure cultures described above (see Materials and Methods), some morphological stability in the vegetative colonies of Pyrobotrys within clones was obtained. In the cultures of P. casinoensis, however, the shape of colonies varied to some extent within a single culture. The cells of the posterior tiers of the colony had some variability in degree of protrusion and attenuation of their posterior ends. They were either ovoid,

with a stumpy end, or pear-shaped with an acute end (Fig. 65; Pl. XXVI A-C). Pringsheim (1960) also reported similar morphological variability in the cells of Chlamydobotrys [Pyrobotrys]. In this study of Pyrobotrys, P. casinoensis was the only species whose cultures could be established even without exchanging the air for nitrogen gas. Therefore, it seems likely that Pringsheim 1960) cultured and observed only one species of Pyrobotrys, P. casinoensis.

Four species of Pyrobotrys could be recognized based on the following differences in cell shape and colony cell number.

P. stellata: colonies eight-celled, cells pear-shaped with a short, acute posterior end (Fig. 63; Pl. XXIV A-D).

P. squarrosa: colonies eight-celled, cells irregularly pear-shaped with a strongly inflated ventral side and and a long posterior tail with a somewhat blunted end (Fig. 63; Pl. XXV A-D).

P. casinoensis: colonies eight- or 16-celled, cells ovoid to pear-shaped; posterior cells often protruding and attenuating posterior ends (Fig. 65; Pl. XXVI A-E).

P. elegans: colonies eight- or 16-celled, cells ovoid to subspherical (Fig. 66; Pl. XXVIII A-D).

Regardless of the species, however, the newly formed colonies were compact and cells were fusiform with an acute posterior end. Therefore, it was not easy to determine which species such juvenile colonies belonged to.

Asexual reproduction. The four species of Pyrobotrys showed essentially the same process of daughter colony formation. The protoplasts of all the cells in a colony divided simultaneously three or four times (only three times in the 8-celled species, P.

stellata and P. squarrosa) within each parental cell wall (Fig. 67; Pl. XXIV E, XXVI F). The first division was longitudinal, perpendicular to the surface of the colony (Fig. 67a). The second division was nearly longitudinal, perpendicular to the first division, forming four nearly parallel cells (Fig. 67b). The two pairs of the two diagonal cells soon began to move apart from each other: one pair to the anterior end of the parental cell, the other pair to the posterior end, forming two alternating tiers of the two cells (Figs. 67c, d). Subsequently, one or two divisions occurred in each of the daughter cells (Fig. 67e) to form an eight- or 16-celled colony. Each cell of the newly formed colony then grew two equal flagella, showed a stigma and two contractile vacuoles, and changed posteriorly to become fusiform. After formation, the colony moved freely.

Sexual reproduction. Except for some heterothallic strains of P. casinoensis from Tokyo (Table 2), all the other strains of the four species of Pyrobotrys in this study were homothallic. The morphological features of sexual reproduction were essentially the same among the different species. After isogamous conjugation, however, the spherical to subspherical form of the planozygote soon changed to the characteristic form of each given species, as follows.

P. stellata: nearly spherical shape with a slight posterior projection (Fig. 68; Pl. XXI, V G, H).

P. squarrosa: elongate-ovoid shape (Fig. 69; Pl. XXV I, J).

P. casinoensis: fusiform with a long, acute posterior end, developing four stumpy radiate processes in the anterior half

when matured (Fig. 70; Pl. XXVI I-L).

P. elegans: ovoid to spherical shape (Fig. 71; Pl. XXVIII G, H).

Because this character was very stable, and its difference among the four species was very distinct, it is thought to be a reliable criterion for distinguishing species of Pyrobotrys.

Pyrobotrys stellata (Korshikov) Korshikov

Vegetative morphology. Colonies were star-shaped and consisted of eight cells compactly disposed in four alternating tiers of two opposed cells each (Fig. 63; Pl. XXIV A-C), measuring up to 40 μm long . The cells were pear-shaped with a short, acute posterior end (Pl. XXIV D; Fig. 63) and measured up to 20 μm long, with two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. The cell wall was delicate and had a small papilla at the base of the flagella. The two flagella were situated on the anterior end of the cell, or somewhat apart from the end. The protoplasts were seldom separated from the cell walls at the posterior end.

The vegetative morphology of this alga agreed well with that of Chlamydobotrys stellata Korshikov (1924). Although Pringsheim (1960) particularly doubted the delimitation between Chlamydobotrys stellata [Pyrobotrys stellata] and Chlamydobotrys gracilis Korshikov [Pyrobotrys casinoensis], these two algae can be clearly distinguished by their colony cell number and form of planozygotes (Figs. 63, 65, 68, 70; Pl. XXIV A-D, G, H, Pl. XXVI

A-E, I-L).

Sexual reproduction. Initially, each protoplast within the cell wall divided into four or eight small cells. These soon grew two equal flagella and dissociated, then were released from the parental cell wall to become individual isogametes. The gametes were spherical to subspherical in shape, measured 5-7 μm in diameter, and had a stigma, a cup-shaped chloroplast without a pyrenoid and two contractile vacuoles at the base of the flagella. They did not bear a tubular mating structure (mating papilla) at the base of the flagella.

Two of the gametes soon paired with their flagellar tips sticking together at the two opposite ends with regard to the two protoplasts. Plasmogamy was initiated by the union of each anterior end of the two protoplasts (Pl. XXIV F), and proceeded laterally and posteriorly to form a quadriflagellate zygotes. The mature planozygotes were 13-15 μm in diameter and nearly spherical with a slight posterior projection (Fig. 68; Pl. XXIV G, H). Although the zygotes at first had two stigmata shared from the gametes, they contained a single large stigma when matured. After swimming for two to three days, the planozygotes settled down and secreted a smooth cell wall. They became brown in color after about one month. The mature aplanozygotes were spherical in shape and measured 15-28 μm in diameter (Pl. XXIV I).

The form of the planozygotes agreed with that described by Strehlow (1929).

Pyrobotrys squarrosa (Korshikov) Korshikov

Vegetative morphology. Colonies were star-shaped and consisted of eight cells disposed in four alternating tiers of two opposed cells each (Fig. 64; Pl. XXV A-C), measuring up to 42 µm long. The cells were irregularly pear-shaped with a strongly inflated, ventral side and a long posterior tail with a somewhat blunted end (Fig. 64; Pl. XXV D) and measured up to 25 µm long, with two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. The cell wall was delicate and had a small papilla at the base of the flagella. The two flagella were situated on the anterior dorsal side, more or less apart from the anterior end of the cell (Fig. 64a; Pl. XXV D). The protoplasts were often separated from the cell walls at the posterior end.

The vegetative morphology of this alga agreed with that described by Korshikov (1928) as Chlamydobotrys squarrosa. According to him, the posterior portions of the vegetative cells are straight, whereas the cells of the cultures in this study were curved slightly backward (Fig. 64; Pl. XXV A-D). This situation is somewhat similar to that in P. incurva Arnoldi (Arnoldi 1916, Korshikov 1938, Bourrelly 1960), P. rostrata (Playfair) Huber-Pestalozzi (Playfair 1918) and P. acuminata Sarma et Shyam (1974). P. squarrosa differs from P. incurva in having only eight, rather than 16, cells in each colony. Furthermore, P. incurva has prominent lateral outgrowths of the cell walls joining the cells together. P. squarrosa also differs from P. rostrata and P. acuminata in the position of the

flagella. The two flagella of P. squarrosa were situated on the anterior dorsal side, more or less apart from the anterior end of the cell (Fig. 64a; Pl. XXV D), while those of the latter two species are located on the anterior end of the cell (Playfair 1918, Sarma and Shyam 1974).

Sexual reproduction. In the initial stage of sexual reproduction, the protoplast of each cell divided twice, three or four times to form a clump of four, eight or 16 daughter cells within the parental cell wall (Pl. XXV E). Each daughter cell then grew two equal flagella and the clump dissociated into individual naked biflagellate cells, which were released from the parental wall and functioned as gametes. These isogametes were spherical to ellipsoidal in shape (Pl. XXV F), measured 5-7 um long, and had a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. Tubular mating structures (mating papillae) were not recognized in the gametes.

The liberated gametes soon underwent conjugation. Two of the gametes paired with their flagellar tips sticking together at the two opposite ends with regard to the two protoplasts. Plasmogamy was initiated by the union of each anterior end of the two protoplasts (Pl. XXV G), proceeding laterally and posteriorly (Pl. XXV H) to form a quadriflagellate zygote. This planozygote was at first spherical to subspherical in shape, but soon became elongate-ovoid (Fig. 69; Pl. XXV I, J) and a thin cell wall was secreted. One of the two stigma shared from its former gametes became prominent, while the other gradually became indistinct.

The planozygote was 9-13 μm long. After swimming for about a half-day, the planozygotes lost their flagella to become non-motile (Pl. XXV K). Several to many of the zygotes then aggregated and gradually accumulated green granules, and increased in size, becoming spherical in shape (Fig. XXV L). The green granules in the zygotes gradually turned reddish brown in color as the zygotes became bigger and their walls thicker, forming mature aplanozygotes (Pl. XXV M) after about one month. They measured 15-28 μm in diameter.

Sexuality in P. squarrosa has not been reported previously.

Pyrobotrys casinoensis (Playfair) Silva

Vegetative morphology. Colonies were mulberry-shaped and consisted of eight or 16 cells disposed in four alternating tiers of the two or four opposed cells each, respectively (Fig. 65; Pl. XXVI A-D), measuring up to 53 μm long. The cells were ovoid to pear-shaped and measured up to 21 μm long, with two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. The cells in the posterior one or two tiers often protruded and were attenuated the posterior ends (Fig. 65; Pl. XXVI A-C). The cell wall was delicate and had a papilla in median size at the base of the flagella (Pl. XXVI E). The two flagella were situated on the anterior end of the cell. The protoplasts were sometimes separated from the cell walls at the posterior end.

The cells in the posterior tiers were variable in their degree of attenuation of the posterior ends (Fig. 65; Pl. XXVI

A-C). These cells were often pear-shaped with a protruded posterior end (Pl. XXVI B, C), but at other times might be ovoid with a stumpy one (Pl. XXVI A). In the latter case, the vegetative morphology agreed with Uva casinoensis Playfair (1914) and Chlamydobotrys gracilis Korshikov (1924). In the former case, however, the colonial appearance was similar to that of P. elongata Korshikov (1938b), although the cells in the most posterior tier of P. elongata have an enormous stigma, nearly one third of the cell length.

Sexual reproduction. In the first stage, each protoplast of the cells divided three, four or five times to form a clump of eight, 16, or 32 daughter cells within the parental cell wall (Pl. XXVI G). Each daughter cell then grew two equal flagella and the clump dissociated into individual, naked biflagellate isogametes, which were released from the parental wall. The gametes were ellipsoidal to spherical in shape, measured 4-9 μm long, had a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid (Pl. XXVI H). They lacked a tubular mating structure (mating papilla) in the anterior region (Pl. XXVI H).

The liberated gametes soon underwent conjugation. Two of the gametes paired and plasmogamy was initiated by the union of each anterior end of the two protoplasts, and proceeded laterally and posteriorly to form a quadriflagellate zygote. The newly formed planozygote was ovoid to subspherical in shape but soon became fusiform with a long, acute posterior end (Pl. XXVI I) and a thin cell wall was secreted. During motile period of three to

10 days, the planozygote developed four, radiate, stumpy processes in the anterior half as it accumulated green granules and increased in size (Pl. XXVI J-L). This mature planozygote had a single large stigma and measured up to 28 μm long (Fig. 70). The zygotes then settled down, lost their flagella, and became spherical in shape, and the cell walls became thick. They turned reddish brown in color after about one month, measuring 16-24 μm in diameter (Pl. XXVI M).

By use of abundant numbers of aplanozygotes of heterothallic strains, zygote germination was observed for the first time in the family Spondylomoraceae. The zygotes began to germinate one to three days after they were transferred from the darkness to the usual illuminated and anaerobic conditions.

Initially, part of the zygote protruded, becoming pear-shaped (Pl. XXVII A). The protoplast in the zygote wall then underwent two divisions, probably meiotic, to form four cells of nearly equal size (Pl. XXVII B, C). Subsequently, the tip of the protruded wall ruptured and the four biflagellate gone cells were released gradually and separately, leaving the empty cell wall behind (Pl. XXVII D-F). The gone cell was ovoid to elongate-ovoid in shape (Pl. XXVII G) and secreted a thin cell wall.

After swimming for several hours, the protoplast of the gone cell within the cell wall divided successively to form a gone colony, as in asexual reproduction (Pl. XXVII H). During colony formation, the two flagella provided by the original gone cell remained functional and the developing embryo within the cell wall swam with these flagella. The gone colony was usually eight-celled (Pl. XXVII I).

The form of planozygotes agreed with that described by Strehlow (1929), Behlau (1935), Silva and Papenfuss (1953) and Balakrishnan (1966), and also with that of the vegetative cells of the unicellular green alga, Chlorobrachis gracillima Korshikov (1925). Heterothallic sexuality in the genus Pyrobotrys has not been reported previously.

Pyrobotrys elegans (Behlau) Nozaki comb. nov.

Vegetative morphology. Colonies were mulberry-shaped and consisted of eight or 16 cells disposed in four alternating tiers of two or four opposed cells each, respectively (Fig. 66; Pl. XXVIII), measuring up to 48 µm long. The cells were ovoid to subspherical in shape (Fig. 66; Pl. XXVIII A-D) and measured up to 21 µm long, and had two equal flagella, a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. The cell wall was delicate and had a prominent papilla at the base of the flagella (Pl. XXVIII D). The two flagella were situated on the anterior end of the cell. The protoplasts were usually separated from the cell walls at the posterior end.

Behlau (1935) mentioned several differences in the vegetative morphology between Chlamydobotrys elegans Behlau [Pyrobotrys elegans (Behlau) Nozaki] and Chlamydobotrys gracilis Korshikov [Pyrobotrys casinoensis (Playfair) Silva], with regard to the frequency of the colony cell number eight or 16, the papillae of the cell walls, the bottom of the cup-shaped chloroplast and

the accumulation of starch. However, these differences in the present cultures were very small or unclear. Nonetheless, the shape of the vegetative cells of the two species were different from each other. Those of Pyrobotrys elegans were ovoid to subspherical with a stumpy posterior end and have nearly the same shape from anterior to posterior tiers of the colony (Fig. 66; Pl. XXVIII A-C), while the cells of P. casinoensis were pear-shaped to ovoid and had some variability in the degree of attenuation of their posterior ends in posterior tiers of the colony (Fig. 65; Pl XXVI A-D).

Sexual reproduction. Initially each protoplast of the cells divided twice, three or four times successively to form a clump of four, eight or 16 small cells within the parental cell wall. Each of the daughter cells then grew two equal flagella and the clump dissociated into individual, naked biflagellate isogametes, which were soon released from the parental wall. The gametes were ellipsoidal to spherical in shape, measured 6-10 μm long, and had a stigma, two contractile vacuoles at the base of the flagella and a cup-shaped chloroplast without a pyrenoid. They did not bear a tubular mating structure (mating papilla) at the base of the flagella.

Two of the gametes soon paired with their flagellar tips sticking together at the two opposite ends with regard to the two protoplasts. Plasmogamy was initiated by the union of each anterior end of the two protoplast (Pl. XXVIII E), and proceeded laterally and posteriorly (Pl. XXVIII F) to form a quadriflagellate zygote. The mature planozygotes were spherical to ovoid in shape (Fig. 71; Pl. XXVIII G, H) and measured 8-15 μm

in diameter. After swimming for several days, many of the zygotes aggregated and settled down to become aplanozygotes. The mature aplanozygotes were spherical in shape, had a smooth cell wall and contained brown granules. They measured 9-19 μm in diameter (Pl. XXVIII I).

The form of the planozygotes agreed well with that described by Behlau (1935) as Chlamydobotrys elegans. However, rather than stressing differences in the vegetative morphology between Pyrobotrys elegans and P. casinoensis, the major distinguishing feature in this study is the form of the planozygotes specific to each species (Figs. 70, 71; Pl. XXVI I-L, Pl. XXVIII G, H).

Taxonomic treatment. This species was originally described by Behlau (1935) under the name Chlamydobotrys elegans, on the basis of vegetative morphology and planozygotes and has not since been recorded by other workers. Therefore, a new combination is proposed here as follows.

Pyrobotrys elegans (Behlau) Nozaki comb. nov.

Basionym: Chlamydobotrys elegans Behlau, Beitr. Biol. Pfl. 23: 148, fig. 5, pl. 3, E, 1935.

TYPE LOCALITY: Between Gliwice and Tozek, Poland.

Conclusions

I. Comparative morphology of sexual reproduction in the colonial Volvocales.

A. Gametogenesis and conjugation.

Since isogamy is generally thought to be the most primitive form of sexual reproduction, gametogenesis and conjugation of the isogamous algae in the colonial Volvocales are first compared with one another. Pyrobotrys has a unique gametogenesis among the colonial Volvocales. The gametes of this alga are produced by successive divisions of the cells of the vegetative colony and their size is much smaller than the vegetative cell. Because of the small size of the isogametes, it may be expected that the plano- or aplanozygotes of Pyrobotrys increase in size conspicuously after gametic union (Pl. XXV K-M, Pl. XXVI I-M). Furthermore, these gametes have no anterior, tubular mating structure (mating papilla) (Pl. XXV F, Pl. XXVI H) which has been observed in other isogamous volvocacean and astrephomenacean algae. This situation may relate to the large motility effected by the small gametes; small gametes can move and pair rapidly.

On the other hand, the isogametes of the Volvocaceae and Astrephomenaceae are produced by the direct escape of the vegetative cells from the gelatinous matrix of the colony (Fig. 25; Pl. II B, C, Pl. IV E, Pl. VII D, Pl. IX G, Pl XI H, Pl. XXIII B). Therefore, the volume and organelles of these gametes are essentially the same as the cells in the vegetative colony.

Such big isogametes are thought to swim slowly and may have difficulty attaching their anterior regions to each other for conjugation. It is suggested, therefore, that these gametes bear an anterior tubular mating structure (mating papilla) (Figs. 5, 6, 9, 26; Pl. II D, Pl. IV, G, Pl. VII F, Pl. IX H, Pl. XI J, Pl. XX K, Pl. XXIII D; Figs. 5, 6, 9, 26) in order to facilitate the successful initial contact between the anterior regions of the two big protoplasts.

However, the form of the isogametes of Gonium sociale (Dujardin) Warming is different from the other members of the isogamous Volvocaceae and Astrephomenaceae. Excluding Gonium sociale, in the other members of these two families, each of the two conjugating gametes bears a mating papilla at the base of the flagella (Fig. 6; Pl. II F) and plasmogamy is initiated by the union of the tips of the two papilla (Figs. 9, 26; Pl. II G, H, Pl. VII K, Pl. XII A). In contrast, only one of the two conjugating gametes of G. sociale bears an anterior mating papilla (Pl. V A; Fig. 5) and plasmogamy is initiated by the union of the tip of the papilla and the anterior region of the other gamete, this not bearing a mating papilla (Pl. V B). Therefore two terms are proposed, "unilateral mating papilla" and "bilateral mating papilla" for the papilla of G. sociale and that of the other members of volvocacean and astrephomenacean algae, respectively, on the basis of the difference between their roles in plasmogamy.

Although the unilateral mating papillae exist only in Gonium sociale among the Volvocaceae and Astrephomenaceae, a similar

mating structure has been observed by electron microscopy in conjugating gametes of Chlamydomonas reinhardtii Dang. (Friedmann et al. 1968, Triemer and Brown 1975, Cavalier-Smith 1975, Goodenough and Weiss 1975). In C. reinhardtii, only one of the two conjugating gametes bears a tubular mating structure, which is termed "fertilization tubule" (Friedmann et al. 1968) or "gamosomal tubule" (Cavalier-Smith 1975).

Anisogamy and oogamy are exhibited only in a single family, Volvocaceae, among the colonial Volvocales. The trend from isogamy through anisogamy to oogamy in the Volvocaceae seems to be closely related to the increase of colony cell number (Table 4). The anisogamous conjugation in Eudorina elegans Ehrenberg is initiated by the entrance of the anterior end (including the flagellar base) of the male gamete into the anterior region of the female gamete and plasmogamy proceeds laterally and posteriorly (Figs. 42-46). This type of anisogamous conjugation is similar to that in isogamous algae, with particular regard to the mode of plasmogamy. These results suggest that the slender cytoplasmic protrusion at the base of the flagella of the male gamete of E. elegans (Fig. 41; Pl. XIII I, J) might be homologous to the bilateral mating papilla, which is also located at the base of the flagella of the isogametes and initiates plasmogamy. The genus Eudorina is thought to be closely related to Pandorina unicocca Rayburn et Starr, which also has a bilateral mating papilla, based on the similarity of colonial organization and asexual reproduction (see the discussion of P. unicocca).

On the other hand, the sperm of oogamous Volvox carteri Stein does not bear such a tubular structure at the base of the

flagella (Pl. XX D) and plasmogamy seems to be specialized (Figs. 55-58), lacking the lateral fusion observed in the isogamous and anisogamous algae.

B. Zygote germination.

Pyrobotrys (Spondylomoraceae) and Gonium sociale (Dujardin) Warming (Volvocaceae) exhibit a similar mode of zygote germination. The germinating zygotes of both algae give rise to four biflagellate gone cells separately (Pl. VI D-G, Pl. XXVII C-F). However, this mode of zygote germination seems primitive, because the four meiotic products of the germinating zygote are thought to become four gone cells directly, and various members of the unicellular Volvocales [e.g. Haematococcus (Pocock 1959), Chlamydomonas (Bold and Wynne 1978) and Pseudocarteria (Suda personal communication)] usually exhibit this type of zygote germination. Therefore, the similarity of zygote germination in Pyrobotrys and G. sociale does not necessarily indicate a close phylogenetic relationship between the two algae.

In the volvocacean algae, however, G. sociale is unique in having this type of zygote germination. The genera characterized by having spheroidal colonies in the Volvocaceae (Pandorina, Volvulina, Eudorina and Volvox) and the Astrephomenaceae have germinating zygotes which give rise to a single gone cell (Pl. VIII B, Pl. X D, Pl. XII G-I, Pl. XV A-D, Pl. XX N, Pl. XXI O, Pl. XXIII G-I), except for some taxa of Eudorina (Pl. XVI H-K). Zygote germination of this type results from a concentration of

food material from the zygote into only a single cell among the meiotic products, in order to ensure the optimum development of the gone colony. This situation seems to be similar to oogenesis in the higher animals. Therefore, the hyaline bodies observed in zygote germination (Figs. 13, 14, 32, 33; Pl. X C, Pl. XII G, Pl. XV A, D, E, Pl. XVI E, F, Pl. XX N, Pl. XXIII G, H) are thought to have the same role as the polar bodies observed in the oogenesis in the higher animals. The situation of two viable gone cells from a single germinating zygote in Eudorina (Pl. XVI G-K) may be caused by the fact that the accumulation of food material from anisogamous conjugation can fully serve the development of two gone colonies with a relatively small cell number, up to 32.

The processes of gone colony formation in the members of the Volvocaceae are essentially the same with regard to the fate of the two flagella and the gelatinous envelope provided by the gone cell. During colony formation, the gelatinous envelope containing the developing embryo swims by means of the two flagella, which are retained intact from the original gone cell (Figs. 15-20, 34-39, 47-53)

II. Phylogenetic relationships within the colonial Volvocales.

Based on essential differences in the gametogenesis and the mode of conjugation among the isogamous colonial Volvocales, two large phylogenetic groups can be recognized. One of them is the Spondylomoraceae, including Pyrobotrys. The isogametes of this alga are produced by the successive divisions of the colonial

cells. They are small and bear no tubular mating structure (mating papilla). In addition, the colonial morphology (colonies lacking an encompassing gelatinous matrix) and the mode of asexual reproduction (Fig. 67) are essentially different from those of the other two families. Therefore, the Spondylomoraceae should be placed in a phylogenetic position largely separated from the other members of the colonial Volvocales, the Volvocaceae and the Astrephomenaceae.

Astrephomene belonging to the Astrephomenaceae, Gonium pectorale Müller, Pandorina and Volvulina have essentially the same mode of gametogenesis and bilateral mating papillae. Lang (1963) observed, by electron microscopy, the vegetative cells of the Volvocaceae (from four-celled Gonium sociale to Volvox) and Astrephomenaceae, and concluded that the ultrastructure of the vegetative cells of these algae were essentially the same with regard to pyrenoid morphology and arrangement of organelles in the cell. Therefore, algae with bilateral mating papillae may constitute a closely related phylogenetic group, with colonies consisting of many (not less than 16) cells.

Furthermore, this group is considered to be subdivided into two phylogenetic groups, based on differences in the mode of daughter colony formation and the structure of the gelatinous matrix. One group consists of Gonium pectorale and Astrephomene. These algae exhibit essentially the same mode of daughter colony formation with particular regard to the eight-celled embryo (cells arrange in two rows of four each) (Pl. I C, Pl. XXI H, Pl. XXII H) and new flagellar elongation (each daughter cell of the

colony at first grows only one flagellum) (Pl. I D-F, Pl. XXI J, Pl. XXII J). In addition, each cell of the vegetative colonies of G. pectorale and Astrephomene is surrounded by a tripartite boundary of the gelatinous matrix at the ultrastructural level (Hoops and Floyd 1982a, Greuel and Floyd 1985).

The other group consists of Pandorina and Volvulina. This group is characterized by having the same mode of daughter colony formation [cells arrange cruciately in eight-celled stage (Figs. 18, 38; Pl. VIII H, Pl. X I, Pl. XI E) and inversion occurs]. Especially, P. morum Bory and Volvulina have essentially the same mode of asexual and sexual reproduction (see the discussion of Volvulina). They also have the same ultrastructure of the gelatinous matrix of the colony (Fulton 1978, Nozaki et al. 1987): the tripartite boundary of the gelatinous matrix surrounds the entire colony and the innermost zone of the matrix penetrates between the cells into the central region of the colony, forming keystone-shaped spaces observed by light microscopy (Pl. VII B, Pl. XI C-F). Therefore these two algae are thought to be especially closely related phylogenetically with each other. It is speculated that Volvulina evolved from algae similar to Pandorina morum, only by changing the cell shape to lenticular.

Although Eudorina and Volvox are anisogamous and oogamous, respectively, both of the genera exhibit essentially the same mode of daughter colony formation as Pandorina and Volvulina. In addition, Eudorina is thought to be closely related to Pandorina unicocca Rayburn et Starr, based on the similarity of colonial organization and asexual reproduction (see the

discussion of P. unicocca). Therefore, the anisogamous Eudorina is thought to have evolved from the organisms similar to P. unicocca, by acquisition of anisogamous sexual reproduction with sperm packets. The oogamous Volvox is thought to have evolved from Eudorina-like algae by acquisition of somatic cells and oogamous sexual reproduction, in relation to an increase of colony cell number (Table 4).

Although Gonium sociale is unique among the Volvocaceae and Astrephomenaceae in having a unilateral mating papilla, there is no essential difference between G. sociale and the other members of these two families, based on the observations of ultrastructure of the vegetative colonies (Lang 1963, unpublished data) as well as on the studies of the sensitivity to cell wall lytic enzyme and cell wall antigenicity in the Volvocaceae and Astrephomenaceae (Matsuda personal communication). Therefore, G. sociale should not be separated from the other members of these algae phylogenetically. It seems to be a organism which retained the most primitive types of colonial organization (four-celled colony) and zygote germination (Pl. VI A-G).

The suggested phylogenetic relationships within the three families of the colonial Volvocales presented here are represented in Fig. 72.

Summary

Morphological details of sexual reproduction at light microscope level in the seven genera of the three families, the Volvocaceae, the Astrephomenaceae and the Spondylomoraceae, of the colonial Volvocales (Chlorophyta) were observed using clonal cultures under controlled laboratory conditions, with particular regard to conjugation between gametes and zygote germination. In Gonium pectorale Müller (Volvocaceae), each of the two conjugating gametes bore a tubular mating structure (bilateral mating papilla) at the base of the flagella and a germinating zygote gave rise to four biflagellate gone cells, joined in a colony (germ colony). In contrast, only one of the two conjugating gametes of G. sociale (Dujardin) Warming bore a tubular mating structure (unilateral mating papilla) and four biflagellate gone cells were released separately from a germinating zygote of this alga. Based on these two differences in sexual reproduction, it is suggested that G. sociale should be placed in a genus different from G. pectorale (the type species of Gonium) and a new combination is proposed: Tetrabaena socialis (Dujardin) Nozaki comb. nov.

Pandorina and Volvulina (Volvocaceae) had isogametes with bilateral mating papillae and germinating zygotes which produced a single biflagellate gone cell. The details of anisogamous conjugation was observed in Eudorina elegans Ehrenberg (Volvocaceae). Fusion of this alga was initiated by entrance of the anterior end (including the flagellar base) of the male gamete, and proceeded laterally and posteriorly. Male gametes of

E. elegans bore a slender cytoplasmic protrusion at the base of the flagella and this structure is thought to be homologous to the bilateral mating papilla of the isogamete. Germinating zygotes of E. elegans produced one or two, viable biflagellate gone cells and gone colony formation was observed in detail. Fertilization between sperm and egg was observed for the first time in Volvox, using strains of V. carteri Stein f. kawasakiensis Nozaki f. nov. Fertilization was initiated by the entrance of the anterior end of the sperm into the anterior region of the egg and the sperm penetrated the egg from anterior to posterior portions. The sperm of Volvox bore no tubular cytoplasmic protrusions, like those observed in the male gametes of Eudorina.

