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Life History and Taxonomy of *Cricosphaera roscoffensis* var. *haptonemofera*, var. nov. (Class Prymnesiophyceae) from the Pacific*

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A new coccolithophorid *Cricosphaera roscoffensis* var. *haptonemofera* is described by means of electron microscopy and with the aid of laboratory culture. The living specimens, which were in the motile phase, were collected in Okinawa, a subtropical region in Japan, and a unialgal culture was established in the laboratory. The life history was observed, starting from the motile cells. The life history consists of two phases: the motile phase and the benthic filamentous phase. The former is unicellular and presumably a diploid sporophyte, whereas the latter is similar to an alga previously known as "*Apistonema submarinum*" described by Dangeard (1934), and probably a haploid gametophyte. The motile cells bear two acronematic flagella and one short haptonema. The scales attached on the surface of the motile cells are of two kinds: one is an organic thin scale, and the other is a cricolith, a kind of coccolith, the latter being structurally almost identical with that of *Cricosphaera roscoffensis* studied by Gayral and Fresnel (1976). Because this species has neither haptonema nor a benthic filamentous phase, we propose, at present, to treat this alga as a new taxon at the rank of infraspecies, naming it *Cricosphaera roscoffensis* var. *haptonemofera* Inouye et Chihara.

Since the findings by Stosch (1955) of the occurrence of an *Apistonema*-phase in certain members, the life history and taxonomy of the coccolithophorids (Class Prymnesiophyceae) have been the subject of recent studies using laboratory culture and the electron microscope. Most of these studies, however, were on species from the western Atlantic, and very little information is available about those from the Pacific. Therefore, one of the objective of this research was to determine whether a flora and the life history of this algal group within our boundaries of water are similar to those described in Europe. For the last several years special attentions have been given to this group of algae as well as to collecting living specimens along the coast of Japan. The present paper describes the results obtained from a study of a new coccolithophorid, *Cricosphaera roscoffensis* var. *haptonemofera*, var. nov. The study was made using the laboratory cultures and the electron microscope.

Materials and Methods

Specimens used in the present study were collected as motile cells from a tide pool in a coral reef at Minatogawa, Okinawa, which is located in the subtropical region of Japan.

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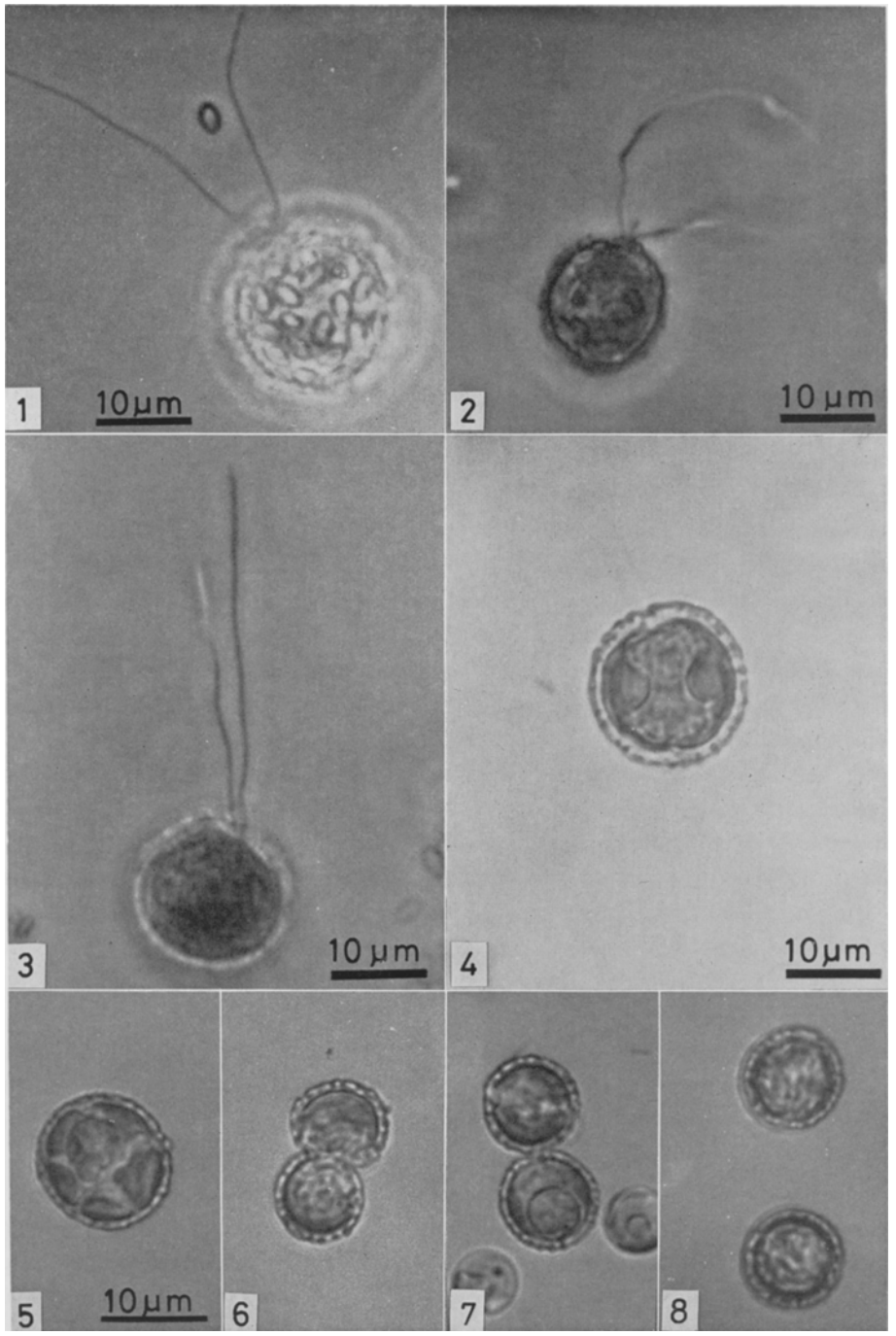
The isolation was made by the micropipette method and the alga was cultured in SW2 (Iwasaki, 1961). The culture was maintained either in SW2 or in ASP7 (Provasoli, 1968) for establishing unialgal cultures. The culture conditions were at about 15 C, with alternating 16 hr light/8 hr dark at a light intensity of about 2500 lux provided by cool white fluorescent tubes. For the induction of the benthic filamentous phase from the motile ones, the cells were usually inoculated upon acidified solid medium, with a pH ranging from 6.0 to 7.0, and containing 0.8% agar. For the induction of the release of swarmers from the benthic form, a filament was inoculated into a drop of fresh ASP7 medium hanging under a cover slip, and then the cover slip was placed on a depression slide, so that the filaments within a drop faced the concavity of the glass. For continuous observation, the cover slips were usually sealed with vaseline to avoid desiccation.

