CHAPTER 4

MOLECULAR PHYLOGENY

4-1 Phylogenetic position of *Heterocapsa* among dinoflagellates

In order to assume the phylogenetic position of the genus *Heterocapsa*, the molecular phylogenetic analysis using small subunit ribosomal RNA gene sequences was performed. For this analysis, 72 dinoflagellates including 60 species of the Peridiniales, Prorocentrales and Gymnodiniales as well as 12 of the Gonyaulacales as outgroups were provided. Based on the DNA sequences determined in this study and referred from the DDBJ / EMBL / Genbank database, two kinds of phylogenetic trees were constructed by neighbor joining (NJ) and maximum parsimonious (MP) methods to represent the phylogenetic relationships.

As shown in Figure 4-1, the NJ tree indicated that twelve species belonging to the Gonyaulacales were firstly branched and others made a huge clade, which is so-called as the Peridiniales / Prorocentrales / Gymnodiniales complex (GPP complex, 90% of bootstrap value). The genus *Heterocapsa*, presently examine four species made a small clade with *Cachonina hallii* in the GPP complex. From the result obtained, it can be referred that the genus *Heterocapsa* is monophyletic, and the phylogenetic position is uncertain, unfortunately. Other members of the complex made many smaller monophyletic clades, which are consisted of the planktonic species of *Prorocentrum*, benthic *Prorocentrum*, *Symbiodinium* with some other gymnodinioids, *Scrippsiella* with other marine peridinioids, *Heterocapsa*, and *Karenia* and *Karlodinium* containing fucoxanthin derivative as accessory pigments. Peridinioid species were separated into roughly four clades and several species diverged solitary (e.g. *Scrippsiella nutricula* and *Peridinium umbonatum* Stein). The clade of *Heterocapsa* species was far from the clade including freshwater species of *Peridinium*. Around the *Heterocapsa* clade, some

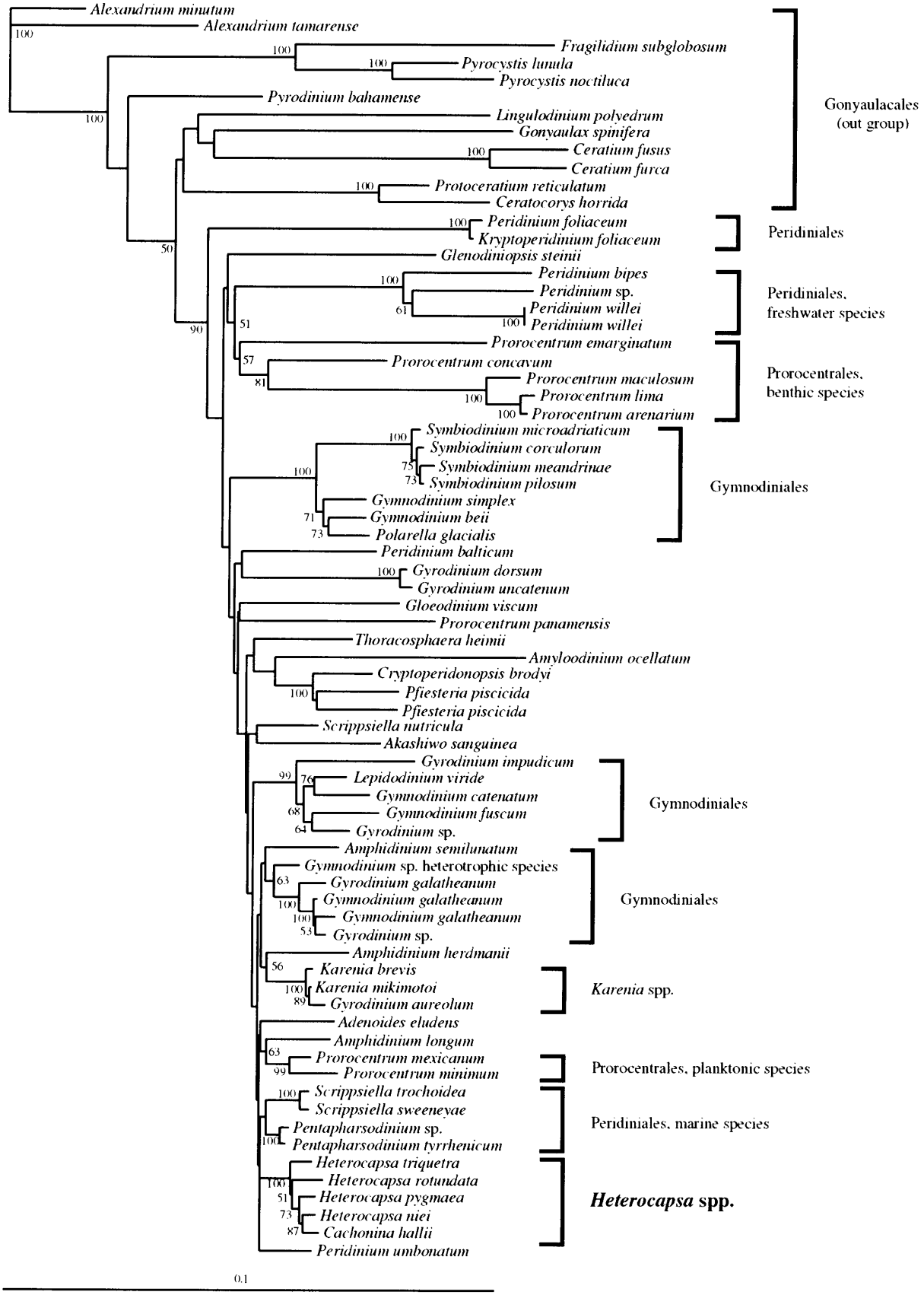

Figure 4-1. Phylogenetic tree constructed by neighbor-joining of complete SSU rRNA gene sequences from 72 dinoflagellates. Bootstrap values are shown above the internodes when higher than 50 %.

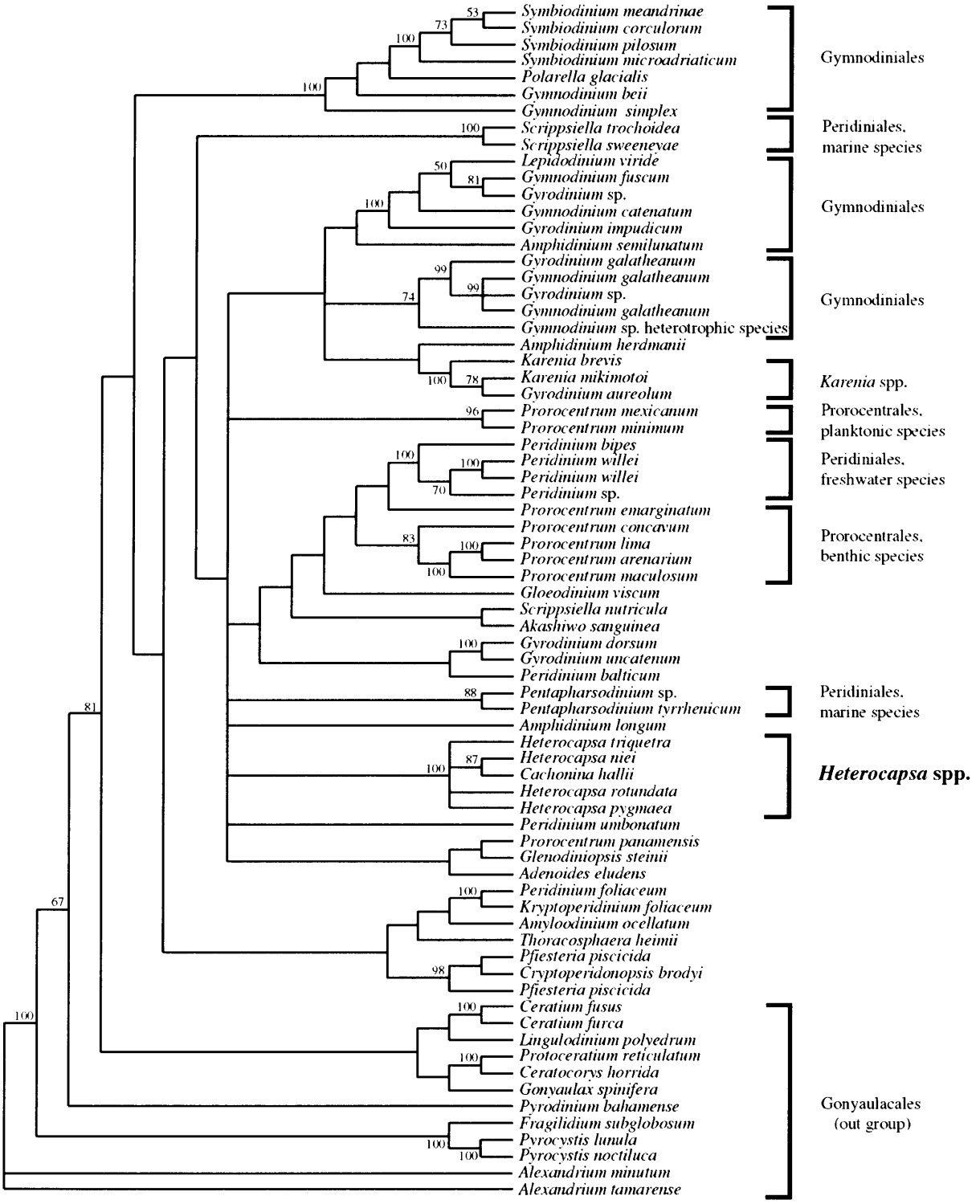

Figure 4-2. Phylogenetic analysis of SSU rRNA gene sequences. The strict consensus tree was constructed from 78 of equal maximum parsimonious trees. The percentage of bootstrap resamplings are shown at each internodes when higher than 50% (1000 replicates).

