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Ultrastructure and taxonomy of *Pyramimonas lunata*, a new marine species of the Class Prasinophyceae*

Isao INOUYE, Terumitsu HORI and Mitsuo CHIHARA

Institute of Biological Sciences, University of Tsukuba, Sakura-mura, Ibaraki, 305 Japan

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A new species of *Pyramimonas P. lunata* is described based on the specimens collected in Kesen-numa, Miyagi Prefecture, one of the places in Japan, where phytoplankton blooms have often been observed. Unialgal cultures were examined with the transmission electron microscope with particular emphasis on scale morphology and the ultrastructure of cell organelles.

This species is characterized by the presence of trichocysts, the morphology of the pyrenoid and newly-described body scales. The body scales of the intermediate layer are box-like and consist of a square base perforated in a characteristic pattern and four sides made up of slender rods. Scales of the outermost layer are, as in many other species of the genus, coronate and composed of slender rods. However, these scales are particularly large (520 nm high and 680 nm wide). Scale morphology and cellular ultrastructure are compared with those of other species previously studied and their validity as diagnostic characters is discussed.

Key Index Words: *Flagellar apparatus*; *Prasinophyceae*; Pyramimonas lunata *sp. nov.*; *Scale morphology*; *Taxonomy and ultrastructure of Pyramimonas.*

For the last 20 years red tides have frequently occurred in various places in Japan and caused significant damage to the local fisheries. To date many taxonomic studies of red tide organisms have established the basic data useful for further studies to resolve this serious economic and social problem. However, most investigations have been restricted to the Bacillariophyceae, Dinophyceae and Raphidophyceae. Other groups of phytoflagellates such as the Prasinophyceae, Prymnesiophyceae, Cryptophyceae and Chrysophyceae have not been included as sujects of red tide research. Among the Prasinophyceae, only two species were reported as organisms which cause phytoplankton bloom in the coastal basins around Japan, viz. *Pyramimonas* aff. *amylifera* (as *Asteromonas propulsum*) and *P. disomata* (ADACHI 1972), despite the further species of this class are apparently a common component of the microalgal flora of coastal waters and have undoubtedly caused blooms more often than has been recorded.

Recently we have directed our research towards the genus *Pyramimonas*, the taxonomy of which at species rank is still confused. We have isolated more than twenty strains of the genus from seawater samples collected from various places around the coast of Japan and examined them using both light and electron microscopy in an attempt to clarify the diagnostic characters.

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This paper is the first of the series and deals with the bloom-causing species *Pyramimonas lunata* sp. nov. isolated from the Bay of Kesen-numa, Miyagi Prefecture, Japan. Both light and electron microscope observations are described and various features including scale and cell ultrastructure are compared with those of other species.

Materials and Methods

Pyramimonas lunata was first collected by Mr. Noritaka Fujita of Kesen-numa Prefectural Fisheries Experimental Station from the Bay of Kesen-numa, Miyagi Prefecture in August 1978. According to him, this species occurred as one of major components of a phytoplankton bloom in the bay during August and October, 1978 and June to September, 1979.

Seawater samples were collected on a monthly basis from April, 1978, to February, 1980. The specimen used in this work was originally isolated from a sample collected on March 11, 1979. Cells were inoculated in Erd-Schreiber medium (FØYN 1934) and were maintained in the same medium and GPM medium (LOEBLICH III 1975) at 15C with exposure to 2000–6000 lux light in alternating 16/8 LD cycle.

Shadowcd materials were prepared by placing single drops of medium containing actively swimming cells on colloidin coated grids and fixing them in osmium tetroxide vapour for 30 seconds. After drying in a desiccator, grids were washed carefully with distilled water to remove salt and dried again. Grids were then shadowed with platinum-palladium at a low angle of 20–40 degree.

