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Ultrastructure of gametes and gametic fusion in *Bryopsis maxima* Okamura (Chlorophyceae)

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The fine structure of gametic fusion in the coenocytic, anisogamous green alga, *Bryopsis maxima* Okamura, is described and compared with those of the isogamous and other anisogamous green algae. Female gametes possess a putative mating structure similar in ultrastructure to that found in many other green algae so far studied. This is a structure absent in male gametes. The general mode of gametic conjugation is by fusion of male gametes at or near the mating structure of the female gamete, but other modes of fusion occur without involvement of this structure. These observations suggest that the mating structure does not function properly in the higher green algae, especially in the anisogamous groups. The mating structure in these algae may thus represent a vestigial sexual organ.

Nuclear fusion occurs at an early stage of zygote formation (25 minutes after mixing gametes), but fusion or disintegration of chloroplasts and/or mitochondria does not occur at this stage. The behaviour of the flagellar apparatuses in the early zygote from both mating types of gametes is discussed.

Key Index Words: Bryopsis maxima—*Chlorophyceae—conjugation—gamete—gametic fusion—green alga—mating structure.*

The first ultrastructural study of fertilization in green algae was made in the oogamous alga *Prasiola stipitata* (Manton and Friedmann 1960). Only a limited number of genera (*Chlamydomonas* and *Ulva*) have since been studied in detail. Although it is generally accepted that *Ulva* is anisogamous (e.g. Bold and Wynne 1985), the difference in cell size between female and male gametes is slight (e.g. Melkonian 1980b, Koeman 1985), and the general mating behaviour and the structure of gametes are similar to those of *Chlamydomonas* (Melkonian 1980b).

I have studied the fine structure of gametes and the fertilization processes of anisogamous green algae, as well as some other isogamous green algae, as a prelude for analyzing mechanism of maternal inheritance in green plants. The main reasons for selecting *B. maxima* were, 1) this species shows extreme anisogamy and is dioecious, 2) female gametes possess a large chloroplast with an eyespot and a pyrenoid while male gametes possess a reduced chloroplast with fewer thylakoids and neither eyespot nor pyrenoid, 3) behaviour of both male- and female-derived chloroplasts in a zygote can be easily followed by light and electron microscopy. A part of the studies showing that both male chloroplast-DNA and male mitochondrial-DNA of this alga are preferentially digested during the late period of gametogenesis has already been reported (Kuroiwa and Hori 1986). In contrast to this, in isogamous species of *Chlamydomonas*, the preferential degradation of male chloroplast-DNA occurs in young zygotes after gamete conjugation (Kuroiwa *et al.* 1982, Kuroiwa *et al.* 1985, Tsubo and

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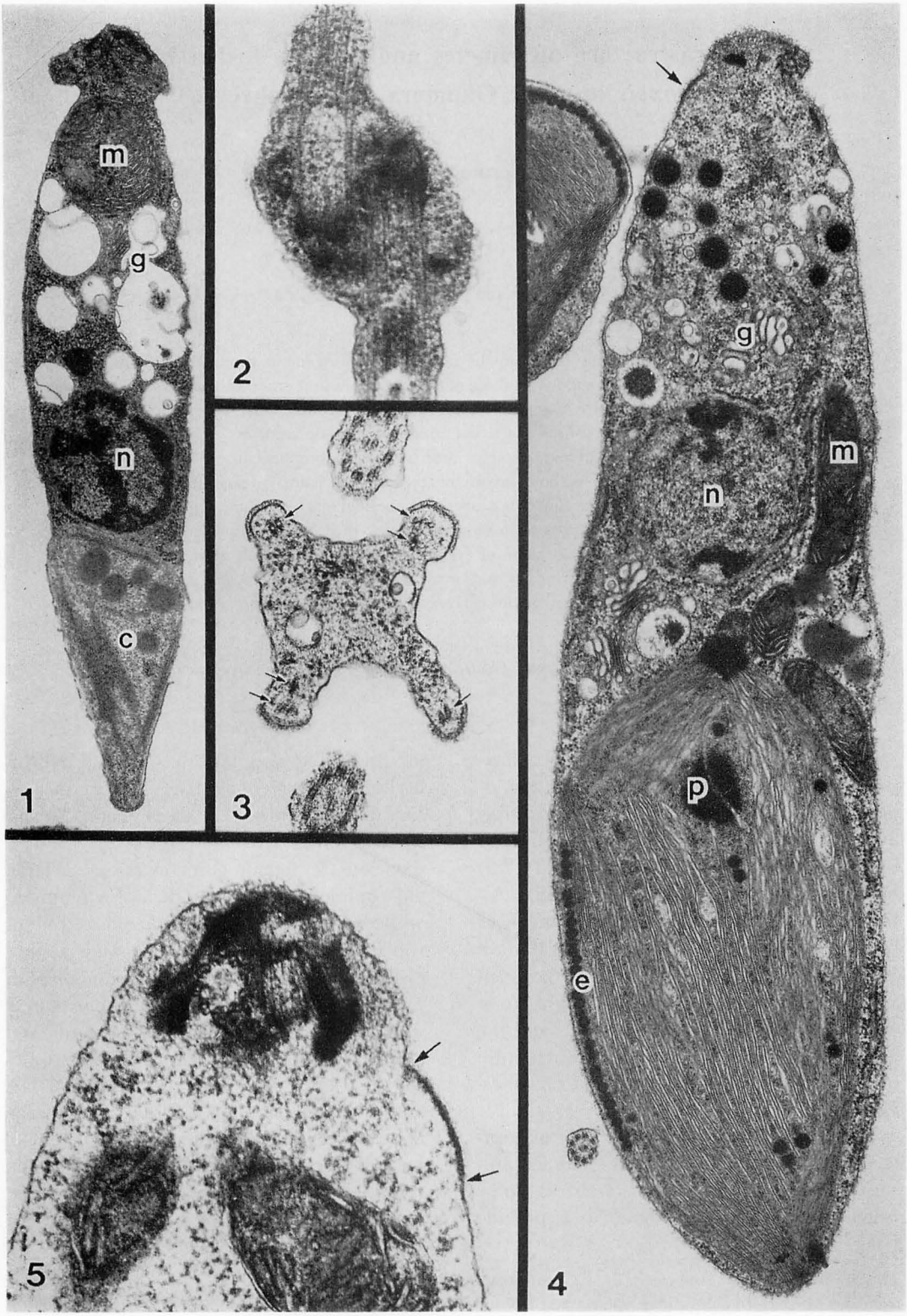
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MATSUDA 1984, COLEMAN and MAGUIRE 1983).