clades such as *Karenia* / *Karlodinium* spp., planktonic *Prorocentrum* spp., *Scrippsiella* spp. and *Peridinium umbonatum* were diverged, although relationships between *Heterocapsa* clade and each of these clades were also not supported. Therefore, sister group of *Heterocapsa* could not clarified from this NJ tree.

From the maximum parsimonious analysis by using SSU gene sequences, 78 of equal parsimonious trees were gained (Fig. 4-2). One strict consensus tree is given in Fig. 4-2. It is obvious that the topology and smaller groups in the huge clade of GPP complex are not so different between them.

4-2 Phylogenetic relationships of each species

Since sister group of the genus *Heterocapsa* could not be clarified by molecular systematic method using SSU rDNA gene sequences, several dinoflagellates belonging to *Prorocentrum* were employed as outgroups for phylogenetic analysis among species in the genus *Heterocapsa* based on ITS region sequence data. Topologies of both phylogenetic trees using NJ and MP methods were almost same (Fig. 4-3, 4-4). Consequently, all *Heterocapsa* species were well supported by high bootstrap value (100%) as a lineage of ingroup in the NJ tree (Fig. 4-3). Among the species of the genus, *H. illdefina* first diverged and the other *Heterocapsa* species were allied to one lineage (75%). In this *Heterocapsa* lineage, species made some clades; that is *H. horiguchii* and *H. ovata* (Clade 1), *H. triquetra* and *H. pseudotriquetra* (Clade 2) and *H. arctica*, *H. lanceolata* and *H. rotundata* (Clade 3). All these were supported by high bootstrap value (> 70%). Other two species, *H. pygmaea* and *H. circularisquama* made each conspecific lineage. Of these, Clade 1 diverged first, and then both pair of Clade 2 and *H. pygmaea*, and Clade 3 and *H. circularisquama* were weakly related, respectively. Phylogenetic relationships between these two pairs were unclear.

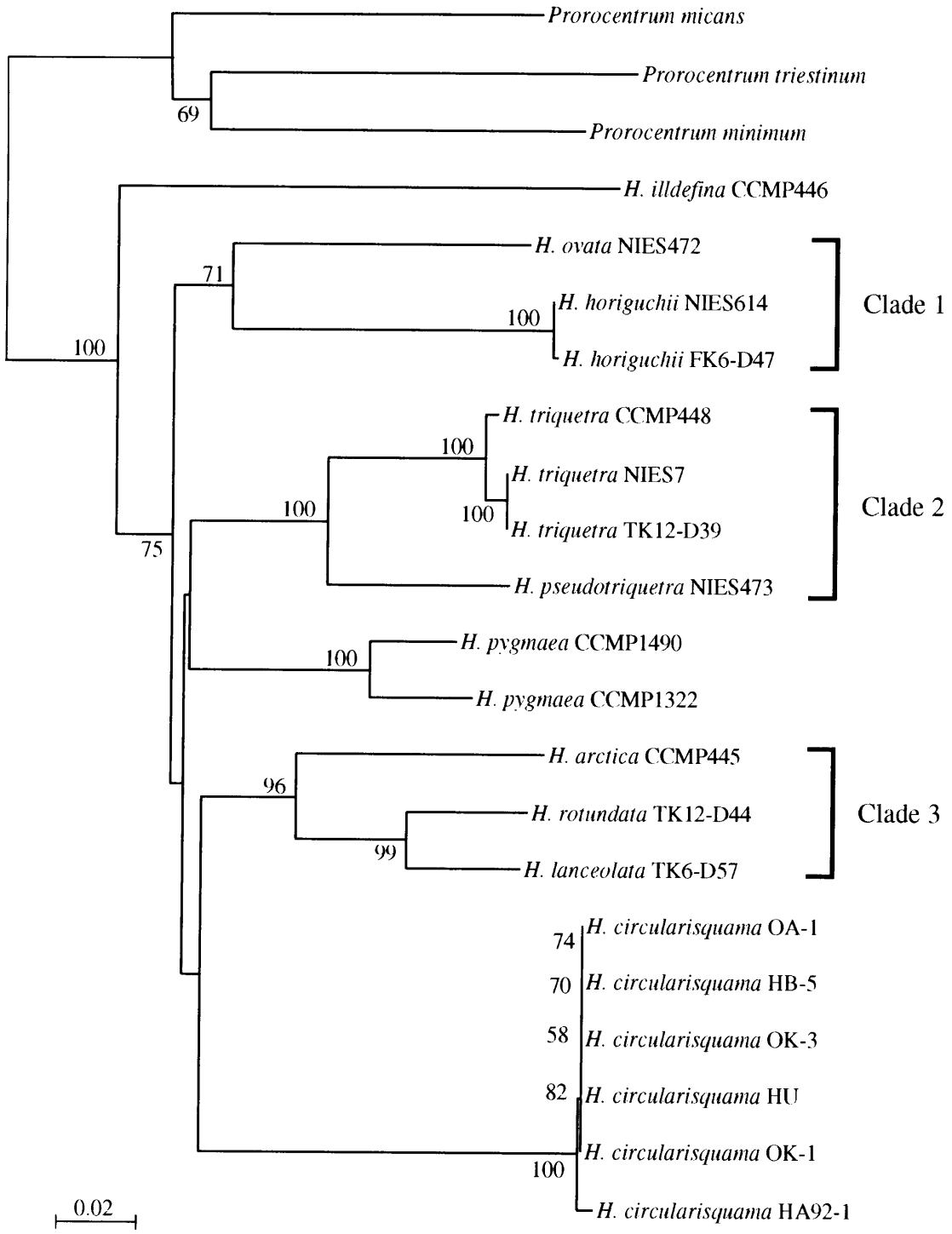

Figure 4-3. Neighbor-joining tree constructed from the ITS region alignment of *Heterocapsa* species. Numbers at internal branches indicate percentage of bootstrap support (1000 replicates).