The following procedures were used in direct preparations for electron microscopy. A drop of medium containing swimming cells was exposed to osmium tetroxide vapor for about 30 sec and then the drop was placed, by micropipetting, on a carbon-coated grid. Shadowcasting was not carried out in the present examination. For sectioning, cells were fixed for 30 min in 5% glutalaldehyde with ASP7 medium. This was followed by several washes in ASP7 medium and postfixed for 3 hr by 2% osmium tetroxide, or followed by washes in phosphate buffer at pH 7.0 and postfixed by 2% osmium tetroxide for 1 hr. These were dehydrated by an ethanol series and finally embedded in 'Epon'. Sections were cut with glass knives and were double stained with uranyl acetate and lead citrate. Electron microscopy was carried out using a JEM-7 and JEM-100C electron microscopes. Fixation for scanning electron microscopy was made by Lugol's solution, since this solution had produced good results, preventing the removal of coccoliths from the cells. Fixed materials were washed several times in distilled water and then dehydrated in ethanol. This was followed by the replacement of amylalcohol for application to critical point drying techniques. Observations were made using a JEOL JSM-25 scanning electron microscope. For light microscopy, both motile cells and the filaments were examined alive in most cases, but sometimes they were fixed in Lugol's solution or 2% osmium tetroxide.