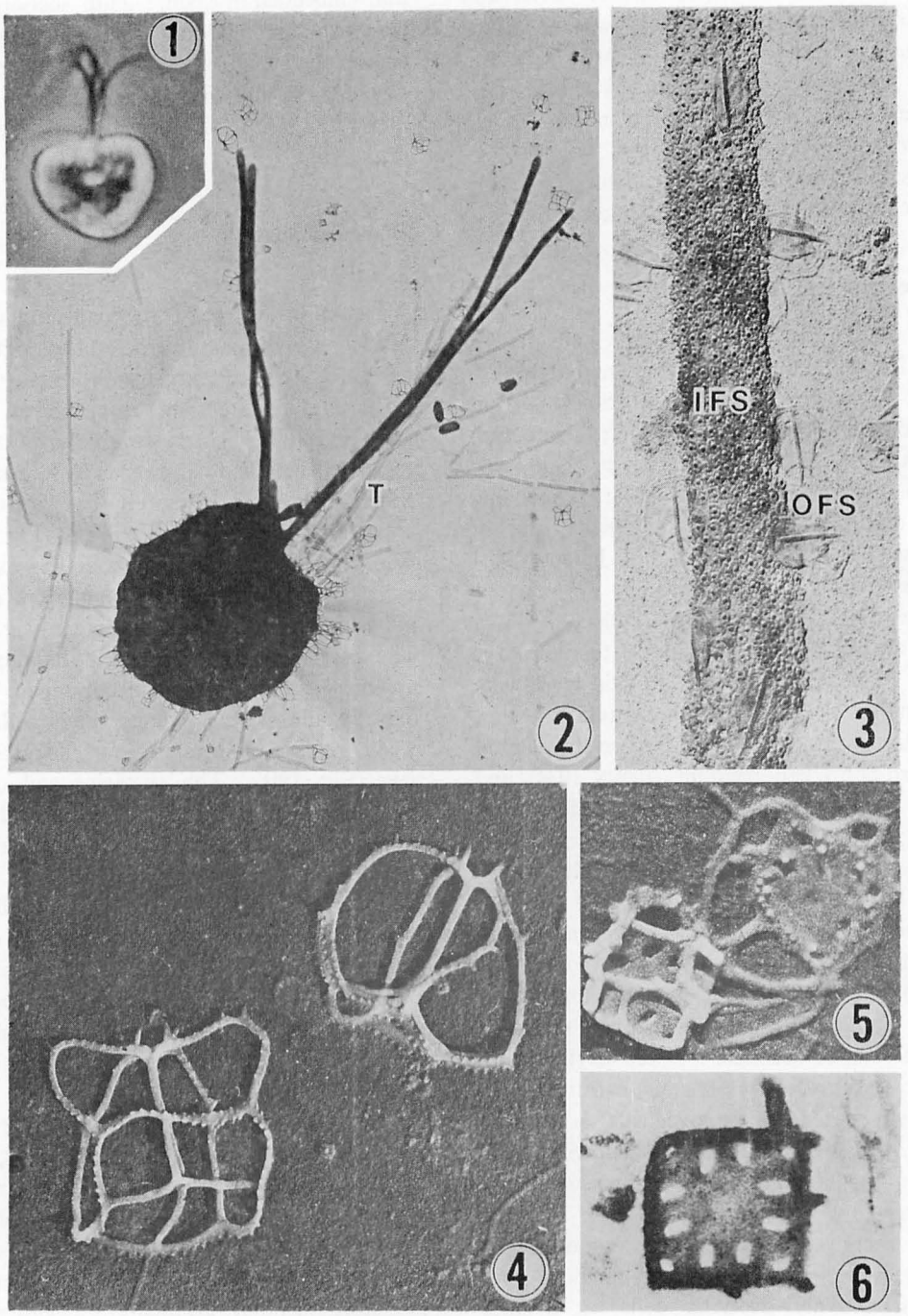
For sectioned materials, 50% aqueous glutaraldehyde was added to the medium containing actively growing cells, at a final concentration of 3%. After 1 hr fixation at room temperature, cells were rinsed several times in the same medium and postfixed in 2% aqueous osmium tetroxide in 0.2 M phosphate buffer (pH 7.0) for 2 hr. Then cells were dehydrated in graded ethanol series and embedded in Epon. Thin sections were cut with diamond knives, double stained with uranyl acetate followed by lead citrate (REYNOLDS 1963) and viewed with Hitachi H-12A and JEOL JEM 100C transmission electron microscope.