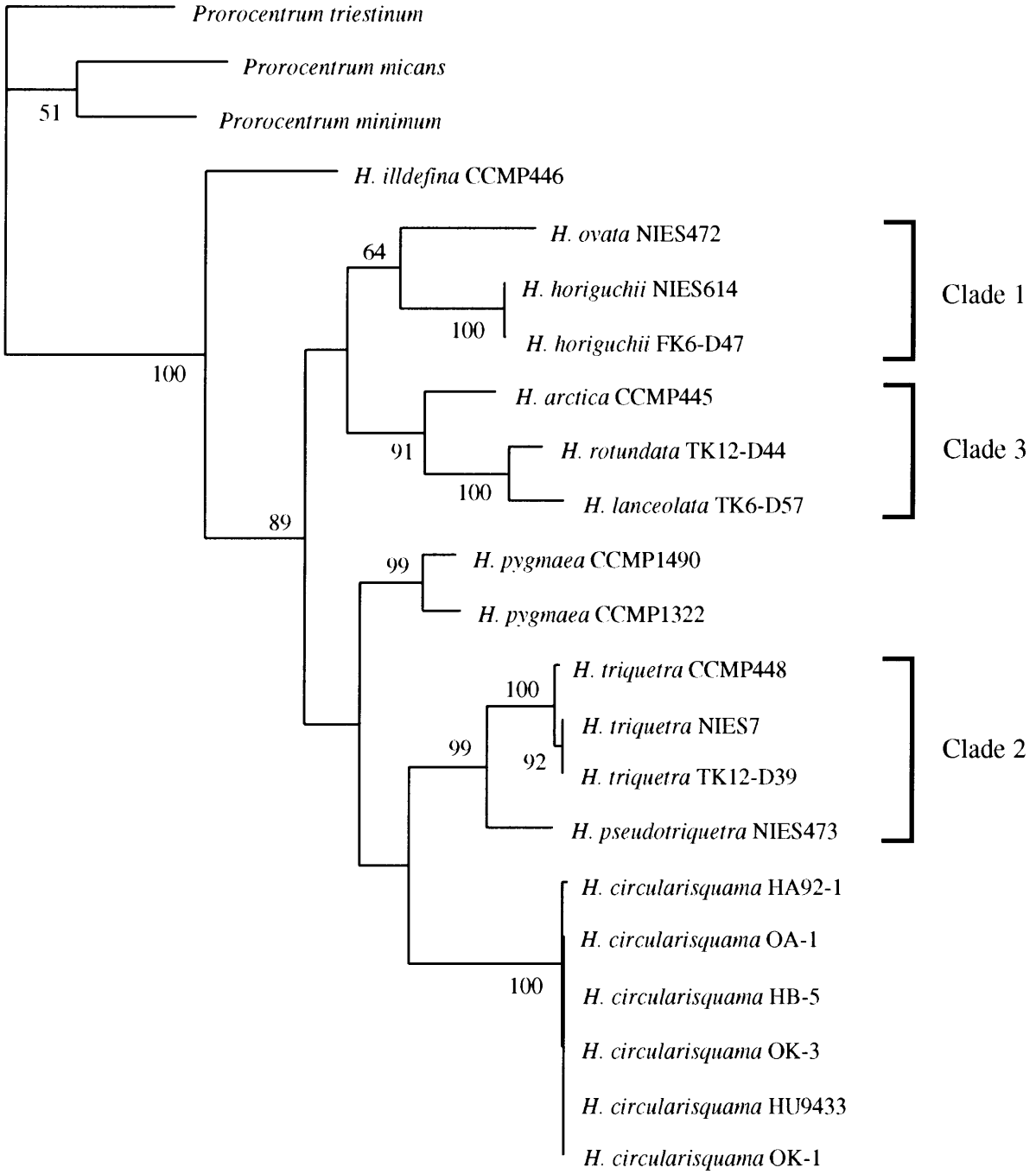

Figure 4-4. Maximum parsimony phylogenetic tree constructed from ITS region from 19 *Heterocapsa* species and 3 *Prorocentrum* species as outgroups. Bootstrap value are shown above the internodes when higher than 50% (1000 replicates).

In MP tree, *Heterocapsa* species also formed a generic lineage as well as each Clade found in NJ tree (Fig. 4-4). In the lineage of *Heterocapsa*, Clade 3 was weakly related to Clade 1 instead of lineage of *H. circularisquama*. Clade 2 was diverged together with *H. circularisquama*. These relationships between each clade were rarely supported (< 50%).

CHAPTER 5

GENERAL DISCUSSION

5-1 Taxonomy and phylogeny of the genus *Heterocapsa*

5-1-1 Taxonomic and phylogenetic position of the genus

Taxonomically, the genus *Heterocapsa* has been assigned to the class Peridiniales based on the tabulation of its thecal plates. However, polyphyly of this order has been revealed by recent molecular phylogenetic analysis using SSU rDNA gene sequences (Saunders *et al.* 1997; Saldarriaga *et al.* 2001) and partial LSU rDNA gene sequences (Daugbjerg *et al.* 2000). Among dinoflagellates, namely, enigmatic species such as *Noctiluca scintillans* (Macartier) Kofoid & Swezy and *Crypthecodinium cohnii* Seligo are firstly branching and a distinct clade of species of the Gonyaulacales secondly diverged. Most of the other dinoflagellates assigned to three orders, the Gymnodiniales, the Peridiniales and the Prorocentrales, are then mingling together, and this assemblage is called as GPP complex (Sounders *et al.* 1997). This relationship, particularly existing GPP complex, indicated polyphyly of these three classes including the Peridiniales.

In the present study, the phylogenetic trees showing the similar topology to these studies was gained based on SSU rDNA gene sequences. The order Peridiniales including *Heterocapsa* was also separated into several groups. Among them, species of *Heterocapsa* made a definite lineage, which is separated from the clade including the freshwater species of *Peridinium*, viz. the Peridiniales *sensu stricto*, as well as the clade of the marine peridinioid genus *Scrippsiella*. Morphological characteristics among marine and freshwater species of Peridiniales are more or less similar, but some thecal plates characters are different between

them. For instance, the number of the cingular plate series clearly differs in groups of the Peridiniales. The cingular plate number of the freshwater peridinioid species is five, while the marine species such as *Scrippsiella* and *Heterocapsa* possess six cingular plates. The group of marine peridinioids should be separated from the order Peridiniales *sensu stricto* based on this morphological characteristic, however, monophyly among *the genera* of marine peridinioids is still unclear based on the molecular phylogenetic tree. Based on the molecular data, each marine peridinioid group, viz. *Heterocapsa* and *Scrippsiella*, should be transferred from the order Peridiniales into new order. Since the relationships between *Heterocapsa*, *Scrippsiella* and other groups in the GPP complex are still unclear, the genus *Heterocapsa* will be treated as a member of the Peridiniales *sensu lato* until information of this group will be accumulated.

Species of *Heterocapsa* share some unique morphological characters, such as organic body scales and conspicuous pyrenoids, as well as thecal plate arrangement; Po, cp, 5', 3a, 7", 6c, 5-8s, 5'", 0-1p, 2"" (Horiguchi 1997). In the present study, molecular phylogenetic tree of NJ method weakly indicated several expected sister group, e.g. *Scrippsiella* and planktonic species of *Prorocentrum*. These dinoflagellates do not possess body scales and similar thecal plate arrangement to *Heterocapsa*. In addition, most of these species lack the pyrenoids. It suggests that these characters have been obtained in the common ancestor of the genus *Heterocapsa*.

Several authors have discussed the phylogenetic relationship of thecal plate arrangements among dinoflagellates (e.g. Bujak & Williams 1981; Saunders *et al.* 1997). According to Saunders *et al.* (1997), following three schematic hypotheses were proposed; 1) the plate increase model, 2) the plate reduction model and 3) the plate fragmentation model. Among these slightly related groups, the most assumptive sister group based on thecal plates is *Scrippsiella*, which possess thecal plates less than only two plates of *Heterocapsa*. If the genus *Scrippsiella* is considered as a sister group of *Heterocapsa*, it can be explained that the thecal plate arrangement of *Heterocapsa* would be conducted to be added an apical and sulcal plate to

those of *Scrippsiella*, following the plate increase model proposed by Saunders *et al.* (1997).

5-1-2 Taxonomy and interspecific relationship of *Heterocapsa*

Phylogenetic trees based on ITS region (Fig. 4-3, 4-4) including 5.8S rDNA gene sequence also indicate the strong affinity among *Heterocapsa* species. Four species, *H. circularisquama*, *H. horiguchii*, *H. pygmaea* and *H. triquetra*, were analyzed by using several culture strains with different localities. Each of these species made an well supported monophyletic lineages (100% of bootstrap value). These molecular phylogenetic results seem to be consistent with the taxonomy of *Heterocapsa* species based on their morphological data.

These phylogenetic trees also revealed several clades in the genus, namely, *H. horiguchii* and *H. ovata* (Clade 1), *H. triquetra* and *H. pseudotriquetra* (Clade 2), and *H. arctica*, *H. lanceolata* and *H. rotundata* (Clade 3). These relationships were supported in both NJ and MP trees. Since each clade was also supported by several common phenetic characters as well as this genetic support. Therefore, it is likely that these three groups are natural group in the genus *Heterocapsa*.

In the Clade 1, cell shapes of *H. horiguchii* and *H. ovata* do not resemble with each other, but some morphological characteristics indicate their affinity. One of common features is the position of nucleus and pyrenoid in cytoplasm. Both species possess a spherical nucleus in the epitheca, and the pyrenoid is located below the nucleus (Fig. 5-1). Another feature of body scale ultrastructure is also common. Number of peripheral uprights of their body scales is six (Fig. 5-2). In other species, this number was found only from *H. circularisquama*. But *H. circularisquama* was not allied to them in the molecular phylogenetic tree (Figs 4-3, 4-4). These facts suggest that these characters are stable among the genus. These characters are also shared by *H. horiguchii* and *H. ovata*, although tubular invagination in pyrenoid is present only

in *H. ovata*.