Observations and Results

Motile phase

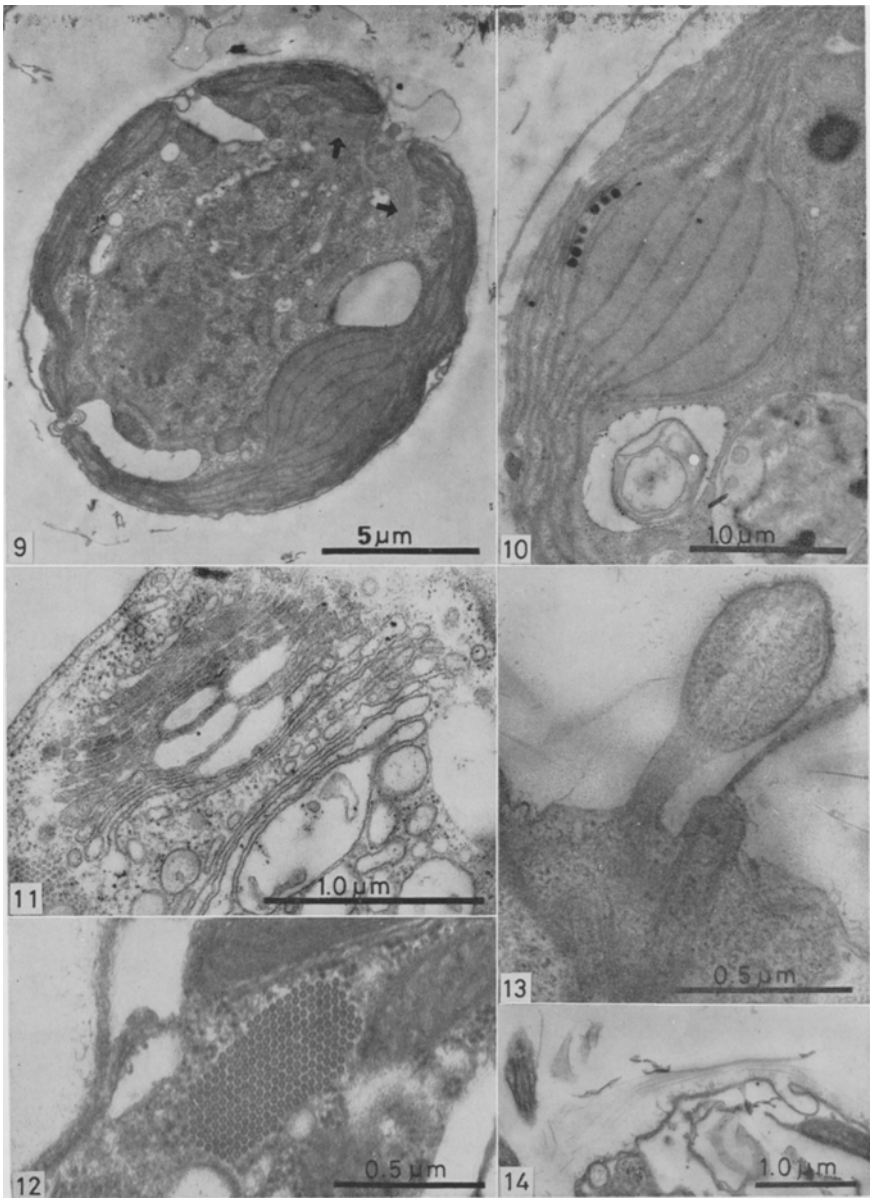
Light microscopy. The motile cells, which bear small, flat ellipsoidal coccoliths on the surface (Fig. 1), are spherical to ovoid or pyriform in shape, measuring 9.5 to 15.5 μm in length and 9.5 to 10.5 μm in width. They have two flagella arising from the anterior end of the cell in the mediam plane of symmetry (Fig. 2). The two flagella are long, though they are not equal in length, one measuring 19.5 to 21.5 μm and the other 27.5 to 30.5 μm long (Fig. 3). A very short haptonema is present, measuring about 1 μm long. The haptonema can be clearly observed by phase contrast microscope after the coccoliths have been detached from the cells by treating with dilute acetic acid, or they can be observed directly when the cells bear few coccoliths in number (Fig. 2).



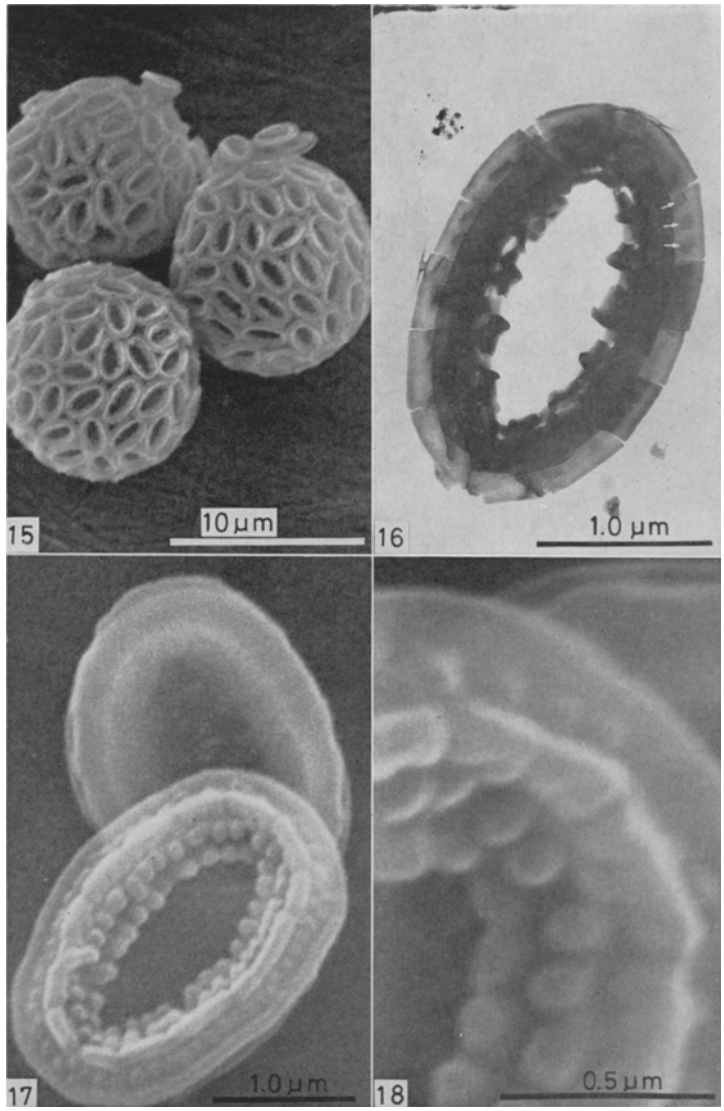
Figs. 1–8. *Cricosphaera roscoffensis* var. *haptonemofera*. 1: Motile cell, showing two flagella and coccoliths on the cell surface. 2: Young motile cell with a short haptonema visible between two long flagella. 3: Motile cell, showing two flagella of unequal length. 4: Motile cell in optical cross section, showing coccolith layers and two chloroplasts, each possessing bulging pyrenoid. 5: Motile cell just before fission takes place. Chloroplasts already divided are shown. 6, 7: Motile cells undergoing fissions. 8: Two resultant daughter cells. (Figs. 5–8 are shown in the same scale).