This paper presents the fine structure of gametes and the early stage of zygote formation.

Materials and Methods

Bryopsis maxima is dioecious. Gametophyte fronds were collected a few nights before full and new moon at Kimiga-hama, Chyoshi, Chiba and brought back to the laboratory, where they were kept in a tank of seawater provided with aeration. Close to full or new moon, the fronds become fertile and change colour to orange (male) and dark-green (female). At this stage the female and male fronds were separated and maintained as above.

In gametic fusion experiments two volumes of a suspension of female gametes and one volume of male gametes were mixed and fixed for electron microscopy at 0.5, 2, 5, 10, 15 and 25 minutes after mixing.

Initial fixation was done by adding 50% glutaraldehyde to gamete suspensions, giving a final glutaraldehyde concentration of 0.5%. The fixed cells were stored no longer than 12 hr before further processing. After centrifugation the zygotes were further fixed in 3% glutaraldehyde (made up in 0.28 M sucrose in 0.1 M cacodylate buffer, pH 7.1) for 1 hr at room temperature. The cells were then washed in a series of 0.1 M cacodylate buffer solutions containing 0.28, 0.14, 0.07 M sucrose and no sucrose, each step taking 15–20 minutes. Post-fixation was made in 1% OsO_4 (prepared as a 2% solution and mixed with equal volumes of 0.2 M cacodylate buffer, pH 7.1) overnight at 4° C. The fixed cells were centrifuged and stained for 8 minutes in a saturated solution of uranyl acetate in distilled water. After dehydration through a graded series of ethanol, the cells were embedded in Spurr's resin. Sections were cut with a diamond knife and stained with lead citrate. Observations were made using a JEOL 100 CXII electron microscope.

Observations

Dioecious fronds of *Bryopsis maxima* produce different types of gametes which can be clearly distinguished from each other by cell size and intracellular structure. The fine structure of these gametes is quite different and their components are useful as definitive morphological markers for analyzing the behaviour of cell organelles in zygotes.

I. *Male gamete*

The biflagellate male gametes of *B. maxima* are pear-shaped, measuring 4–7 μm in length and around 2 μm in width. The cell anterior differentiates into a papilla in which two basal bodies overlap at their most proximal ends (Fig. 2). They continue with the flagellar proper, which are 15–17 μm in length, slightly shorter than the female flagella (mean length 18 μm).

The fine structure of the male flagellar apparatus has already been described in detail for *B. maxima* (HORI 1977) and *B. lyngbyei* (MELKONIAN 1980a) and will not be

Figs. 1–3. Male gamete of *Bryopsis maxima*. 1. Median longitudinal section showing relative positions of organelles; mitochondrion (m), Golgi-body (g), nucleus (n) and a reduced chloroplast (c). The posterior end of the cell body is overlain by one of four ridges that extends down from the papillae. ×19,000. 2. Cross-section of the flagellar apparatus showing the overlapping basal bodies which are displaced in a counter-clockwise direction. ×52,000. 3. Cross-section just under the flagellar apparatus showing four ridges. In the upper left and lower right ridges a two-membered microtubular root can be seen; and a five-membered root can be seen in the upper right and lower left ridges (arrows). ×40,600.

Figs. 4–5. Female gamete of *B. maxima*. 4. Near-longitudinal section of the cell showing relative positions of organelles; the putative mating structure (arrow), many electron dense granules, Golgi body (g), nucleus (n), mitochondrion (m), a large chloroplast with pyrenoid (p) and eyespot (e). Note the location of the mating structure and eyespot on the same side of the cell. ×19,000. 5. Enlargement of the putative mating structure (arrows). ×48,000.