Two *Heterocapsa* species *H. pseudotriquetra* and *H. triquetra* composed the Clade 2. Compared morphological characters, cell shapes of these species are rather different. Cell of *H. pseudotriquetra* is subspherical with rounded apex and antapex, whereas *H. triquetra* is rhomboid with antapical horn on the plate 2"" (Fig. 5-1). However, both species possess a spherical nucleus in the epitheca, and a pyrenoid is situated below the nucleus. Tubular invaginations in pyrenoid matrix are present in both species. Moreover, ultrastructures of their body scale are approximately same (Fig. 5-2). It implies that the ultrastructure of body scale is a relatively stable character than the cell shape in the phylogenetic line of *Heterocapsa* species.

Members of the Clade 3 share some morphological characters such as the relatively larger epitheca than hypotheca and body scale ultrastructures. Among *Heterocapsa* species, *H. arctica*, *H. lanceolata* and *H. rotundata* are the only species possessing large epitheca (Fig. 5-1). Body scale of *H. arctica* is slightly variable, but numbers of uprights and spines of the mature body scale are same as that of *H. rotundata* (Fig. 5-2). This morphological characteristic also indicates the affinity between these two species. Moreover, the central hole of the basal plate is present only in *H. lanceolata* and *H. rotundata*. In the phylogenetic tree (Figs 4-3, 4-4), *H. arctica* branched first and the other two species made a clade (99% and 100% in NJ and MP, respectively) in Clade 3. The central hole in the basal plate supports the monophyly of *H. lanceolata* and *H. rotundata*. These similarities of cell morphology and body scale fine structure strongly support the interspecific affinity in this clade.

Two culture strains of *H. horiguchii*, FK6-D47 (Fukuyama) and NIES614 strain (Kashiwazaki) with similar cell morphologies were used in this molecular analysis. Fine structure of their body scales is almost same with an exception of the fineness of the mesh of the basal plate. Basal plate texture of FK6-D47 strain is quite fine reticulation, while that of NIES614 strain consists of a rough network (Plate 35). These characteristics appear to be stable in each culture, and are tentatively named as *Heterocapsa* sp.2 and *Heterocapsa* sp.4,

respectively. However, their molecular sequences of ITS region are almost identical, namely only 1 base are substituted in 594 base pairs. Cell morphology, molecular phylogeny, and number of uprights and spines of body scale did not show clear differences enough to differentiate them as two independent species. They are thus regarded as the same species, *H. horiguchii*. Differences in basal plate texture, viz. fine and rough meshes, were also found in *H. rotundata*. Taxonomic significance on the basal plate texture will be also discussed in the following section (see Section 5-2-5).

Phylogenetic relationships between these clades and other species were less supported and could not be solved from the phylogenetic tree using ITS region. Therefore the relationships among *H. illdefina*, *H. ovata* and *H. pygmaea* are still uncertain. Topology of this tree indicates the first branching of *H. illdefina*, but it may be caused by selection of sister group.

5-1-3 Evolutionary relationships of morphological characters

Species of the genus *Heterocapsa* have been differentiated based on several morphological characters, such as cell size, cell shape, position of nucleus and pyrenoid, tubular cytoplasmic invaginations in pyrenoid, thecal plate arrangement and ultrastructure of body scale. Here in this section, phylogenetic relationships of these characters are discussed based on the molecular phylogenetic analysis.

Cell sizes are significant for characterizing each species, however, no implication to show the interspecific relationship exists. For example, such smaller species as *H. rotundata* and *H. pygmaea* are not allied in the tree. Contrary, small species of *H. rotundata* and large *H. arctica* are well affined together.

Cells of *H. arctica*, *H. lanceolata* and *H. rotundata* have the large epitheca, and their

phylogenetic affinity is also suggested from molecular analysis. Therefore, this character appears to be obtained only by one lineage through the divergence in the genus. It may imply that another *Heterocapsa* species possessing the large epitheca, *H. minima*, also allies to these three species. Of these *H. arctica* is quite large and occurs in Arctic Sea, therefore *H. minima* may relate with *H. lanceolata* or *H. rotundata*.

Cells of *H. orientalis*, *H. ovata* and *H. pseudotriquetra* are spherical or ovoid, and relatively large among *Heterocapsa* species, but they emerge in different branches in the phylogenetic tree. Actually, *H. triquetra* and *H. horiguchii* are related with the rounded species, *H. pseudotriquetra* and *H. ovata*, respectively, in the topology of molecular analysis. These major cell shapes, viz. spherical or ellipsoid, must be polyphyletic character in the genus.

The tubular invaginations in pyrenoid matrix have been reported in *H. triquetra* (Dodge 1968), *H. illdefina* (Herman & Sweeney 1976) and *H. arctica* (Horiguchi 1997). Presence or absence of this character was employed as a discernable feature of *H. circularisquama* from *H. illdefina* in the original description (Horiguchi 1995). The invaginations are also present in *H. orientalis*, *H. ovata* and *H. pseudotriquetra*. Therefore, this character has been confirmed from six *Heterocapsa* species so far. This means a half of the *Heterocapsa* species possess this character. As these species are separately emerged in the tree, monophyly of this character was not supported by the molecular data. In the lineage including *H. pseudotriquetra* and *H. triquetra* (Clade 2), both species possess the tubular invaginations in the pyrenoid matrix. But in the other clade containing *H. arctica*, *H. lanceolata* and *H. rotundata* (Clade 3), this character is present only in *H. arctica*. Since the character is found in species with relatively large cell size, this structure may act as physical transportation in the cytoplasm. If the ancestor of *Heterocapsa* species is relatively large and rounded species such as *H. orientalis*, *H. ovata* or *H. pseudotriquetra* (or *H. illdefina*, from the topology), the tubular invagination could be a primitive character that simultaneously obtained with the pyrenoid itself. The phylogenetic position of *H. illdefina*, which possesses tubular invaginations, actually accords this view. But

the reliability of this topology especially in shallower branching is still ambiguous.

Thecal plate arrangement of *Heterocapsa* species are almost same, regardless the cell size and cell shape, that is Po, cp, 5', 3a, 7", 6c, 5s, 5'", 2"". Significant differences of plate formula among the species are not detected in the present study. Therefore, this plate arrangement has been obtained in the common ancestor of *Heterocapsa*.

Body scales of *Heterocapsa* present an appearance of triradiate construction and superficially resemble among all *Heterocapsa* species. They consist of a basal plate, a central spine, three radiating bars, peripheral uprights, horizontal bars that connects peripheral uprights and radiate bars, and spines. Numbers of these parts are basically different from the species, but some of them are shared similarities. That is *H. pseudotriquetra* and *H. triquetra*, and matured scale of *H. arctica* and *H. rotundata*. Monophyly of each of these two groups are well supported by the molecular data (100% of bootstrap value in both trees). Moreover, a central hole on the basal plate is found only in two species, *H. lanceolata* and *H. rotundata*, which make a well supported clade by the phylogenetic analysis. This suggests that the body scales would be gradually modified in each lineage, that is to say, the fine structure of *Heterocapsa* body scale indicates affinity of each species. Body scale of *Heterocapsa* species could be roughly separated into two groups based on number of the peripheral uprights. Some scales have six peripheral uprights, and the other own nine. The former group includes *H. circularisquama*, *H. horiguchii*, *H. ovata* and *H. pygmaea*, and the latter does *H. arctica*, *H. illdefina*, *H. lanceolata*, *H. orientalis*, *H. pseudotriquetra* and *H. triquetra*. The interspecific relationships among these species could not be elucidated using ITS sequence data, hence these affinities are still unclear, unfortunately.

5-2 Taxonomic characteristics of *Heterocapsa*

5-2-1 Thecal plates

Thecal plate arrangement of *Heterocapsa* species seems to be well conserved despite the diversity in their cell size and shape. These species usually possess 35 thecal plates, and the arrangement is Po, cp, 5', 3a, 7", 6c, 5s, 5"', 2"". This arrangement involves distinctive features of the genus on the position of the canal plate, the first apical plate and the anterior sulcal plate. The canal plate of *Heterocapsa* is small and contact with the apical pore plate, similar to the X plate of *Scrippsiella*. It is located between the first apical plate (1') and the fifth apical plate (5'), viz. the 1' plate also adjacent to the apical pore plate (Po). The 1' plate stops at the middle of the epitheca, then contacts with the anterior sulcal plate (as). These positioning in ventral view have not been reported from the other extant marine dinoflagellates. Therefore, thecal plate arrangement, especially the ventral view is a rather useful character for the generic identification.