The motile cells swim actively, showing slightly positive phototaxis. They contain two chloroplasts, each possessing a single pyrenoid. The chloroplasts are golden brown in color and peripherally situated in the cell (Fig. 4). Many oil drops located in the periphery of the cells were usually observed. The motile cells are capable of reproduction by binary fission either in its motile flagellated form or in a form in which the cell loses its flagella. The process in which asexual reproduction takes place, resulting in the formation of two daughter cells, is shown in a series in Figs. 5 to 8.

Electron microscopy. The microanatomy of the motile cells cultured at 20 C and pH 8.0 to 9.0 was examined using the electron microscope. Sections of the cells show the distribution of major organelles (Fig. 9), similar to those of *Hymenomonas* and *Cricosphaera*, both of which have already been studied by Manton and Peterfi (1969). The nucleus is situated in the region behind the center of the cell and a single Golgi body is present in the region above the nucleus. The Golgi body has peculiar dilations in the middle part of the cisternae, a feature considered to be characteristic of the Prymnesiophyceae (Hibberd, 1976) (Fig. 11). The chloroplast contains many three thylakoid lamellae and one pyrenoid, the latter bulging laterally from the inner face of the chloroplast. The pyrenoid is usually traversed by two layers of thylakoids (Fig. 10). The flagellar root is located at the anterior end of the cell (Fig. 9). A longitudinal section through an almost median plane of the cell shows a flagellar root consisting of bundles of microtubules packed in a hexagonal outline (Fig. 12). The haptonema is bulbous as is that of *Hymenomonas roseola* (Manton and Peterfi, 1968) and *Cricosphaera carterae* (Manton and Peterfi, 1968; Leedbeater, 1971) and contains at least three microtubules. (Fig. 13). The surface of the cell body is covered with two kinds of scales. One type is thin, delicate, and round in shape and attached directly to the surface of the cell body. The other is three-dimensional and architecturally elaborate (Fig. 15), and situated on the top of the thin scale (Fig. 14). The latter scale is a "cricolith", a kind of coccolith, described by Braarud *et al.* (1955). The cricolith is pulley-shaped in outline, consisting of 6 to 14 rim elements arranged in a circle (Fig. 16). The proximal side of the cricolith, which attaches to the thin scale, seems to be flat and smooth, whereas the distal side is not flat, but canaliculate with a rough texture, owing to the presence of continuous upheavals produced on the sides, as well as many protuberances on the bottom of the depressions (Fig. 17). These protuberances are usually three in number per each rim element, forming a line at the center of the bottom, and thus, as a whole, forming a streak within a circle (Fig. 17). On the inner surface of the cricolith ring, there are two kinds of protuberances: one is larger, conspicuous and four in number per each rim element, one pair above the other pair, arranged in two tiers in horizontal direction, whereas the other is small, less conspicuous and two or three in number per each rim element. They are situated just above the former tier, forming a horizontal line (Fig. 18). Since these features described above were revealed by scanning and transmission electron microscopes, it is reasonable to consider that these are not artifacts occurring during the process of the preparation.



Figs. 9–14. *Cricosphaera roscoffensis* var. *haptonemofera*. 9: Motile cell in longitudinal section, showing the distribution of major organelles. Nucleus, bulging pyrenoid on the chloroplast, two flagella roots (arrows) and a haptonema arising from apical depression are visible. 10: Motile cell in section, showing a part of chloroplast. Pyrenoid is traversed by two layers of thylakoid bands while other parts of chloroplast are by three ones. 11: Motile cell in section, showing Golgi body which possesses peculiar dilation of cisternae. 12: Cross section of flagellar root consisting of bundle of microtubules packed as a hexagonal outline. 13: Longitudinal section of bulbous haptonema. At least three tubules are visible in it. 14: Motile cell in section, showing two kinds of scales. Several layers of thin scales and a single cricolith with thin scale on the bottom are shown.