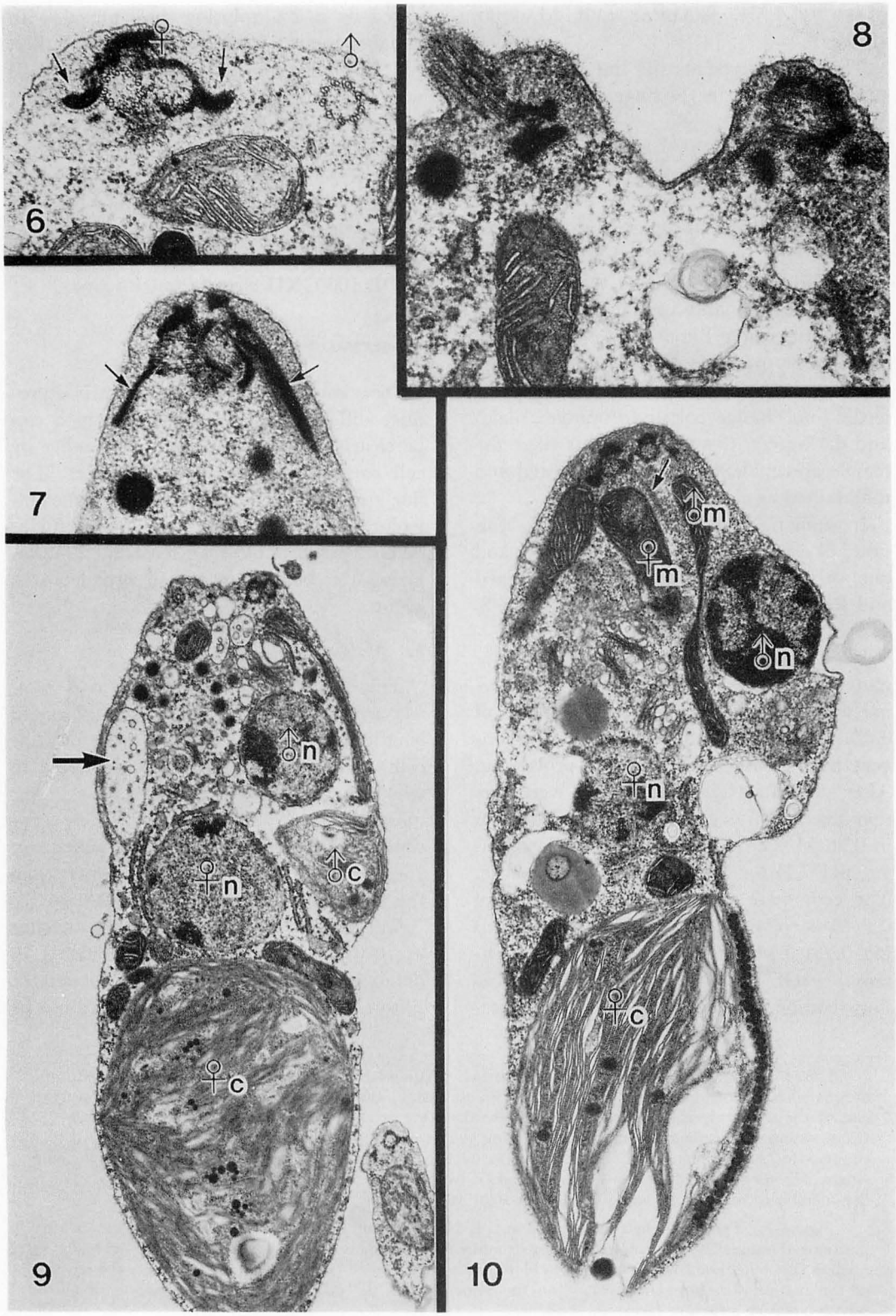
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Fig. 3 is a section just under the flagellar apparatus in which are discernible the basal part of a cruciform papilla composed of four ridges. The ridges originate from this point and extend downward. Some of them taper at an uncertain point in the posterior half of the cell before they reach the cell end, but others reach it (Fig. 1). Along the whole length of each ridge overlies a meniscus-shaped membranous material which is connected to the cell membrane by regularly-shaped bridges (Fig. 3). Microtubular flagellar roots pass along the bulge of each ridge (Fig. 3 arrows, Fig. 11b small arrows). In the upper left and lower right ridges of Fig. 3 (arrow) a two-membered root can be seen, and a five-membered root can be seen in the upper right and lower left ridges (Fig. 3, arrows).

A longitudinal section of a male gamete (Fig. 1) reveals relative positions of major cell organelles; under the flagellar apparatus is the cross profile of a giant U-shaped mitochondrion, the bottom of which is close to the posterior surfaces of the basal bodies. Two mitochondrial arms extend downwards beneath the cell membrane. In the cytoplasm surrounded by these mitochondrial arms are one or two Golgi-bodies and small vacuoles (Fig. 1). The posterior half of the cell body is occupied by a nucleus with condensed chromatin material (compare to female gamete in Fig. 4) and a small chloroplast. The chloroplast has a few thylakoids but no pyrenoid or eyespot.