Among peridinioid dinoflagellates, number of cingular plate series has been considered as the useful character, which subdivides the Peridiniales into several groups. For example, freshwater *Peridinium* species usually possess five cingular plates, whereas *Protoperidinium* species has only three. On the contrary, six cingular plates are supposed to indicate the affinity among marine peridinioid species such as *Scrippsiella* and *Heterocapsa*. Comparing the typical thecal plate number of the genus *Scrippsiella*, *Heterocapsa* has more or less larger number in the apical and the sulcal series.

In dorsal view, the anterior intercalary plate (2a) is usually seven-sided and its posterior end contacts with three precingulars viz. 3", 4" and 5". The plate 2a sometimes changes to six-sided and the posterior part borders only two precingulars. These plate formula variations are present in many *Heterocapsa* species. As this variation has been reported by several authors (e.g. Hansen 1995, Horiguchi 1995), it can be regarded as a common and natural variation among *Heterocapsa* species. The 2a plate of only one species, *H. triquetra*, differs from the

common arrangement of *Heterocapsa*. The posterior end of the 2a plate usually borders with two precingulars, 4" and 5". It is likely to be a specific variation in this species. The anterior end of the 2a plate of *Heterocapsa* borders with the apical plates 3' and 4'. On the other hand, that of *Scrippsiella* borders with only the 3' plate. It is assumed that the plates 3' and 4' are homologous with the plate 3' of *Scrippsiella*.

Since thecal plate arrangement is quite similar among *Heterocapsa* species, it is suitable to use it for generic delimitation. Some variations on plate arrangement were found in each culture strain in the present study. For example, the las plate of *H. horiguchii* is relatively larger than the lps plate, whereas that of *H. ovata* is smaller than the lps plate. The large 5' plate is sometimes divided into two or three small platelets in the large species such as *H. ovata* and *H. pseudotriquetra*. However, it is quite difficult to determine whether the variation occurred naturally or artificially. This is probably attributed to the employment of culture strains for taxonomic studies on *Heterocapsa*. Thecal plates of many *Heterocapsa* species, e.g. *H. niei* (Stosch 1969), *H. illdefina* (Balech 1977), *H. triquetra* (Morrill & Loeblich III 1981), *H. rotundata* (Hansen 1995), *H. circularisquama* (Horiguchi 1995) and *H. arctica* (Horiguchi 1997), have been determined by using culture strains. Of these, *H. arctica* and *H. illdefina* could be presently obtained only from culture collection. In fact, thecal plates of cultured materials are often variable, and variations of plate number were reported (e.g. Stosch 1969; Balech 1977; Morrill & Loeblich III 1981). Stable specific differences of thecal plate should be observed from natural materials. However, *Heterocapsa* species have been finally identified based on ultrastructural characteristics by use of culture strains. These were frustrated for settlement as specific criterion based on thecal plates.

5-2-2 Cell size and shape

Cell size and shape of *Heterocapsa* species are variable. The cell length ranges from 7.4 μm (*H. minima*) to 45 μm (*H. pacifica*). These size ranges of most species often overlap each other, therefore it is difficult to identify only from their cell sizes.

Cell shape seems to be stable in each species. Many of the species exhibit ellipsoidal or spherical outline in dorsoventral view and it is difficult to discern them under light microscope. However, several species possess their specific features on the shape such as the large epitheca or antapical horn. For these species, cell shape is a significant feature of species identification. For example, *H. triquetra* is rhomboid with antapical horn, *H. arctica* is elongated with the large epitheca, *H. lanceolata* is lanceolate with the large epitheca and antapical hone, and *H. rotundata* is quite small with large epitheca. These four species can be easily identified under light microscope. On the contrary, *H. orientalis*, *H. ovata* and *H. pseudotriquetra* are almost spherical. They can be distinguished from the ellipsoidal species, however, difficult to be distinguished with each other on species level. Other ellipsoidal species are also hard to identify under light microscope.

Morrill *et al.* (1981) and Loeblich III *et al.* (1981) discriminated among *H. pygmaea*, *H. niei* and *H. illdefina* from their cell size and considered them as three independent species. In fact, other characters such as body scale ultrastructure, invaginations in pyrenoid and positions of organelle also agree to this view. But the cell size is incomplete character to identify only from it, because cell sizes sometimes overlap each other. Consequently, it can be referred that the cell size and shape are still a useful character for distinguishing several species but it cannot apply to all.

5-2-3 Nucleus and pyrenoid

Some organelles such as the nucleus, chloroplast and pyrenoid can be observed under high magnification of light microscope. In particular, the pyrenoid is one of the common characters of the genus. Positions of the nucleus and pyrenoid are stable among *Heterocapsa* species, and their differences can be useful for differentiation of some species. Namely, the species in which nucleus was located in the epitheca usually possessed pyrenoid in the hypotheca, and *vice versa* (Fig. 5-1). Using this characteristic combined with its cell shape, several nondescript species such as ellipsoidal and spherical species can be distinguished. For example, *H. orientalis*, *H. ovata* and *H. pseudotriquetra* are spherical and rather similar to each other, but only *H. orientalis* possesses the nucleus in the hypotheca. Among the ellipsoidal species, the nuclei of *H. niei* and *H. pygmaea* are located in the hypotheca, whereas *H. horiguchii* holds its nucleus in the epitheca. Especially the nucleus of *H. niei* is conspicuous in the hypotheca, the species is sometimes identified only by this character. However, the nuclei of *H. circularisquama* and *H. illdefina* are elongated through the cell bodies, therefore the position of these species are difficult to observe. *H. circularisquama* is quite similar to *H. horiguchii* under light microscope, because of the ambiguous organelle position of *H. circularisquama*.

Consequently, about a half of *Heterocapsa* species could be distinguished based on the morphological characters such as cell shape and position of organelles, viz. *H. arctica*, *H. lanceolata*, *H. niei*, *H. orientalis*, *H. rotundata* and *H. triquetra*. Since these characters can be observed under light microscope, they appear to be quite useful characters for identification of *Heterocapsa* species. However, these could not be employed to all species.

5-2-4 Tubular invaginations into pyrenoid matrix

Table 5-1. Comparison of selected characters in *Heterocapsa* species.

species	Length (μm, mean)	Width (μm, mean)	Nucleus shape	Nucleus position	Pyrenoid position	Pyrenoid invaginations	Scale	Strain
H. arctica	22.5-37.5 (29.6)	10.0-15.0 (11.6)	ellipsoid	middle	posterior	present	triangular	CCMP445
H. circularisquama	16.0-26.4 (23.7)	11.2-19.2 (15.5)	ellipsoid	middle	middle	absent	circular	HCHS95
H. illdefina	23.2-35.2 (29.2)	16.8-24.0 (19.8)	ellipsoid	middle	middle	present	triangular	CCMP446
H. niei	18.4-27.2 (21.7)	12.0-19.2 (15.9)	spherical	posterior	anterior	absent	triangular	TG607-1
H. pygmaea	[illegible] (15.0)	[illegible] (10.8)	[illegible]	posterior	[illegible]	absent	circular	CCMP1322
H. rotundata	8.8-12.8 (10.4)	7.2-9.6 (8.5)	ellipsoid	middle	anterior	absent	triangular	TK12-D44
H. triquetra	15.2-26.4 (22.1)	10.4-20.8 (15.0)	spherical	anterior	posterior	present	triangular	TK12-D40
H. lanceolata	16.4-25.0 (18.9)	10.0-15.0 (11.6)	ellipsoid	middle	anterior	absent	hexagonal	TK6-D57
H. horiguchii	13.2-20.8 (17.2)	10.0-13.6 (11.6)	spherical	anterior	posterior	absent	circular	FK6-D47
H. ovata	23.6-33.2 (26.9)	18.4-28.0 (21.4)	spherical	anterior	posterior	present	triangular	KZHt-1
H. pseudotriquetra	18.4-27.2 (22.2)	14.4-21.6 (17.2)	spherical	anterior	posterior	present	triangular	NIES 473
H. orientalis	18.4-34.4 (25.7)	16.0-24.0 (19.6)	spherical	posterior	anterior	present	triangular	D-127-C-1

(Continued)

Table 5-1.

species	Length (µm, mean)	Width (µm, mean)	Nucleus shape	Nucleus position	Pyrenoid position	Pyrenoid invaginations	Scale	Reference
H. minima	7.4-10.0	5.4-6.8		posterior				Pomroy (1989)
H. pacifica	45	30		middle ?				Kofoid (1907)