Since Pocock (1953) erected the family Astrephomenaceae, only a single species, Astrephomene gubernaculifera Pocock, has been identified. However, I collected two morphologically distinct colonies of Astrephomene from Japan. One was identified with A. gubernaculifera, while the other was believed to be a new species, A. perforata Nozaki sp. nov. Sexual reproduction of these two species was essentially the same, featuring isogametes with bilateral mating papillae and germinating zygotes which gave rise to a single gone cell.

Sexual reproduction in the four species of Pyrobotrys (Spondylomoraceae) was observed in detail using anaerobic pure cultures, the methods for establishment of which have not been previously described for green algae. These four species showed essentially the same unique mode of sexual reproduction, with

having small isogametes which were produced by successive divisions of colonial cells. These gametes bore no tubular mating structures (mating papillae), like those observed in the isogamous volvocacean and astrephomenacean algae. Zygote germination was observed for the first time in the Spondylomoraceae using abundant aplanozygotes produced by heterothallic strains of Pyrobotrys casinoensis (Playfair) Silva. Germinating zygotes of this alga released four biflagellate gone cells separately. Heterothallic sexuality has not been previously reported in Pyrobotrys.

Based on essential differences in gametogenesis and mating structures in the isogamous algae in these colonial green flagellates, two large, distinct phylogenetic groups are recognized. One of the two is the Spondylomoraceae, including Pyrobotrys, characterized by small isogametes bearing no mating papillae. The other includes the Volvocaceae and the Astrephomenaceae with the isogametes bearing mating papillae. Furthermore, the latter group may be subdivided into two subgroups based on differences in the mode of asexual reproduction and the structure of the gelatinous matrix: the Gonium pectorale-Astrephomene group and the Pandorina-Volvulina group. It is speculated that anisogamous Eudorina has evolved from organisms similar to Pandorina unicocca Rayburn et Starr and oogamous Volvox from Eudorina-like algae. Gonium sociale seems to be an organism which retains the primitive types of colonial organization and sexual reproduction.

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Table 1. Samples from which strains of the colonial Volvocales were established in Japan

Taxon	Sample	Habitat and locality	Date	No. of strains isolated
Volvocaceae				
Gonium pectorale	Water	Small pond, Hiyoshi, Kohoku-ku, Yokohama-shi, Kanagawa	Apr. 6,'79	8
Gonium sociale	Soil	Small pond, Hiyoshi, Kohoku-ku, Yokohama-shi, Kanagawa	Aug.24,'82	9
Pandorina morum	Water	Kawai Dam, Yamanaka-cho, Ishikawa	Aug.20,'77	5
Pandorina unicocca	Water	Pond, Nobi, Yokosuka-shi, Kanagawa	May 5,'79	5
Volvulina steinii	Soil	Paddy field, Nagae, Hayama-cho, Kanagawa	Dec. 4,'80	10
Eudorina elegans var. *elegans*	Water	Moat of the Imperial Palace, Chiyoda-ku, Tokyo	Sep.14,'77	12
Eudorina elegans var. *synoica*	Soil	Paddy field, Miho-cho, Midori-ku, Yokohama-shi, Kanagawa	Jan.10,'80	7
Volvox carteri f. *kawasakiensis*	Soil	Minamikase, Nakahara-ku, Kawasaki-shi, Kanagawa	Jan.31,'84	7
Astrephomenaceae				
Astrephomene gubernaculifera	Soil	Paddy field, Yoshidashima, Kaisei-machi, Kanagawa	Apr.26,'81	9
Astrephomene perforata	Soil	Paddy field, Nagae, Hayama-cho, Kanagawa	Dec. 4,'80	12

Table 1. Continued

Taxon	Sample	Habitat and locality	Date	No. of strains isolated
Spondylomoraceae				
Pyrobotrys stellata	Soil	Paddy field, Ichibu, Tomiyama-machi, Chiba	Oct.19,'80	6
	Soil	Paddy field, Nagae, Hayama-cho, Kanagawa	Dec. 4,'80	6
	Soil	Midorogaike Pond, Kita-ku, Kyoto-shi, Kyoto	Oct. 4,'83	4
	Soil	Paddy field, Manda,	Apr.26,'81	8
Pyrobotrys squarrosa	Soil	Paddy field, Nagae, Hayama-cho, Kanagawa	Dec. 4,'80	3
Pyrobotrys casinoensis [homothallic]	Soil	Paddy field, Dainaka, Kimitsu-shi, Chiba	Oct.19,'80	6
	Soil	Paddy field, Manda, Hirastuka-shi, Kanagawa	Apr.26,'81	4
Pyrobotrys casinoensis [heterothallic]	Soil	Rain water pool, Izumi, Komae-shi, Tokyo	Apr.14,'84	6
Pyrobotrys elegans	Soil	Paddy field, Nagaoka, Tendou-shi, Yamagata	Sep.28,'80	5
	Soil	Small pond, Hiyoshi, Kohoku-ku, Yokohama-shi, Kanagawa	Aug.24,'82	8
	Soil	Paddy field, Kotoi,	Mar.25,'85	4

Table 2. Composition of media and culture conditions of the colonial Volvocales

Component	1)* Growth medium	2)** Growth medium	3) Mating medium	4) Mating medium	5)** Growth medium	6) Mating medium
$Ca(No_3)_2 \cdot 4H_2O$	-	118 mg	40 mg	40 mg	118 mg	118mg
KNO_3	101 mg	-	-	-	-	-
NH_4NO_3	-	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	49 mg	40 mg	40 mg	40 mg	40 mg	40 mg
$CaCl_2 \cdot {}_2O$	70 mg	-	50 mg	50 mg	-	-
KCl	-	50 mg	50 mg	50 mg	50 mg	50 mg
Na_2-β-glycerophostate	153 mg	50 mg	50 mg	50 mg	50 mg	50 mg
Glycylglycine	132 mg	500 mg	500 mg	500 mg	500 mg	500 mg
L-Histidine	155 mg	-	-	-	-	-
Na-acetate$\cdot 3H_2O$	200mg	200 mg	-	200 mg	200 mg	400 mg
Glucose	200 mg	-	-	-	-	-
Vitamin mixture***	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
P IV metals****	3 ml	3 ml	3 ml	3 ml	3 ml	3 ml
Distilled H_2O	995 ml	995 ml	995 ml	995 ml	995 ml	995 ml
pH	7.6	7.4	7.6	7.6	7.4	7.4

*M3-medium (Rayburn and Starr 1974) modified.
**Volvox-medium (Provasoli and Pintner 1959) modified.
***Vitamin mixture solution, which contains the following (in mg/l): Vitamin B_1, 25; Vitamin$_2$, 2.5; Vitamin B_6, 10; Vitamin$_{12}$, 0.1; Biotin, 5.
****P IV metals stock solution, which contains the following (in mg/l): Na_2EDTA, 1500; $FeCl_3 \cdot 6H_2O$, 194; $MnCl_2 \cdot 4H_2O$, 82; $ZnCl_2$, 10; Na_2MoO_4, 8; $CoCl_2 \cdot 6H_2O$, 4.
1): Gonium pectorale, Pandorina, Volvulina and Astrephomene [20° C, 14:10 LD (4000 lux)]
1) and 3): Gonium sociale [20°C, 14:10 LD (4000 lux)].
2) and 4): Eudorina elegans [23°C, 15:9 LD (4000 lux)].
2): Volvox carteri [25°C 16:8 LD (10000 lux)].
5) and 6): Pyrobotrys [20°C, 14:10 LD (7000 lux)].

Table 3. Comparison of features in six forms of *Volvox carteri*

Form	f. *carteri*	f. *weismannia*	f. *manilana*
Asexual colony			
Cell number	2100-6100	2000-7500	up to 11400
Size	480-770 µm	640-800 µm	720-760 µm
Number of gonidia	usually 8 (up to 10)	usually 10 (up to 12)	usually 8 (up to 12)
Male colony			
Cell number*	510-630	200-500	1900-2600
Number of androgonidia (sperm packets)	74-103	ca. 50	120-200
Ratio of somatic cells to androgonidia	5-6:1	3-9:1	8.5-21:1
Female colony			
Number of eggs	21-50	8-25	24-40
References	Carter (1859) Shaw (1922a) Smith (1944)	Powers (1908) Shaw (1922a) Kochert (1968)	Shaw (1922a)

*Total number of somatic cells and androgonidia (sperm packets).

Table 3. Continued

Form	f. *nagariensis*	f. *hazenii*	f. *kawasakiensis*
Asexual colony			
Cell number	5000-8000	4000-5000	500-3000
Size	up to 1100 µm	500-758 µm	up to 750 µm
Number of gonidia	usually 15-16 (up to 16-21)	characteristically 8	usually 8-14 (up to 30)
Male colony			
Cell number*	up to 512	600-1100	16-256
Number of androgonidia (sperm packets)	up to 256	ca. 50	16-128
Ratio of somatic cells to androgonidia	only 1:1	11-21:1	1:1 and 0:1
Female colony			
Number of eggs	20-64	8-23	42-81
References	Iyengar (1933) Starr (1969) Present data	Metzner (1945)	Present data

*Total number of somatic cells and androgonidia (sperm packets).

Table 4. Characteristics of genera of Volvocaceae

Genus	Colony cell number	Cellular differentiation	Sexual reproduction
Gonium	16, 8 or 4	all reproductive cells	isogamous
Pandorina	16 or 8	all reproductive cells	isogamous
Volvulina	16 or 8	all reproductive cells	isogamous
Eudorina	32 or 16	all reproductive or *rarely facultatively somatic in only anterior four cells	anisogamous
Pleodorina	128, 64 or 32	anterior somatic and posterior reproductive cells	anisogamous
Volvox	50000-500	almost all somatic cells	oogamous

*E. illinoisensis.

Figs. 1-3. Instruments for establishing anaerobic pure cultures of **Pyrobotrys**.

Fig. 1. Double-cap (left) and screw-cap (right) tubes.

Figs. 2, 3. Screw-cap tubes enclosed within double-cap tubes. d, double-cap tube; s, screw-cap tube; m, liquid medium.

Figs. 4-6. Sexual reproduction in two species of **Gonium.**

Fig. 4. Newly released gone cells of G. sociale (Dujardin) Warming. (a) front view; (b) side view.

Figs. 5, 6. Diagrams of conjugating gametes. All at same magnification.

Fig. 5. G. sociale. Arrow indicates unilateral mating papilla.

Fig. 6. G. pectorale Müller. Arrow indicates bilateral mating papilla.

Figs. 7-9. *Pandorina morum* Bory.

Fig. 7. 16-celled vegetative colony. Scale represents 20 μm.

Fig. 8. Asexual reproduction. Daughter colony before secretion of new gelatinous matrix is embedded in each keystone-shaped space of parental gelatinous matrix. Note two equal flagella in each cell of the colony. Scale represents 10 μm.

Fig. 9. Gamete clumping and conjugating gametes in sexual reproduction. Note mating papillae (mp) and cytoplasmic bridge (cb) formed by the mutual papillae. Arrow indicates joined flagellar tips of the gametes. Scale represents 20 μm.

Figs. 10-12. *Pandorina unicocca* **Rayburn et Starr.** All at same magnification. Scale represents 20 µm.

Fig. 10. 32-celled vegetative colony.

FIg. 11. 16-celled vegetative colony.

Fig. 12. Asexual reproduction. Daughter colony with apparently uniflagellate cells is embedded in each transparent vesicle in confluent gelatinous envelope of the parental colony.

Figs. 13-21. Sexual reproduction in **Pandorina unicocca** Rayburn et **Starr.** All at same magnification. Scale represents 20 μm.

Figs. 13-15. Zygote germination.

Fig. 13. Germinating zygote with its protoplast (gone cell) growing two equal flagella into space formed by thin-walled protuberance. Note hyaline bodies in this space.

Fig. 14. Biflagellate gone cell escaping from zygote wall. Note three hyaline bodies.

Fig. 15. Biflagellate gone cell with gelatinous envelope.

Figs. 16-21. Gone colony formation. Note both flagella and gelatinous envelope provided by gone cell persisting during the colony formation

Fig. 16. Two-celled stage.

Fig. 17. Four-celled stage.

Fig. 18. Eight-celled stage.

Fig. 19. 16-celled stage.

Fig. 20. Inversion stage of 16-celled plakea.

Fig. 21. 16-celled gone colony in gelatinous envelope. Note the colony having apparently uniflagellate cells.

Figs. 22-24. Volvulina steinii Playfair. All at same magnification. Scale represents 20 μm.

Fig. 22. 16-celled mature vegetative colony in four-day-old culture. Note pyrenoid (py) in the brim of the cup-shaped chloroplast of each cell.

Fig. 23. 16-celled colony in one-day-old culture. Each cell has no pyrenoid. Note stigma (s) in one of the cells of the third tier.

Fig. 24. Asexual reproduction. Daughter colony is embedded in keystone-shaped space formed in gelatinous matrix of the parent. Note the colony having biflagellate cells and a stigma (s) in one of cells of the colony.

Figs. 25-40. Sexual reproduction in **Volvulina steinii Playfair.** All at same magnification. Scale represents 20 μm.

Fig. 25. Gamete release.

Fig. 26. Gamete clumping and gametic union. Note mating papilla (pa) and cytoplasmic bridge (cb).

Fig. 27. Quadriflagellate motile zygote.

Fig. 28. Zygotes settling down with their flagella shortening.

Fig. 29. Young, green zygotes with cell walls and pyrenoids.

Fig. 30. Reddish brown mature zygotes.

Figs. 31-33. Successive stages of zygote germination. Note hyaline bodies (hb).

Fig. 34. Biflagellate gone cell.

Figs. 35-40. Gone colony formation. Note both flagella and gelatinous envelope provided by gone cell persisting during the colony formation.

Fig. 35. Gone cell with gelatinous envelope before cell divisions.

Fig. 36. Two-celled stage.

Fig. 37. Four-celled stage.

Fig. 38. Eight-celled stage.

Fig. 39. Inversion stage of 8-celled plakea.

Fig. 40. Newly formed 8-celled gone colony in gelatinous envelope.

Figs. 41-46. **Eudorina elegans Ehrenberg var. elegans.**

Fig. 41. Male gamete. Note slender cytoplasmic protrusion (cp) (putative mating structure) at the base of the flagella, stigma (s) and contractile vacuole (cv).

Figs. 42-46. Diagrams of stages of conjugation. All at same magnification. cg, confluent gelatinous envelope of female colony; ff, flagellum of female gamete; fs, stigma of female gamete; mf, flagellum of male gamete; ms, stigma of male gamete; tv, transparent vesicle cast by female gamete.

Fig. 42. Male gamete landing on the lateral anterior portion of female gamete just before plasmogamy.

Fig. 43. Initial stage of plasmogamy.

Fig. 44. Middle stage of plasmogamy.

Fig. 45. Late stage of plasmogamy.

Fig. 46. Quadriflagellate zygote just after plasmogamy.

Figs. 47-54. Gone colony formation in **Eudorina elegans Ehrenberg var. elegans.** All at same magnification. Note transparent strand connecting the two flagella and one of the inner daughter cells, and gelatinous envelope surrounding the developing embryo in each stage.

Fig. 47. Biflagellate gone cell.

Fig. 48. Gone cell with gelatinous envelope.

Fig. 49. Two-celled stage.

Fig. 50. Four-celled stage.

Fig. 51. Eight-celled stage.

Fig. 52. 16-celled stage.

Fig. 53. Lateral view of 32-celled plakea.

Fig. 54. 32-celled gone colony in gelatinous envelope.

Figs. 55-58. Diagrams of stages in fertilization of **Volvox carteri f. kawasakiensis Nozaki.** All at same magnification. s, sperm; e, egg.

Fig. 55. Sperm attaching to the lateral anterior portion of egg before fertilization.

Fig. 56. Sperm retracting its posterior tail just before entering the egg.

Fig. 57. Middle stage of fertilization.

Fig. 58. Late stage of fertilization showing posterior tail (arrow) of the entering sperm.

Figs. 59-62. Vegetative phase of two species of **Astrephomene** Pocock. cv, contractile vacuole; s, stigma; p, pyrenoid.

Figs. 59, 60. A. gubernaculifera Pocock.

Fig. 59. 64-celled colony.

Fig. 60. Lateral view of reproductive cell.

Figs. 61, 62. A. perforata Nozaki.

Fig. 61. 64-celled colony.

Fig. 62. Lateral view of reproductive cell.

Figs. 63-66. Line drawings of vegetative colonies of four species of **Pyrobotrys**. All at same magnification.

Fig. 63. P. stellata (Korshikov) Korshikov.

Fig. 64. P. squarrosa (Korshikov) Korshikov.

Fig. 65. P. casinoensis (Playfair) Silva.

Fig. 66. P. elegans (Behlau) Nozaki.

Fig. 67. Diagrams showing asexual reproduction in **Pyrobotrys stellata (Korshikov) Korshikov.** (a) two-celled stage, (b) early four-celled stage, (c) middle four-celled stage, (d) late four-celled stage, (e) eight-celled stage. pc, parental cell wall; ps, parental stigma.

Figs. 68-71. Line drawings of planozygotes of four species of *Pyrobotrys*. All at same magnification.

Fig. 68. *P. stellata* (Korshikov) Korshikov.

Fig. 69. *P. squarrosa* (Korshikov) Korshikov.

Fig. 70. *P. casinoensis* (Playfair) Silva. (a) lateral view, (b) bottom view.

Fig. 71. *P. elegans* (Behlau) Nozaki.

Fig. 72. Schematic representation of phylogenetic relationships within the three families of the colonial Volvocales.

Plate I. Gonium pectorale Müller.

Scale in Fig. C and Fig. F represent 20 um and applies to Figs. A-C and Figs. D-F. D-F: Phase contrast.

A: 16-celled vegetative colony.

B: Eight-celled vegetative colony.

C: Asexual reproduction. Arrow indicates eight-celled embryo.

D: 4-celled daughter colony with uniflagellate cells within parental gelatinous cellular sheath (arrows).

E: Newly formed colony with 16 uniflagellate cells.

F: Eight-celled young colony. Arrow indicates developing secon flagellum.

Plate II. Phase contrast micrographs of sexual reproduction in *Gonium pectorale* Müller.

Scale in Fig. K represents 20 µm and applies to Figs. A-D, F-K. Scale in Fig. E. represents 20 µm.

A: Dissociated individual cell surrounded by gelatinous sheath (arrows).

B, C: Gamete escaping from its cellular sheath (arrows).

D: Biflagellate gamete. A slender cytoplasmic protrusion (arrow) is at the base of the flagella (arrow heads).

E: Gamete clumping and fusing gametes (arrow head). Arrow indicates sticking flagellar tips of the clumping gametes.

F: Pair of gametes just before plasmogamy. Both gametes bear a slender cytoplasmic protrusion (arrow).

G: Two gametes with the tips of the protrusions connected.

H: Two gametes forming cytoplasmic bridge between them.

I: Two gametes fusing with their anterior regions.

J: Late stage of plasmogamy.

K: Quadriflagellate zygote.

Plate III. <u>Gonium pectorale</u> **Müller.**

All at same magnification. Scale in Fig. F represents 10 µm. A, B, E: Phase contrast.

A: Mature aplanozygotes. Each zygote bears a gelatinous wall (arrow head).

B-F: Zygote germination.

B: Two-celled stage of germinating zygote.

C: Four-celled stage of germinating zygote.

D: Four gone colony joined in a colony (germ colony) escaping from the zygote wall.

E: Empty zygote wall after liberation of germ colony.

G: Germ colony just after liberation. Ink preparation.

Plate IV. Gonium sociale (Dujardin) Warming.

B-H: Phase contrast.

A: Vegetative colony. Ink preparation.

B: Asexual reproduction.

C: Newly formed daughter colony within the parental gelatinous sheath. Note each cell growing two equal flagella.

D-H: Sexual reproduction.

D: Dissociated individual cell surrounded by gelatinous sheath (arrow heads).

E: Gamete escaping from the cellular sheath (arrow heads).

F: Biflagellate gamete lacking cytoplasmic protrusion.

G: Gamete bearing a slender cytoplasmic protrusion (arrow) at the base of the flagella (arrow heads).

H: Gametes aggregating in a clump with their flagellar tips sticking together at the two opposite ends (arrow heads). Arrow indicates fusing gametes.

Plate V. Sexual reproduction in **Gonium sociale (Dujardin) Warming.**

A-G: Phase contrast. Scale in Fig. F applies to Figs. A-F, and that in Fig. J applies to Figs. G-J.

A: Pair of gametes. Note one of the two bearing a slender cytoplasmic protrusion (arrow).

B: Two gametes forming cytoplasmic bridge (arrow) between them.

C: Two gametes with their anterior regions touching by shortening the cytoplasmic bridge.

D: Two gametes fusing with their anterior portions.

E: Late stage of plasmogamy.

F: Quadriflagellate zygote.

G: Young zygote with gelatinous wall (arrow head) soon after settling down.

H: One-day-old zygotes showing pyrenoids.

I: Optical section of eight-day-old mature zygotes

J: Surface view of eight-day-old mature zygotes.

Plate VI. Zygote germination and gone colony formation in *Gonium sociale* (Dujardin) Warming.

All at same magnification. C-K: Phase contrast.

A: Two-celled stage of germinating zygote.

B: Four-celled stage of germinating zygote.

C: Germinating zygote with its four gone cells separating from one another within the protruded wall.

D: Germinating zygote just prior to the liberation of gone cells.

E: Four gone cells escaping separately from the zygote wall.

F: The last biflagellate gone cell escaping from the zygote wall.

G: Empty zygote wall after escape of gone cells.

H: Biflagellate gone cells.

I-L: Gone colony formation. Arrow heads indicate gelatinous envelope.

I: Gone cell settling down just before cell divisions.

J: Two-celled stage.

K: Four-celled stage.

L: Gone colony 24 hours after its formation resembling typical colony of *G. sociale*.

Plate VII. *Pandorina morum* **Bory.**

Scales in Figs. A, B, D, E, H-K represent 20 μm. Scale in Figs. F, G are 10 μm, and scale in Fig. C is 100 μm. B, E-I: Phase contrast.

A: 16-celled vegetative colony.

B: Asexual reproducing colony showing developing embryos and internal structure of parental gelatinous matrix. Stained with methylene blue.

C-K: Sexual reproduction.

C: Colony clumping.

D: Gamete release.

E: Gamete clumping and fusing gametes (arrow).

F: Biflagellate gamete bearing a mating papilla (arrow) at the base of the flagella.

G: Two conjugating gametes forming a cytoplasmic bridge (arrow) between them by the union of mating papillae.

H: Quadriflagellate zygote.

I: Zygotes aggregating and settling down.

J: Two-day-old aplanozygotes.

K: 10-day-old mature zygotes.

Plates VIII. Zygote germination and gone colony formation in **Pandorina morum Bory.**

All at same magnification. Scale in Fig. A represents 20 µm. D-F, H, I: Phase contrast.

A: Initial stage in zygote germination.

B: Gone cell escaping from zygote wall.

C. Empty zygote walls and zygote.

D: Biflagellate gone cell.

E-I: Gone colony formation. Stained with methylene blue.

E: Gone cell with gelatinous envelope before cell divisions.

F: Two-celled stage.

G: Four-celled stage.

H: Eight-celled stage.

I. Gone colony within gelatinous envelope provided by the original gone cell.

Plate IX. Pandorina unicocca Rayburn et Starr.

Each scale represents 20 μm, and that in Fig. A or Fig. J applies to Figs. A-E, G, I or Figs. J-L, respectively. C-E: Stained with methylene blue. C-K: Phase contrast.

A: Optical section of 32-celled vegetative colony.

B: Surface view of 32-celled vegetative colony.

C, D: Asexual reproduction.

C: Early stage. Note each two- to eight-celled plakea surrounded by a transparent vesicle within parental confluent gelatinous envelope.

D: Late stage.

E: 32-celled young colony with apparently uniflagellate cells.

F-L: Sexual reproduction.

F: Colony clumping.

G: Gamete release.

H: Biflagellate gamete bearing a mating papilla (arrow) at the base of the flagella (arrow heads).

I: Gamete clumping.

J: Quadriflagellate zygote.

K: Planozygotes aggregating.

L: Aggregated young aplanozygotes before secretion of cell walls. Note each having two pyrenoids.

Plate X. Pandorina unicocca Rayburn et Starr.

All at same magnification. Scale in Fig. L represents 20 µm. C-E, G-L: Phase contrast. E, G-L: Stained with methylene blue.

A: Young zygotes showing pyrenoids.

B: Mature, reddish brown zygotes.

C: Germinating zygote showing hyaline bodies in thin-walled protuberance (arrow).

D: Biflagellate gone cell escaping from zygote wall.

E: Biflagellate gone cell with gelatinous envelope.

F: Empty wall and zygotes.

G-L: Gone colony formation. Note each developing embryo or gone colony surrounded by gelatinous envelope.

G: Two-celled stage.

H: four-celled stage.

I: Eight-celled stage.

J: 16-celled stage.

K: Inversion stage.

L: Newly formed gone colony.

Plate XI. Volvulina steinii Playfair.

C-F: Stained with haematoxylin. H-K: Phase contrast.

A: Surface view of vegetative colony in two-day-old culture. Note stigma (s) and contractile vacuoles (v).

B: Vegetative colony in four-day-old culture. Each cell has a pyrenoid (py) in the brim of the cup-shaped chloroplast.

C: Surface of vegetative colony showing flagella (f) and structure of gelatinous matrix.

D: Optical section of vegetative colony showing structure of gelatinous matrix. Note small hollow in the center.

E, F: Asexual reproduction.

E: 8-celled plakeal stage showing structure of parental gelatinous matrix.

F: Newly formed daughter colonies within the keystone-shaped spaces formed in the parental gelatinous matrix.

G-K: Sexual reproduction.

G: Colony clumping.

H: Gamete release.

I: Empty colonial matrix after escape of gametes.

J. Biflagellate gamete bearing a mating papilla (pa) at the base of the flagella.

K: Gamete clumping. Arrow indicates adhering flagellar tips of gametes.

Plate XII. Sexual reproduction in **Volvulina steinii Playfair.**

All at same magnification. Scale in Fig. J represents 20 μm. A-C, I: Phase contrast. J-L: Ink preparation.

A: Pair of gametes forming cytoplasmic bridge (cb) by the union of both of mating papillae in the initial stage of plasmogamy.

B: Late stage of plasmogamy.

C: Quadriflagellate zygote.

D: Zygotes just after settling down.

E: Two-day-old zygotes with cell walls.

F: Reddish brown mature zygotes.

G: Germinating zygote. Note hyaline body in thin-walled protuberance (arrow).

H: Empty wall after liberation of gone cell.

I: Biflagellate gone cell.

J-L: Gone colony formation. Note gelatinous envelope surrounding developing embryo or gone colony.

J: Two-celled stage.

K: Four-celled stage.

L: Newly formed 16-celled gone colony.

Table XIII. Eudorina elegans Ehrenberg var. elegans.

Each scale represents 20 μm. B, C, H-J: Phase contrast.

A: 32-celled vegetative colony.

B: Asexual reproduction. Note each daughter colony surrounded by transparent vesicle (arrow head).

C-J: Sexual reproduction.

C: Male colony producing sperm packets.

D: Upper view of sperm packet.

E: Lateral view of sperm packet.

F: Sperm packets surrounding female colony.

G: Sperm packet (arrow head) dissociating nearby female colony. Note individual male gametes (arrow) penetrating female colony.

H: Mature female gamete in confluent gelatinous envelope (arrows) of female colony. Note transparent vesicle (arrow head) behind the gamete.

I, J: Various shapes of male gametes. Note slender cytoplasmic protrusion (arrow) at the base of the flagella (arrow heads).

Plate XIV. Sexual reproduction in **Eudorina elegans Ehrenberg var. elegans.**

Each scale represents 20 μm.

A: Male gamete (arrow head) landing on lateral anterior portion of female gamete just prior to plasmogamy.

B: Middle stage of plasmogamy. Note anterior portion of the male gamete fusing with female gamete. Arrow head indicates posterior end of the male gamete.

C: Late stage of plasmogamy. Arrow head indicates lateral side of the fusing male gamete.

D: Quadriflagellate zygote. Note two longer flagella (arrow head) and two shorter ones (arrow). Phase contrast.

E: One-day-old zygotes with cell walls.

F: Mature zygotes with reddish brown granules.

Plate XV. Sexual reproduction in **Eudorina elegans Ehrenberg var. elegans.**

All at same magnification. Scale in Fig. G represents 20 µm.

A-F: Zygote germination. Phase contrast.

A: Initial stage. Note hyaline body in thin-walled protuberance (arrow).

B: Gone cell with two equal flagella (arrows) squeezing out into thin-walled protuberance

C: Gone cell in thin-walled protuberance just before escape.

D: Gone cell escaping from thin-walled protuberance. Arrow indicates hyaline body.

E: Empty wall after escape of gone cell. Arrows indicates two hyaline bodies .

F: Biflagellate gone cell. Arrow heads indicates two flagella.

G-I: Gone colony formation. Each developing embryo or gone colony is surrounded by gelatinous envelope of the original gone cell. Ink preparation.

G: Four-celled stage.

H: 16-celled stage.

I: 32-celled newly formed gone colony.

Table XVI. Eudorina elegans **Ehrenberg var.** synoica **Goldstein.**

Scale in Fig. A and Fig. F applies to Figs. A-C and Figs. E-L. C, E, F, L: Phase contrast.

A: Surface view of 32-celled vegetative colony.

B: Optical section of 32-celled vegetative colony.

C: Asexual reproduction. Each daughter cell develops within each transparent vesicle (arrow) in parental gelatinous matrix.