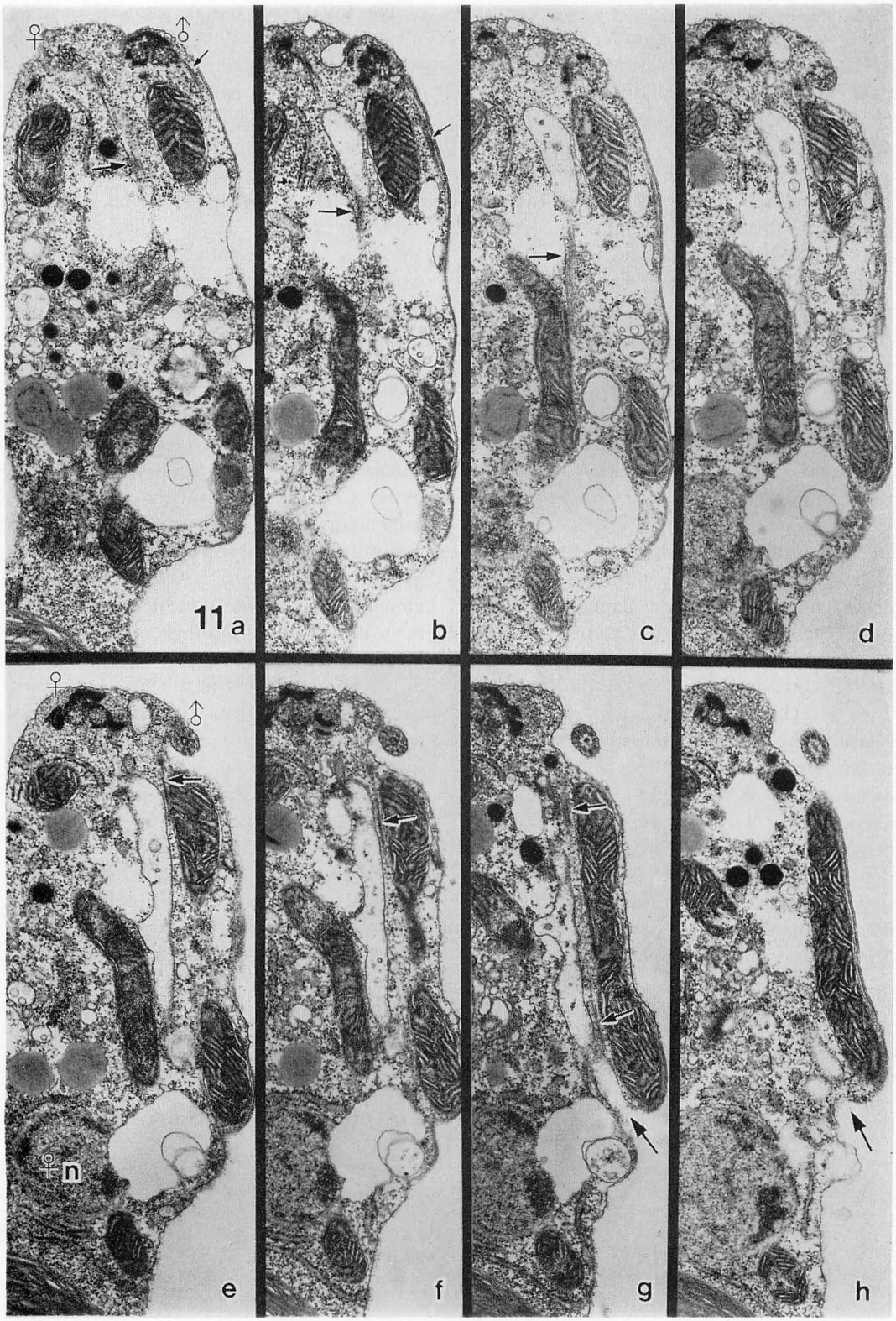
II. *Female gamete*

The biflagellate female gametes measure about 8–14 μm in length and 4–7 μm in width. Two isokont flagella, being 17.5–19.5 μm in length, extend from the papilla. Two basal bodies lie in exactly the same way as those in male gametes. The flagellar apparatus of female gametes has a pair of unusual crescent-shaped bodies (Fig. 6) (see MELKONIAN 1981, ROBERTS *et al.* 1982); a two-membered root is associated with each crescent-shaped body (Fig. 7). These are absent from male gametes and other green coenocytes. Another component associated with the two-membered root is the electron dense material which overlies the root for some distance (Fig. 7). MELKONIAN (1981) demonstrated that in female gametes of *B. lyngbyei* this material forms a cylinder and suggested that it represents a mating structure. Such a configuration is not discernible in the female gametes of *B. maxima*. At the shoulder region under the basal portion of the papilla is an area of thicker cell membrane underlain by an electron-dense material (Figs. 4, 5), which may represent the putative mating structure of this alga. Longitudinal serial sections through this area revealed that it measures 500 nm in length and 240–350 nm in width. The cytoplasm between the flagellar apparatus and nucleus contains four to five Golgi-bodies, small vacuoles and many granules filled with electron-dense material (Fig. 4). Individual granules measure 100 to 300 nm in diameter and sometimes a single limiting-membrane is discernible. These granules are present only in female gametes, and disappear in

Fig. 6. Part of a longitudinal section of conjugating gametes showing the female flagellar apparatus (♀) with crescent bodies (arrows) and one basal body of the male (♂). ×40,600.

Fig. 7. Part of a longitudinal section of the cell anterior of a female gamete showing the electron dense material (arrows) lying over the emerging roots. ×33,500.

Figs. 8–10. Early stage of gametic fusion fixed 2 minutes after copulation. 8. Section illustrating the distinct individuality of each papilla from both female (left) and male (right) gametes. ×49,400. 9. A slightly later stage of gametic fusion than that shown in Fig. 8. Note a vesicle (arrow) that stemmed from the cell gap originally formed between the two conjugating gametes. n, nucleus; c, chloroplast. ×12,000. 10. A more advanced stage than that shown in Fig. 9, showing that the cell anterior becomes more round due to the disappearance of the groove between male and female gamete papillae (compare to Fig. 8). Arrow indicates the flagellar root of the female apparatus extending down along the fusion site. ×16,200.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the zygote cytoplasm after conjugation (Fig. 16).

The female chloroplast contains a pyrenoid and an eyespot (Fig. 4). The eyespot is composed of a layer of granules 80 nm in diameter and is located on the same side of the cell body as the putative mating structure (Fig. 4).

The whole cell surface is covered by a fuzzy material similar to the male gamete (Fig. 4).