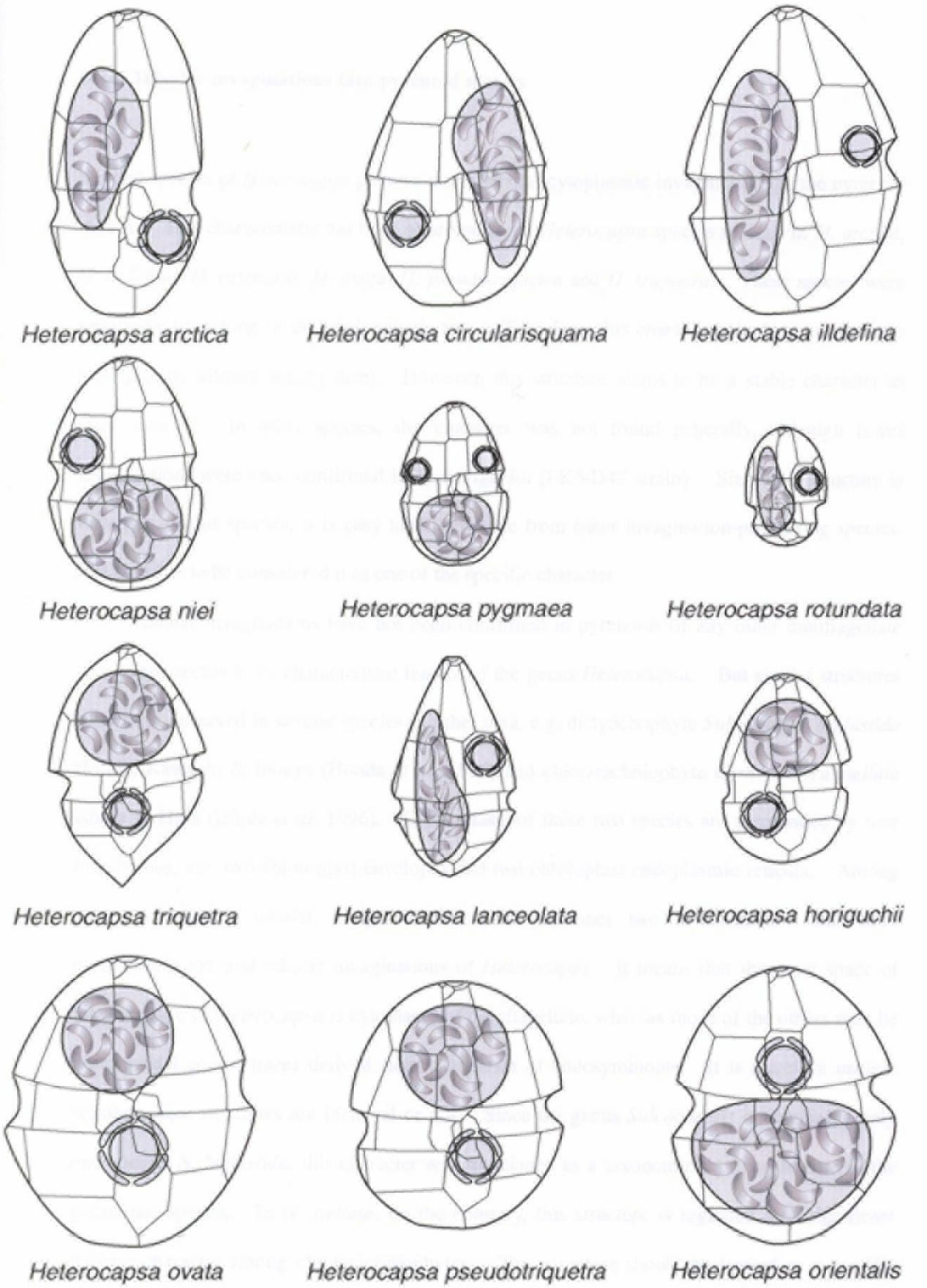

Figure 5-1. Schematic drawings of cell size, shape and position of nucleus and pyrenoid in *Heterocapsa* species.

5-2-4 Tubular invaginations into pyrenoid matrix

Several species of *Heterocapsa* possess many tubular cytoplasmic invaginations in the pyrenoid matrix. This characteristic has been observed in six *Heterocapsa* species so far, viz. *H. arctica*, *H. illdefina*, *H. orientalis*, *H. ovata*, *H. pseudotriquetra* and *H. triquetra*. These species were separately branching in the phylogenetic tree. Therefore, this characteristic does not indicate phylogenetic affinity among them. However, this structure seems to be a stable character in these species. In other species, the character was not found generally, although fewer invaginations were once confirmed in *H. horiguchii* (FK6-D47 strain). Since this structure is very rare in this species, it is easy to differentiate from other invagination-possessing species. It is possible to be considered it as one of the specific character.

Tubular invaginations have not been confirmed in pyrenoids of any other dinoflagellate and it thus seems to be characteristic feature of the genus *Heterocapsa*. But similar structures have been observed in several species of other taxa, e.g. dictyochophyte *Sulcochrysis biplastida* Honda, Kawachi & Inouye (Honda *et al.* 1995) and chlorarachniophyte *Gymnochlora stellata* Ishida & Hara (Ishida *et al.* 1996). Chloroplasts of these two species are surrounded by four membranes, viz. two chloroplast envelopes and two chloroplast endoplasmic reticula. Among these membranes, tubular invaginations consist of inner two membranes. But three membranes surround tubular invaginations of *Heterocapsa*. It means that the inner space of this structure of *Heterocapsa* is cytoplasm of dinoflagellate, whereas those of the others may be periplastidal compartment derived from cytoplasm of endosymbionts. It is therefore unclear whether these structures are identical or not. Since the genus *Sulcochrysis* is containing only one species *S. biplastida*, this character was mentioned as a taxonomic criterion neither in the genus nor species. In *G. stellata*, on the contrary, this structure is regarded as a significant generic character among chlorarachniophytes. This structure should be treated as a specific criterion in the genus *Heterocapsa*.

5-2-5 Body scale

Extracellular body scales are found in all *Heterocapsa* species. Each *Heterocapsa* scale superficially resembles each other with regard to having three-dimensional and triradiate structure. Body scales are well known in several groups of phytoflagellates, such as haptophytes and prasinophytes, but it is quite rare in dinoflagellates. Scales are known in only two unarmored species of two genera, *Lepidodinium viride* Watanabe *et al.* (Watanabe *et al.* 1987; Watanabe *et al.* 1990) and *Oxyrrhis marina* Dujardin (Clarke & Pennick 1972; 1976). The body scale of the former species, *L. viride*, consists of a square basal plate and ornamentations on it and exhibiting basket shape. The latter species bears two types of scale, i.e. rounded body scale and ellipsoidal flagellar scale, both of which are constructed only by the basal plate. Body scales of these two species are easily distinguished from those of *Heterocapsa* species. Moreover, cell body of thecate dinoflagellate *Peridiniella catenata* (Levander) Balech is surrounded by scale-like layer, but the layer does not possess complex structure (Hansen & Moestrup 1998). Hansen & Moestrup (1998) presumed it as a glycocalyx layer. This scale is also easily discerned from *Heterocapsa* body scale.

Body scales of haptophytes, e.g. *Chrysochromulina*, *Isochrysis* and *Prymnesium*, are relatively simpler than *Heterocapsa* scale, it basically consists of only discoid plate, or discoid scale and another scale consisting of basal plate and some spines. Many prasinophytes possess body scales and flagellar scales. The genus *Pyramimonas* retains five kinds of scales viz. two flagellar scales and three body scales. However, basic structures of these scales are usually square, pentagonal or limuloid, and they are thus different from triradiate scale of *Heterocapsa*. Therefore the triradiate scale with three-dimensional ornamentation can be regarded significant characteristic of the genus *Heterocapsa* among phytoplankton.

Although the body scales of *Heterocapsa* have morphological similarity, the body scale of each species can be distinguished by detailed comparison using shape of the basal plate, number

of the uprights, bars, spines and holes. Diagrammatic illustrations of body scale structures are given in Figure 5-2. Although specific identifications of *Heterocapsa* species cannot be always completed by using cell morphology such as cell size, shape, position of organelles, and tubular invaginations in pyrenoid matrix, the body scale are characteristic in almost all species. When the body scale ultrastructure of *H. niei* was first investigated, Morrill & Loeblich III (1983) mentioned that the species specific differences of body scale found in *H. triquetra* and *H. niei* might be also present in the other *Heterocapsa* scales e.g. *H. illdefina* and *H. pygmaea.* Their statement is clearly demonstrated in the present study.

Most *Heterocapsa* species possessed only one kind of scales, but two different structures were found in *H. arctica* and *H. circularisquama*. *H. arctica* had simpler scale consisting of basal plate and spines, and the other scale possessing radiate and peripheral bars in addition to the component of simpler one. Both scales were located on the cell surface of the same culture strain. Since both of them had same outline of basal plate and same number of spines and were observed from same position, it may be immature and mature scale. Spines of the simpler scale are thin, and therefore it seems to be immature one.