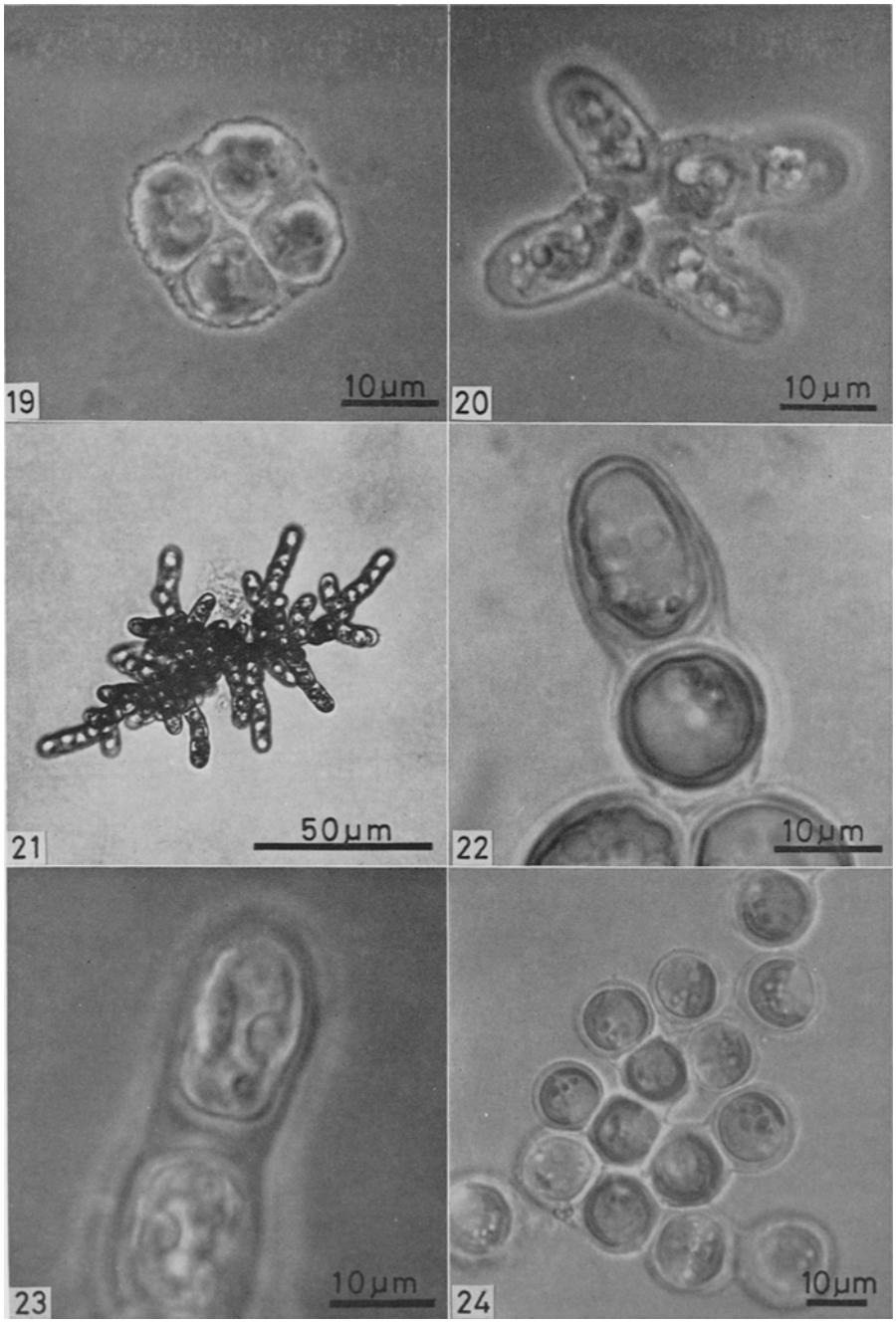
Figs. 15–18. *Cricosphaera roscoffensis* var. *haptonemofera*. 15: Scanning electron micrograph of motile cell with cricoliths covering the entire cell surface. 16: Direct mount preparation of cricolith, showing 13 rim elements which construct the cricolith. Three protuberances (arrows) arranged on the distal side of cricolith are seen. Many protuberances arranged in a circle on the inner side of cricolith are also visible. 17: Two cricoliths; one showing smooth surface of proximal side and the other showing rough surfaces of distal and inner sides owing to the presence of many protuberances. The canaliculate of the distal side possesses continuous upheavals on the sides and protuberances arranged on the bottom. Protuberances are also arranged on the inner surface. 18: A part of cricolith showing three rows of protuberances in horizontal direction; the two rows consisting of large conspicuous protuberances while the other one, which lies just above the two rows, consisting of small and less conspicuous protuberances.