Observations

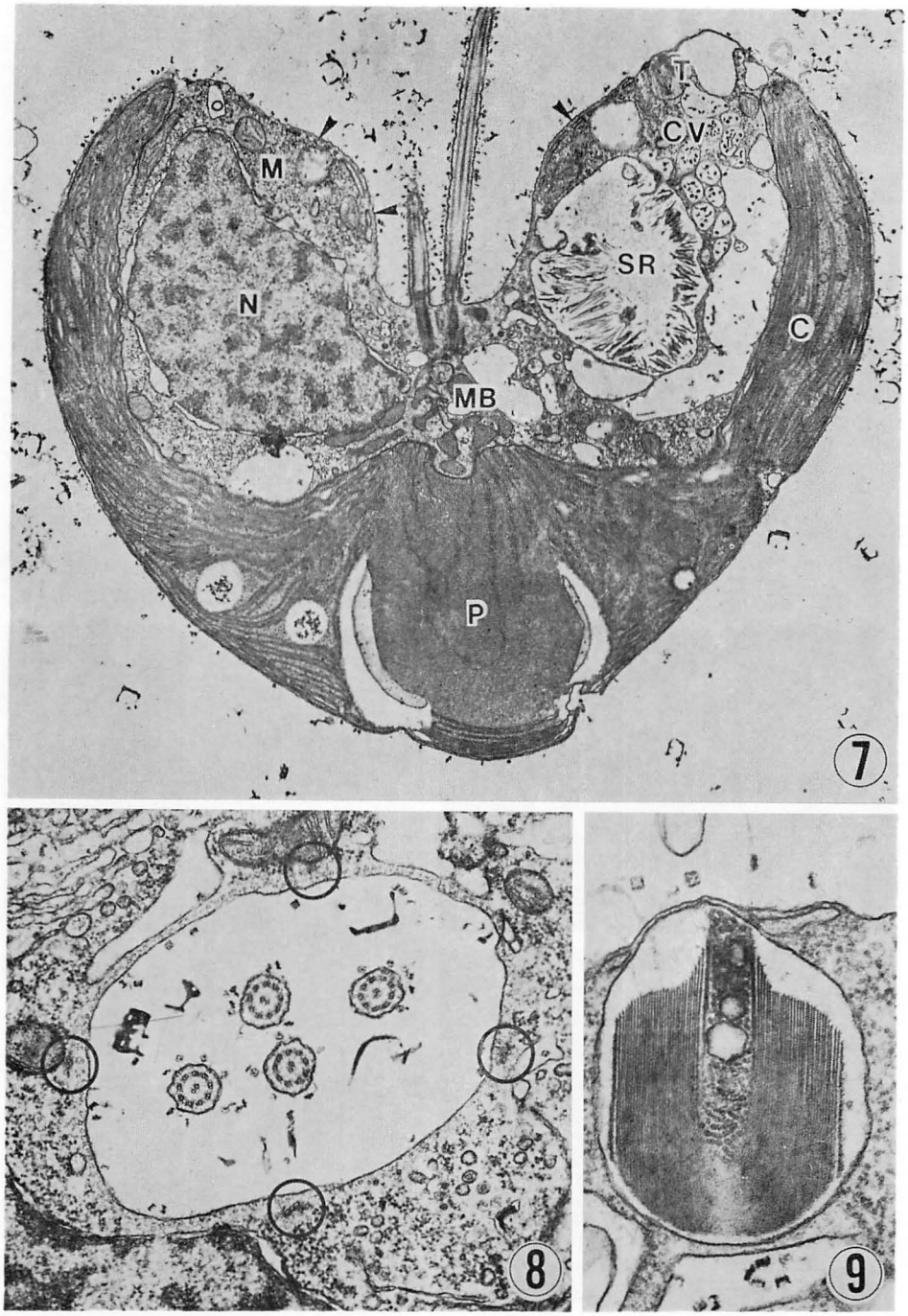
a) Light microscopy

Pyramimonas lunata is broadly obovoid in actively growing cultures. The length and width of the cell are usually almost equal (Figs. 1, 19A), ranging from 12 to 15 μm and 10.5 to 14 μm, respectively. Four anterior lobes extend backwards as four distinct ridges up to approximately two thirds the way along the length of the cell (Fig. 19A). These are most conspicuous at the extreme anterior end so that the polar view appear as a square with rounded corners (Fig. 19B). The anterior lobes surround a flagellar pit, a conical groove, from the bottom of which four flagella arise. Around the flagellar pit, many refractive granules, trichocysts, are seen (Fig. 19B). The posterior end of the cell is usually rounded but sometimes slightly acute in old culture. The single chloroplast is cup-shaped and has eight anterior lobes (Fig. 19B). A conspicuous stigma is located in a median-lateral portion of the chloroplast (Fig. 19A). A pyrenoid, located at the posterior end of the chloroplast, is surrounded by two laterally arranged, collar-shaped starch sheaths which are often obscure under the light microscope.

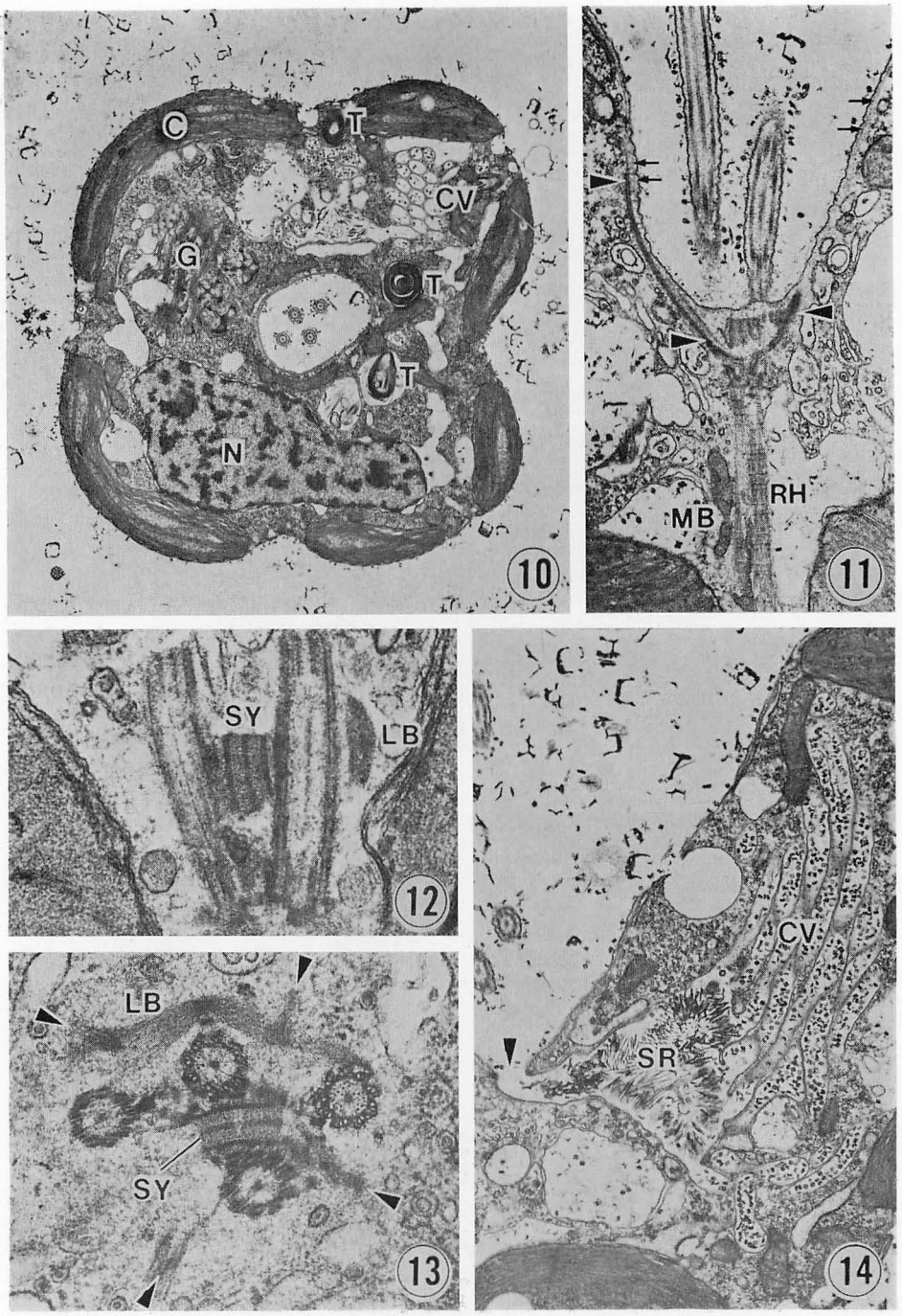
Cells swim rapidly for several seconds rotating around their longitudinal axis, then suddenly stop swimming and attach to the cover slip or glass slide with four vibrating flagella which radiate out in four directions at right angle to one another. When the mounting medium gradually evaporated or was drawn off with filter paper, the cells discharged many thread-like trichocysts. Using phase contrast microscopy many detached body scales appear as small grains floating around the cell body.