Two kinds of body scales were also found in *H. circularisquama*. Most of body scales fit to the original description (Horiguchi 1995), but another type of scales with slender structure were observed. This scale was less in number than the normal scale, but it was found in all specimens of *H. circularisquama*, and may be immature scale like that of *H. arctica*. Based on this finding, body scale development can be presumed. Probably it may also imply evolutional direction of *Heterocapsa* body scale. For example in *H. arctica*, immature scale possessed spines without horizontal bars and uprights. It indicates that the spines developed at first, and horizontal bars viz. peripheral and radiating bars subsequently followed. Each radiating bar is made by each radiating ridge, and peripheral bars are derived from rim of basal plate. They are made up by development of uprights. In *H. circularisquama*, radiating ridges of immature scale do not reach the rim of basal plate, similar to that of *H. horiguchii*, and each spine is

Table 5-2. Characters of body scale ultrastructure in *Heterocapsa* species examined in the present study.

species	Diameter (nm)	Outline of basal plate	Texture of basal plate	Ridges on basal plate	Central hole	Spines on basal plate	Central uprights (or spines)	Peripheral uprights (or spines)	Peripheral bars	Strain
H. arctica *	350	triangular	fibrous	6	0	0	1	12	6	CCMP 445
H. circularisquama *	400	circular	reticulate	6	0	0	1	6	6	HCHS95
H. illdefina	430	triangular	fibrous	6	0	0	1	9	0	CCMP 446
H. niei *	300	triangular	fibrous	3	0	3	1	15	18	NIES 420
H. pygmaea	400	circular	fibrous	6	0	0	1	9 - 12	0	CCMP 1490
H. rotundata	350	triangular	reticulate	6	1	0	1	9	2	TK12-D44
H. triquetra	250	triangular	reticulate	6	0	0	1	9	12	TK12-D40
H. lanceolata	500	hexagonal	fibrous	3	1	0	1	9	12	TK6-D57
H. horiguchii	310	circular	reticulate	6	0	0	1	6	0	FK6-D47
H. ovata	220	triangular	reticulate	6	0	0	1	6	9	KZHt-1
H. pseudotriquetra	240	triangular	reticulate	6	0	0	1	9	12	NIES 473
H. orientalis	300	triangular	reticulate	6	0	0	1	9	12	D-127-C-3

* results on mature scale.

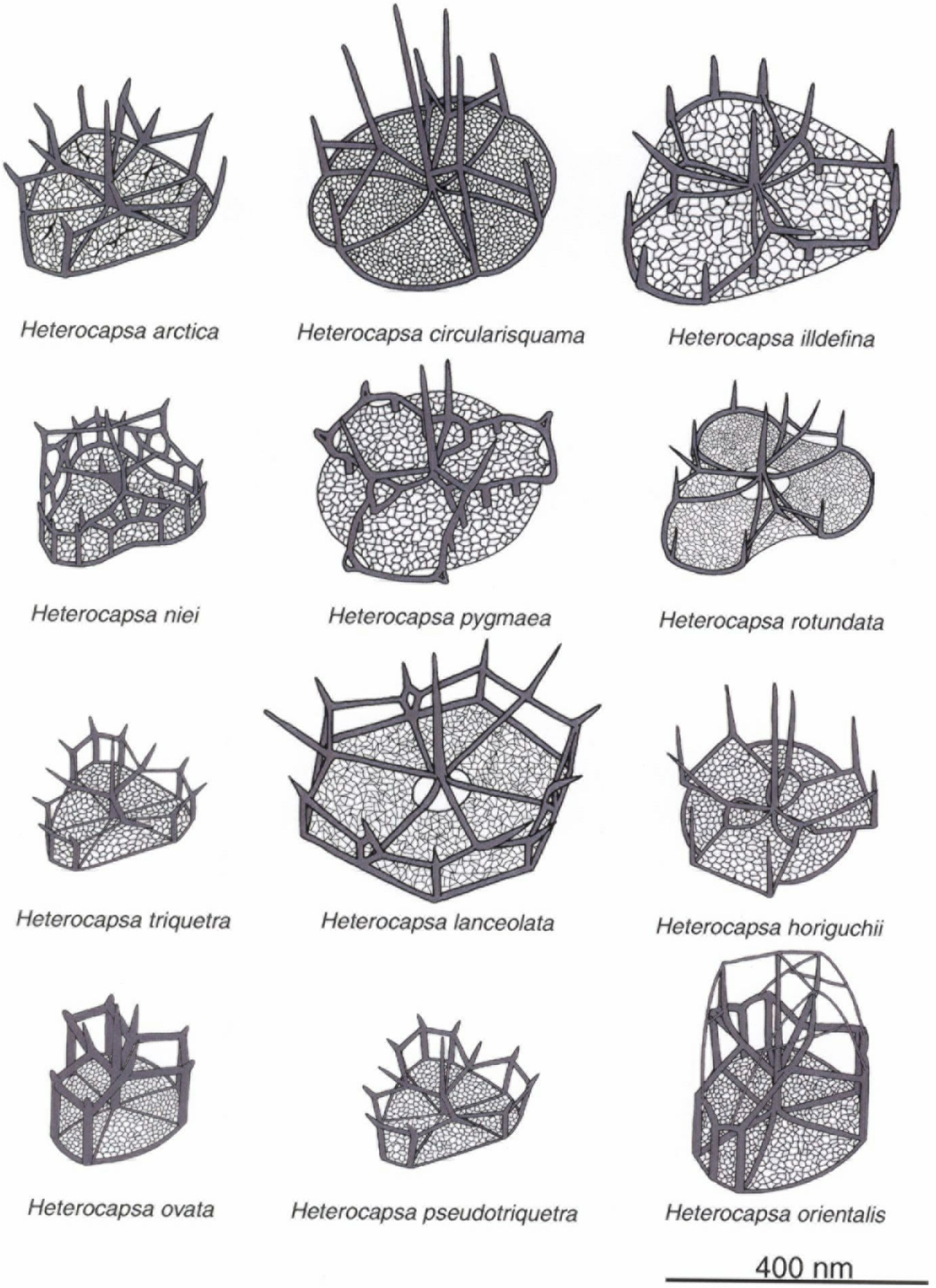

Figure 5-2. Diagrammatic illustrations of body scale fine structure in *Heterocapsa* species.

relatively short.

Textures of basal plate are slightly different among species. Those of *H. circularisquama*, *H. pygmaea*, *H. orientalis*, *H. ovata*, *H. pseudotriquetra* and *H. triquetra* are fine reticulation, whereas it is fine fibrous texture in *H. lanceolata*. The texture of *H. arctica* is relatively fine, but its thickness seems irregular. This texture appeared to be stable in each culture, but it was sometimes different in same species. The clear differences were found among strains of *H. horiguchii* and *H. rotundata*. In *H. horiguchii*, NIES614 strain (Kashiwazaki, Japan) has quite rough texture of basal plate, while that of others is fine. Texture of each culture is rather steady, therefore they were once treated as two separate species *Heterocapsa* sp. 4 and sp. 2, respectively. Two strains of *H. rotundata*, K-479 and K-480 strains (Denmark), also possess body scales with rough texture, but that of other cultures collected in Japanese coastal waters have fine one. Both of the former strains possessing body scale with rough texture are old cultures maintained more than 10 years in the laboratory, while the latter strains were collected in recent years. Differences on cellular morphology and another body scale structure are almost identical in these cultures. Moreover, the ITS region molecular sequences of each *H. horiguchii* are almost equal. This indicates that the texture of basal plates are insecure, especially in the old culture strains, hence it is an unreliable character for specific criterion.

Although ultrastructure of body scale is the most useful specific criterion, yet it has one exception. *H. triquetra* and *H. pseudotriquetra* have a body scale composed of the almost identical constituent. Cell shape of these species is rather different and easily discerned, therefore they are different species evidently. The similarity of body scales apparently implies affinity among them, and this view was also supported by the molecular phylogeny. Their relationship between cellular and scale morphology indicates structural stability of body scale through their evolution. The body scale ultrastructure is a relatively conservative character sufficient for a specific criterion of the genus.