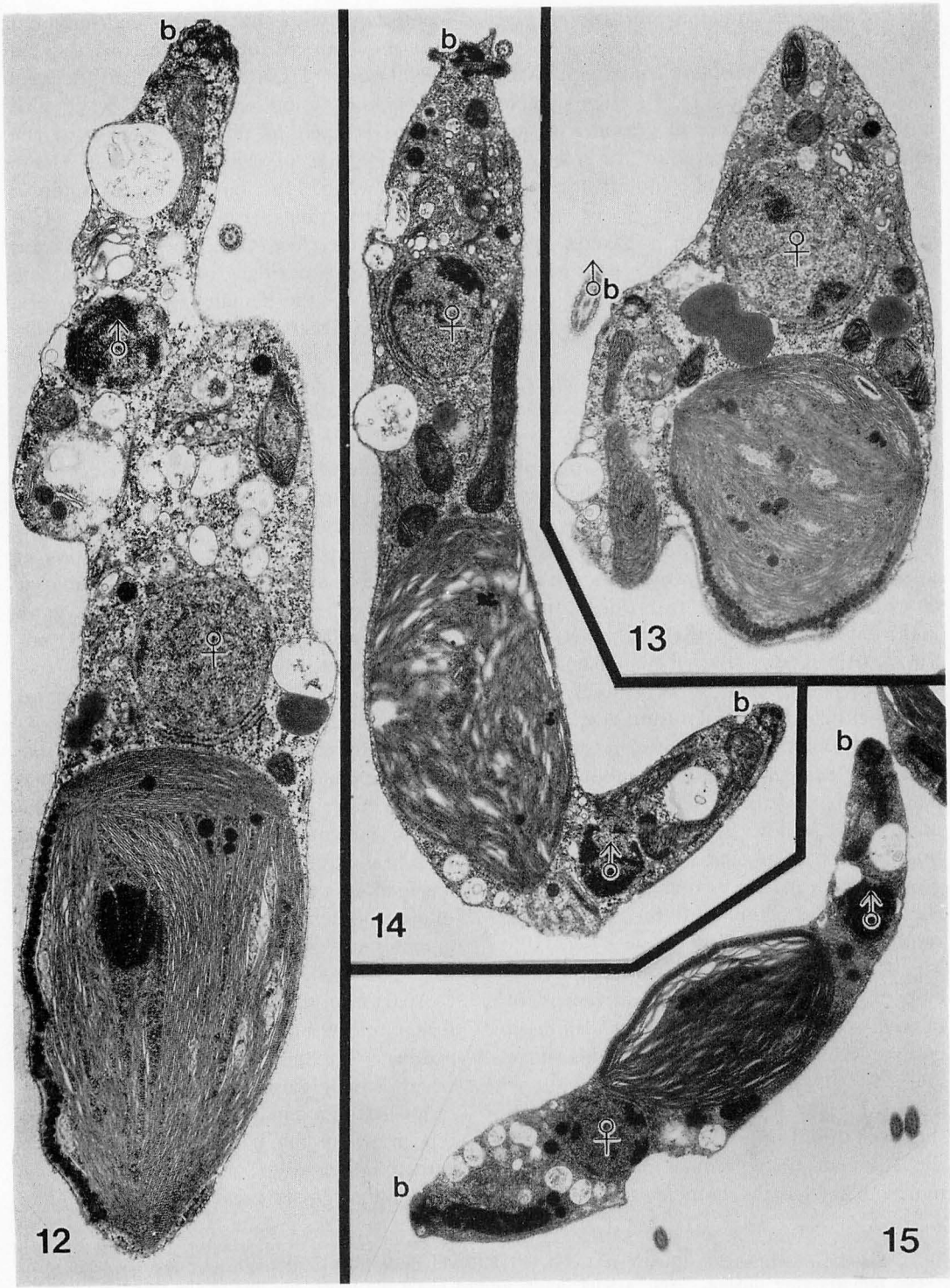
III. *Gametic fusion*

Early zygote fixed 2 minutes after mixing: The most general mode of gametic fusion is initiated at the anterior of female gametes, including the area of the putative mating structure located near the basal bodies (Figs. 8, 9). In many cases the fusion site of the male gamete is restricted to the cell anterior, but sometimes the posterior half or posterior two thirds of the male gamete fuses to the cell anterior of the female body (Fig. 12). The initial fusion process appears to be a quick event, since neither the initial fusion nor activation of the mating structure were detected. At a very early stage of conjugation, papillae from both female (left in Fig. 9) and male (right in Fig. 9) gametes can be distinguished. At a slightly later stage the groove between the two papillae (Fig. 8) rises up as the cytoplasmic fusion proceeds, resulting in a rounded dome (Fig. 10). Fig. 9 shows the two sets of basal bodies close together. The original arrangement of cell organelles found in each unfused gamete is maintained even at this stage (Figs. 9, 10).

Fig. 11 is a set of serial sections which show the initial fusion event of the gametes, the left half being female and the right male. The female gamete cytoplasm is continuous with that of the male gamete, but a cylindrical lumen formed by the cell membranes of both gametes can be seen along the fusion site (Fig. 11b-f). The lumen is open to the cell exterior at the posterior end of the male gamete (large arrow in Fig. 11g), but it is closed again in the next section (arrow in Fig. 11h). It is possible therefore that when the anterior portion of two ridges of the male gamete attaches to the female cell surface, the initial coalescence of gamete membranes begins both around the outer most margin of the male gamete body, which tightly attaches to the female body and to the margin of the lumen formed by the cell surface of the female gamete and the inter-ridge concave cell surface of the male gamete (Fig. 4). The continuity of the cytoplasm expands inwards, resulting in a reduction of lumen size and the formation of vesicles. These vesicles are released into the zygotic cytoplasm (Fig. 9) and later released outside the zygote. Along the cytoplasmic surface of the lumen two flagellar roots extend down, one from the female flagellar apparatus (large arrows in Figs. 11a-c) and one from the male (small arrows in Fig. 11e-g). Fuzzy material on the inner side of the lumen indicates the origin of the lumen membrane from the gamete cell membranes engaged in the cell fusion. The presence of electron-dense granules specific to female gametes was not observed near the fusion site.