Benthic phase

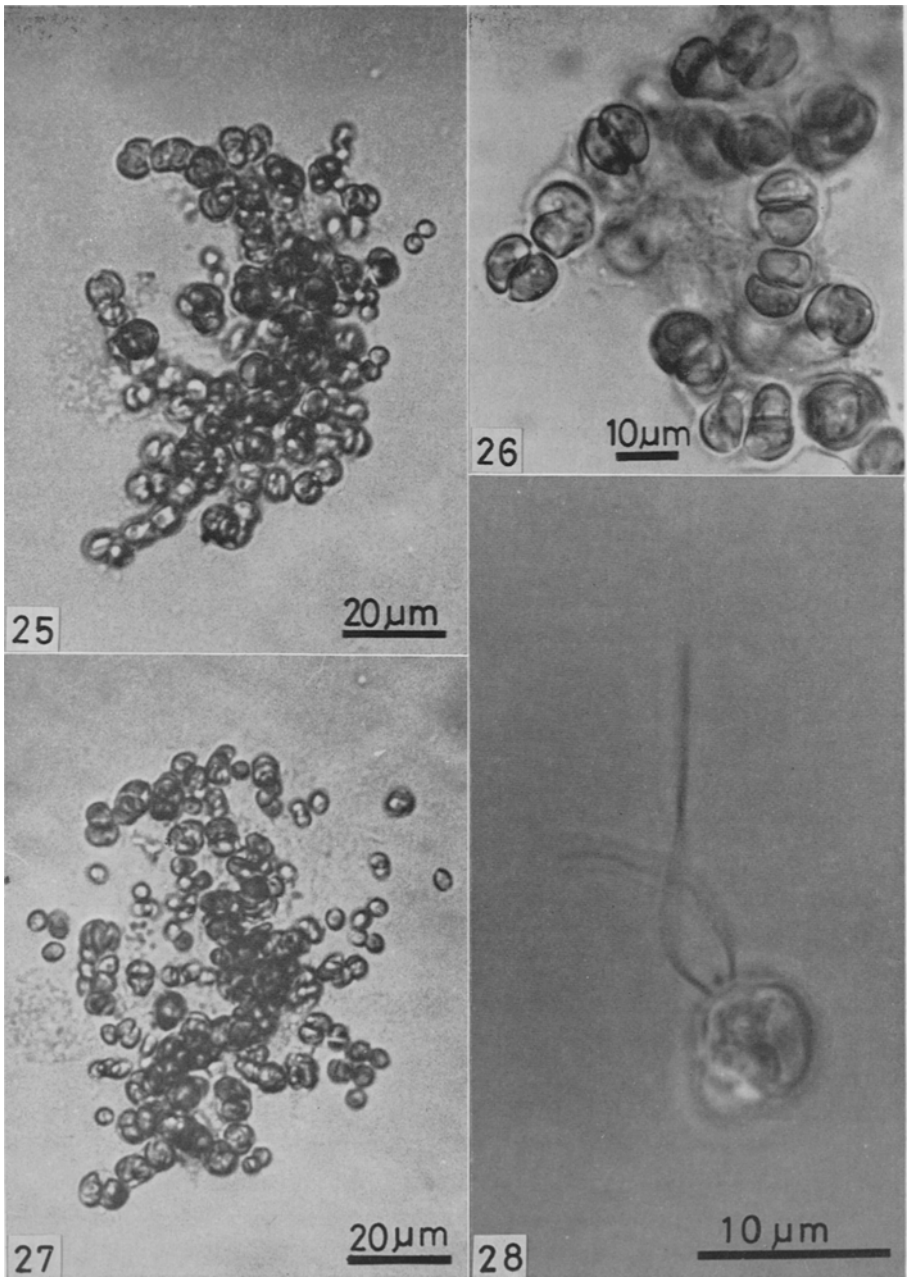
The transformation from the motile cell to the non-motile phase can easily be induced by keeping the culture for 2 weeks, or more, without changing the medium, or inoculating motile cells into medium acidified at pH 6.0 to 7.0. The transformation usually takes place within a few days after the treatment in the latter case. This suggests that the induction of a non-motile phase is probably related to the depletion of major anions, such as phosphate and nitrate.