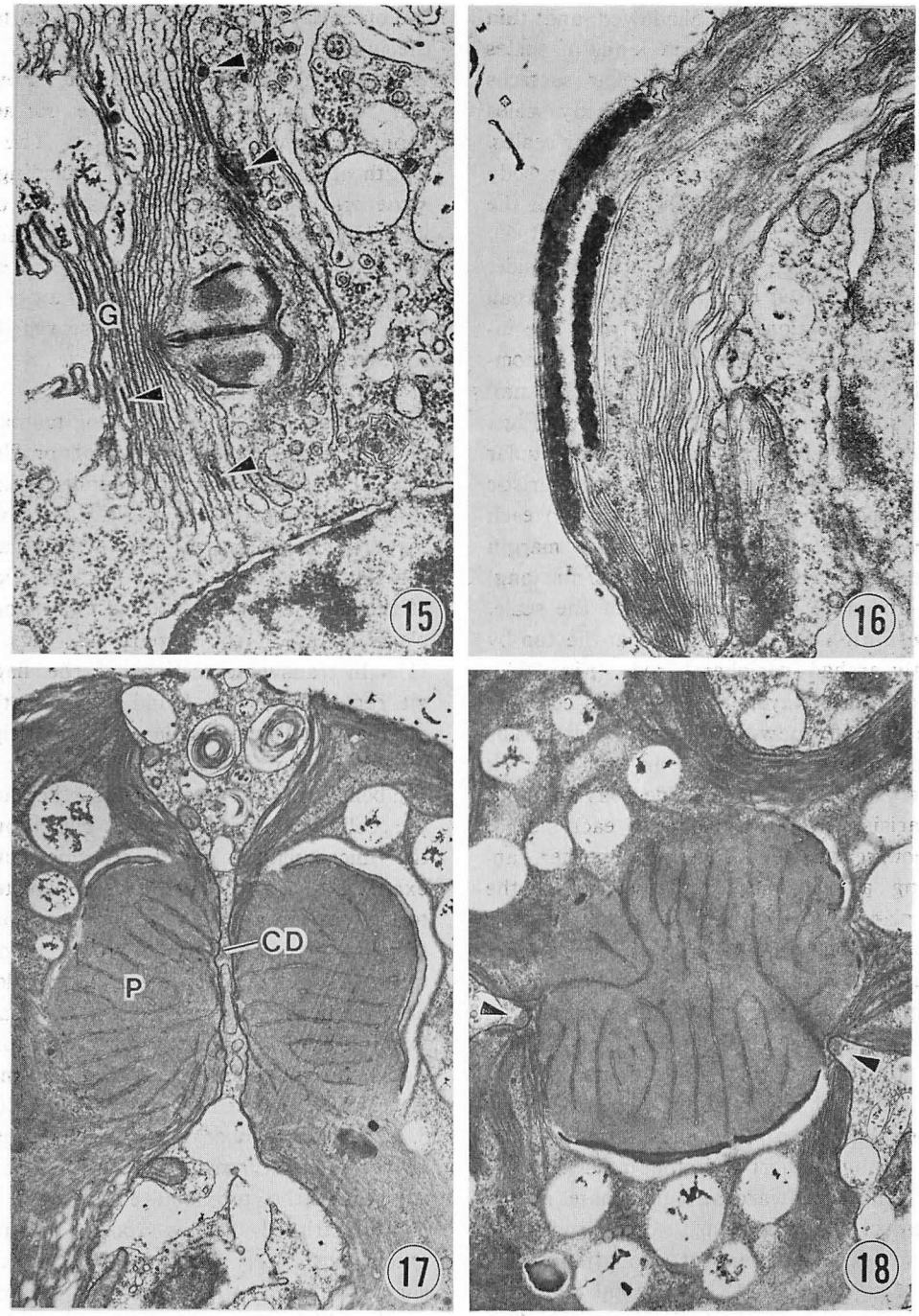
Figures 1-6. *Pyramimonas lunata*. 1. Light micrograph of the cell. The length and width of the cell are nearly same size. ×1,200; 2-5. Electron micrographs of shadowed materials. 2. Whole mount of the cell coated by scales. Thread-like discharged trichocysts (T) are seen. (Holotype). ×3000; 3. Inner (IFS) and outer (OFS) layer flagellar scales. ×33,000; 4. Outermost layer body scales. (Reversed print). ×40,000; 5. Intermediate layer body scales. (Reversed print). ×60,000; 6. Electron micrograph of a base of the intermediate body scale showing characteristic perforations (sectioned material). ×70,000.