D: Sexual colony with sperm packets (arrows) and female gametes (arrow heads).

E, F: Germinating zygotes producing a single gone cell. Arrow head indicates hyaline body.

E: Initial stage.

F: Late stage.

G-K: Germinating zygotes producing two gone cells. Arrow head indicates hyaline body.

G: Initial stage.

H: Just after transverse division.

I: Two gone cells squeezing out into protruding wall.

J: Just prior to liberation of gone cells.

K: Two gone cells escaping from zygote wall (arrows).

L: Empty walls and zygotes.

Plate XVII. Asexual colonies of **Volvox carteri Stein f. kawasakiensis Nozaki.**

A: Colonies.

B: Colony with 12 gonidia.

C: Colony with 30 gonidia.

D-O: Portion of colonies. All at same magnification.

D: Lateral view of somatic cells.

E: Upper view of somatic cells.

F: Part of colony showing individual gelatinous sheaths of cells. Stained with haematoxylin.

G: optical section of gonidium.

H-O: Development of asexual colonies.

H: Four-celled stage.

I: 16-celled stage.

J: Later stage of plakea.

K: Pre-inversion plakea.

L-N: Successive stages of inversion.

O: Newly formed colony after flagellar elongation.

Plate XVIII. Male colonies of **Volvox carteri** Stein f. **kawasakiensis** Nozaki.

Each Scale represents 40 µm.

A: Male colonies within parental colonies.

B-I: Male colonies removed from parent to facilitate observation.

B: Mature 16-celled colony composed of only sperm packets.

C: Mature 32-celled colony composed of only sperm packets.

D: 64-celled colony with a 1:1 ratio of somatic cells to androgonidia.

E: Immature 64-celled colony composed of only androgonidia.

F: Mature 64-celled colony composed of only sperm packets.

G: Portion of colony of Fig. F showing sperm packets.

H: Immature 128-celled colony with a 1:1 ratio of somatic cells to androgonidia.

I: Sperm packet formation in 128-celled colony.

J: Mature 256-celled colony.

K-N: Development of 32-celled male colonies.

K: Pre-inversion plakea.

L: Middle stage of inversion.

M: Late stage of inversion.

N: Newly formed compact colony just after inversion.

Plate XIX. Female colonies of **Volvox carteri Stein f. kawasakiensis Nozaki.**

Each scale represents 40 μm.

A: Colony with 81 eggs.

B: Colony with 64 eggs.

C: Portion of colony in Fig. B showing eggs.

D-G: Development of female colonies.

D: Pre-inversion plakea.

E: Initial stage of inversion.

F: Inversion stage.

G: Female colony just before release from parent.

Plate XX. Sexual reproduction in **Volvox carteri Stein f. kawasakiensis Nozaki.**

A, B: Sperm packet (arrow) attaching to posterior portion of female colony.

C: Sperm packet (arrow) dissociating on the female colony.

D: Phase contrast micrograph of sperm.

E-H: Successive stages of fertilization. All at same magnification.

E: Sperm (arrow) attaching to lateral anterior portion of egg before fertilization.

F: Sperm (arrow) slightly retracting posterior tail just before entering egg.

G: Middle stage of fertilization. Note anterior portion of sperm (arrow) entering egg.

H: Late stage of fertilization. Arrow indicates posterior tail of entering sperm.

I, J: One-day-old zygotes in parental female.

K, L: Seven-day-old mature zygotes in parental female.

M-P: Zygote germination and gone colony formation. All at same magnification.

M: Initial stage of zygote germination.

N: Germinating zygote showing two hyaline bodies (arrow).

O: 16-celled stage in gone colony formation. Arrows indicate two flagella provided by the original gone cell.

P: Newly formed gone colony with four gonidia.

Plate XXI. Astrephomene gubernaculifera Pocock.

Scale in Fig. A represents 50 µm and applies to Figs. A-I, and that in Fig. L does 20 µm and to Figs. L-R. Scales in Figs. J, K represent 20 µm. E, F, I, J, Stained with haematoxylin. G, Q, R: Ink preparation. I-L, N-P, R: Phase contrast.

A: Surface view of mature 64-celled colony.

B: Optical section of colony in Fig. A.

C: Bottom view of 64-celled colony showing four somatic cells.

D: Bottom view of 32-celled colony showing two somatic cells.

E: Mature colony showing cellular sheaths (arrow).

F: Young colony showing cellular sheaths.

G: Colony with no encompassing matrix.

H: Asexual reproduction showing 8-celled embryo (arrow).

I: Asexual reproduction showing four somatic cells and daughter colonies embedded in parental cellular sheaths.

J: Daughter colony in parental cellular sheath (arrow heads) having biflagellate posterior cells (arrows) and uniflagellate other cells.

K-R: Sexual reproduction.

K: Gamete bearing a mating papilla (arrow) at the base of the flagella (arrow heads).

L: Quadriflagellate zygote.

M: Mature aplanozygotes.

N, O: Germinating zygotes.

P: Biflagellate gone cell.

Q: 8-celled embryo in gone colony formation.

R: Gone colony in gelatinous envelope.

Plate XXII. Astrephomene perforata Nozaki.

Scale in Fig. A represents 50 μm and applies to Figs. A-I. Scale in Fig. J is 20 μm. E, F, J: Stained with haematoxylin. G: Ink preparation. J: Phase contrast.

A: Surface view of mature 64-celled colony in two-day-old culture.

B: Optical section of the colony in Fig. A.

C: Colony showing pyrenoids (arrow) in five-day-old culture.

D: 64-celled colony showing two posterior somatic cells.

E: Mature colony showing fenestrations (arrow) formed by the interconnecting cellular sheaths.

F: young colony showing fenestrations.

G: Colony showing encompassing watery gelatinous matrix.

H: Asexual colony showing 8-celled embryo (arrow).

I: Two somatic cells and daughter colonies in 64-celled parental colony.

J: Newly formed colony having biflagellate posterior cells (arrows) and uniflagellate other cells

Plate XXIII. Sexual reproduction in **Astrephomene perforata Nozaki.**

Scale in Fig. A represents 600 μm. Scales in Figs. B-L represent 20 μm. B-D, G-J: Phase contrast. K, L: Ink preparation.

A: Colony clumping.

B: Gamete release.

C: Gamete clumping. Note the gametes aggregating with their flagellar tips sticking together in one center.

D: Biflagellate gamete bearing mating papilla (arrow) at the base of flagella (arrow heads).

E: One-day-old aplanozygotes.

F: Mature aplanozygotes.

G-I: Successive stages in zygote germination. Arrow indicates hyaline body.

J: Biflagellate gone cell.

K: 8-celled embryo in gone colony formation.

L: Newly formed gone colony within gelatinous envelope.

Plate XXIV. Pyrobotrys stellata (Korshikov) Korshikov.

D, F-H: Phase contrast.

A: Front view of colony.

B: Side view of colony.

C: Young colonies.

D: Individual biflagellate cell.

E: Asexual reproduction.

F: Fusing gametes.

G, H: Planozygotes. Arrow head indicates posterior projection.

I: Mature aplanozygotes.

Plate XXV. Pyrobotrys squarrosa (Korshikov) Korshikov.

D, F-K: Phase contrast.

A: Front view of colony.

B: Side view of colony.

C: Young colonies.

D: Individual vegetative cell. Arrow indicates flagellar insertion at the anterior dorsal side of the cell.

E-M: Sexual reproduction.

E: Gametogenesis.

F: Biflagellate gamete.

G: Initial stage of gametic union.

H: Late stage of plasmogamy.

I: Bottom view of planozygote.

J: Lateral view of planozygote.

K: Zygotes just after settling down from motile stage.

L: Young four-day-old zygotes.

M: Mature two-month-old aplanozygotes.

Plate XXVI. Pyrobotrys casinoensis (Playfair) Silva.

E, H-L: Phase contrast.

A-C: Various shapes of 16-celled colonies.

D: Eight-celled colonies.

E: Individual biflagellate cell. Note anterior papilla (arrow) at the base of the flagella (arrow heads).

F: Asexual colony in two- to four-celled stage.

G: Gametogenesis.

H: Various shapes of gametes.

I: Young fusiform planozygote.

J: Relatively mature planozygote.

K: Lateral view of mature planozygote showing radiate stumpy processes (arrow heads) and posterior tail (arrow).

L: Bottom view of mature planozygote showing four radiate stumpy processes (arrow heads).

M: Mature aplanozygotes.

Plate XXVII. Sexual reproduction in **Pyrobotrys casinoensis (Playfair) Silva.**

All at same magnification. G-I: Phase contrast.

A-G: Zygote germination.

A: Initial stage.

B: Two-celled stage in germinating zygote.

C: Four-celled stage in germinating zygote.

D-E: Four gone cells gradually escaping from the zygote wall.

F: Empty wall after escape of gone cells.

G: Biflagellate gone cell.

H, I: Gone colony formation.

H: Gone cell dividing into gone colony as it swims with its two flagella (arrows).

I: Eight-celled gone colony.

Plate XXVIII. Pyrobotrys elegans (Behlau) Nozaki.

D-H: Phase contrast.

A, B: 16-celled colonies.

C: 8-celled colonies.

D: Individual biflagellate cell. Note prominent papilla (arrow) at base of flagella (arrow heads).

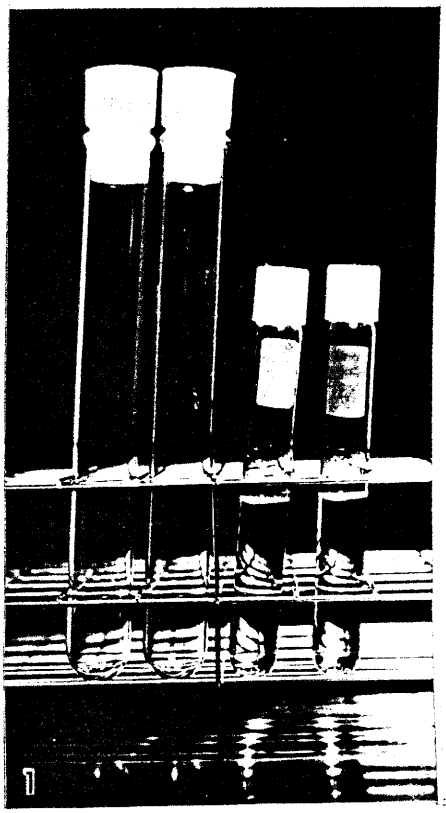
E: Initial stage of gametic union.

F: Late stage of plasmogamy.

G: Lateral view of planozygote.

H: Bottom view of planozygote.

I: Mature aplanozygotes.



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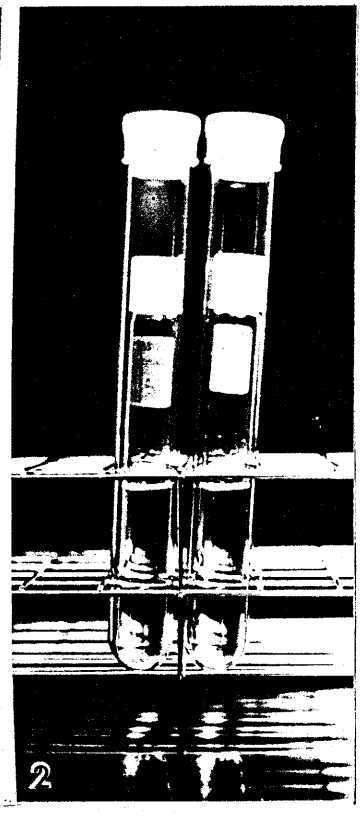

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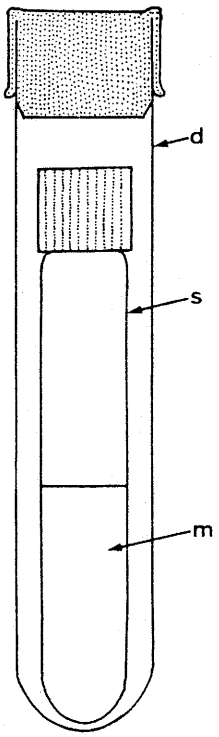

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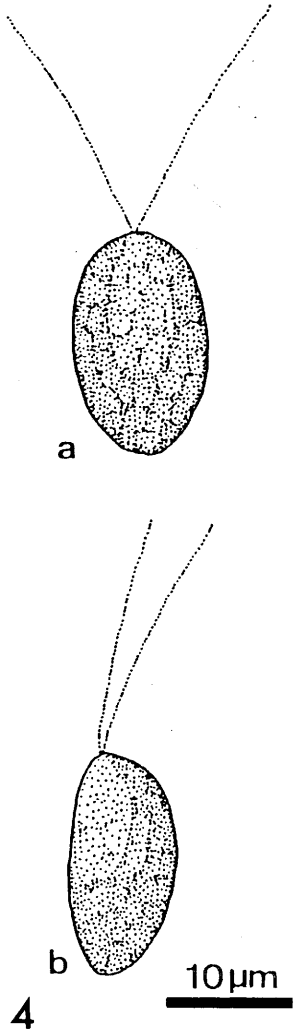

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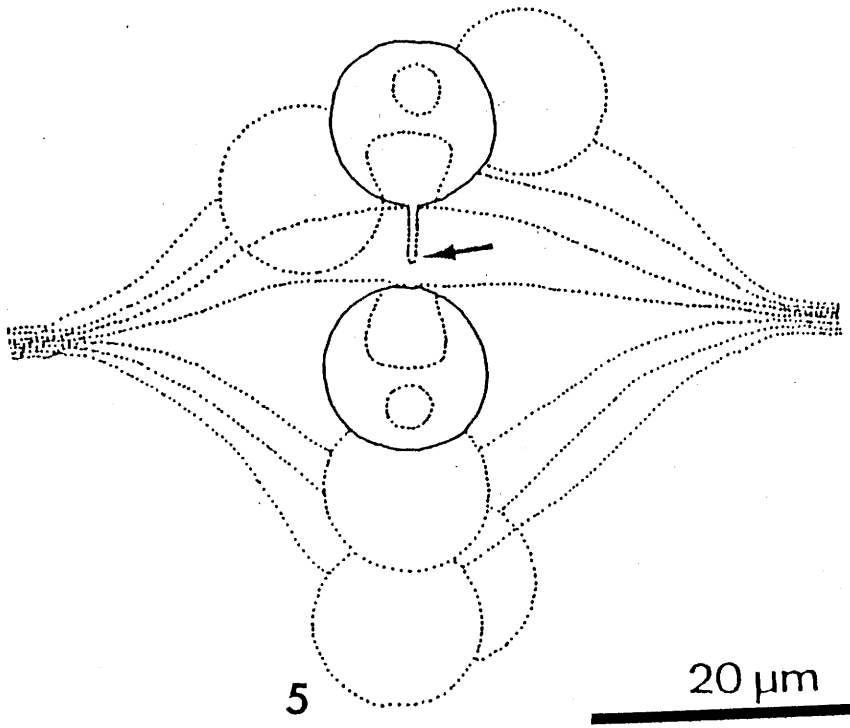

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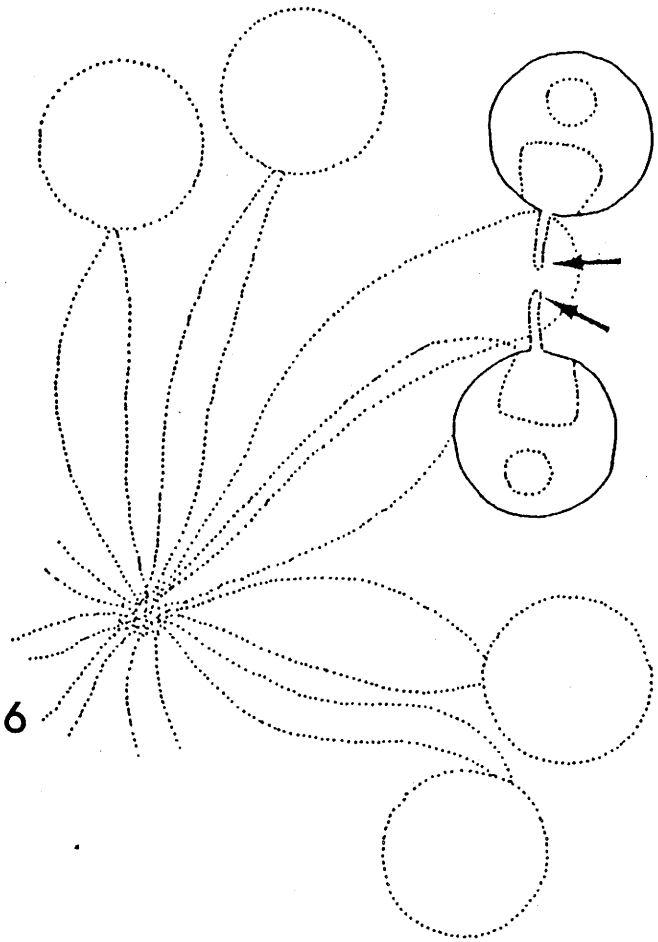

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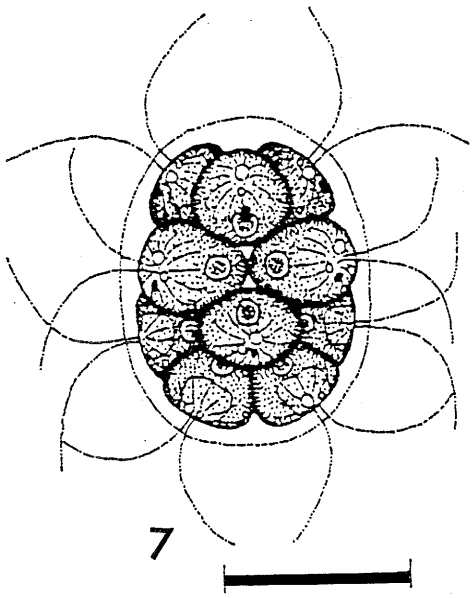

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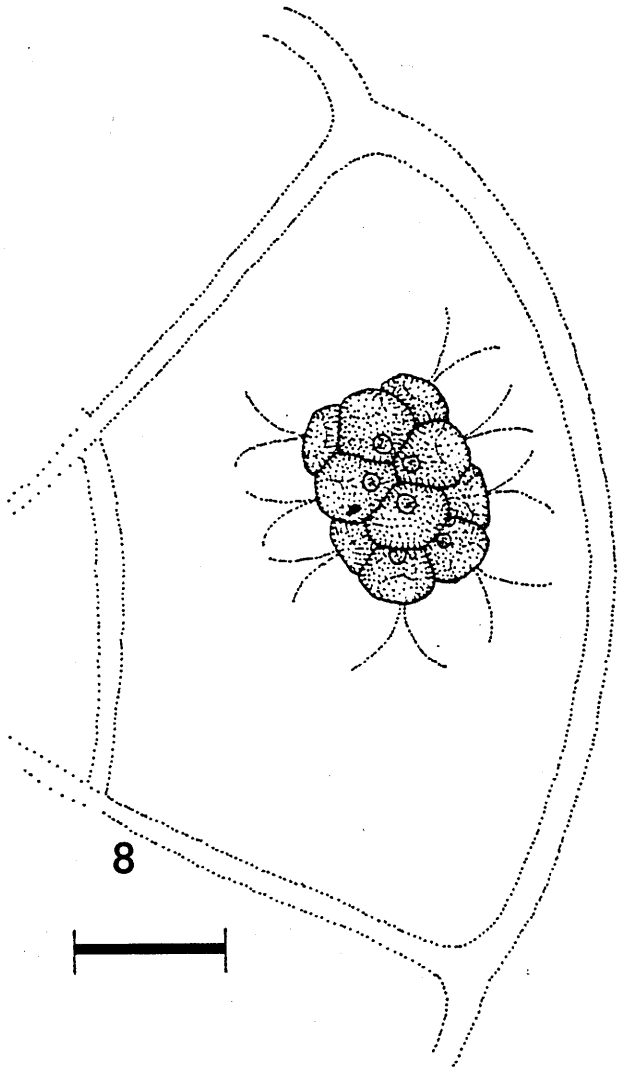

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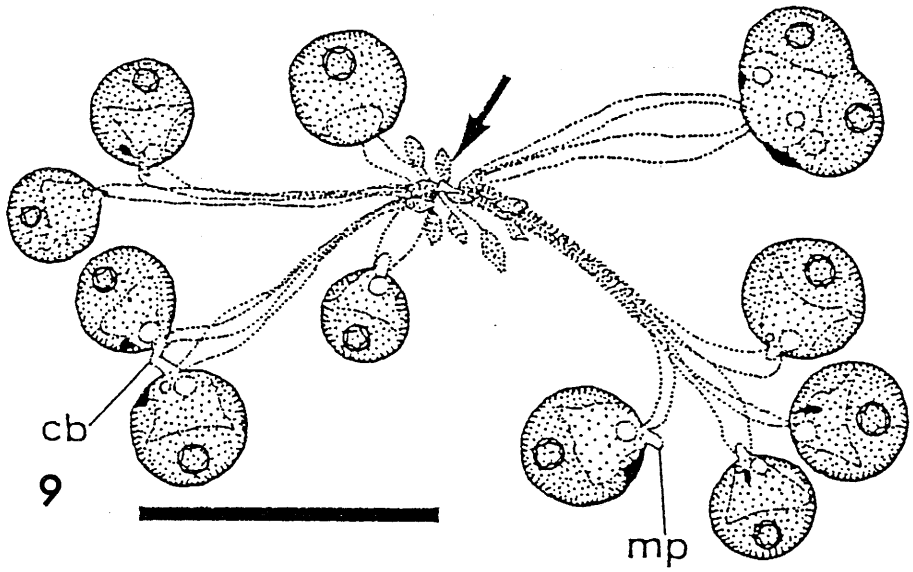

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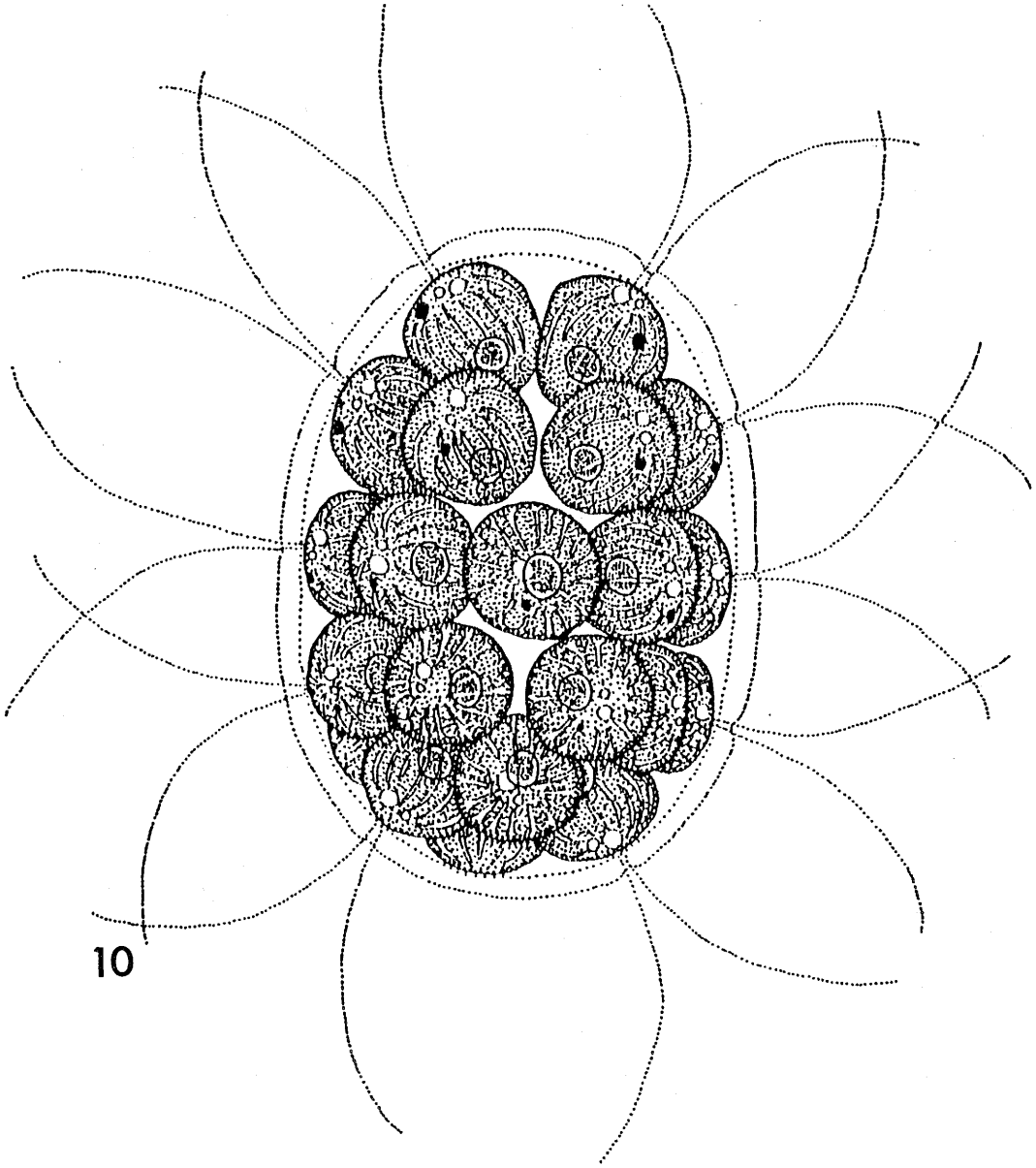

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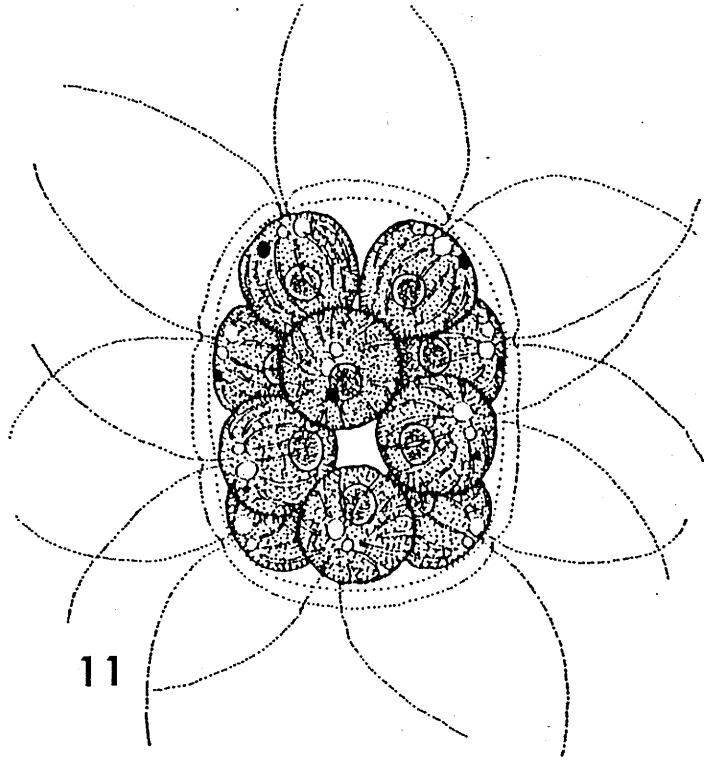

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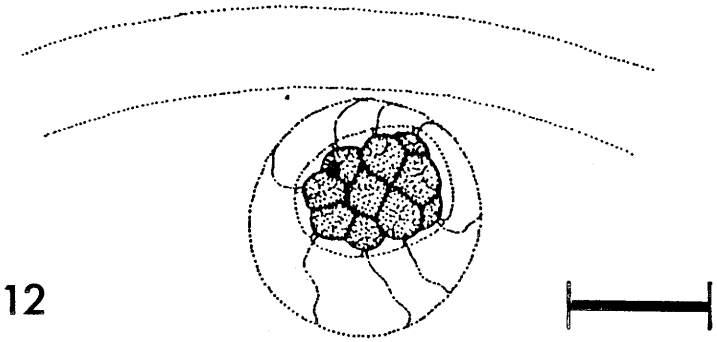