Apart from this common mode of gametic fusion, other modes were observed in *B. maxima*. The first type is shown in Fig. 13, where a male gamete has fused to the posterior half of a female gamete. The second type is where the posterior half of a male gamete attaches to or near the posterior end of the female gamete (Fig. 14). The

Fig. 11. Eight sections (numbers 1, 3, 4, 6, 7, 8, 10, 13) from a series through a young zygote fixed two minutes after mixing. Note a long lumen formed along the fusion site due to the incompleteness of cytoplasmic continuity. It is open to the cell exterior at the posterior end of the male gamete (large arrow in Fig. 11g). Flagellar roots extend down from the female flagellar apparatus (♀) (large arrows Fig. 11a-c) and from the male (♂) (small arrows in Fig. 11a-b, and small arrows in Fig. 11e-g). See details in the text. ×16,000.



Figs. 12–15. Conjugating gametes fixed two minutes after mixing. Figures showing atypical modes of gametic fusion. b, basal bodies; ♂, male nucleus; ♀, female nucleus. 12, ×14,500. 13, ×13,000. 14, ×11,000. 15, ×8,300.

third type is where both gametes fuse to each other at their posterior ends (Fig. 15).

Zygotes fixed 25 minutes after mixing:

Zygotes are spherical at this stage (Fig. 16), but new cell wall has not been formed yet. The most prominent feature of zygotes at this stage is the appearance of variously-sized vacuoles containing small vesicles of about 60 nm in diameter. These vesicles are not present in unfused gametes. The flagellar apparatuses from both gametes are present as separate entities in the periphery of the zygotic cytoplasm (Fig. 16). The origin of each flagellar apparatus is identified by the presence of the crescent-shaped bodies specific to female gametes. The composition of the flagellar apparatus is the same as in unmixed gametes, except that the microtubular roots appear to dissolve as the cytoplasmic coalescence proceeds. The two sets of paired basal bodies are still continuous with their flagellar axonemes, indicating that they are absorbed into the cytoplasm through the cell surface (Fig. 17), similar to *Ulva mutabilis* (BRÅTEN 1971). Another event characteristic to this stage is nuclear fusion. Once the continuity of the outer nuclear membranes of both nuclei is established (Fig. 17), then the inner membranes coalesce (Fig. 18). The fusion or disintegration of chloroplasts and mitochondria was not observed. These events occur in this alga at a later stage of zygote maturation (HORI and KUROIWA in prep.).

Discussion

The mating reaction of the isogamous, biflagellate alga, *Chlamydomonas reinhardtii*, is initiated by an agglutination between the flagellar tips of mating type plus (mt^+) and minus (mt^-) gametes (SAGER and GRANICK 1954, GOODENOUGH and WEISS 1975), and then the extension of the fertilization tubule from the mt^+ cell surface to the mt^- cell, an event mediated by a mating structure of specialized cell membrane (FRIEDMANN *et al.* 1968, CAVALIER-SMITH 1975, GOODENOUGH and WEISS 1975, TRIEMER and BROWN 1975, WEISS *et al.* 1977).

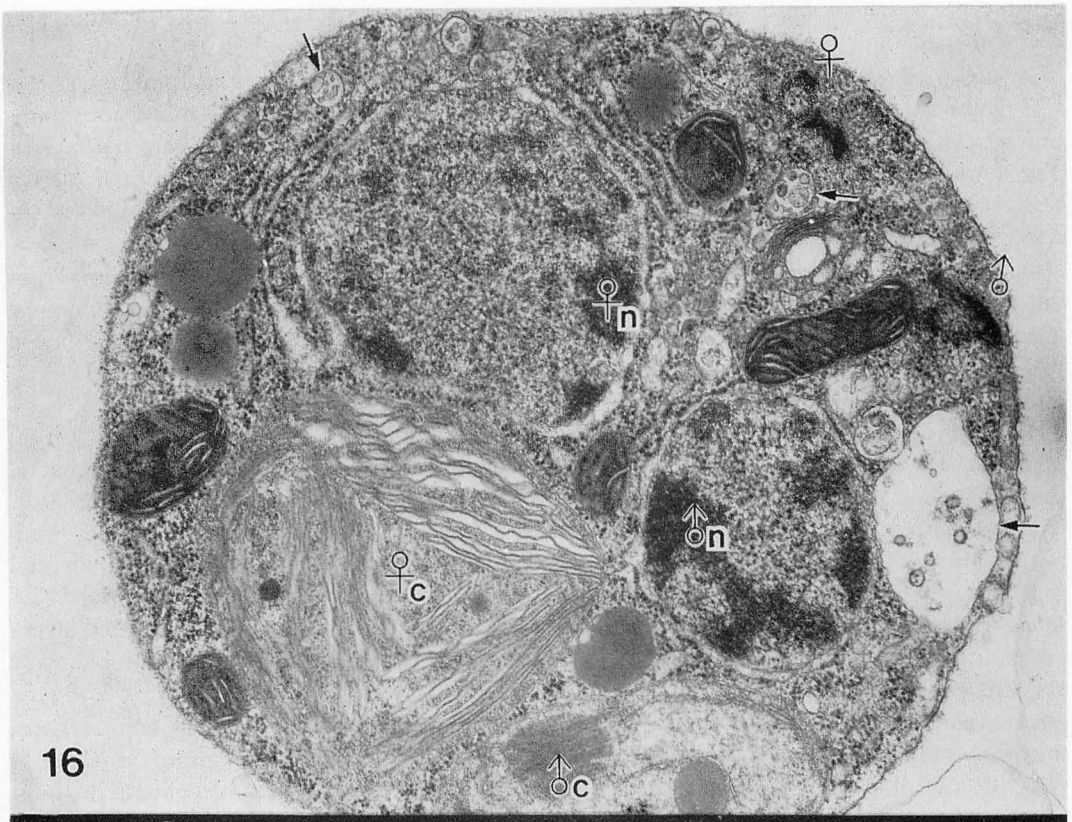
The multicellular green alga, *Hydrodictyon reticulatum*, produces isogamous gametes. One of the mating pair has a specialized structure of cell membrane (apical cap) which mediates gametic fusion (MARCHANT and PICKETT-HEAPS 1972).