Figures 7-9. *Pyramimonas lunata*. Electron micrographs. 7. Median longitudinal section of the cell, showing lunate profile. Major cell components are seen; chloroplast (C), cylindrical vesicles (CV), microbody (MB), mitochondria (M), nucleus (N), pyrenoid (P), trichocysts (T) and pit microtubules (arrow heads). ×9,200; 8. Transverse section of the flagellar pit region. Pit microtubules arranged along the flagellar pit and four microtubular flagellar roots consisting of 4 (bottom), 3 (right), 2 (top) and 2 (left) microtubules with electron dense material are seen (in the circles). ×23,000; 9. Longitudinal section of the trichocyst made up of rolled thin membranous material. Numerous granules are contained in the central core. ×40,000.



Figures 10–14. *Pyramimonas lunata*. Electron micrographs. 10. Transverse section of the anterior region of the cell showing typical arrangements of cell organelles. G : Golgi body. Other abbreviations same as Fig. 7. ×6,000; 11. Longitudinal section through the flagellar apparatus. Flagellar roots (arrow heads) extending from the flagellar base towards the cell anterior along the pit microtubules (arrows) are seen. Rhizoplasts (RH) and associated microbody (MB) are also seen. ×15,000; 12. Two basal bodies connected by the synistosome (SY). Laterally situated fibrous band (LB) is seen. ×45,000; 13. Transverse section of the flagellar apparatus. Three basal bodies linked by a lateral fibrous band (LB) are arranged along the convex margin and the other is attached to the concave side of the synistosome (SY). Four flagellar roots (arrow heads) radiating in a cruciate pattern are also seen. ×40,000; 14. A scale reservoir (SR) containing only small-size scales is continuous with cylindrical vesicles (CV) and the flagellar pit (arrow head). ×10,000.



Figures 15-18. *Pyramimonas lunata*. Electron micrographs. 15. Golgi body in cisternae of which the outermost layer body scale and various other scale types (arrow heads) are being produced. ×26,000; 16. Stigma made up of two layers of plastglobules. ×28,000; 17. Pyrenoid (P) and associated chloroplast ditch (CD), a boader between two chloroplast lobes. 14,000; 18. Pyrenoid and sections of the chloroplast ditch which are occupied by the rhizoplasts (arrow heads). ×12,000.

b) Electron microscopy

Scale morphology: Shadowed and thin sectioned materials show six kinds of scales coating the cell body and flagellar surfaces (Figs. 2, 3). Of these, three are body scales and the remaining three are flagellar scales. Small body scales, the undermost layer body scales, form a layer immediately outside the plasmalemma of the cell body (Fig. 19C). They are square, 45-50 nm on each side, and possess raised marginal rim and a small boss or projection at the centre. The intermediate layer body scale is box-like, composed of a square shaped base (290×290 nm) and four sides made up of slender rods (Figs. 5, 6, 20A). The square base has rectangular perforations arranged in a characteristic regular pattern (Figs. 6, 20A). From each corner and the middle of each side margin of the base, eight upright rods (200 nm long) arise to make up four sides of the scale. These upright rods are linked at the top by slightly arched peripheral rods (Fig. 20A). The outermost layer body scale is coronate made of a framework (Figs. 4, 20B). The base is square, ca. 680 nm on each side. It consists of four slender side rods and four rods arising from the middle of each side of the equare base and meeting together, appearing as a "cross in the square" on the base plane (Fig. 20B). Each rod of the base has numerous small projections pointing downward (Figs. 4, 20B). From the centre of the cross of the base, an upright rod (370-460 nm) arises and on top of it a short rod (100-120 nm) is arranged horizontally with a pair of spines at both ends. A pair of rods arise from each end of the horizontally arranged short rod, four rods all together, bend downward and terminate at the corners of the square base. These rods have tiny spines on the way at two fifth portion from the upper end. The total height of the outermost scale including spines is 400-520 nm.

The inner layer flagellar scale which lies external to the flagellar surface is square to pentagonal in shape, ca. 45 nm on each side, and has a raised rim and a central boss or projection (Fig. 3). The second type of scale, the outer layer flagellar scale, lies on top of the inner layer flagellar scales. This scale is shaped like the horseshoe crab, *Limulus*, and its ornamentation on the surface is similar to a spider's web (Fig. 3). The total length of the Limulus scale, including the spine projecting toward the distal end of the flagella, is ca. 300 nm. Hair scales similar to those described in other species are also present. They are often washed away from flagellar surface but can be observed in the scale reservoir (Figs. 7, 14).