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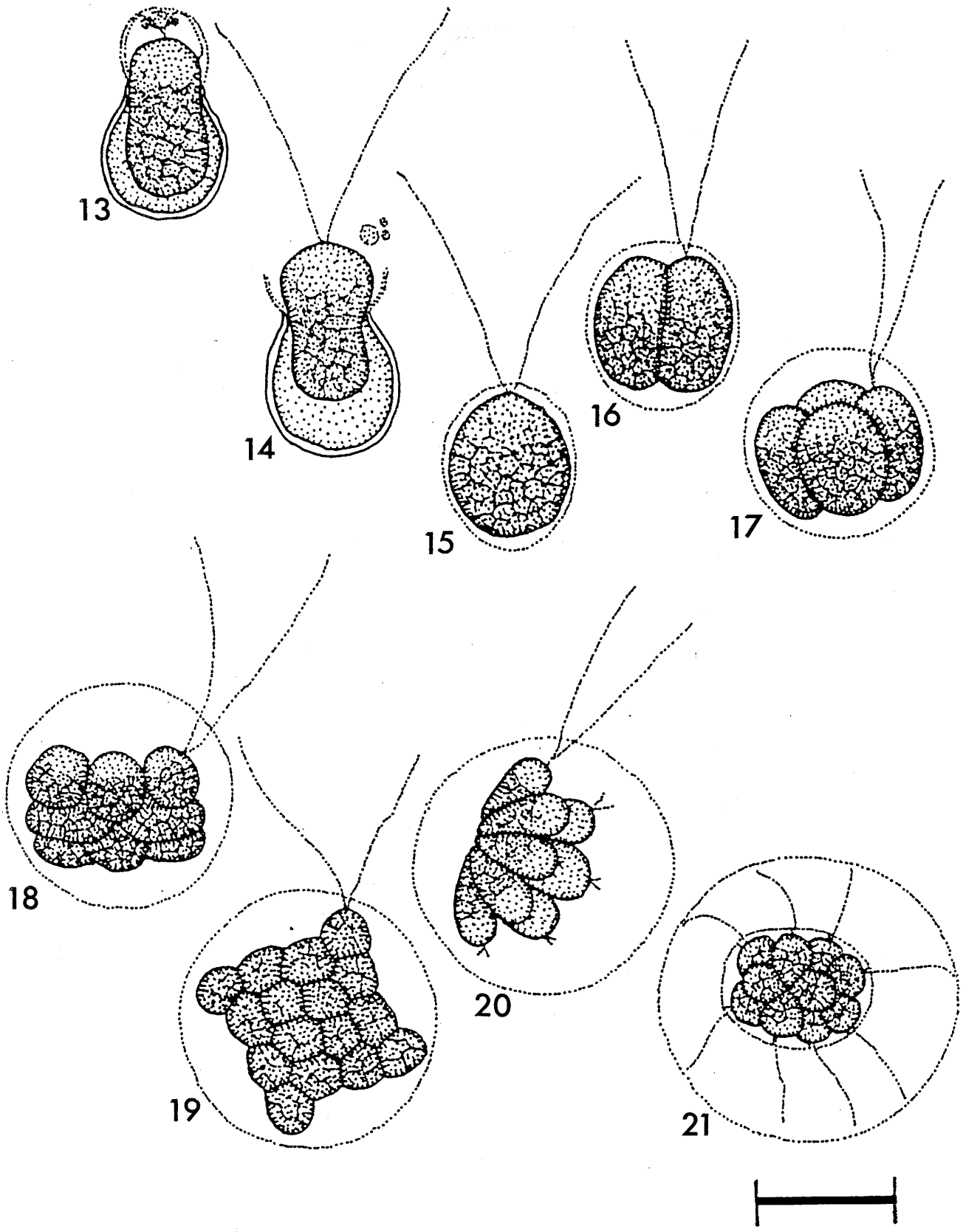
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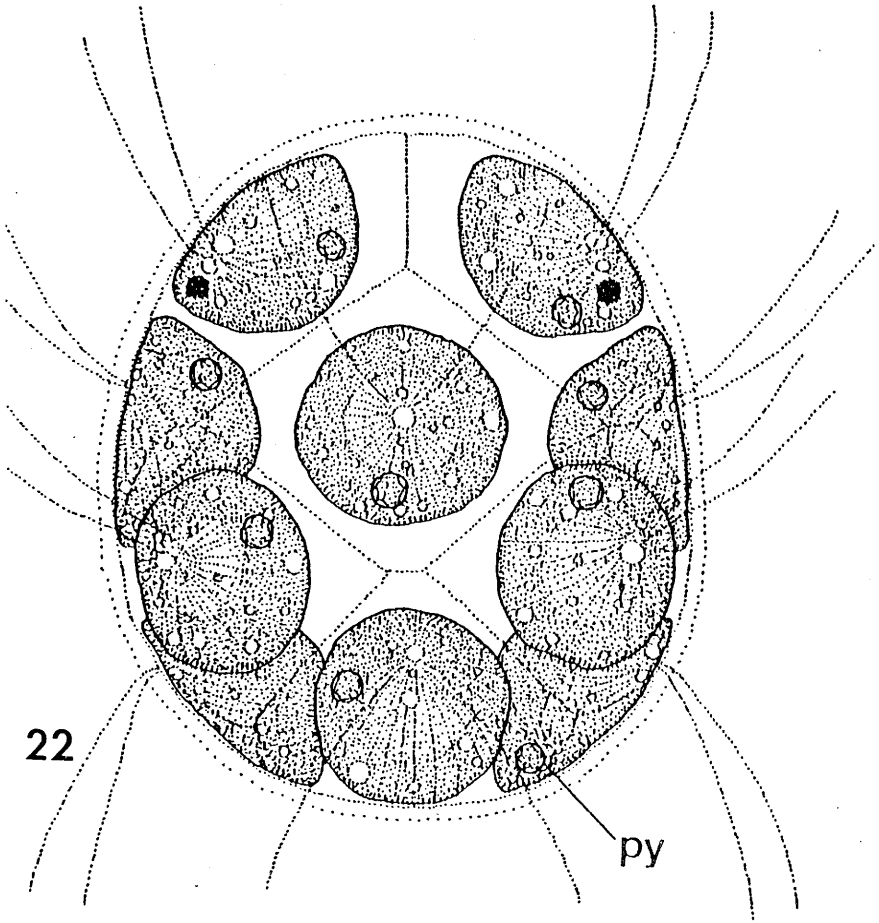

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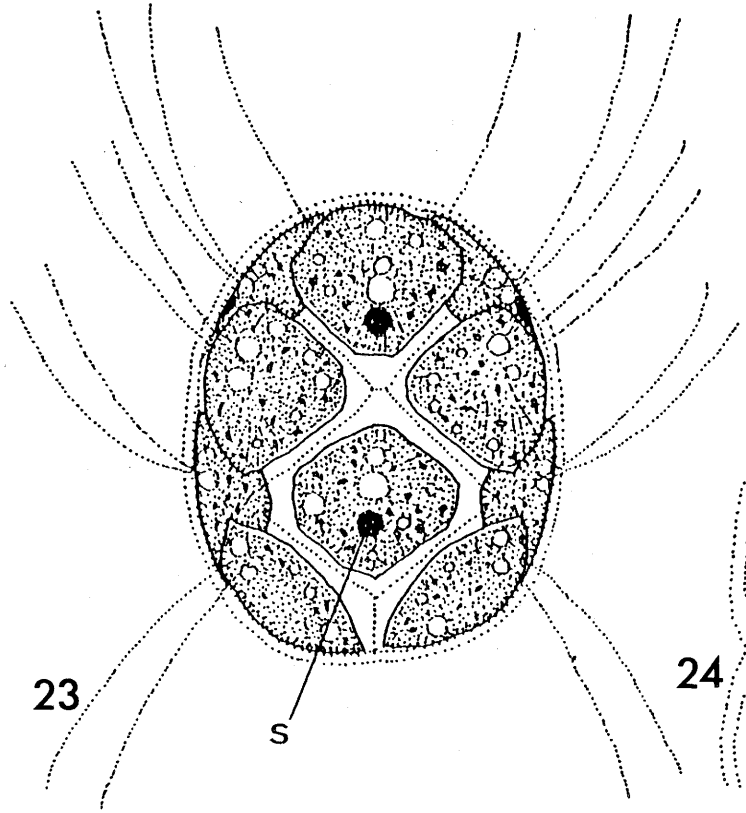

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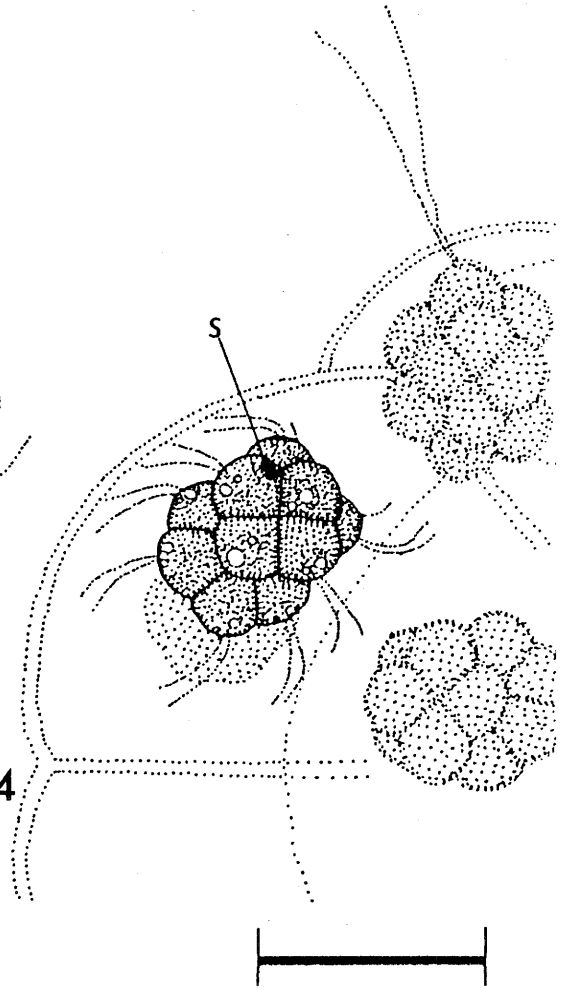

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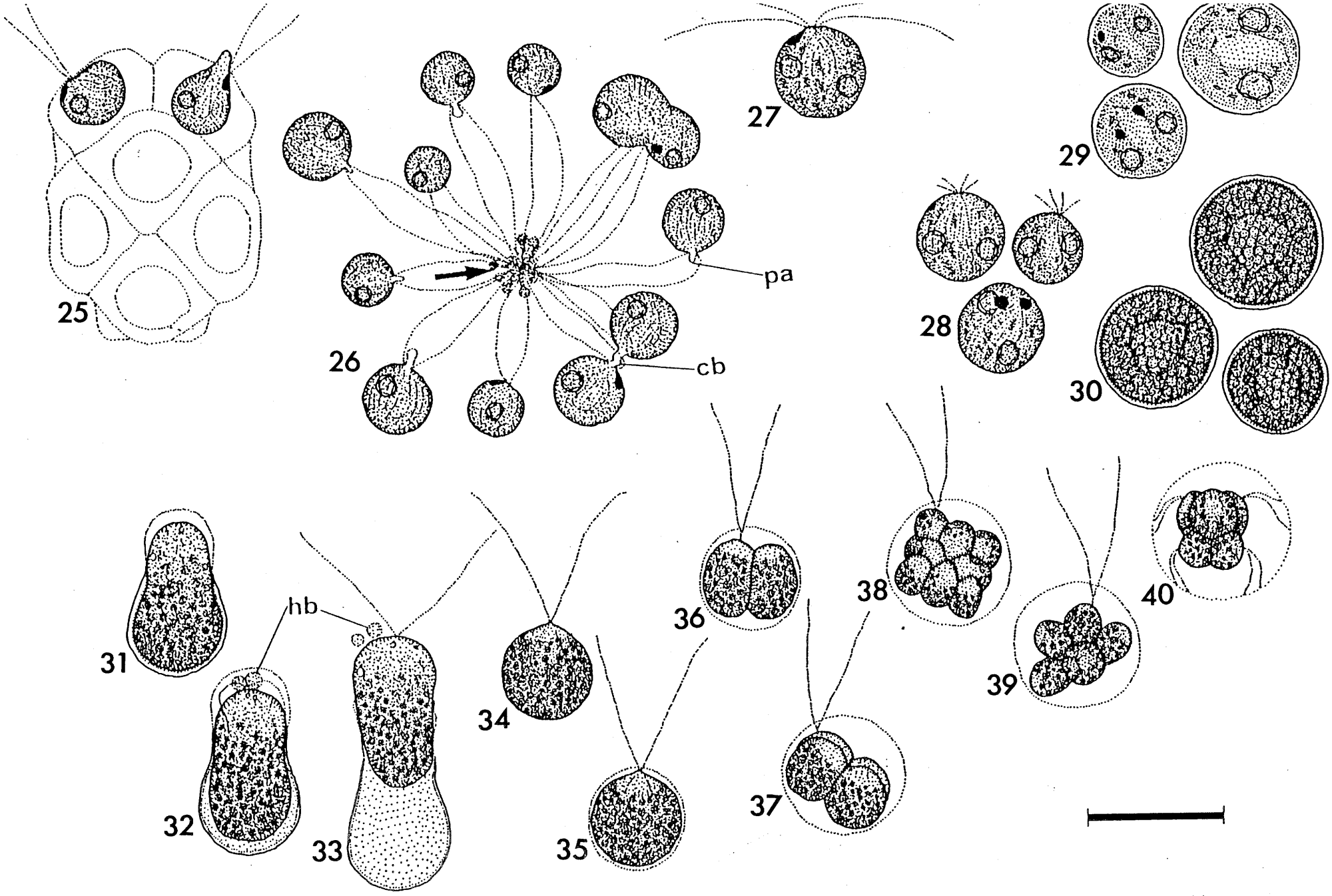
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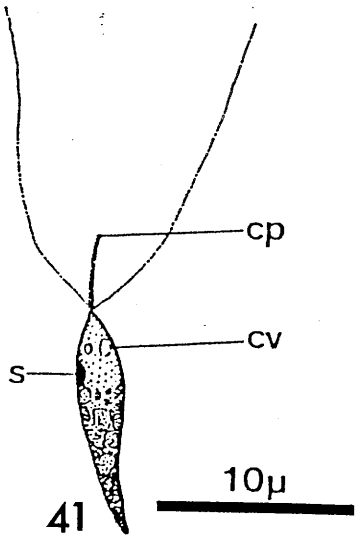

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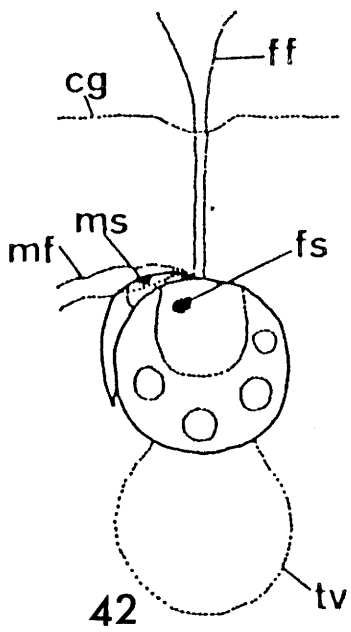

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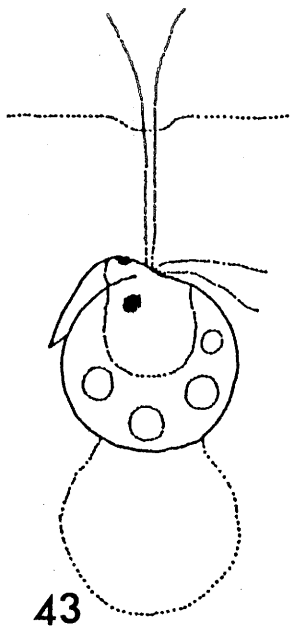

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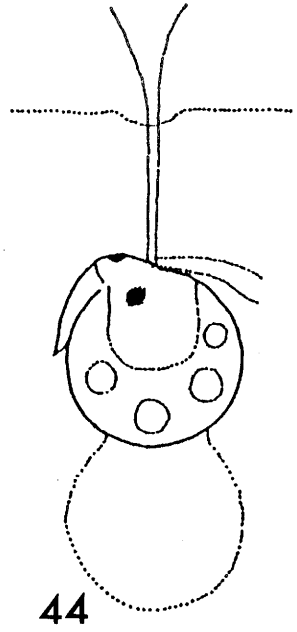

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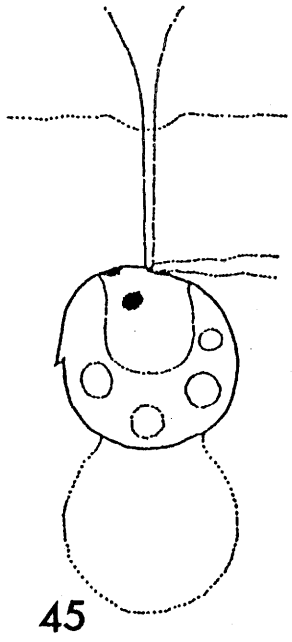

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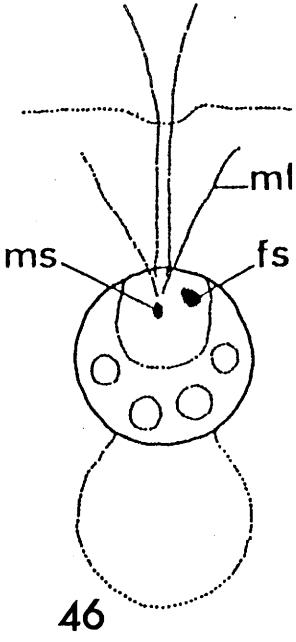

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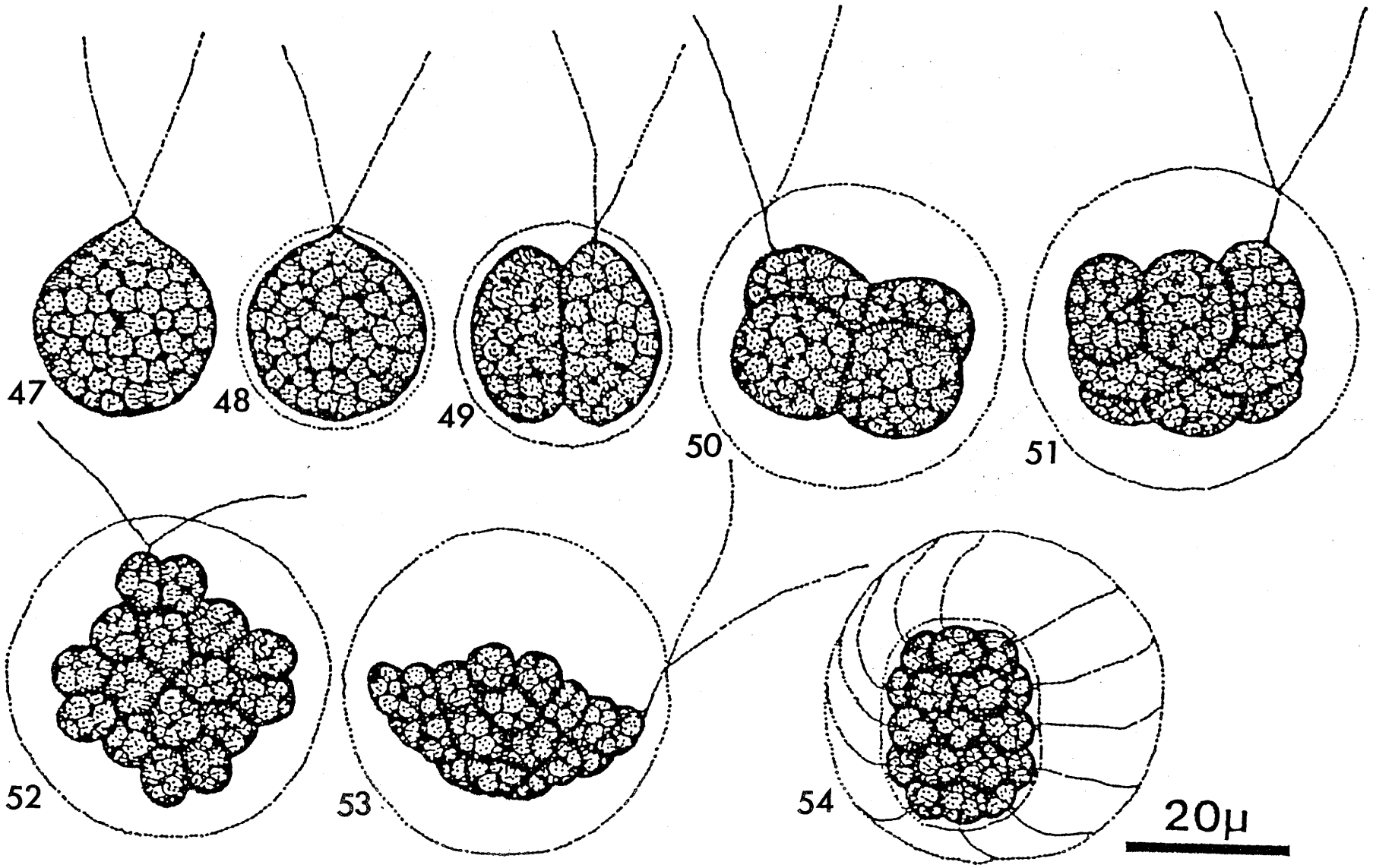
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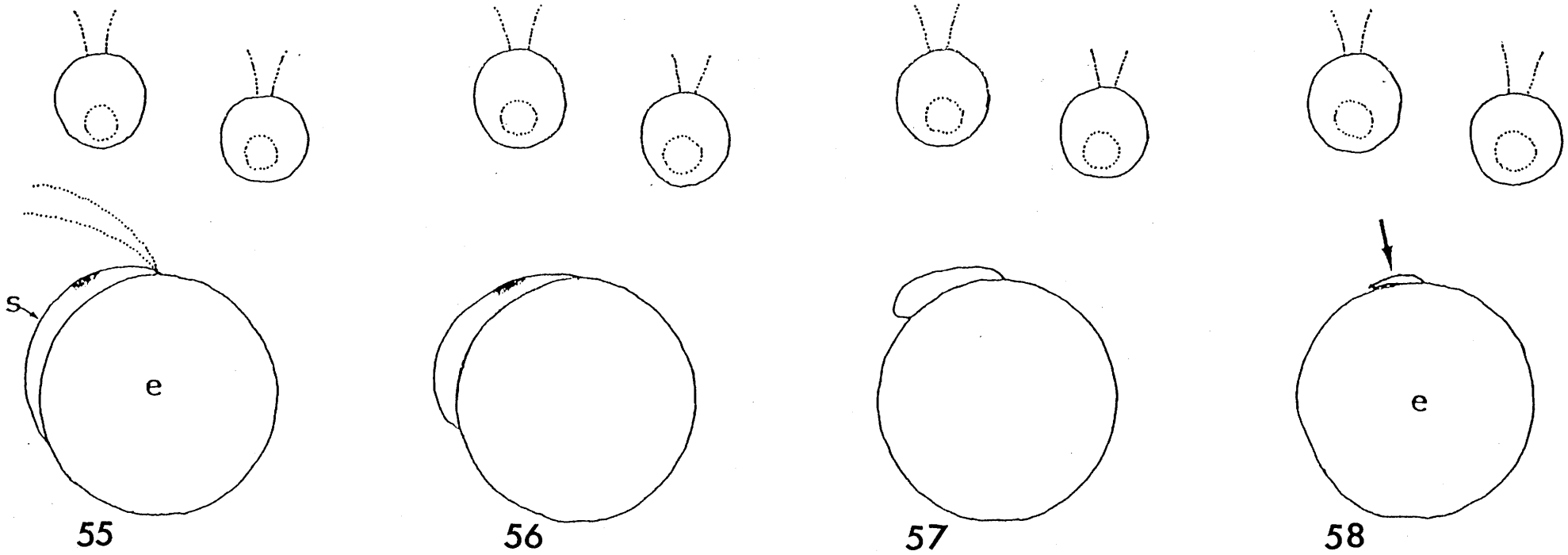
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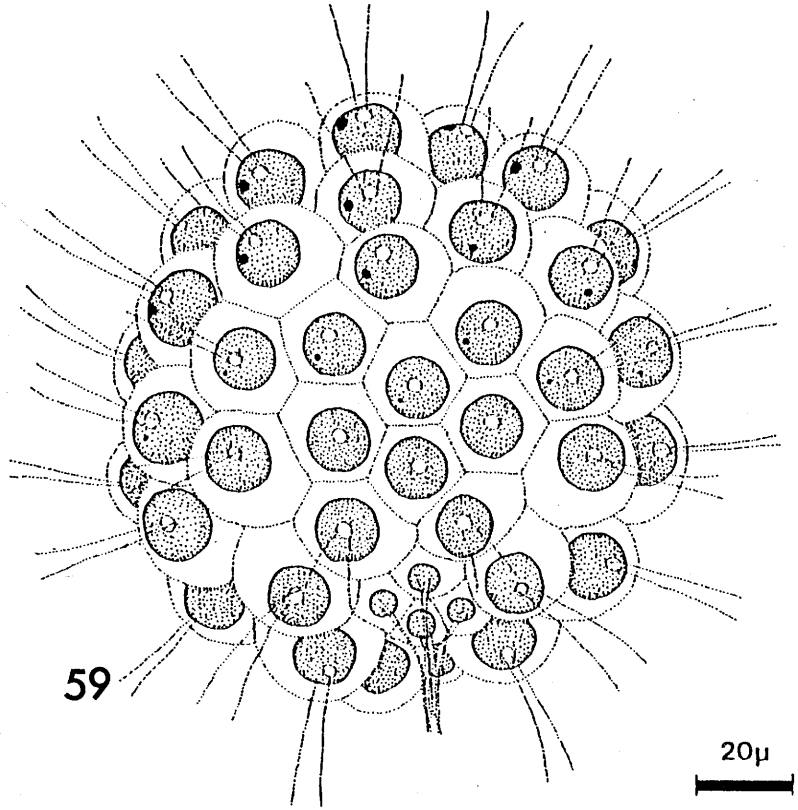

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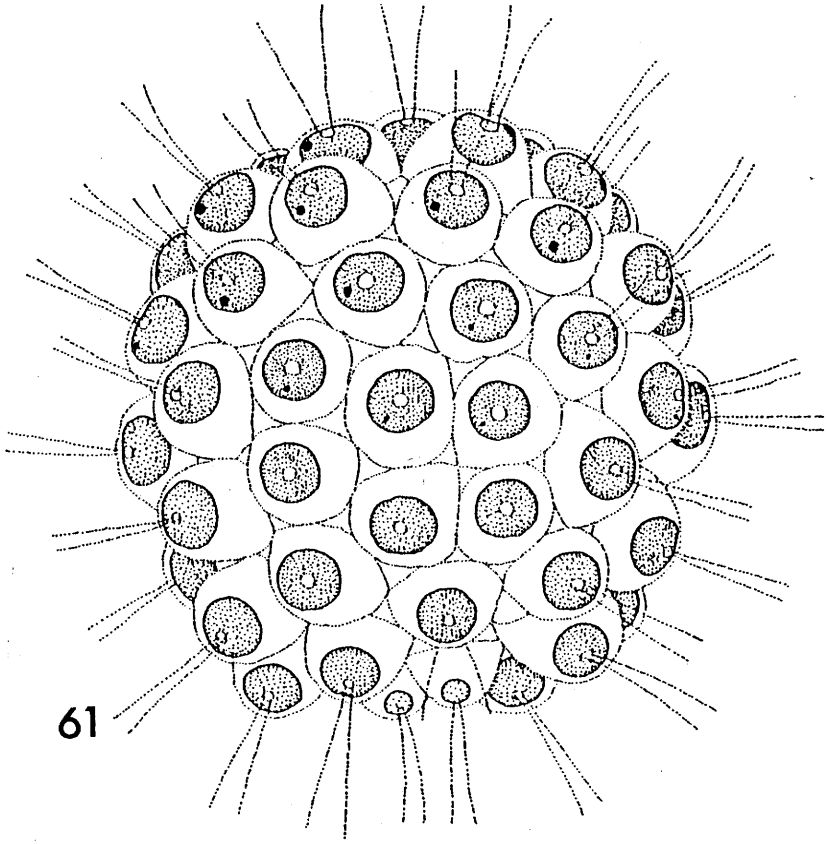

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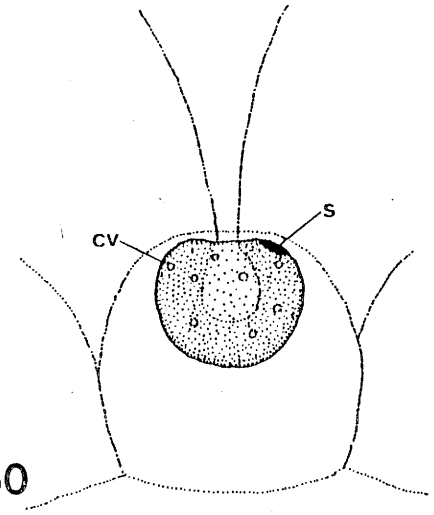
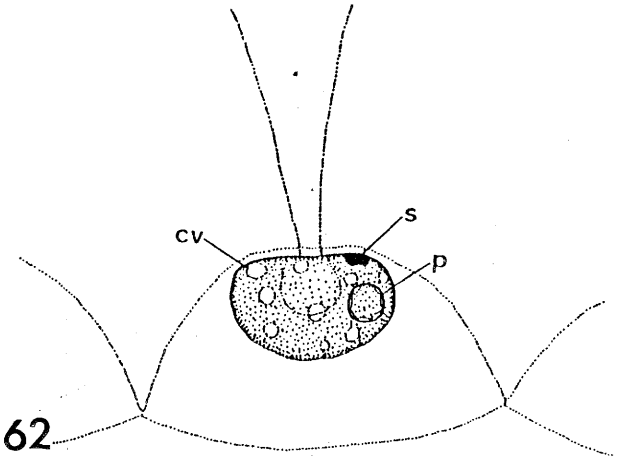
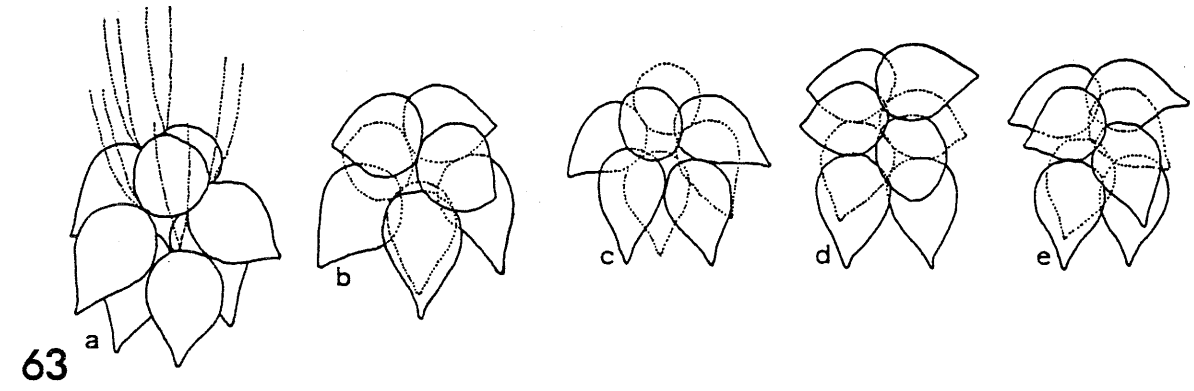