Soon after the motile cell attached to the substratum and lost its flagella, it undergoes the first cell division, which is a kind of desmoschisis (in the sense of Groover and Bold, 1969), passing in most cases through the center of the cell body, forming two cells almost equal in size. Each of these two cells next divides again almost at right angles to the first formed septum, resulting in the formation of four crucitately arranged cells in a tetrad (Fig. 19). After the formation of a tetrad of cells within the mother cell, each cell of the tetrad begins to develop into a uniseriate filament, behaving as a spore germinating *in situ* (Fig. 20). The four filamentous extensions tend to develop radially and soon produce sparse and irregularly arranged branches (Fig. 21). Cells of the filaments vary considerably both in shape and in size, showing cylindrical, ovoid, ellipsoidal or spheroidal shapes. They measure 11.0 to 29.5 μm in length and 9.0 to 12.5 μm in width. No coccoliths were observed on their surfaces. The cells contain one or two chloroplasts, which are parietal in position and golden brown in color, and each is furnished with a pyrenoid (Fig. 22). The pyrenoid, as in the motile cell, bulges laterally from the inner face of the chloroplast (Fig. 23). The seriate filaments thus obtained in the culture are reminiscent of the thallus of certain filamentous brown algae, such as *Streblonema* and *Ectocarpus*. However, close examination reveals that they differ from those algae in having cells which do not touch tightly with one another in the filaments, the cells tending to be spheroidal with the walls becoming gelatinous and sometimes stratified. The seriate filaments are similar to an alga known previously as "*Apistonema submarinum*" described by Dangeard (1934). Some of the germlings obtained in the culture did not become filamentous but the cells became round, resulting in the formation of irregularly shaped colonies consisting of coccoid cells (Fig. 24).

The benthic phase seems to produce three kinds of reproductive cells: zoospores, or male and female gametes. The formation of zoospores can be artificially induced by placing the filaments into a fresh medium of pH 8.0 to 9.0 for a few days. The protoplasts in the cells generally divide several times, with the resultant formation of two to eight zoospores (Figs. 25–27). At maturity, cell walls in the filaments dissolve and the cells break apart and release zoospores (Fig. 27). The zoospores are pear-shaped or spheroidal, being similar to those of *Prymnesium* in form, measuring 6.5 to 11.5 μm in length and 5.5 to 7.5 μm in width and having two flagella, which arise terminally and are rather unequal in length (Fig. 28). They possess a haptonema arising from the site between the two flagella (Fig. 28). They have no coccoliths on the surface of the cell. After swimming ceases, the zoospores germinate and develop into a benthic



Figs. 19–24. *Cricosphaera roscoffensis* var. *haptonemofera*. 19: A tetrad, and initial stage in the development of filamentous phase, derived from a coccolith-bearingmotile cell. 20: Early stage in the development of the germinating tetrad, each of the tetrad tending to grow radially. Coccoliths still remained are visible. 21: Filamentous from derived form the germinating tetrad being similar to *Apistonema submarinum*. 22: A part of filamentous form, showing cells surrounded by thick gelatinous wall. A single parietal chloroplast is visible within the cell. 23: Cells fixed with Lugol's solution, showing bulging pyrenoid on the chloroplast. 24: Coccoid forms derived from the germinating tetrad.



Figs. 25–28. *Cricosphaera roscoffensis* var. *haptonemofera*, showing successive stages in zoosporogenesis. 25: Filament at the stage of zoospore release, showing divided cells of the filament. 26: A part of filament, showing the formation of zoospores. Cell wall that was originally rigid and thick is melting. 27: The same filament as one shown in Fig. 25, the cells becoming unjointed (after about 1 hr). 28: A zoospore discharged from the filament, showing two long flagella and a haptonema between them.

filament, in a manner similar to that of a spore-like cell of the tetrad described above. The filaments derived from zoospores later again produce the same type of zoospores. This evidence suggests the capability of the benthic filament for asexual reproduction.

The formation of sexual reproductive cells may be induced, as in the case of zoospores, by inoculating the filament into a fresh medium of pH 8.0 to 9.0 for a few days. The period for the induction, however, seems to require one or two more days than in the case of zoospores. The process of their conjugation was not observed in the preseent culture, although a quadriflagellate swarmer has been observed several times. It is hoped that the morphology of gametes and the manner of zygote development will be described in detail in the near future.

Discussion

Taxonomy. On the basis of the morphological features described above, it is clear that the organism belongs to *Cricosphaera*, a genus founded by Braarud in 1960 and later redescribed by Gayral and Fresnel (1976). As far as is known, only two species have been described for the genus, *C. carterae* Braaarud et Fagerland and *C. roscoffensis* (Dangeard) Gayral et Fresnel. These two species are similar to each other in many respects. In their study on *Cricosphaera*, Gayral and Fresnel (1976) reviewed the history of the genus and compares the two species in detail. Characters which they used for the comparison are as follows: 1) cell size, 2) flagellar length, 3) presence or absence of an emergent haptonema, 4) morphology of the cricolith, 5) shape and size of the scale, 6) structure of the pyrenoid, and 7) presence or absence of a benthic filamentous phase in the life cycle.