Cell ultrastructure: Median longitudinal sections of cells show a variety of profiles of shapes, viz. boomerang to rounded triangle. The most typical is lunate (Fig. 7) to which the specific epithet refers. Approximately 250-300 microtubules emerge from vicinity of the flagellar bases and ascend along the plasmalemma of the flagellar pit (Figs. 7, 8, 11). In transverse sections of the flagellar pit region, they are found just beneath the plasmalemma in a regular interval (Fig. 8). They disperse along the cell contour (Fig. 11), bend along the lateral cell surface toward the cell posterior. It is not clear how far the microtubules, called pit microtubules, extend posteriorly. They were detected up to the horizontal level of the base of the flagellar pit. Four other groups of microtubules, flagellar roots, each surrounded by electron dense material arise from near the flagellar base and ascend along the proximal side of the pit microtubules (Fig. 11) in four directions creating a cruciate pattern (Figs. 8, 13). The number of microtubules making up the flagellar roots are 4, 3, 2 and 2 (Fig. 8). From the proximal end of the basal bodies striated fibrous roots, rhizoplasts, extend posteriorly passing along the nuclear surface to reach the chloroplast surface beneath which the pyrenoid is situated (Fig. 11). They extend dichotomously along the chloroplast ditch (Fig. 18). A well developed microbody is associated with this root (Fig. 11). Below the flagellar pit four basal bodies are characteristically arranged in the 3-1

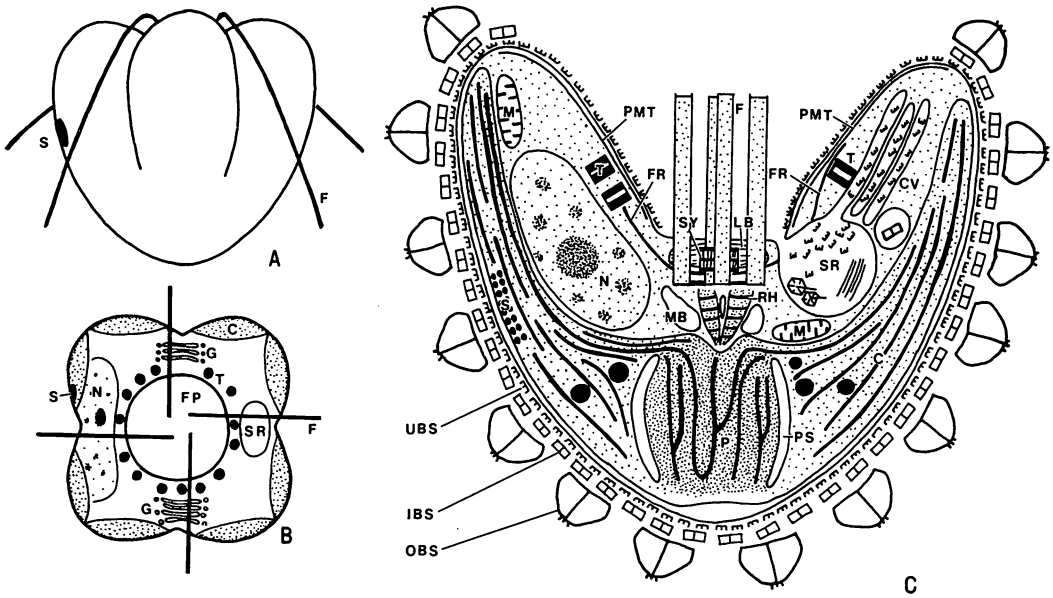


Figure 19. Diagrammatic illustrations of *Pyramimonas lunata*. A. Side view of the cell; B. Vertical view of the cell, showing relative positions of flagella, stigma, nucleus, Golgi bodies, trichocysts and a scale reservoir; C. Longitudinal section of the cell, showing ultrastructural features. Flagellar scales are not illustrated. C: chloroplast, CV: cylindrical vesicles, F, flagella, FP: flagellar pit, FR: microtubular flagellar roots, G: Golgi bodies, IBS: intermediate layer body scales, LB: lateral fibrous band, M: mitochondria, MB: microbody, N: nucleus, OBS: outermost layer body scales, P: pyrenoid, PMT: pit microtubules, PS: pyrenoid starch sheaths, RH: rhizoplasts, S: stigma, SR: scale reservoir, SY: synistosome, UBS: undermost layer body scales. Not to scale.

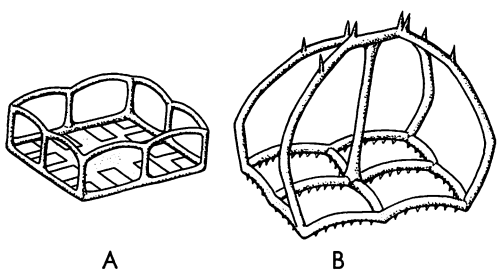


Figure 20. Diagrammed illustrations of the intermediate (A) and the outermost (B) layer body scales. Not to scale.

pattern (Fig. 13). Three lie more or less linearly along the convex margin and the other one is attached to the concave side of the synistosome (Fig. 13). Non-striated fibrous band links the former three basal bodies along their lateral margins (Fig. 13). Several slender connecting bands were also observed between the basal bodies and synistosome (Fig. 13) although their configulations are not described in this context.