60

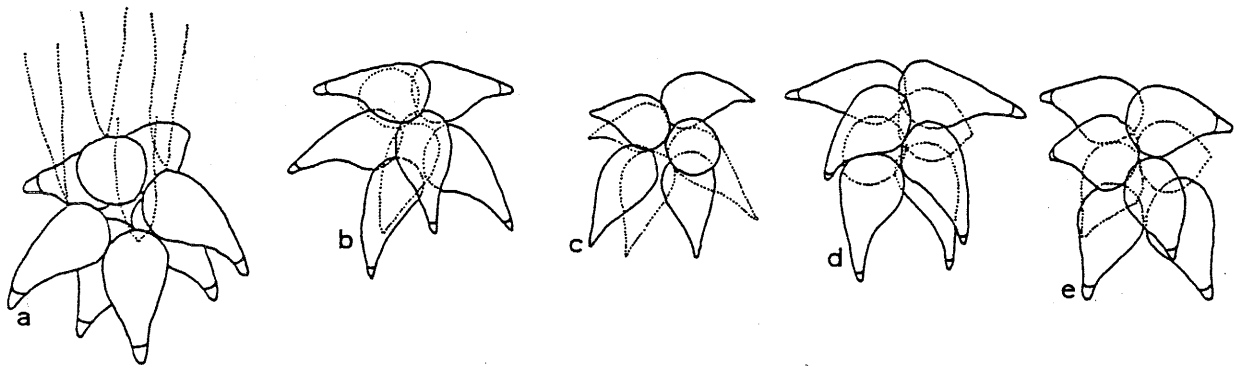
20μ



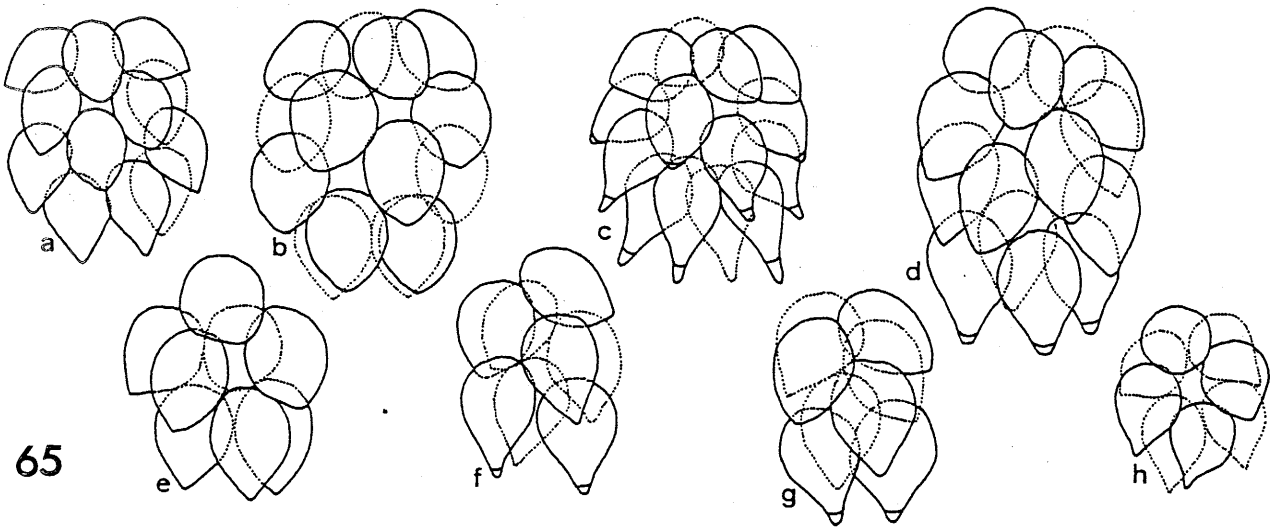
62



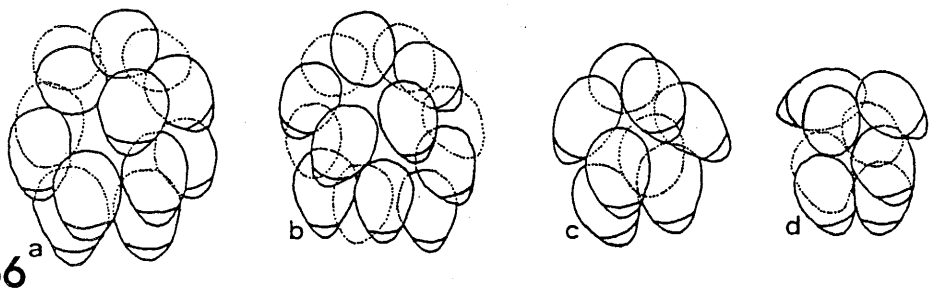
63



64

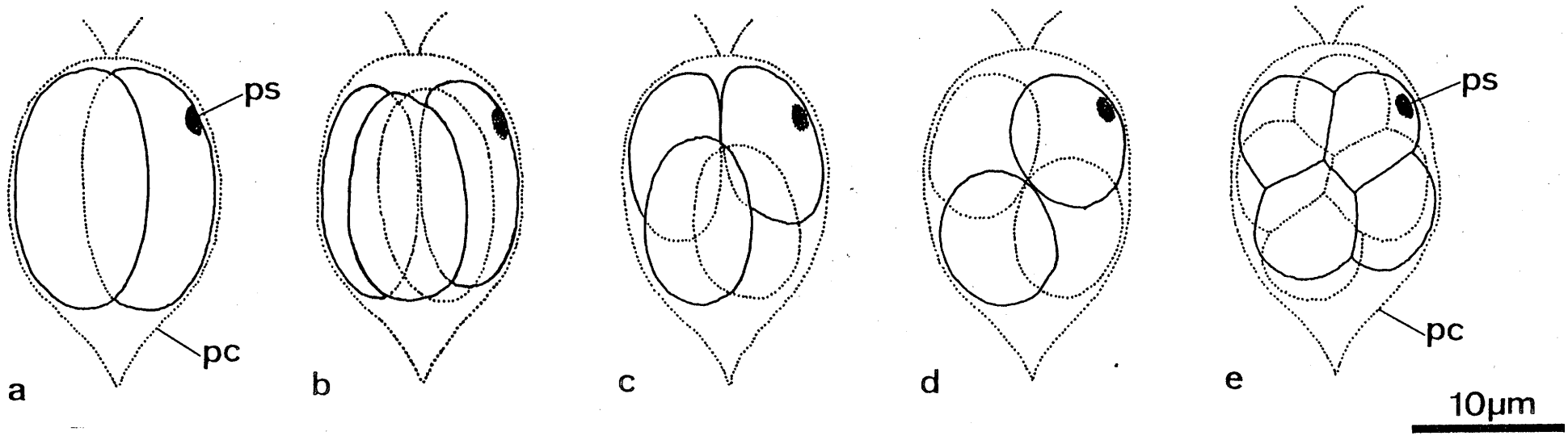


65

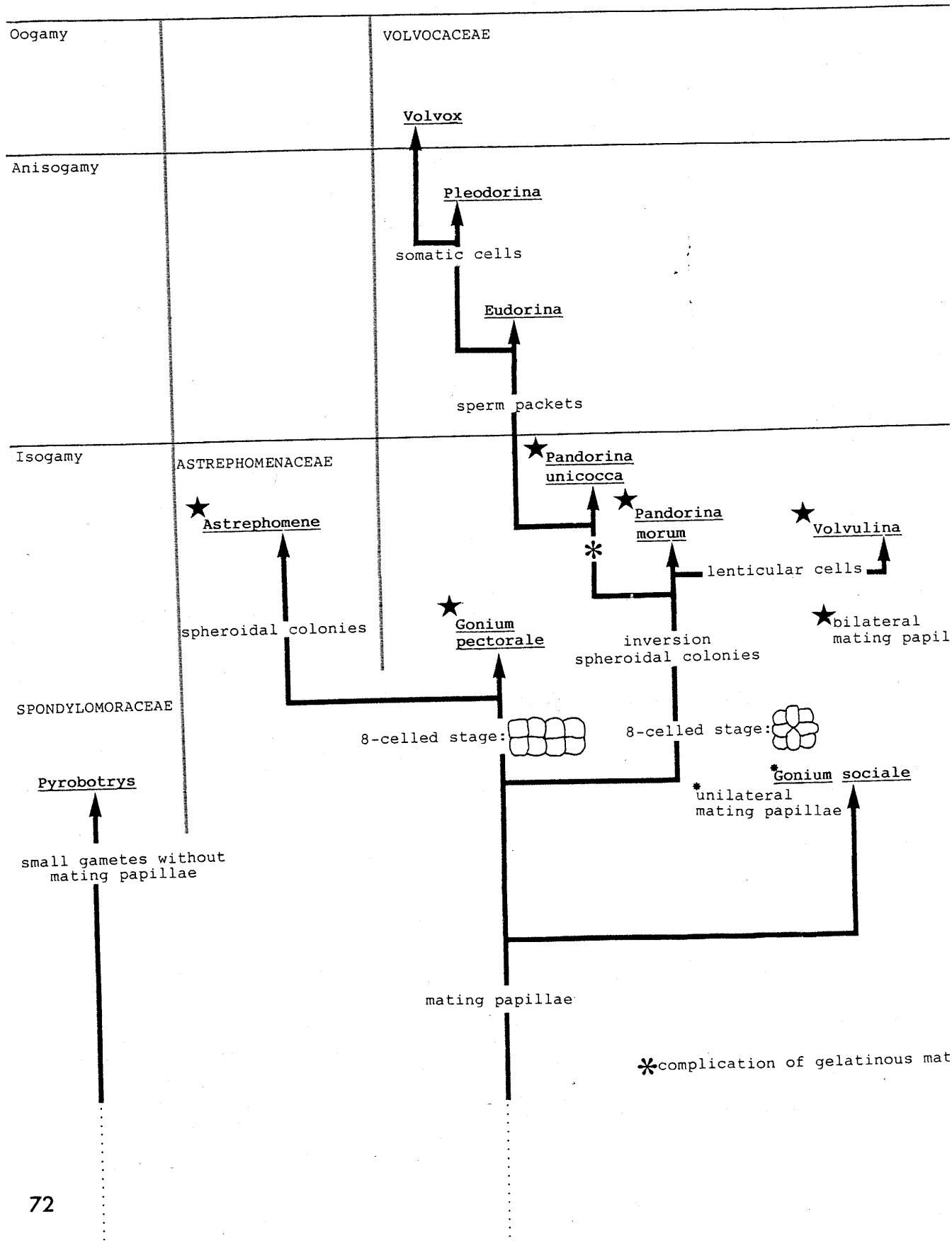


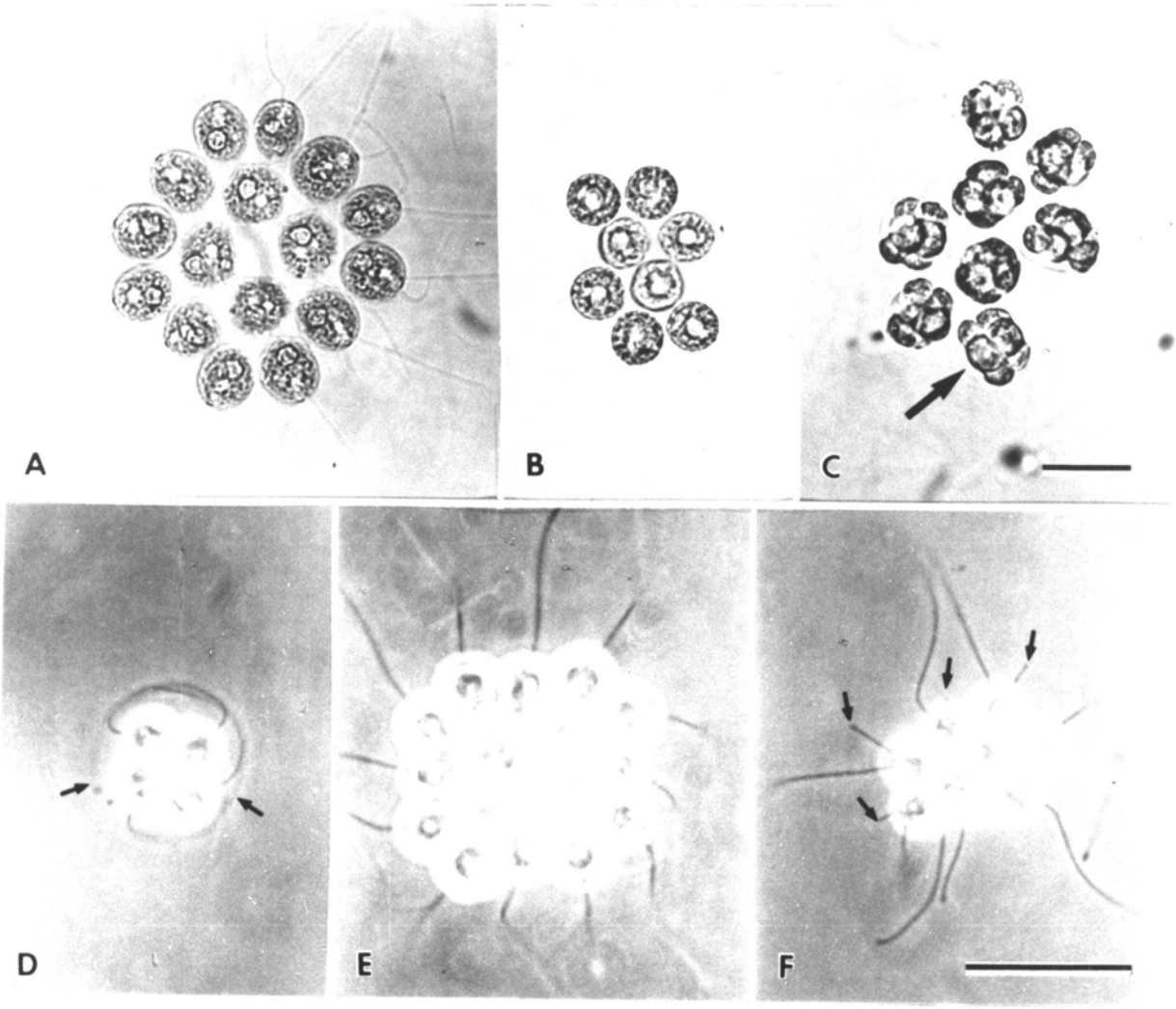
66

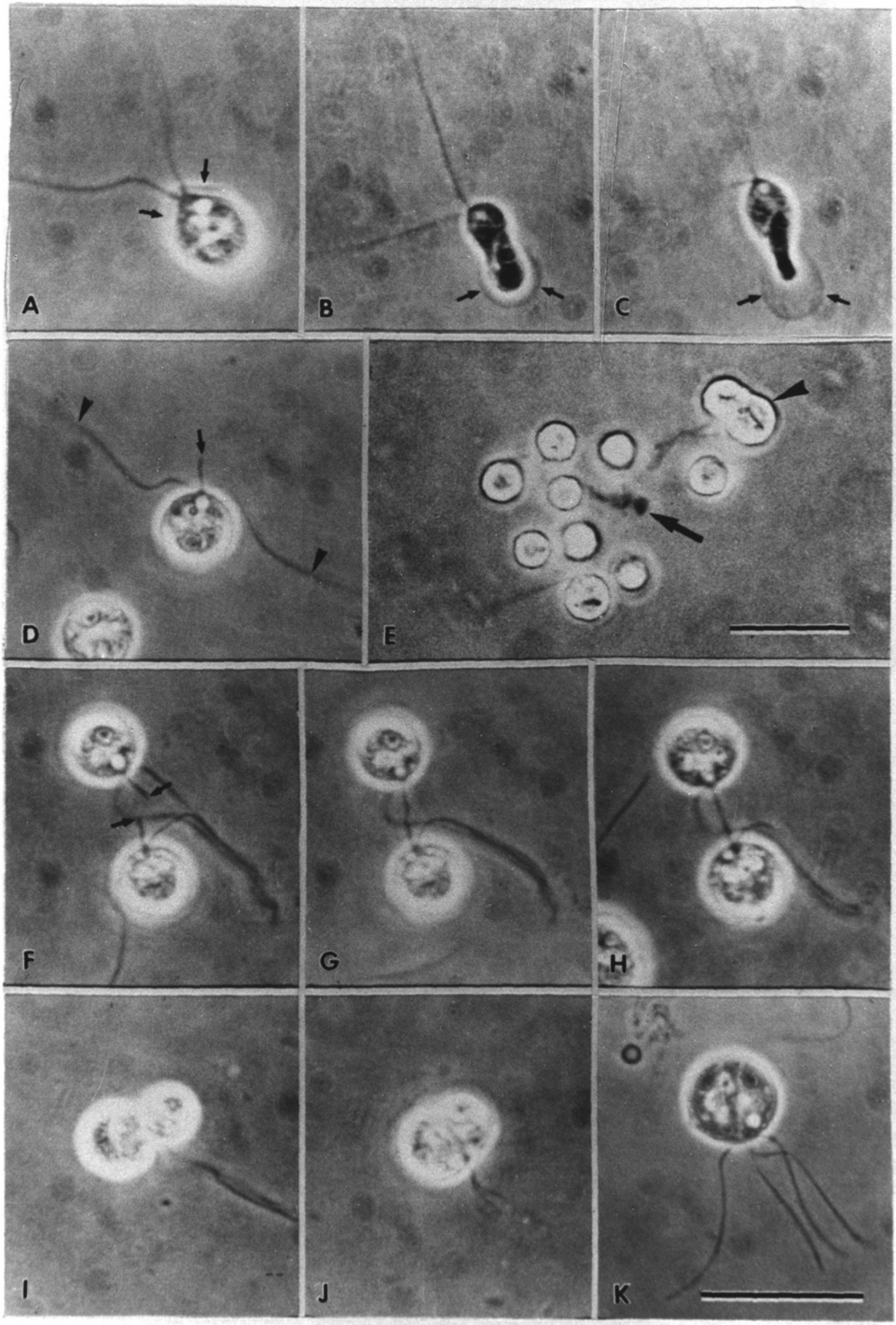
20µm


ps
pc
a
b
c
d
e
ps
pc
10µm

68
69
70
a
b
71
10μm


Oogamy
VOLVOCACEAE
Volvox
Anisogamy
Pleodorina
somatic cells
Eudorina
sperm packets
Isogamy
ASTREPHOMENACEAE
Pandorina unicocca
Astrephomene
Pandorina morum
Volvulina
lenticular cells
spheroidal colonies
Gonium pectorale
inversion spheroidal colonies
bilateral mating papil
SPONDYLOMORACEAE
8-celled stage:
8-celled stage:
Gonium sociale
unilateral mating papillae
Pyrobotrys
small gametes without mating papillae
mating papillae
complication of gelatinous mat


A
B
C
D
E
F


A
B
C
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E
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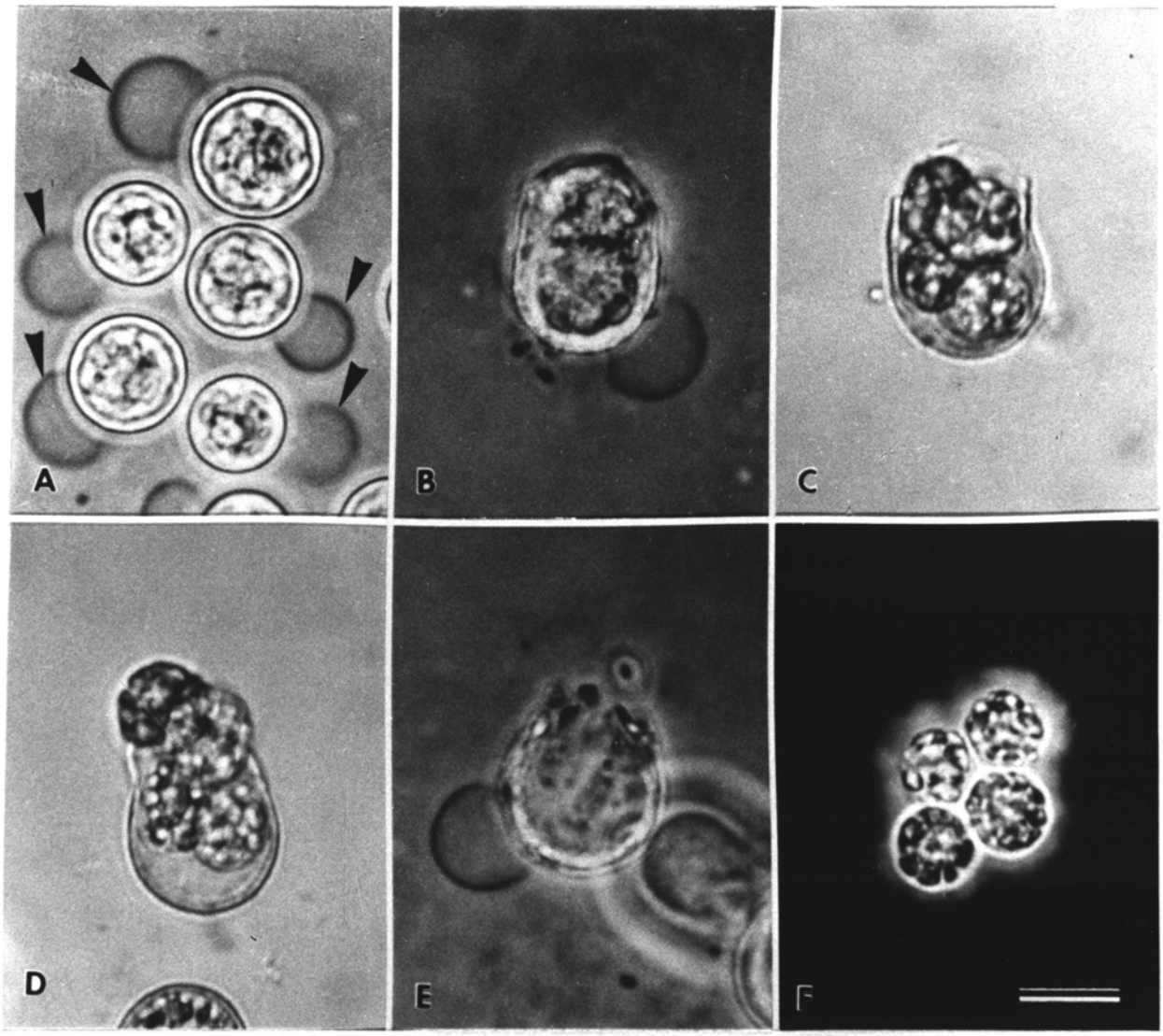
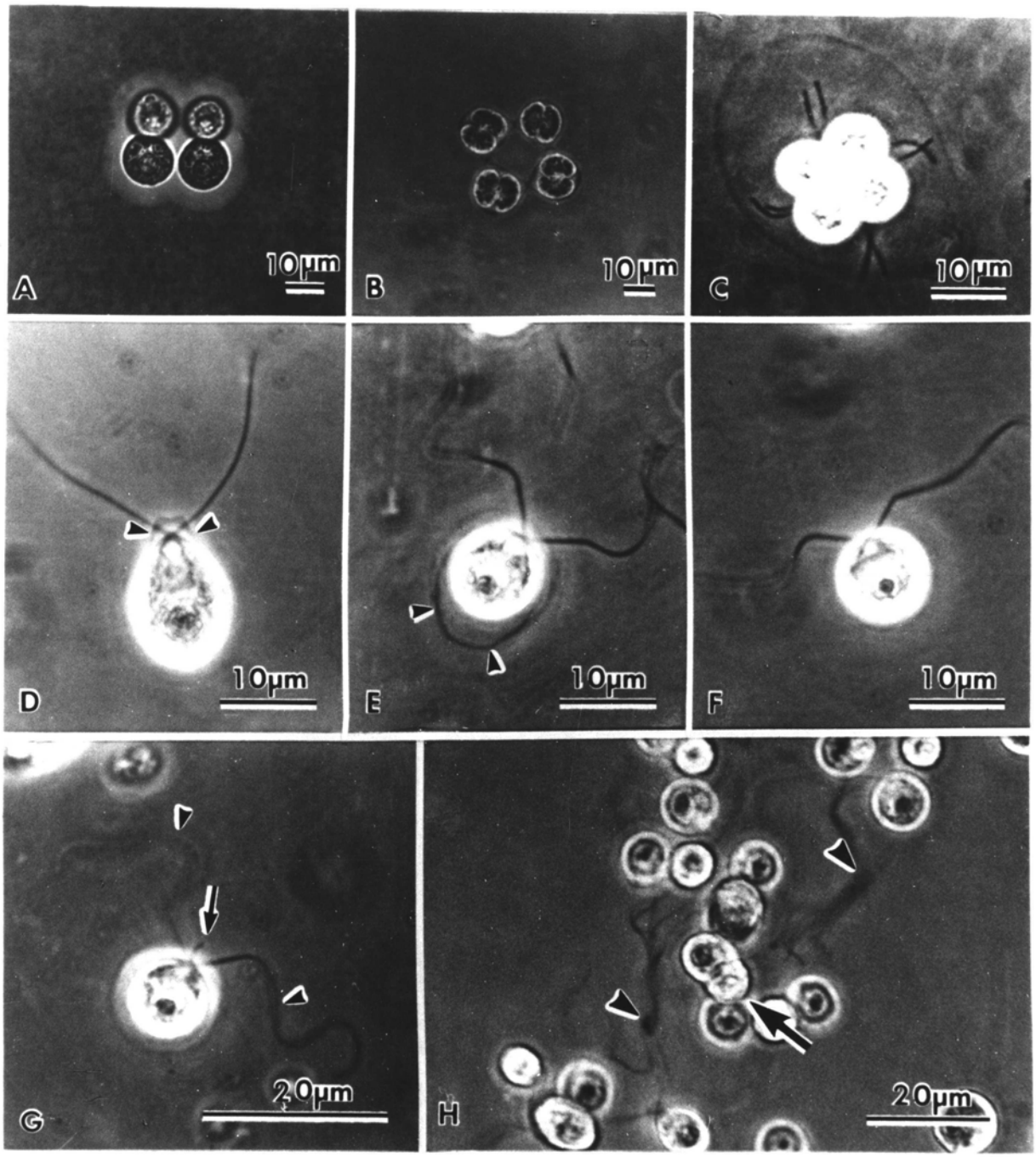
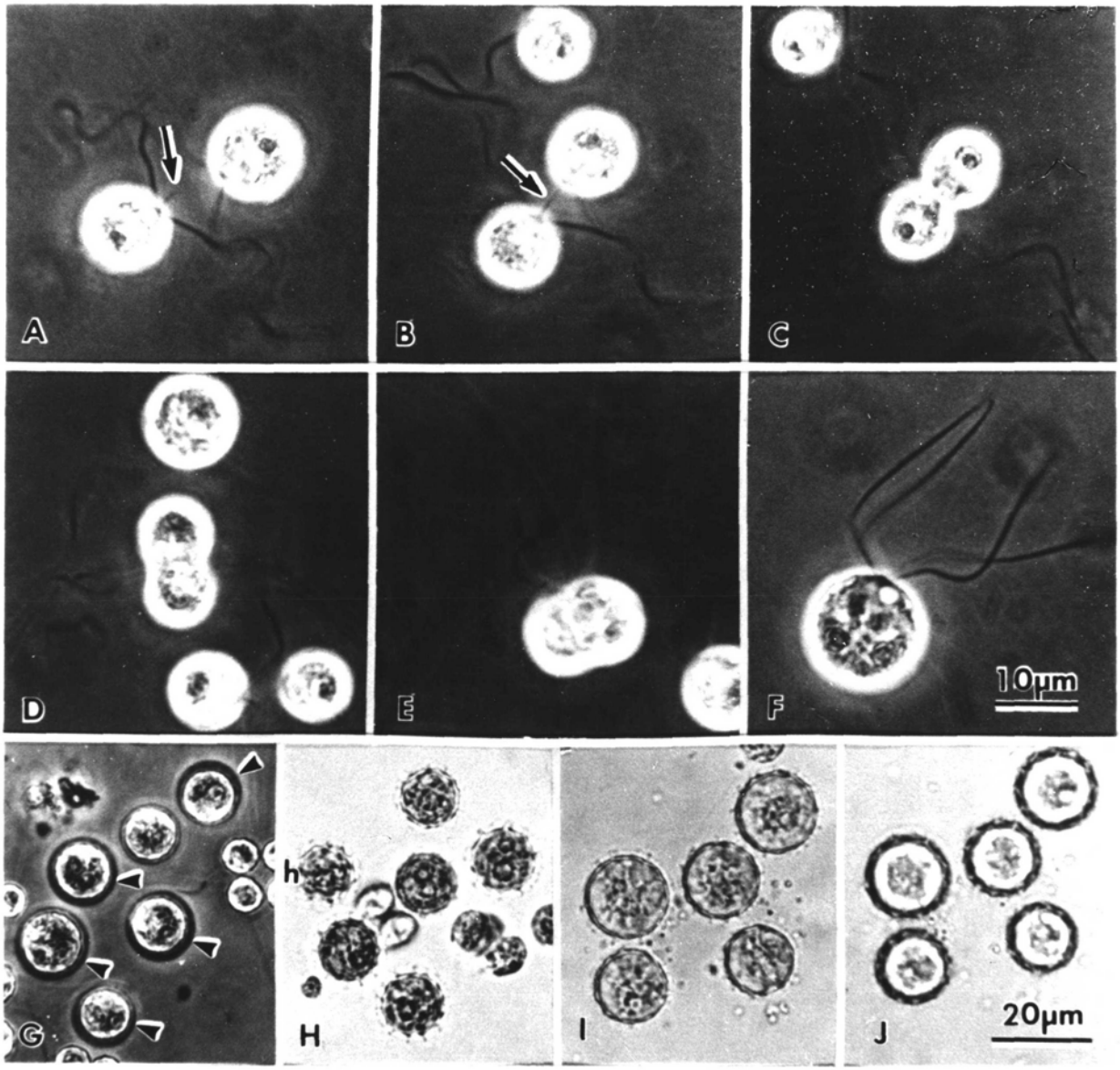
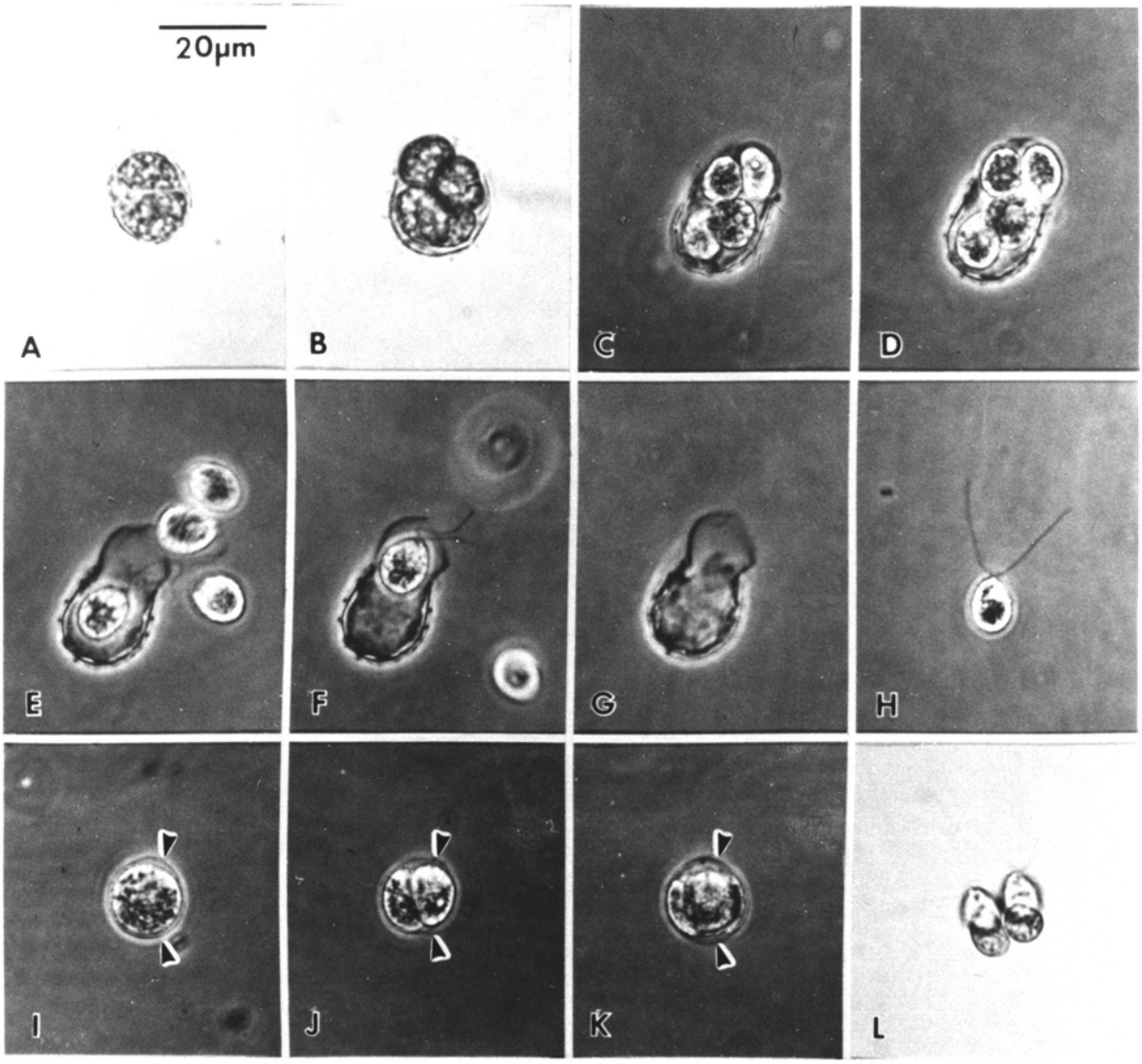
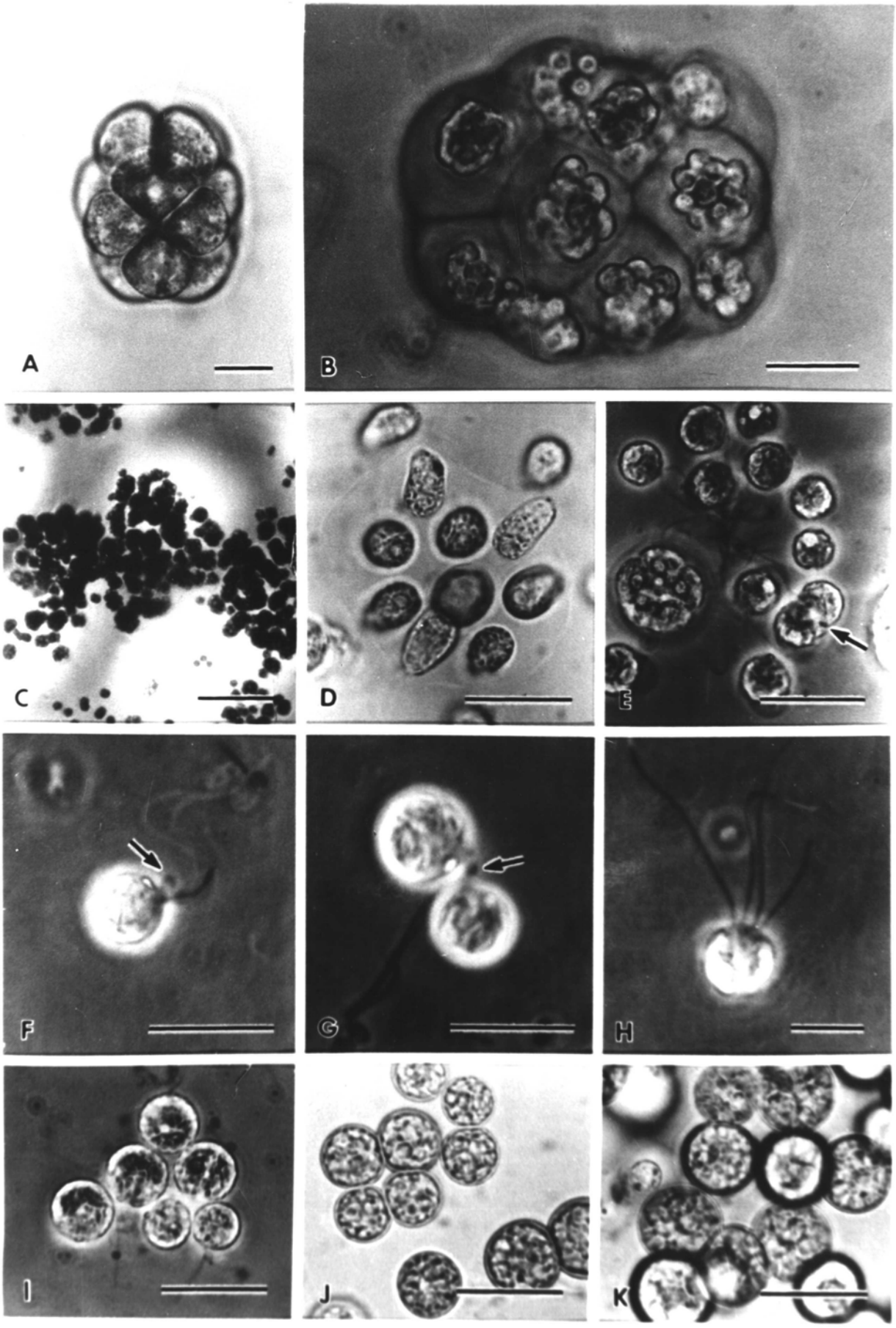
G
H
I
J
K