Most phycologists now use the morphology of coccoliths as the most important diagnostic criterion separating species or genera in the taxonomy of coccolithophorids. The cricoliths, a kind of coccolith, of the organism more closely resemble those of *C. roscoffensis*, in possessing protuberances on the surface of cricolith; *C. carterae*, on the other hand, has neither protuberances nor ornamentation on the cricolith surface (Manton and Leedale, 1969; Pienaar, 1969; Outka and Williams, 1971; Gayral and Fresnel, 1976). As is shown in Figs. 15–18, there are many protuberances on the inner surface and on the distal end of the cricoliths. No critical difference can be found between the alga and *C. roscoffensis* in regard to the structure of the cricolith. When *C. roscoffensis* was described, Gayral and Fresnel (1976) discussed the haptonema from the viewpoint of taxonomy and concluded that the presence or absence of a haptonema was a character of secondary value in the taxonomy. As described previously, the alga possesses a haptonema which can be seen by using a phase contrast microscope or electron microscope. In addition, the alga has a benthic filamentous phase in the life history, whereas in *C. roscoffensis*, such a filamentous phase has not been observed (Gayral and Fresnel, 1976). With regard to these two points, the alga is similar to *C. carterae*, in which a haptonema and a filamentous phase have been observed (Stosch, 1958; Manton and Leedale, 1969; Manton and Peterfi, 1969; Leedbeater, 1970; Boney and Burrow, 1968) and thus it would be reasonable to consider the alga as an intermediate

taxon between the previously described two species. The question arises concerning which character should be used as the primary criterion in the taxonomy of the alga. Boney and Burrow (1968) made a laboratory study with *C. carterae*, using benthic filaments and, as a result, they recognized the benthic filaments as a phase being a stage tolerant to extreme environmental conditions, such as high salinity, desiccation, and high or low temperatures. We have also conducted similar experiments with the alga and obtained results which support their interpretation. Although a benthic filamentous phase has not been reported for *C. roscoffensis*, it seems likely that this species may produce this advantagenous phase, growing as a certain species of this algal group does at the high to the middle water marks. More detail analytical experiments are necessary before the presence or absence of the filamentous stage can be used as a diagnostic character. In view of the above consideration, we would like to adopt at present the morphology of coccolith as the primary criterion at the specific rank and, on the basis of the similarities referred to, we would like to regard the present organism as an infraspecific taxon of *C. roscoffensis*, naming it *C. roscoffensis* var. *haptonemofera* Inouye et Chihara, var. nov.

Cricosphaera roscoffensis (Dangeard) Gayral et Fresnel, Phycologia **15**: 339–255, f. 1–24. 1976.

var. **haptonemofera** Inouye et Chihara, var. nov.

Diagnosis: Haptonemibus brevissimis, eminentibus et generationibus filamentosis differt. Ceterum ut in typo.

Holotype: Figures 1–28.

Type locality: Minatogawa, Okinawa-ken, Japan.

Distribution: Possibly widely distributed in the warm seas around Japan.

A culture of the new variety is deposited in the Culture Collection of Algae, The University of Tsukuba.

Life history. From the observations obtained in the present cultural work, it becomes clear that the life history of *Cricosphaera roscoffensis* var. *haptonemofera* Inouye et Chihara consists of two phases: a unicellular motile phase and a benthic filamentous phase. The unicellular motile phase is capable of reproduction by binary fission as well as production of the benthic filamentous phase through the formation of a tetrad, which is possibly a kind of asexual reproductive structure and one which is the result of meiosis of the mother cell, as has already been pointed out by Stosch (1967) and Rayns (1962) with *Cricosphaera carterae* (called *Syracosphaera carterae* by them). According to Stosch (1967), the tetrad produces a motile asexual cell, whereas Parke (1962) observed the occurrence of a non-motile asexual cell in the tetrad in the same species as Stosch used. In the present cultural works, non-motile cells from the tetrad have often been observed but never motile cells. This difference is probably dependent on the cultural conditions. As far as is known, only one paper is available dealing with the occurrence of sexual reproduction in this algal genus, that of Stosch (1967) with *C. carterae*. However, Stosch and his co-workers did not succeed in observing the details of the gametes, their conjugation and the germination of the zygote. For this

reason, they have given a scheme showing a life cycle of *Cricosphaera carterae* (called *Syracosphaera carterae* by them) in which sexual reproduction is questioned. As described in the preceding chapter, we have several times observed in culture a quadriflagellate swarmer, which is probably a zygote produced by gametes derived from the benthic filament, but we have failed to confirm the details of its origin. Since our observations on the life history of the present alga are being continued in the laboratory, it is hoped that a detailed life cycle will be reported in the near future.

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