BRÅTEN (1971) described *Ulva mutabilis* as isogamous and no morphological difference can be found between the two sex types of gametes even at the ultrastructural level. Another species, *U. lactuca*, produces a female gamete slightly larger than the male, but their ultrastructure is similar to that of *U. mutabilis* gametes (MELKONIAN 1980b). The mating structure of both gamete types of *U. lactuca* is a special region of the cell membrane. This region is oval-shaped (1.1 –0.7 μm) and is a rather complicated structure composed of four different elements (MELKONIAN 1980b).

Another more conspicuous feature common to this isogamous group of algae is that their gametes can germinate without fertilization (MARCHANT and PICKETT-HEAPS 1971, KOCHERT 1982, KOEMAN 1985).

In the oogamous alga, *Prasiola stipitata*, the membrane of one of the two spermatozoid flagella coalesces with the membrane of the egg, and the axoneme of that flagellum is incorporated into the egg protoplast (MANTON and FRIEDMANN 1960).

Male gametes of the coenocytic, anisogamous green algae, *Bryopsis maxima* (HORI 1977) and *B. lyngbyei* (MELKONIAN 1980a) have no mating structure. In female gametes of *B. lyngbyei* a cylindrical structure which is linked to the basal bodies overlies the two-membered microtubular root for some distance. This cylinder is suggested to represent a mating structure (MELKONIAN 1980b), but it has yet to be confirmed that it functions as such during gametic fusion because of an insufficient number of gamete pairs studied by electron microscopy (MELKONIAN 1980b). In the posterior region of the cylindrical structure, however, there is



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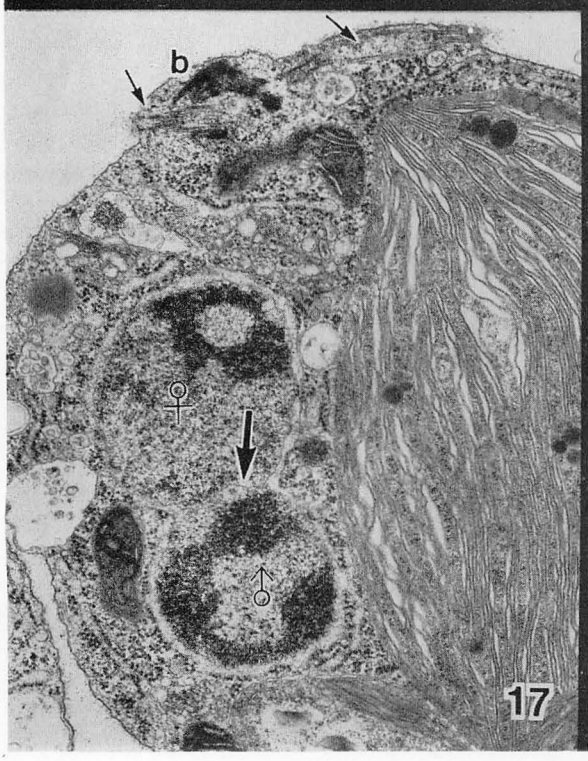

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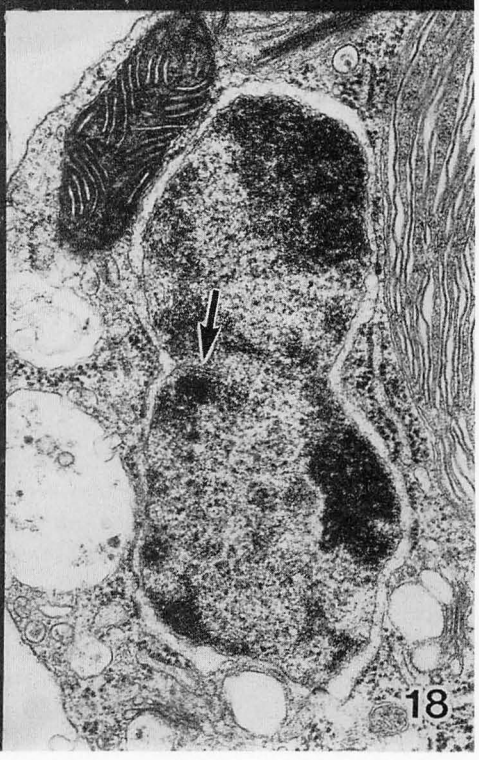

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an area of thickened cell membrane (which measures 300–400 nm in cross section) adjacent to the cell membrane overlaying the cylindrical structure. This specialized area of cell membrane resembles the mating structure found in the isogamous algae discussed above and in *B. maxima* (as well as in other ulvalean algae).

The gametic fusion of *B. maxima* usually occurs in a certain area of the female gamete membrane, which corresponds to the putative mating structure. In this alga, however, gametic fusion also occurs elsewhere on the female cell body. The male gametes do not have a putative mating structure and their fusion with the female gametes commonly occurs at an area near the basal part of the papilla, though it does occur at other areas of the male cell body too. This is conspicuously different from that in isogamous algae described above. Female and male gametes of *B. maxima* are not able to germinate without fertilization (TATEWAKI 1973).

Light microscopists have shown that anisogametes of coenocytic green algae can fuse with each other at any site as well as at the cell anterior; examples are found in *Pseudobryopsis* sp. (Figs. 262–3, 5a in OLTMANNS 1922), *Codium tomentosum* (Fig. 258 in OLTMANNS 1922), *C. fragile* (Fig. 1–7 in ARASAKI *et al.* 1956), *Halimeda cuneata* (Figs. 4M, N, P in CHIHARA 1956) and some other halimedean algae (Plate II-I in KAMURA 1966).