A
B
C
D
E
F


A
10μm
B
10μm
C
10μm
D
10μm
E
10μm
F
10μm
G
20μm
H
20μm


A
B
C
D
E
F
10μm
G
h
H
I
J
20μm


20μm
A
B
C
D
E
F
G
H
I
J
K
L


A
B
C
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G
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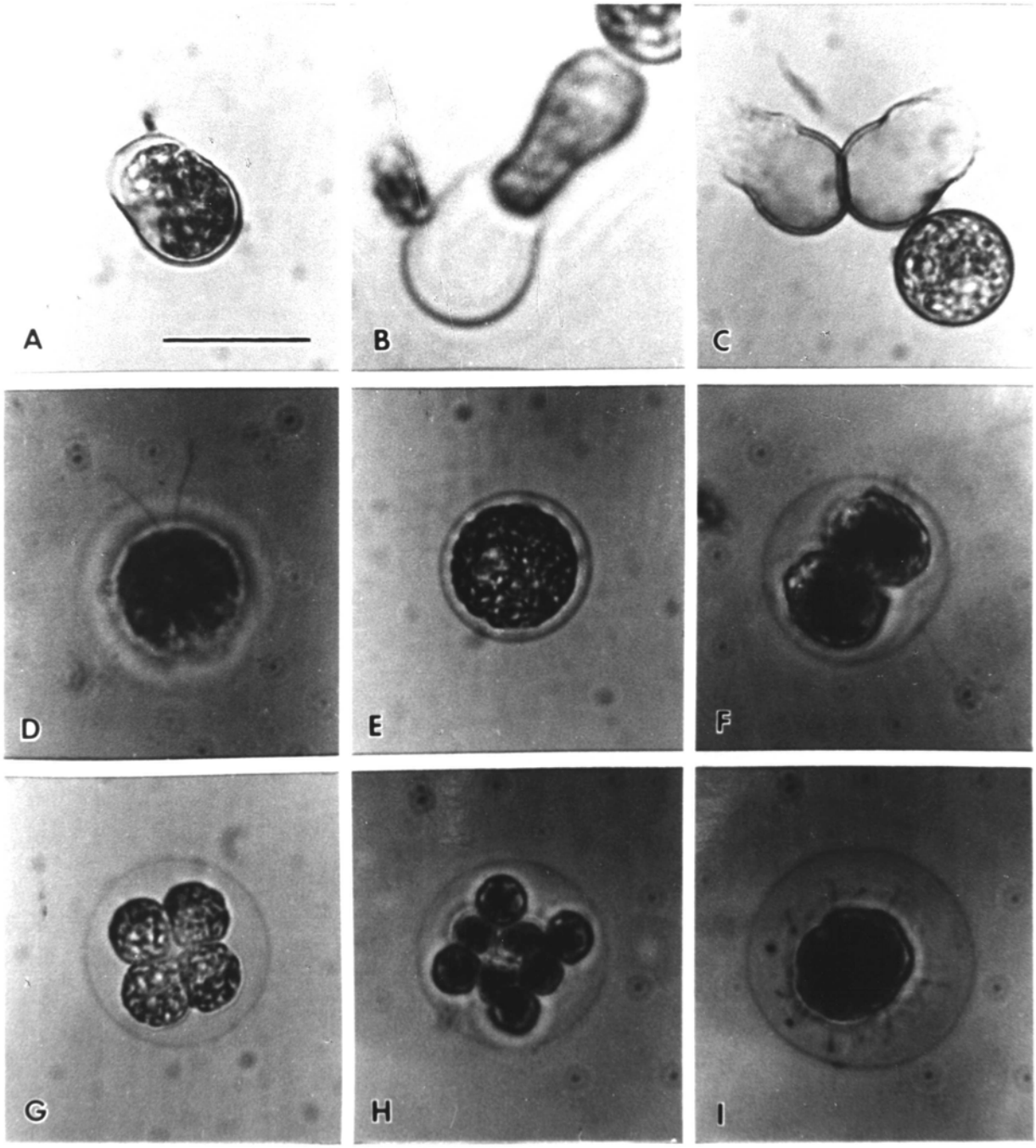
I
J
K


A
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G
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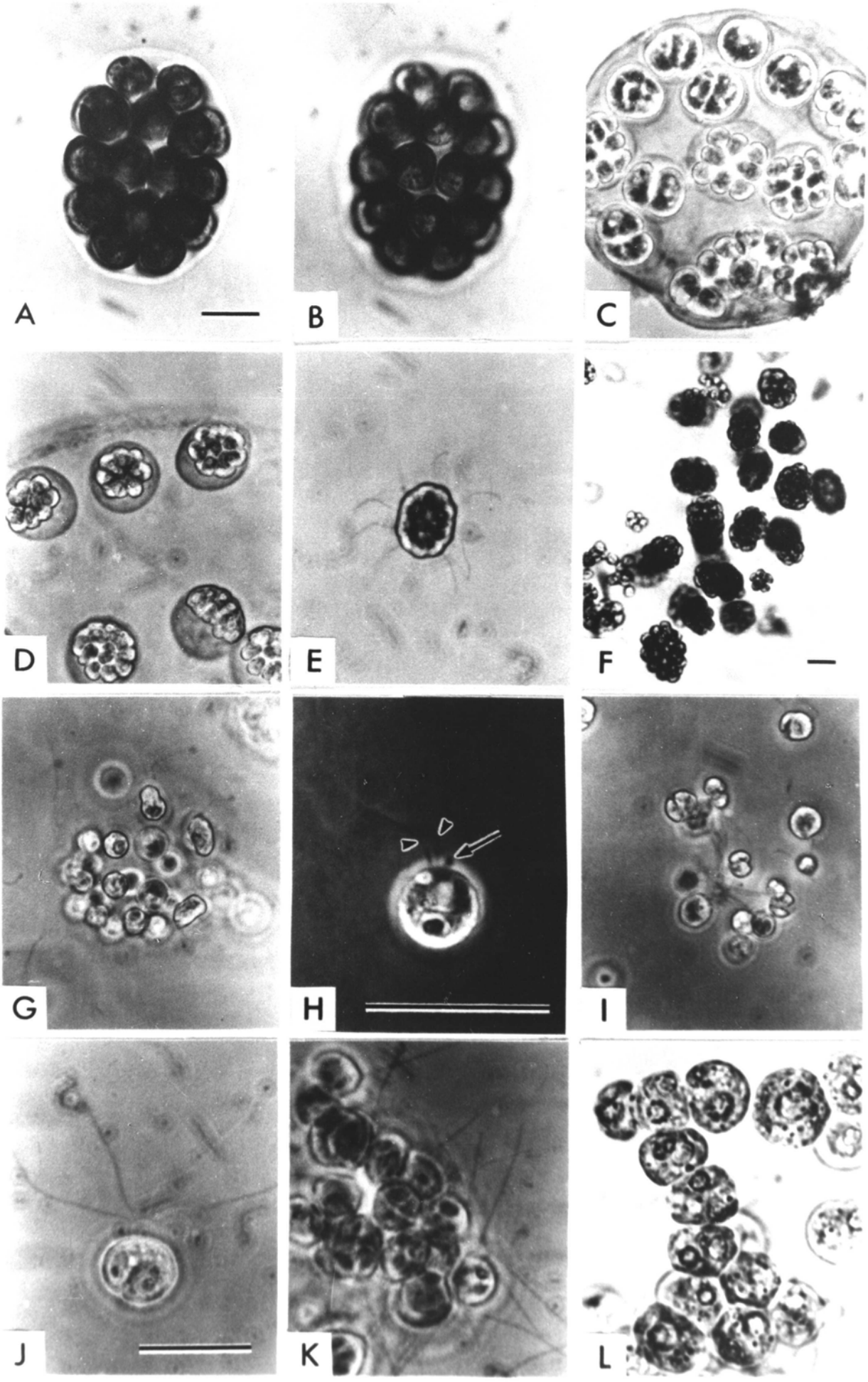
I


A
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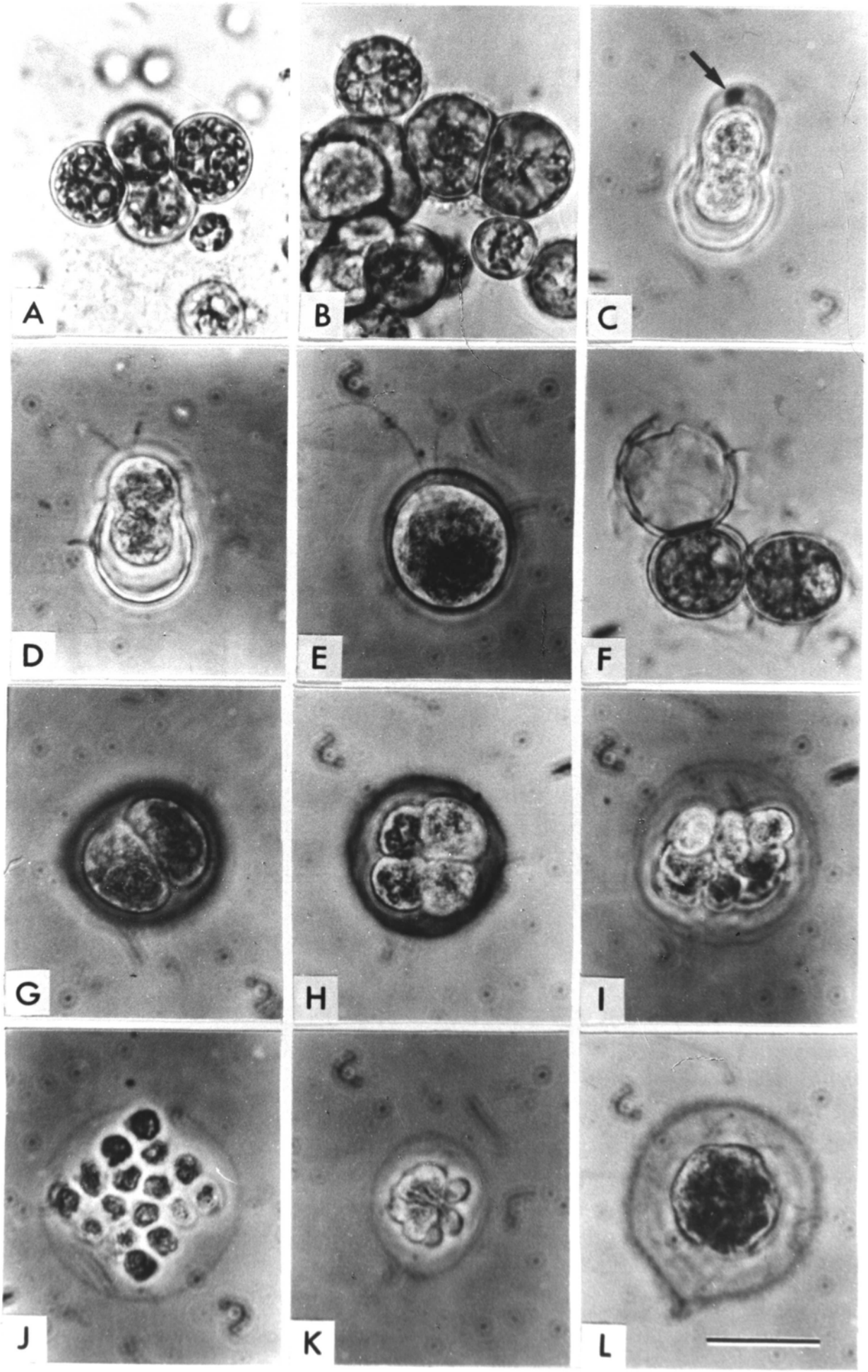
A
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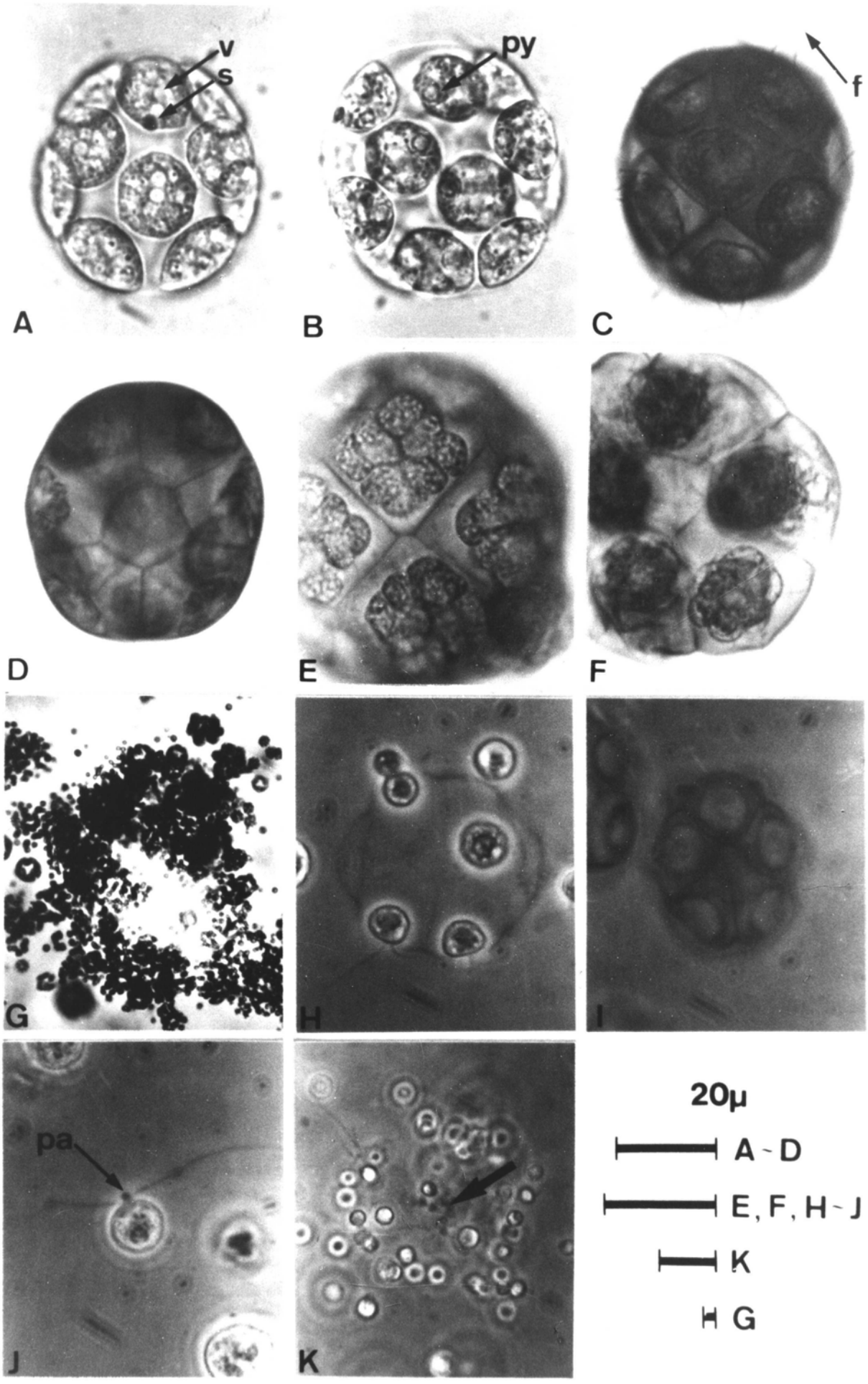
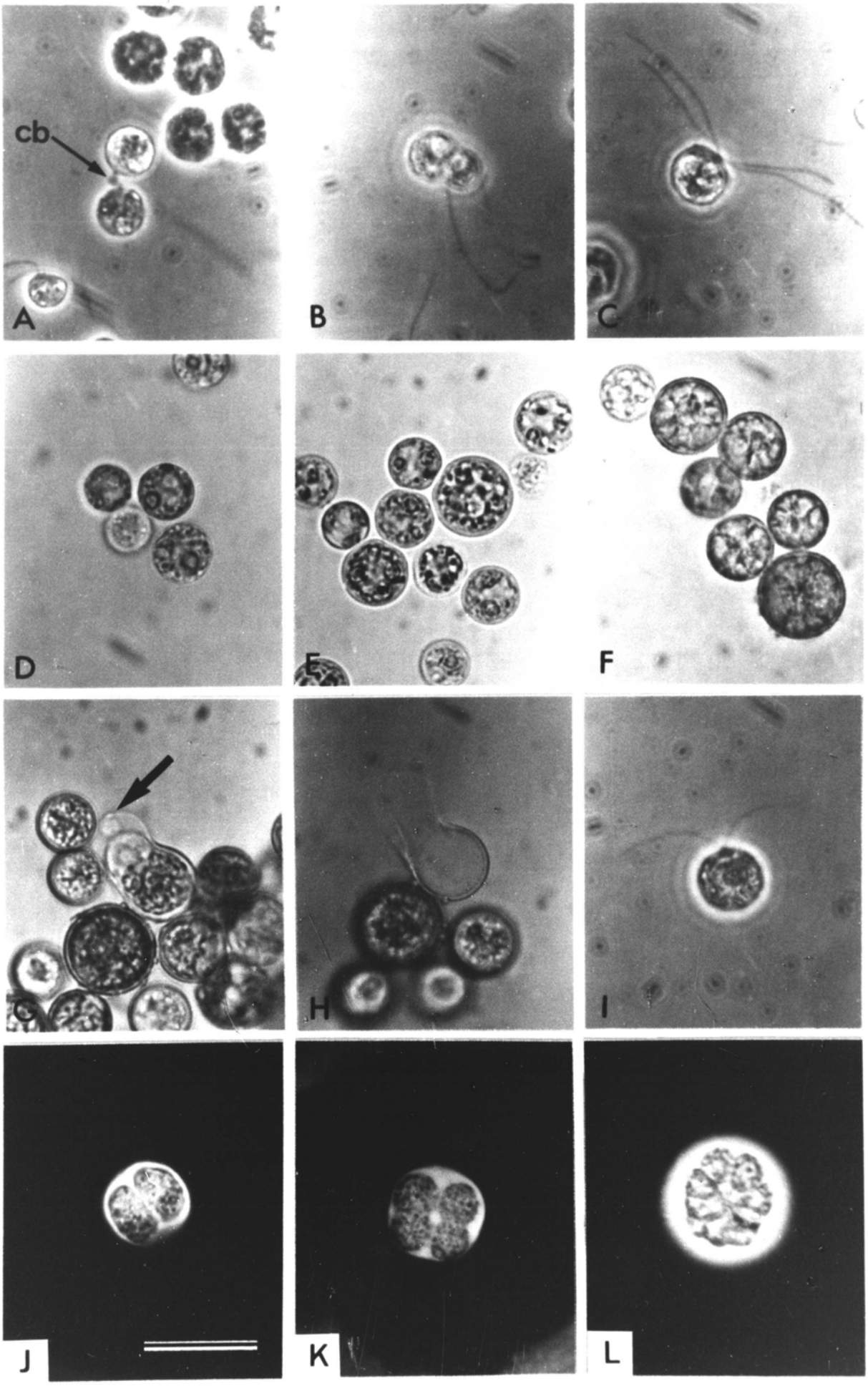

v
s
A
py
B
f
C
D
E
F
G
H
I
pa
J
K
20µ
A - D
E, F, H - J
K
G


cb
A
B
C
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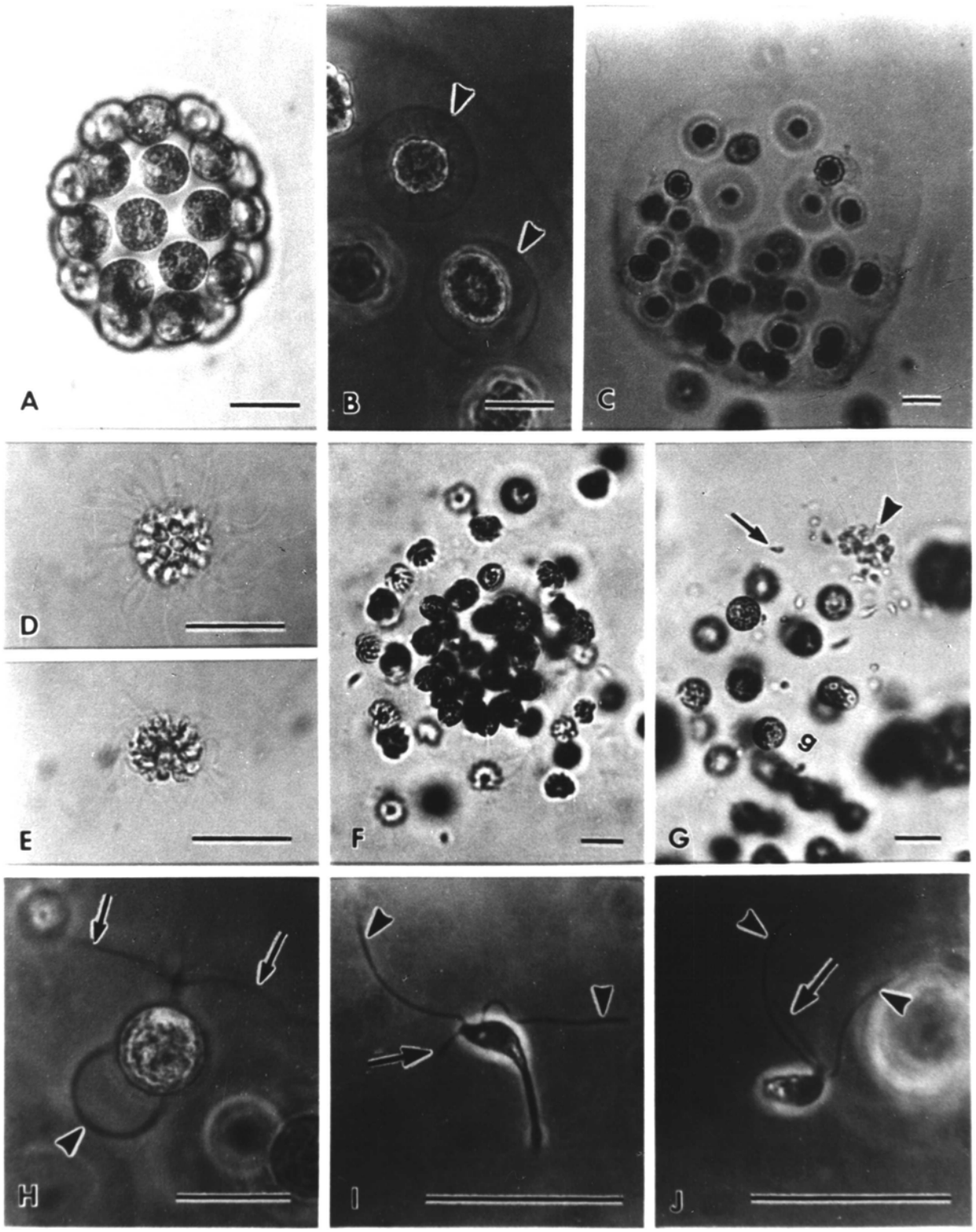
I
J
K
L