The chloroplast is single and cup-shaped. It is deeply lobed into four sections in the cell anterior. Each lobe extends anteriorly along the ridge of the cell body and lobes once again into two sections anteriorly (Fig. 10) so that eight small lobes are formed. The pyrenoid is spheroid to oval in shape. On the chloroplast surface, there is a dimple-like cavity (Fig. 7) from which the chloroplast ditch extends posteriorly in opposite directions (Fig. 17). Many thylakoid bands enter into the matrix (Fig. 7). Although they penetrate deeply, they never traverse the matrix. Some bands terminate near the posterior portion of the matrix, while others bend and switch back toward the anterior end or anastomose with other thylakoids. Two laterally arranged collar-like starch sheaths surround either the posterior half or the more or less posterior two thirds of the pyrenoid matrix (Fig. 7). No inter-

vening membranous elements between the matrix and starch sheath have been observed. The dimple-like cavity above the pyrenoid surface is occupied by the rhizoplast and a microbody which is surrounded by a single unit membrane (Fig. 7). The stigma lies in a median position in one of the lobes of the chloroplast to which the nucleus is closely appressed (Fig. 16). It consists of one to two layers of linearly arranged lipid droplets. Many peculiar globules which are not bounded by membranes lie between the thylakoid bands (Figs. 17, 18). Their contents may have dissolved or washed away during the preparation of electron microscopy, but occasionally electron dense material remains.

Trichocysts are present mainly around the flagellar pit region (Fig. 10). They are threads-like when viewed in shadowed material (Fig. 2). They consist of rolled membranous material and contain numerous small globules in the centre (Figs. 9, 10).

Usually two Golgi bodies (rarely three) are present in the anterior half of the cell body. Both flagellar and body scales are produced in the Golgi cisternae (Figs. 10, 15). A large scale reservoir and many cylindrical vecicles, which are continuous to each other (Fig. 14), were observed opposite to the nucleus (Figs. 7, 14). The reservoir is well developed and contains three kinds of scales including the Limulus, hair and small square-shaped scales (Figs. 7, 14). Large scales, intermediate and outermost layer body scales, may usually be released singly to the cell surface.

The ultrastructure of the cell is diagrammed in Fig. 19C.

Discussion

The intermediate body scales of *P. lunata* are characteristic so that they could be used as diagnostic characters of this species. The perforation pattern of the base of the scale is stable feature and does not change regardless of the age and condition of culture. The outermost layer body scale could also be considered distinctive for this species because of its large size. It is much larger than that in any other species previously examined. The largest scale previously described in *Pyramimonas* (325 nm wide in *P. occidentalis*, PENNICK 1982) is half the size of the outermost scale of *P. lunata*.

PENNICK *et al.* (1978) studied scale morphology in 12 strains allied to *P. orientalis* and concluded that scale morphology is constant in each strain but there are consistent differences between strains. This raises a serious problem as to whether scale morphology is a reliable taxonomic character for the species level. However, in the extensively examined species *P. amylifera* no distinct differences have ever been observed in scale morphology among the various strains (e.g. compare figures given by MANTON *et al.* 1963 and NORRIS and PIENAAR 1978). In three strains of *P. grossii* isolated from various localities in Japan, no differences have been observed in scale morphology (unpublished observation). Therefore, we opine that the morphology of the scale is a useful taxonomic character in spite of the fact that strain difference on scale morphology exist in certain species of the genus. It could be more useful when combined with other features such as the ultrastructure of the flagellar apparatus, stigma, pyrenoid and presence or absence of trichocysts.

The number of pit microtubules has been estimated as 250-300 in *P. lunata*, approximately 150 in *P. parkeae* (NORRIS and PEARSON 1975), probably more than 250 in *P. tetrarhynchus* (MANTON 1968, estimated from figs. 7, 11), approximately 150 in *P.* aff. *amylifera* (unpublished observation), 25-30 in *P. orientalis* (MOESTRUP and THOMSEN 1974) and approximately 100 in *P. grossii* (unpublished observation). Among these species, the first four are large representatives (more than 10 μm) and the latter two small representatives (less than 10 μm). The number of pit microtubules is reliably different from species to species but it is not correlated with cell size. For instances, *P. orientalis* and *P. grossii* are similar in size but have a very different number of pit

microtubles. The taxonomic significance of such differences is still uncertain but should receive attention in future investigations.

It is likely that there are at least two different types of flagellar apparatus in four flagellated species of the genus *Pyramimonas*. The first has four basal bodies arranged in a 3-1 pattern and the linearly arranged three are connected to each other by a lateral non-striated fibrous band. *P. lunata*, *P. parkeae* (NORRIS and PEARSON 1975), *P. grossii* and *Pyramimonas* spp. (Strain Samekawa, Strain Udo, unpublished) belong to this group. Whereas the second has basal bodies arranged in a distinct diamond-shaped pattern and the lateral fibrous band is absent. *P. orientalis* (MOESTRUP and THOMSEN 1974), *P. obovata* (MELKONIAN 1981) and *P. longicauda* (unpublished observation) can be classified to this group. MELKONIAN (1981) described detailed three dimensional structure of the second type of flagellar apparatus. Because ultrastructure of the flagellar apparatus is considered as a useful taxonomic character in various groups of algae, further details of the ultrastructure of the first type should be investigated. Previously examined species possessing the 3-1 pattern of basal bodies have more than 100 pit microtubules while those possessing diamond pattern basal bodies have few. The arrangement of basal bodies and the number of pit microtubules appear to be distinctive to each species.