It has been observed by electron microscopy that female gametes of *Pseudobryopsis* sp. possess a dense staining region at cell membrane, and an electron-dense tubular extension along one of the outer microtubular roots (ROBERTS *et al.* 1982). These structures correspond to the features which MELKONIAN (1981) considered to be a putative mating structure in the female gamete of *B. lyngbyei*. The male gamete does not have these structures. *Derbesia* is also anisogamous, but both gamete types lack the mating structure and eyespot (ROBERTS *et al.* 1981). MELKONIAN (1981) suggested that the absence of a mating structure in the female gamete of *Derbesia* might be due to its lack of an eyespot.

The genus *Halimeda* is anisogamous and from light microscope studies female gametes are known to have an eyespot, but not male gametes (CHIHARA 1956, KAMURA 1966). GORI (1979) studied *Halimeda tuna* by electron microscopy, but he mentioned nothing about the presence or absence of a mating structure in the male gamete. If MELKONIAN's suggestion mentioned above is correct, the female gametes of *Halimeda* possess a putative mating structure as in other green siphons having eyespots. The male gametes of coenocytic green algae have no mating structure (ROBERTS *et al.* 1982), and no species is known in which the male gametes have an eyespot.

The putative mating structure has since been found in many other green algae by electron microscopy (O'KELLY and FLOYD 1983, FLOYD and O'KELLY 1984, O'KELLY *et al.* 1984, MIYAJI and HORI 1984). In all these cases the structure is similar to an area of thickened cell membrane as found in *U. lactuca* and *Chlamydomonas*. The position of the structure is restricted to a region close to the flagellar apparatus. At present, there is no evidence that the mating structure can move freely in the cell membrane. It has also been suggested that the mating structures are fixed at a certain position in the cell by the flagellar roots (GOODENOUGH and WEISS 1978, MELKONIAN 1980b). If the conjugation property of the mating structure of the coenocytic or anisogamous green

Figs. 16–18. Zygotes fixed 25 minutes after mixing. 16. Female (♀) and male (♂) nuclei (n), chloroplast (c) and basal bodies are present. Arrows indicate vesicles which specifically appear in the zygote cytoplasm after copulation. ×25,000. 17. Part of a zygote illustrating the uptake of the flagellar axonemes (small arrows) through the cell surface. Note nuclear fusion where the outer nuclear membrane is continuous, but the inner one (large arrow) is still intact. ×20,900. 18. A later stage of nuclear fusion than in Fig. 17. Arrow indicates remnants of the inner membrane. ×52,200.

algae functions properly as in isogamous algae, their gametes will fuse only at the cell anterior, at least in the female gametes. However, note that gamete fusion of anisogamous algae often occurs at a region far away from the putative mating structure of female gametes. Thus, it may be concluded that the mating structure, even if it occurs, does not function properly in the green siphons, or more generally, in anisogamous green algae. The mating structure found in female gametes of these algae probably represent a vestigial sexual organ.

The young zygotes of *U. lactuca* contain two sets of flagellar apparatuses originated from both sexes of gametes. They lie side by side within the zygote (see Fig. 29 in MELKONIAN 1980b). This arrangement indicates that the gametes fuse parallel to each other by using their mating structures. As the process ensues, zygotes become spherical. At present, the fate of these two sets of flagellar apparatuses at a much later stage of zygote maturation in *U. lactuca* is not known.

The two flagellar apparatuses in the early zygote of *B. maxima* lie in the same way as in *U. lactuca*. However, they soon separate in the zygote cytoplasm of *B. maxima* and later disappear. According to preliminary observations on early zygotes of *Monostroma latissimum* (unpublished observation) and *U. nitidum* (MOTOMURA personal communication) (both were fixed 30 minutes after mixing), four basal bodies originated from the two gamete types are arranged in the same manner as in quadriflagellate zoospores of *U. lactuca* (MELKONIAN 1979). At a very early stage of zygotic fusion (20 seconds after mixing) two sets of the flagellar apparatuses in *M. latissimum* lie in the same way (unpublished observation) as in the early zygotes of *B. maxima* and *U. lactuca*, that is, gamete pairs lie side by side with their longitudinal axes parallel to one another. These observations strongly suggest that the arrangement of the two sets of basal bodies is changed and rearranged as the zygotic maturation proceeds. Although the basal body arrangement in quadriflagellate zoospores of *M. latissimum* and *U. nitidum* has not been observed, they probably have the similar arrangement as that of the zoospores of *U. lactuca*.

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堀　輝三：オオハネモ（緑藻綱）の配偶子および配偶子接合の微細構造

異型配偶子緑藻オオハネモの配偶子，接合および接合子初期を微細構造的に解析し，他の異型および同型配偶子緑藻のそれと比較した。雌性配偶子は，他の多くの緑藻の配偶子でも知られているものと微細構造的に類似した配偶子接合装置をもつが，雄性配偶子はこれを欠く。配偶子の接合は雌性配偶子の接合装置か，あるいはそれに近傍の部位で起るのが最も一般的な様式である。しかし，本藻においては接合装置が関与しない接合もあるこ

とがわかった。これは，配偶子接合装置が同型配偶子緑藻における程には有効に機能しなくなっていることを示唆するものと考えられる。従って，より進んだ緑藻，特に異型性のグループでは接合装置が有性生殖に関わる一種の痕跡器官となっているのであろう。

接合子形成の初期（配偶子混合25分後まで）で，既に雌・雄核の融合は起るが，葉緑体，ミトコンドリアの融合または分解は起らない。接合子中における雌・雄配偶子由来の鞭毛装置構造の挙動についても論議した。

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