A
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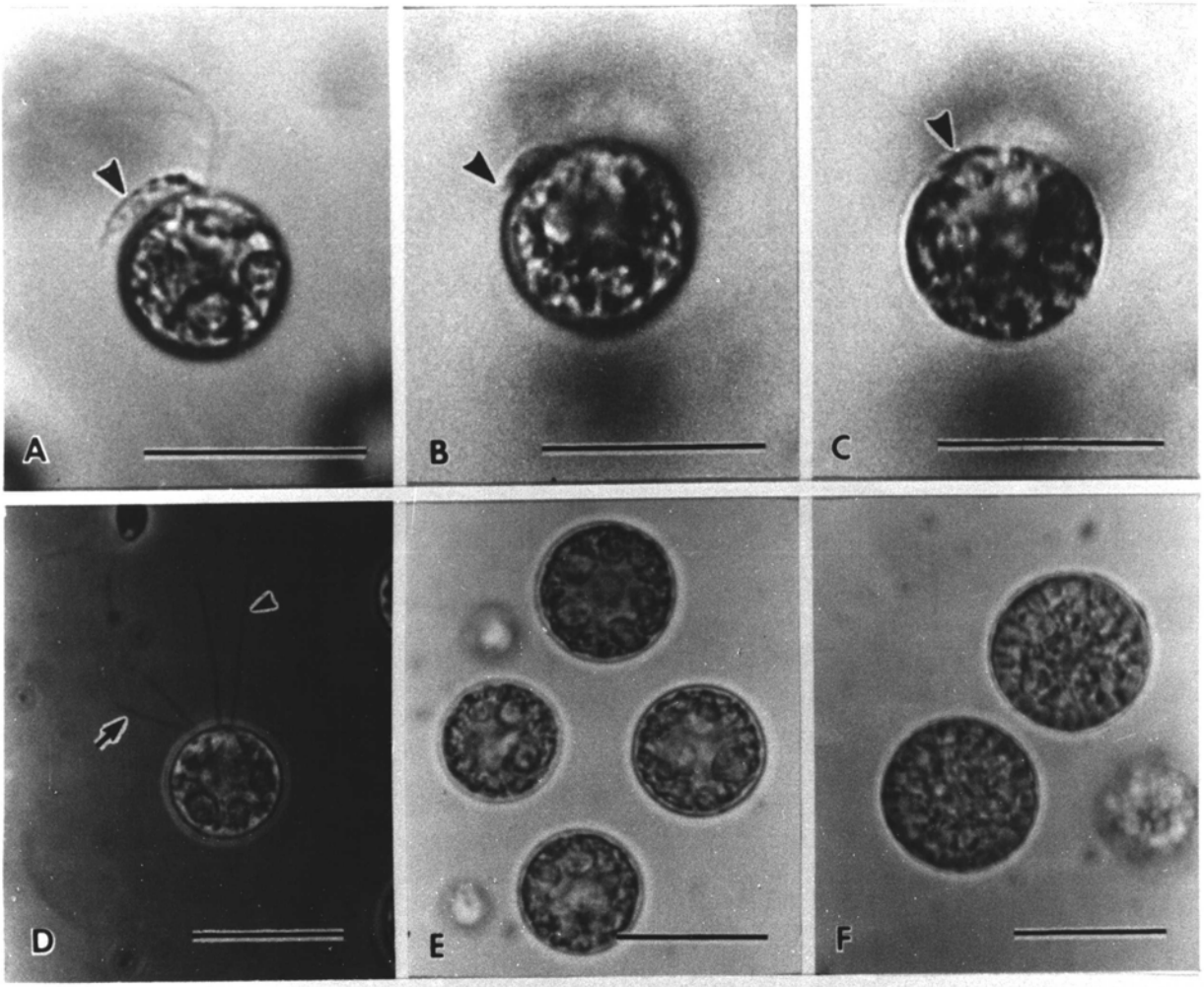
A
B
C
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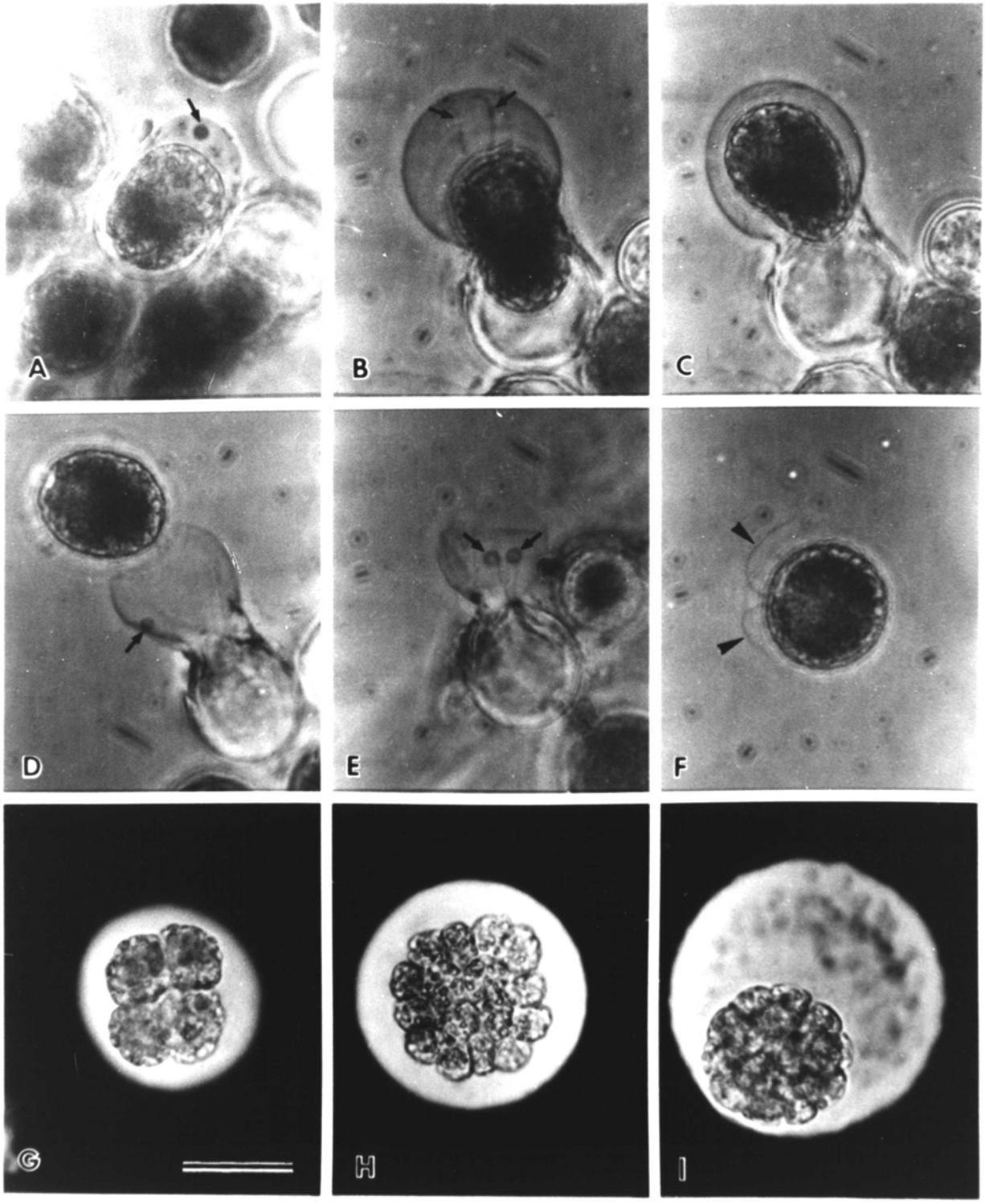
E
F


A
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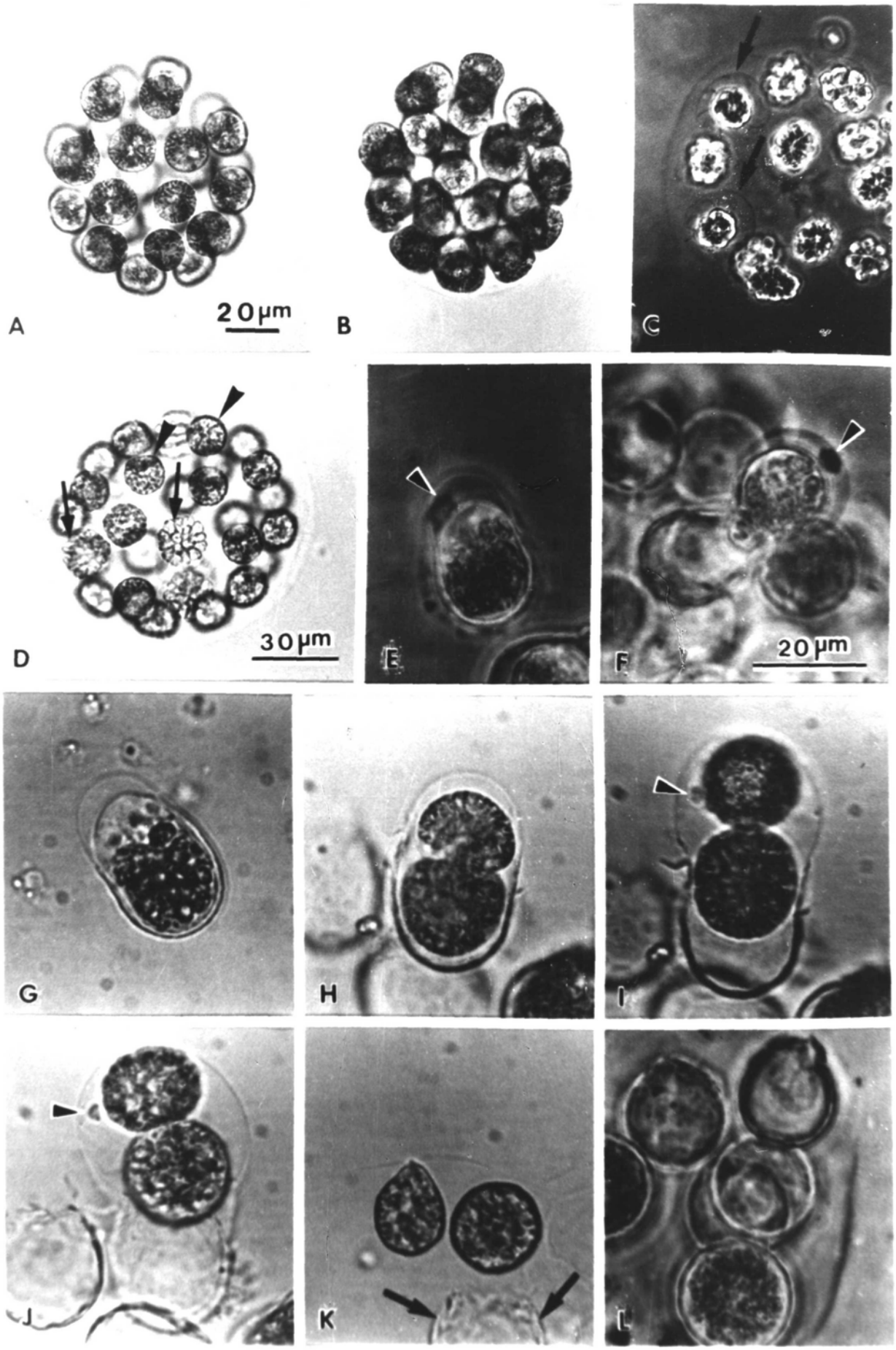
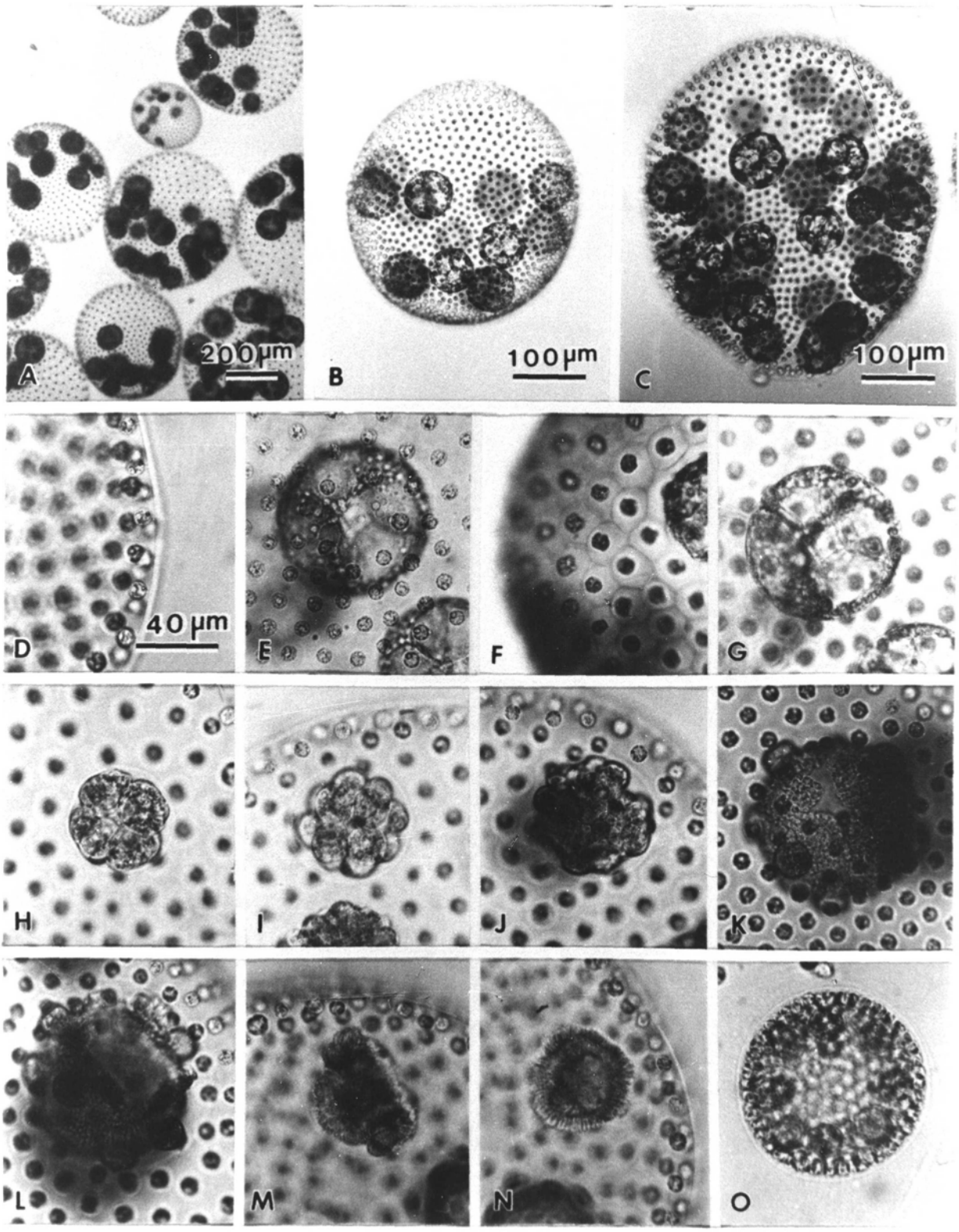
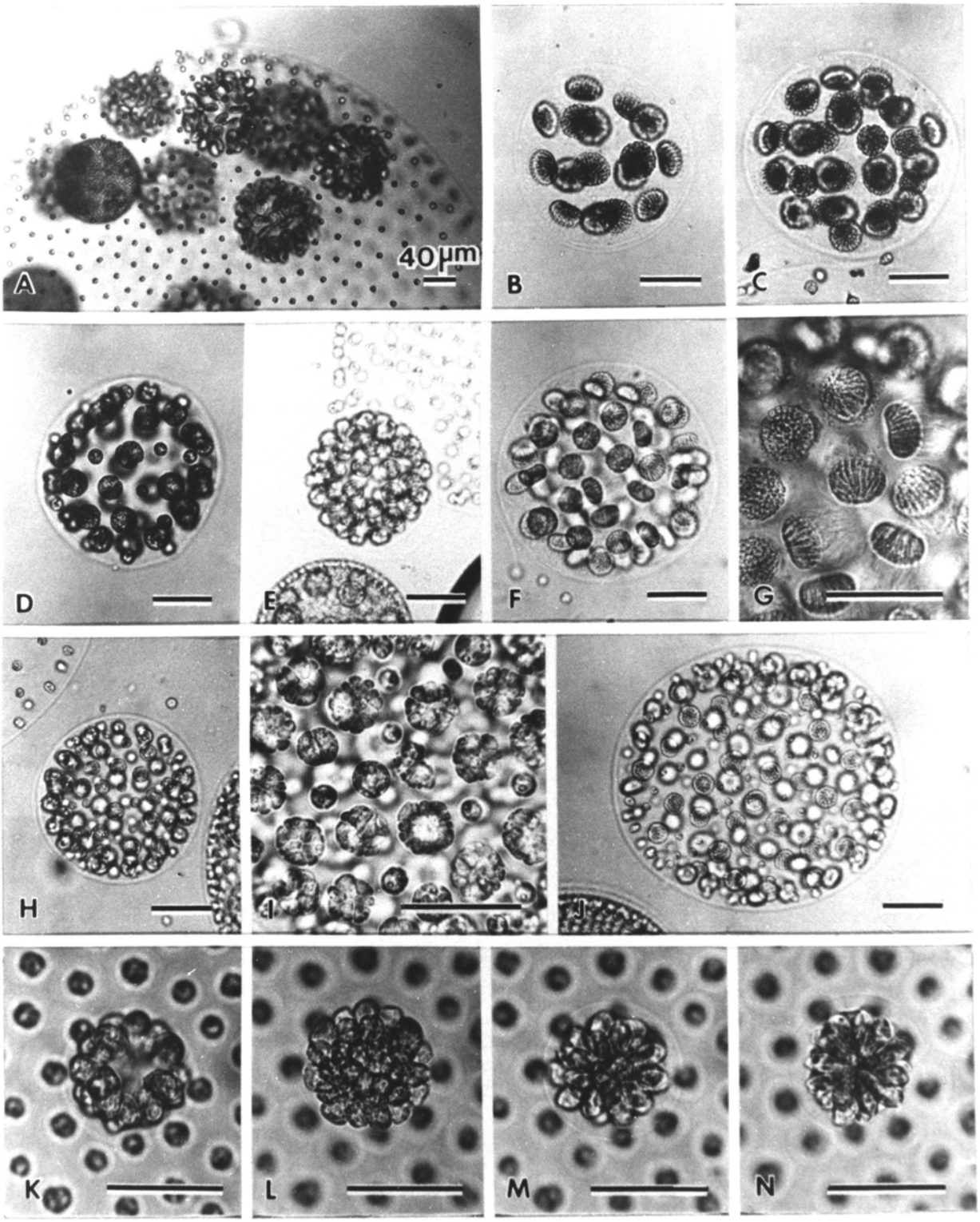
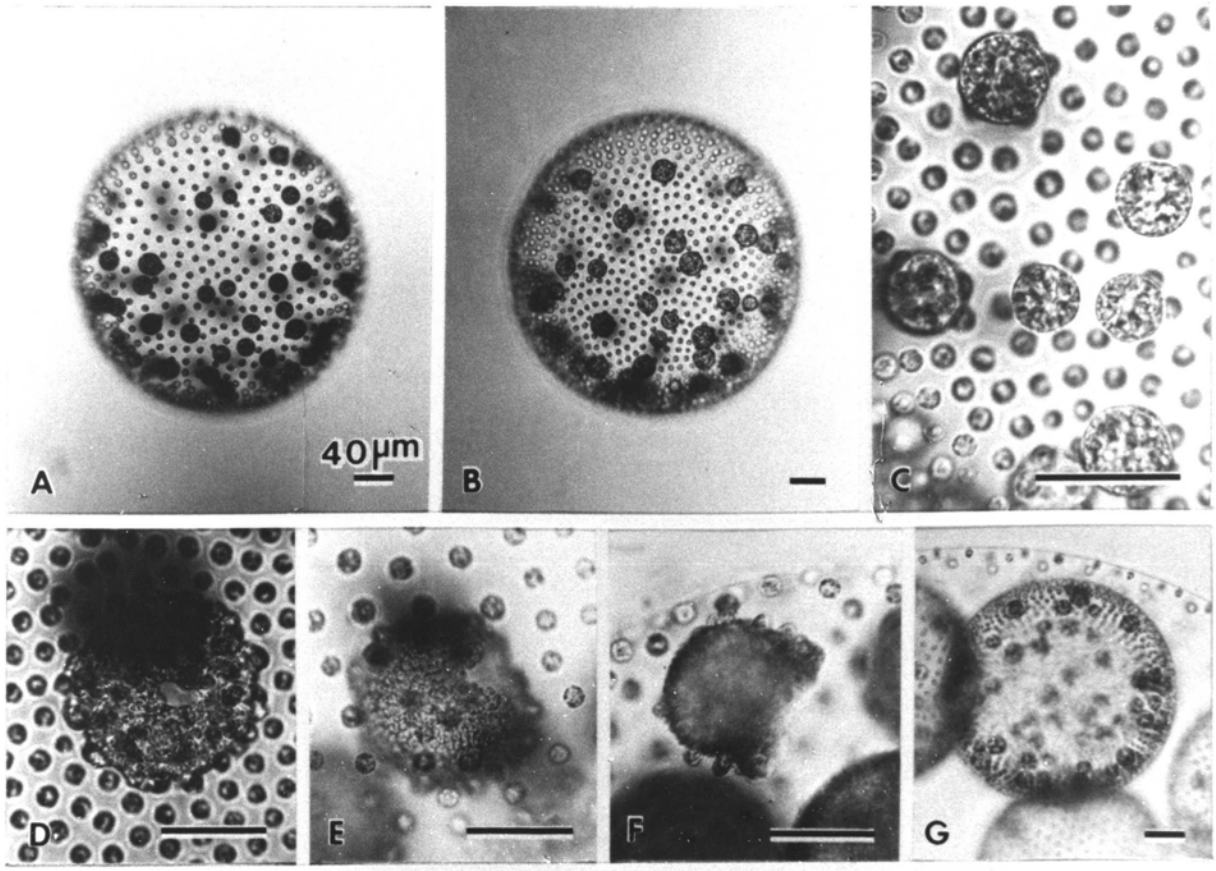
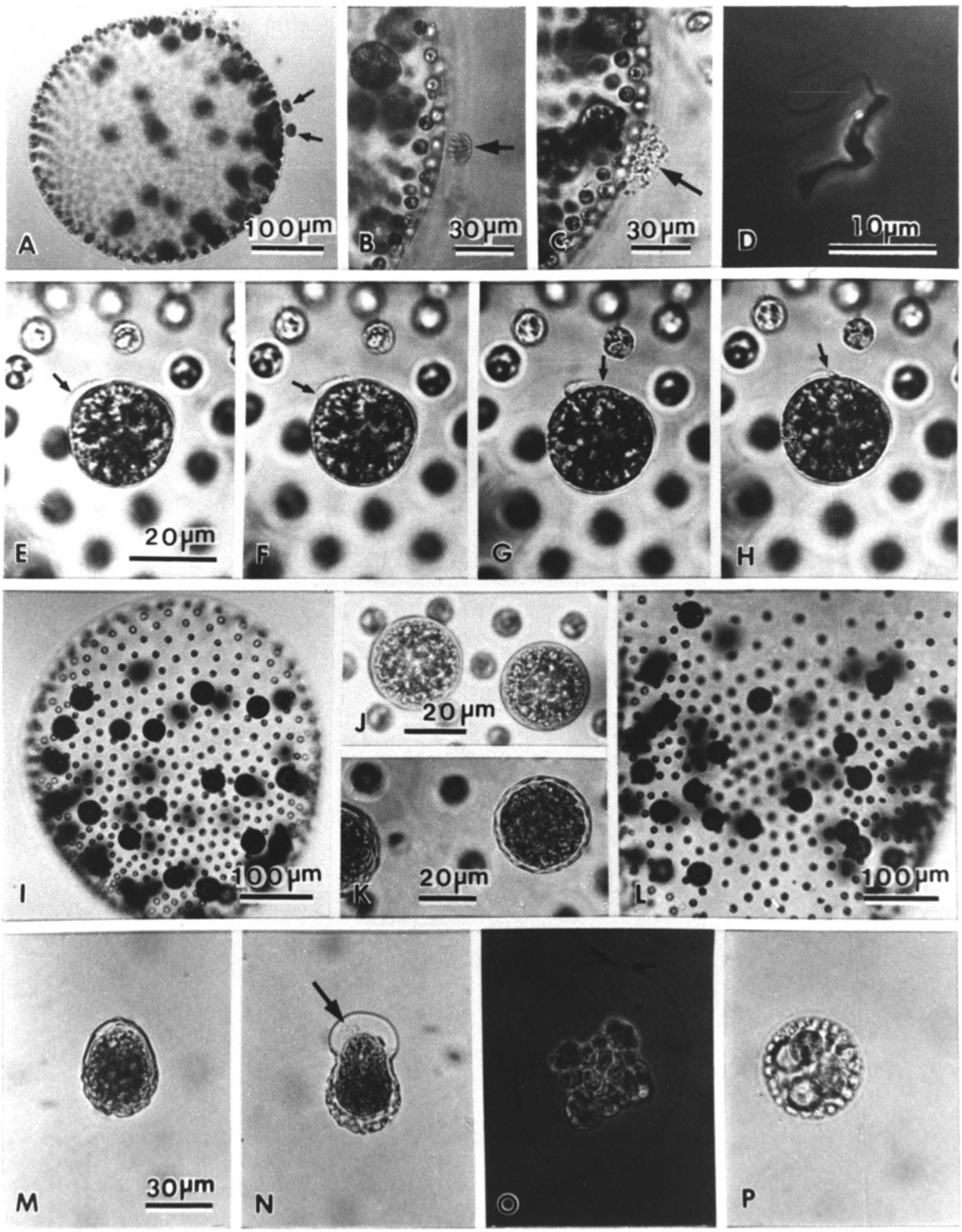
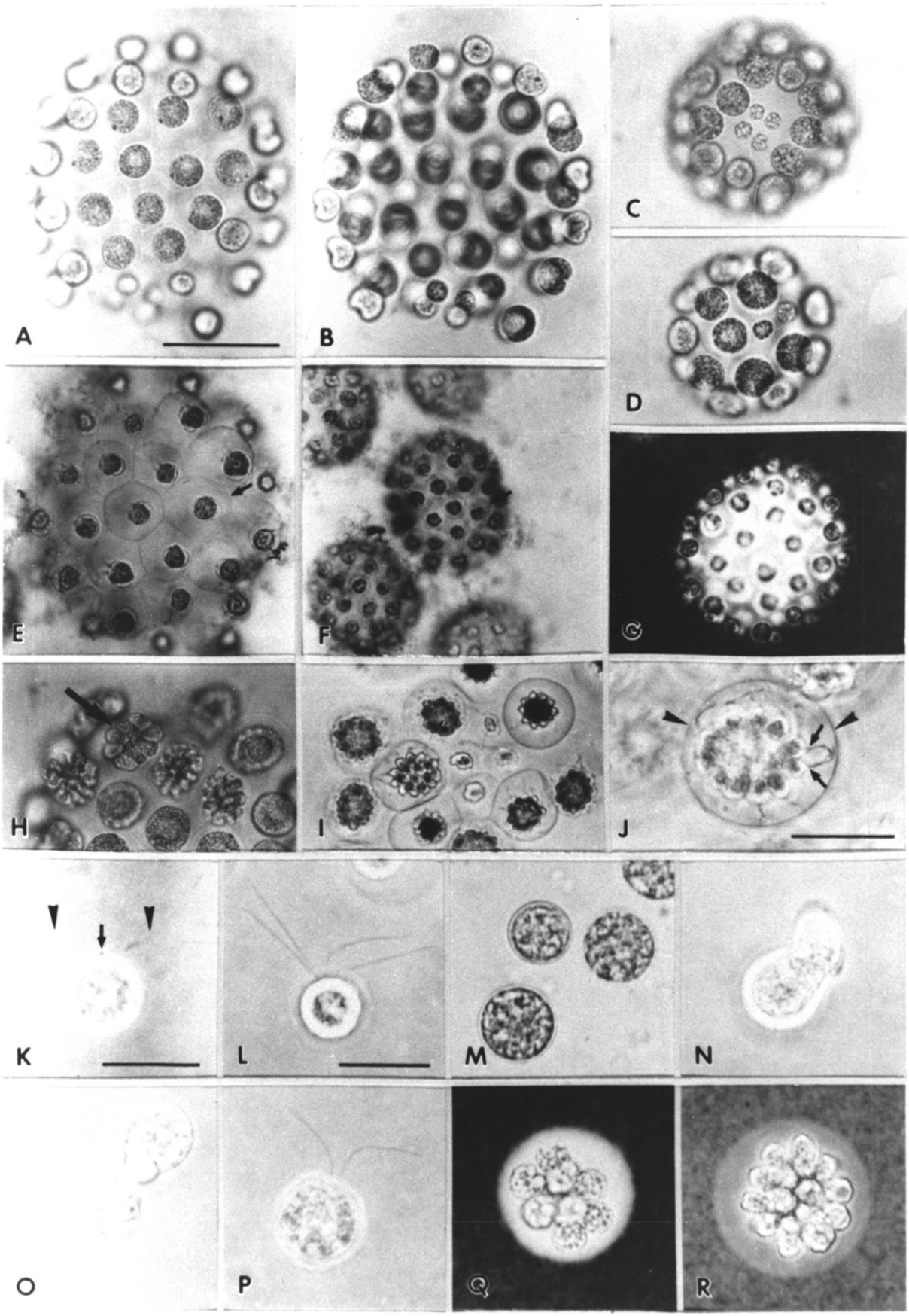