The trichocyst is not a common organelle in the Prasinophyceae and seems to be restricted to very few species of *Pyramimonas*, to date only five, including *P. lunata*. Since this organelle is infrequent, it can be adopted as one of the most important characters to delineate species.

We believe that the number of chloroplast lobes is constant within species. Eight lobes at the anterior end are known at present in *P.* aff. *amylifera* (unpublished observation) and in *P. lunata*, while four lobes are commonly observed in many other species.

P. lunata is the only species with a pyrenoid invaded by thylakoids from only the anterior side and surrounded by collar-like starch sheaths. *P. orientalis* (MOESTRUP and THOMSEN 1974, Fig. 14), *P.* aff. *disomata* (NORRIS and PIENAAR 1978) and *P. obovata* (PENNICK *et al.* 1976, Fig. 1; BELCHER *et al.* 1974, Fig. 11) have a similar pyrenoid invaded by thylakoids from the anterior end. But the starch sheath is cup-shaped and the thylakoids in the matrix are many fewer than in *P. lunata*.

At the light microscope level the following four species are similar to *P. lunata*, viz. *P. extravagans*, *P. cruciata*, *P. inflata* (CONRAD and KUFFERATH 1954) and *P. adriaticus* (SCHILLER 1925), that is, in all of these species length and width of the cell are nearly the same size. However, these species are distinguished from *P. lunata* in the following characteristics: *P. extravagans* has three large anterior lobes; *P. cruciata* is acute posteriorly and has four pyrenoids; *P. inflata* has two elongated bodies in the chloroplast; *P. adriaticus* has no pyrenoid. None of these species has been studied by electron microscopy so it is not possible at present to compare their detailed cellular features with those of *P. lunata*.

Diagnosis

Pyramimonas lunata sp. nov.

Cells wide obovoid, 12-15 μm long, 10.5-14 μm wide, lunate in longitudinal section, motile with 4 flagella, possessing 4 conspicuous anterior lobes and a conical flagellar pit; chloroplast single, green, 4 lobed subanteriorly and finally 8 lobed; stigma single, laterally situated; pyrenoid single, located posteriorly, surrounded by two collar-shaped starch sheaths; trichocysts present.

Cells covered by 6 types of scales: 3 body and 3 flagellar scales; intermediate body scales box-shape; base plate, 290×290 nm, perforated with rectangular holes; outermost body scales, coronate, large, 680 nm wide and 400-520 nm high.

Holotype: Figure 2.

Type locality: Kesen-numa, Miyagi Prefecture, Japan.

Distributions: Kesen-numa, Miyagi Pre-

fecture; Shioya-zaki, Fukushima Prefecture, Japan (collector: T. Horiguchi).

Habitat: estuary.

Pyramimonas lunata sp. nov.

Cellulae obovoideae, 12–15 μm longae, 10.5–14 μm latae, lunatae in sectione longitudinale, mobiles cum 4 flagellis, 4 lobos conspicuos anticos et foveam conicam flagelli habentes; chloroplastus unus, viridis, subantice tetralobus et extremum octolobus; stigma unum in parte postico cellulae sita, 2 vaginis amili collumiformis circumcincta; trichocystae adsunt.

Cellulae squamis 6 typorum obtectae: 3 squamae corporum et 3 squamae flagellorum; squamae corporum intermediae buxiformae; laminae basi, 290×290 nm, perforatae cum cavis; squamae corporum extimae coroniformes, magnae, 680 nm latae et 400–520 nm altae.

Holotypus: Figura 2.

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Refereces

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井上　勲・堀　輝三・千原光雄：　海産プラシノ藻の一新種 *Pyramimonas lunata* の微細構造と分類

宮城県気仙沼湾でブルームを形成するプラシノ藻 *Pyramimonas* の一種を培養し，形態学的，分類学的検討を加えた。本種は幅広い倒卵形の側面観，丸みをおびた四辺形の頂面観を有し，細胞前部には4本鞭毛を生じる円錐形の鞭毛溝を持つ。鞭毛溝のまわりには多数のトリコシストがある。葉緑体は1枚で緑色，杯状，細胞最前端では8片葉にわかれる。体長および鞭毛表面は形態の異なる6種類の鱗片に被われる。これらのうち，体表中層鱗片は箱形で底盤に長方形の孔をもつ点で，また体表外層鱗片は冠形で，680 nm に達する大型のものである点で既，知種の鱗片から明白に区別される。ピレノイドは前端部からチラコイドの侵入をうける。鞭毛基部は3-1パターンで配列し，シニストゾーム・繊維状ベルト・多数の有紋繊維によって結合する。リゾプラストと，4, 3, 2, 2 の微小管からなる交叉型微小管根系を有する。以上のような光顕・電顕レベルの形質を既知種と比較し，またそれらの識別形質としての有効性について考察した結果，本藻を新種と判断し，*Pyramimonas lunata* の名前を与えた。(305 茨城県新治郡桜村天王台，筑波大学・生物科学系)