A
20μm
B
C
D
30μm
E
F
20μm
G
H
I
J
K
L

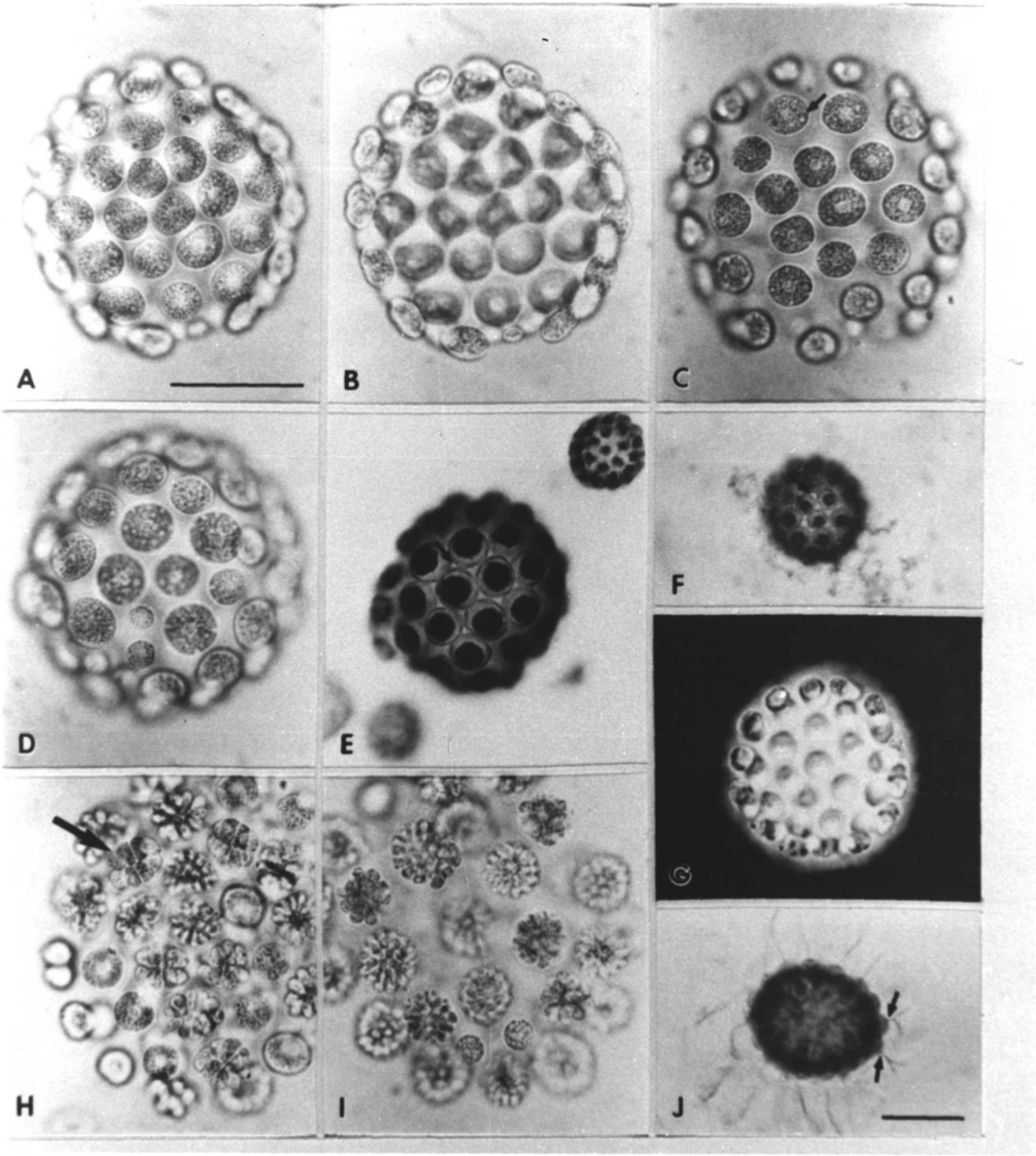

A
200μm
B
100μm
C
100μm
D
40μm
E
F
G
H
I
J
K
L
M
N
O


40µm
A
B
C
D
E
F
G
H
I
J
K
L
M
N


40μm
A
B
C
D
E
F
G


A
100μm
B
30μm
C
30μm
D
10μm
E
20μm
F
G
H
I
100μm
J
20μm
K
20μm
L
100μm
M
30μm
N
O
P


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R


A
B
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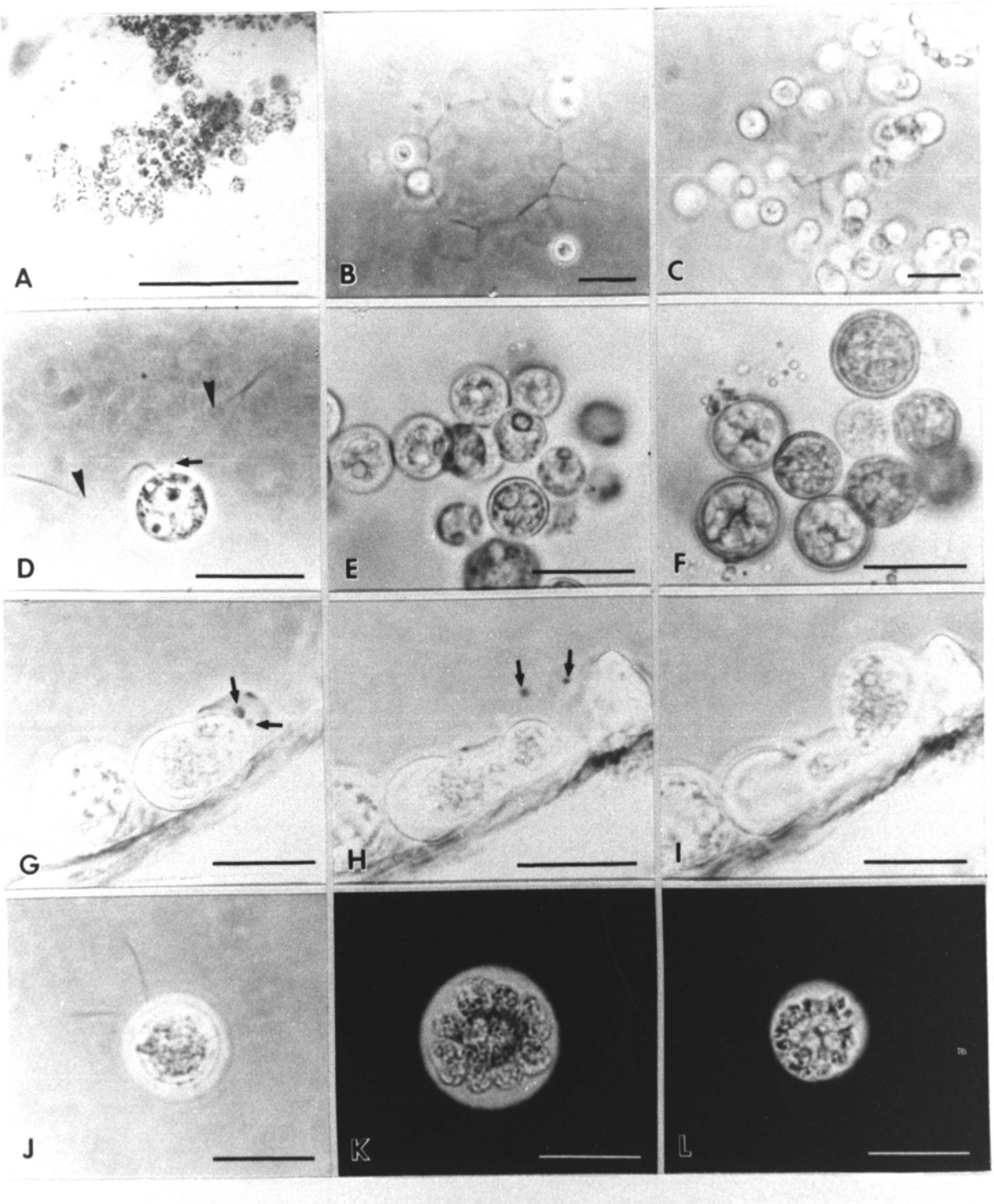
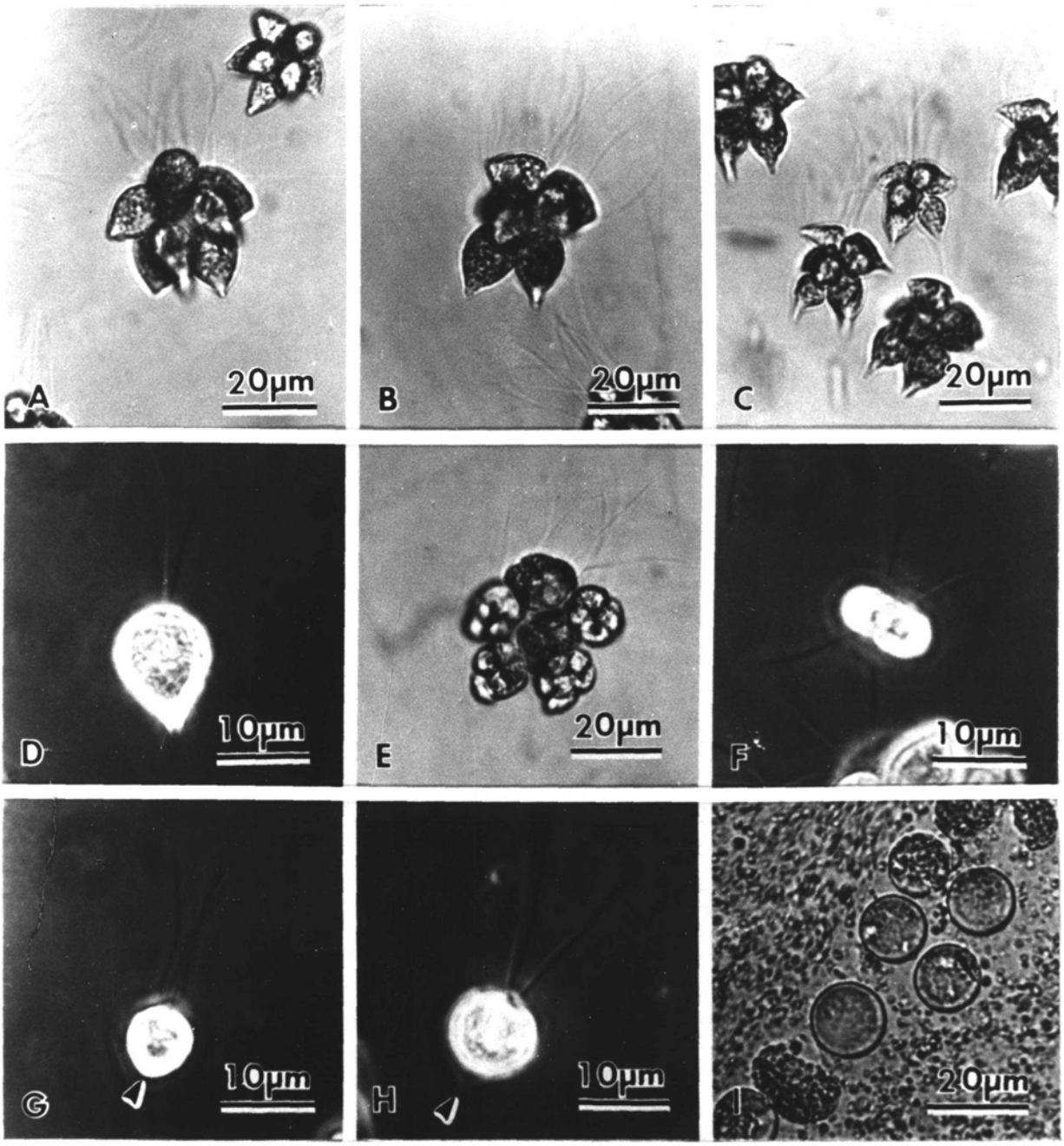
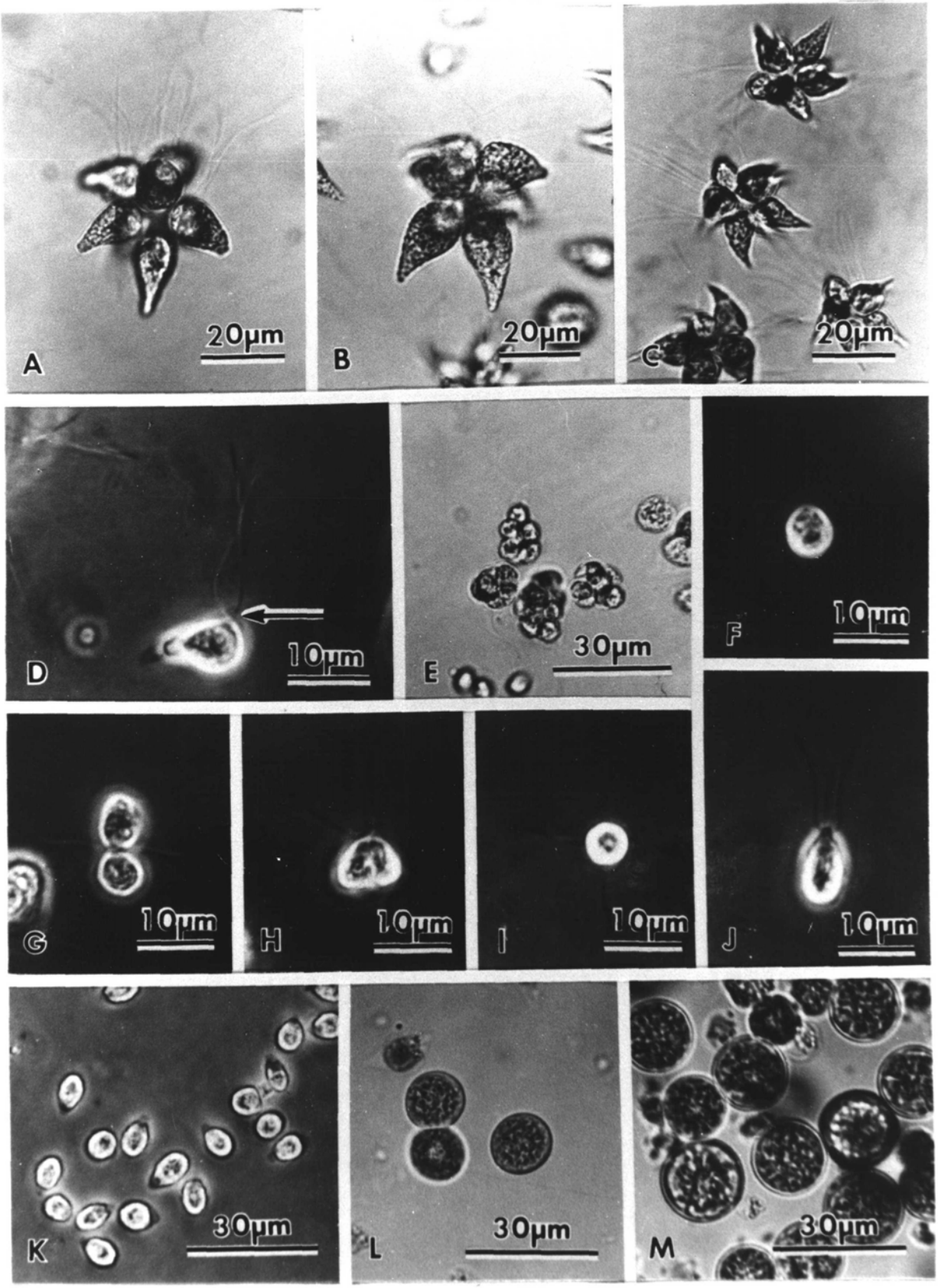
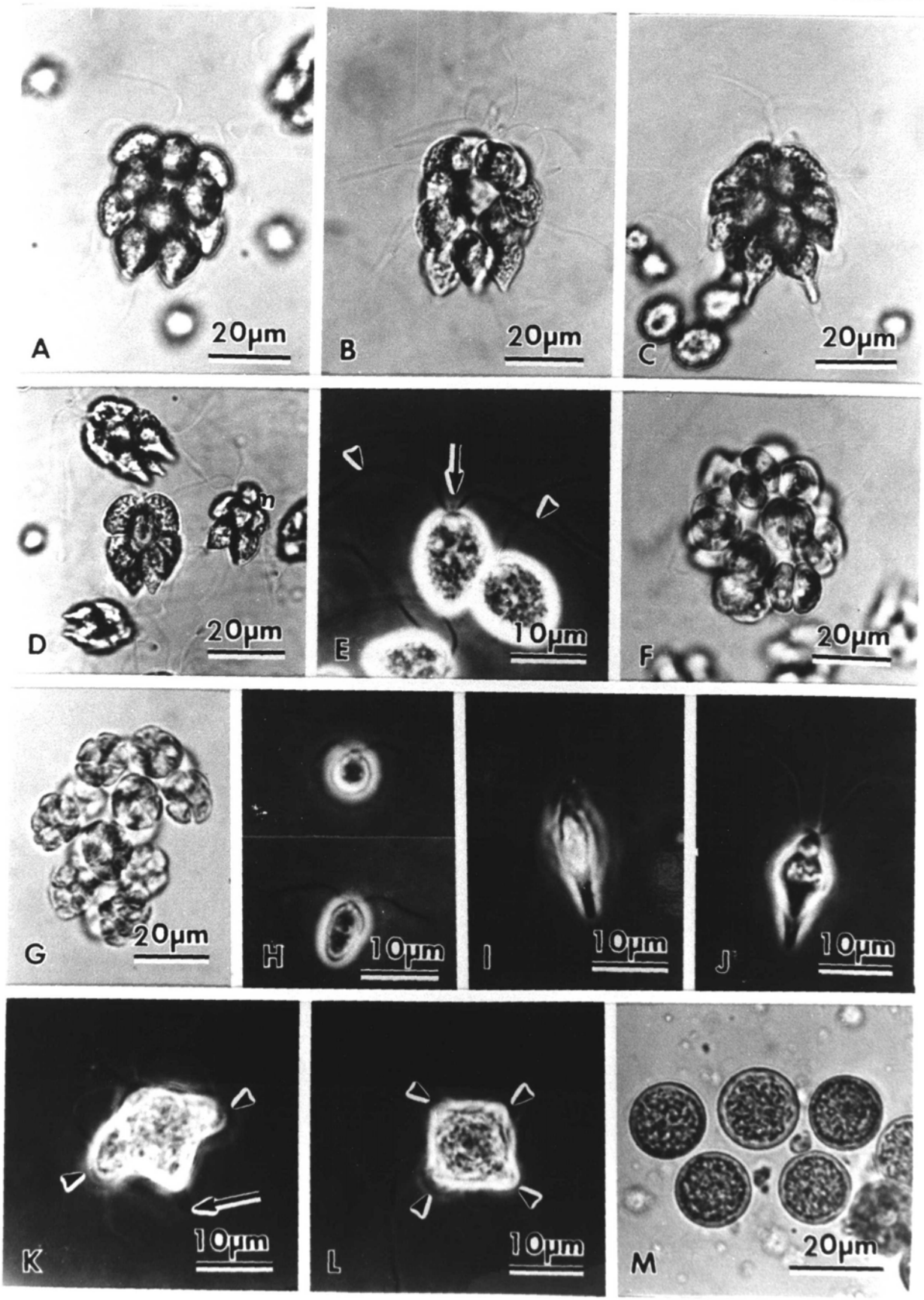
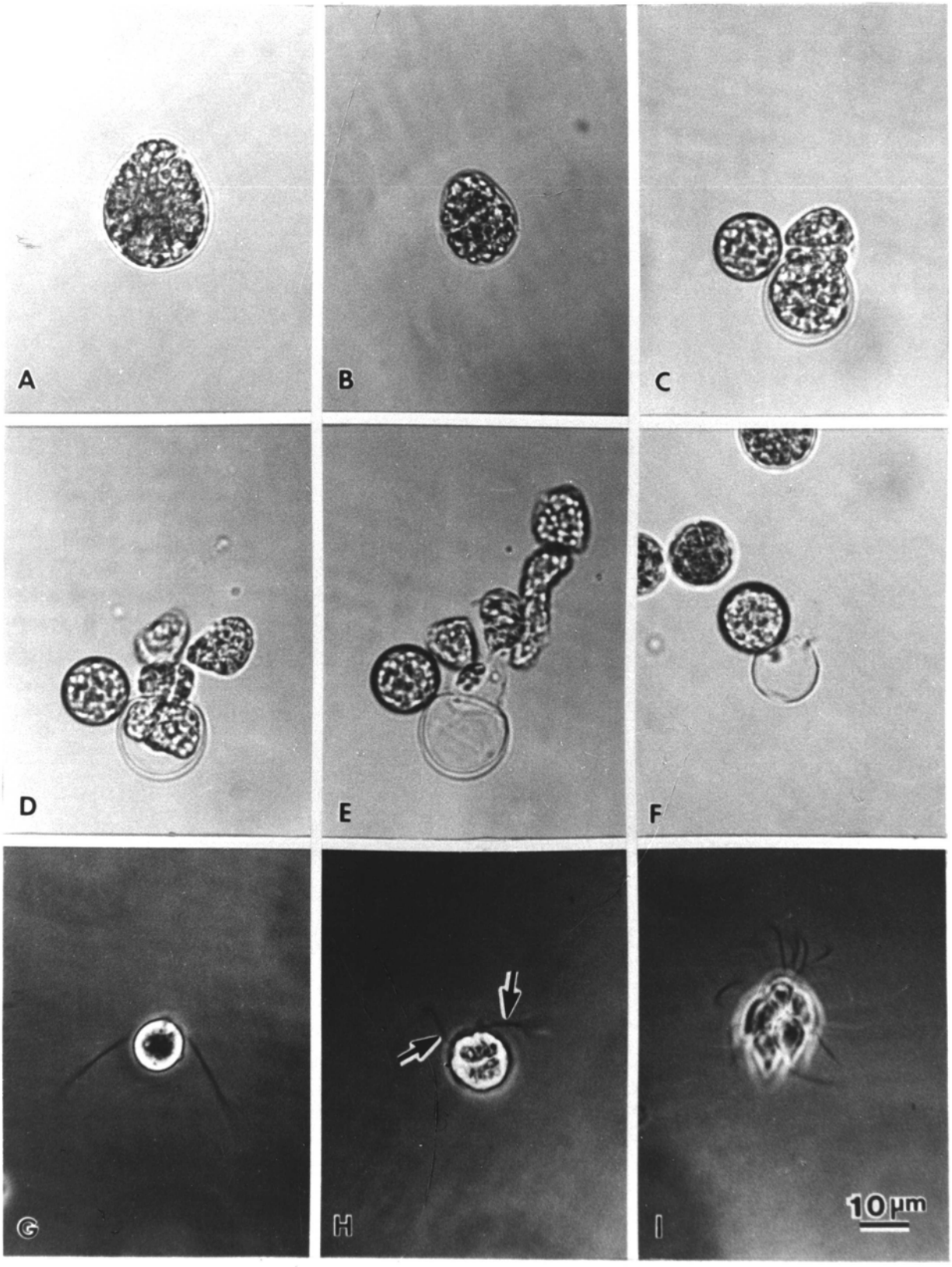
D
E
F
G
H
I
J

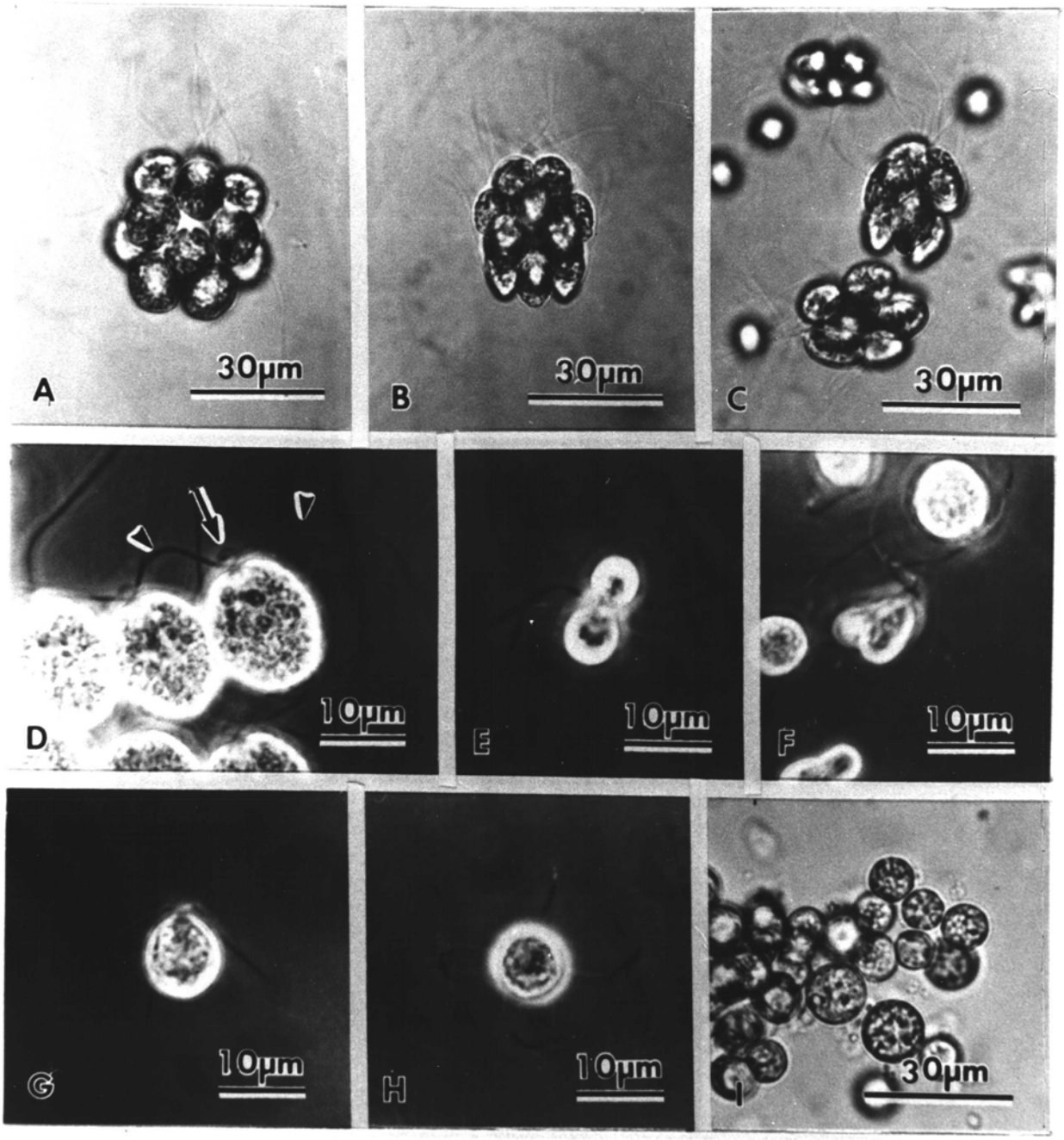

A
B
C
D
E
F
G
H
I
J
K
L


A
20μm
B
20μm
C
20μm
D
10μm
E
20μm
F
10μm
G
10μm
H
10μm
I
20μm


A
20μm
B
20μm
C
20μm
D
10μm
E
30μm
F
10μm
G
10μm
H
10μm
I
10μm
J
10μm
K
30μm
L
30μm
M
30μm


A
20μm
B
20μm
C
20μm
D
20μm
E
10μm
F
20μm
G
20μm
H
10μm
I
10μm
J
10μm
K
10μm
L
10μm
M
20μm


A
B
C
D
E
F
G
H
I
10µm


A
30μm
B
30μm
C
30μm
D
10μm
E
10μm
F
10μm
G
10μm
H
10μm
I
30μm