5-3 Conclusions

Twelve *Heterocapsa* species including seven described and five undescribed species could be investigated in the present study. From morphological and molecular characteristics, it is obvious that *Heterocapsa* species are well allied in dinoflagellates. Five new species are described as new *Heterocapsa* species, *H. lanceolata*, *H. horiguchii*, *H. ovata*, *H. pseudotriquetra* and *H. orientalis*, although new species status have not been given in this thesis in order to avoid taxonomic confusion in original description and to follow the recommendation of the International Code of Botanical Nomenclature. Among the five new ones, cells of three species *H. ovata*, *H. pseudotriquetra* and *H. orientalis* are ovoid or spherical; and dissimilar to other previously described *Heterocapsa* species. Probably these species have not been identified as *Heterocapsa* but considered so far as some other thecate dinoflagellate group such as *Glenodinium*. A similar species to *Heterocapsa* was indeed reported under the name of *Glenodinium* sp. from Nagasaki Harbor (Uchida 1975), and Loeblich III *et al.* (1981) presumed it to be *Heterocapsa* species. Unfortunately, preserved samples for reinvestigation of body scales are not available any more. This may be the reason why the ovoid *Heterocapsa* species had not been reported until now. All new *Heterocapsa* species were collected in Japanese coastal waters, therefore, it is thus possible that other undescribed *Heterocapsa* species may be still present in other areas.

For the taxonomy of *Heterocapsa*, several characters have been employed as specific and generic criteria. The present study evaluated their characters and revealed that 1) the number and arrangement of thecal plates are common among *Heterocapsa* species (except for *H. triquetra* in its arrangement), and they are useful for delimiting the genus *Heterocapsa*, however, not useful for the specific identification, 2) cell shapes, positions of nucleus and pyrenoid, and tubular invaginations into the pyrenoid matrix are suitable characters for species differentiation, and 3) possession of the triradiate body scales is a generic significant character, and their fine

structures are the most reliable character for classification of *Heterocapsa* species.

The phylogenetic position of *Heterocapsa* in dinoflagellates and systematic relationships among each *Heterocapsa* species were also considered using SSU rRNA and ITS region sequence data. These molecular data also indicated monophyly of *Heterocapsa* as a natural group. They also revealed several lineages of *Heterocapsa* species, by which some evolutional relationships of each morphological character were supposed. The problem remained in the present study is taxonomic position of the genus in dinoflagellates. Based on molecular data, the genus *Heterocapsa* is included in *Gymnodinium* / *Peridinium* / *Prorocentrum* complex and far from the 'real' Peridiniales including type species of the order. To clarify this phylogenetic relationship and settle a new systematics, it is requisite to elucidate each lineage in GPP complex.

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PLATES

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Plate 1 – 12. Light and fluorescence microscopy.
Plate 13 – 23. TEM of cell ultrastructure.
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Abbreviations

Thecal plates.

Po, apical pore plate; cp, canal plate; 1'-5', first-fifth apical plate; 1a-3a, first-third anterior intercalary plate; 1"-7", first-seventh precingular plate; 1c-6c, first-sixth cingular plate; as, anterior sulcal plate; rs, right sulcal plate; las, left anterior sulcal plate; lps, left posterior sulcal plate; ps, posterior sulcal plate; 1"'-5"', first-fifth post cingular plate; 1""-2"", first-second antapical plate.

Light and electron microscopy.

BB, basal body; BS, body scale; Ch, chloroplast; G, Golgi; L, lipid globule; M, mitochondrion; N, nucleus; Pu, pusule; Py, pyrenoid; S, starch grain; T, trichocyst.

Plate 1. *Heterocapsa arctica* (CCMP445 strain).

1. Light micrographs of living cell in ventral view.

2. Optical section of cell showing a pyrenoid in the upper part of hypotheca and an elongate nucleus in the epitheca.

3. Temporary cyst.

4-8. Fluorescence microscopy with calcofluor staining in ventral view.

9-16. Dorsal and lateral view, seven-sided 2a (9, 10) and six-sided 2a (11-13).

Plate 2. *Heterocapsa circularisquama* (HCHS95 strain).

1, 2. Light micrographs of living cell in ventral view.

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5. Temporary cyst.

6. Scanning electron microscopy showing body scales on the cell surface.

7-10. Fluorescence microscopy with calcofluor staining in ventral view.

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Plate 3. *Heterocapsa illdefina* (CCMP446 strain).

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Plate 4. *Heterocapsa niei* (1-3, CCMP447; 4, NIES420; 5-16, TG607-1 strains).

1. Light micrographs of living cell in ventral view.

2, 3. Optical sections of cell, showing a pyrenoid in the epitheca and a spherical nucleus in the hypotheca.

4. Scanning electron microscopy showing body scales on the cell surface.

5-8. Fluorescence microscopy with calcofluor staining in ventral view.

9-16. Dorsal and lateral view, seven-sided 2a (10, 11) and six-sided 2a (11-13, 15) were found.

Plate 5. *Heterocapsa pygmaea* (CCMP1322 strain).

1, 2. Light micrographs of living cell in ventral view.

3-5. Optical sections of cell showing pyrenoids in the epitheca and a spherical nucleus in the hypotheca.

6. Spherical temporary cyst.

7-10. Fluorescence microscopy with calcofluor staining in ventral view.

11-16. Dorsal and lateral view, seven-sided (11-13) and six-sided (14) 2a are visible.

Plate 6. *Heterocapsa rotundata* (TK12-D40 strain).

1. Light micrographs of living cell in ventral view.

2-3. Optical sections of cell showing a pyrenoid in the epitheca and an elongate nucleus throughout the epitheca to the hypotheca.

4. Scanning electron microscopy in dorsal view.

5-8. Fluorescence microscopy with calcofluor staining in ventral view.

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1, 2. Light micrographs of living cell in ventral view.

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5. Temporary cyst.

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Plate 8. *Heterocapsa lanceolata* mss. (TK6-D57 strain)

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3. Scanning electron microscopy showing body scales surround whole of the cell surface.

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8, 9. Lateral view.

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1. Light micrographs of living cell in ventral view.

2. Optical sections of cell showing a pyrenoid in the hypotheca and a spherical nucleus in the central epitheca.

3. Temporary cysts.

4-5. Fluorescence microscopy with calcofluor staining in ventral view.

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9-12. Dorsal, view, seven-sided 2a (9, 10) and six-sided 2a (11, 12).

13, 14. Lateral view.

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Plate 13. *Heterocapsa arctica* (CCMP445 strain).

1, 2. Transmission electron micrographs of cell in thin sections, showing a pyrenoid with many tubular invaginations in the matrix. Several lipid globules were found in apical and antapical part of the cell.

3. TEM of a thick section.

4. Thin section with an elongated nucleus.

Plate 14. *Heterocapsa circularisquama* (HCHS95 strain).

1, 2. Transmission electron microscopy, tubular invaginations were not found in the pyrenoid matrix (2).

Plate 15. *Heterocapsa illdefina* (CCMP446 strain)

1. Transmission electron microscopy, a pyrenoid and an elongated nucleus are laterally located.

2. Many tubular invaginations are present in the pyrenoid matrix.

Plate 16. *Heterocapsa niei* (TG607-1 strain).

1. Transmission electron microscopy of thin section showing a pyrenoid in the epitheca and a nucleus in the hypotheca.

2. The pyrenoid has a connection with the chloroplast, tubular invaginations are absent in the pyrenoid matrix.

Plate 17. *Heterocapsa pygmaea* (CCMP1322 strain).

1. Transmission electron microscopy of thin section, several pyrenoids are laterally located.

2. Pyrenoids without tubular invaginations are surrounded by several starch grains and possess a stalk connecting with chloroplast.

Plate 18. *Heterocapsa triquetra* (TK12-D40 strain).

1. TEM of a cell showing a pyrenoid in the hypotheca and a spherical nucleus in the epitheca. Several lipid globules are situated at the apical part of the cell. Many tubular invaginations are present in the pyrenoid matrix.

Plate 19. *Heterocapsa lanceolata* mss. (TK6-D57 strain)

1. Transmission electron microscopy of cell, an elongated nucleus are laterally located.

2. Pyrenoid is laterally located and possesses a stalk, which sometimes elongated. Tubular invaginations were not found.

Plate 20. *Heterocapsa horiguchii* mss. (FK12-D47 strain)

1. Transmission electron microscopy of longitudinal section showing a pyrenoid in the hypotheca and a spherical nucleus in the epitheca.

2. Chloroplast with three appressed thylakoid.

3. Pusules are situated nearby basal body and include many membranous body.. Showing mitochondria with tubular cristae.

4. Pyrenoid without tubular invaginations connects with several lobes of chloroplast. Note body scales beside Golgi.

Plate 21. *Heterocapsa ovata* mss. (KZHt-1 strain)

1. TEM of longitudinal section with a pyrenoid in the upper part of hypotheca and a spherical nucleus in the epitheca. Thin lobes of chloroplast are peripherally located.

2. Chloroplast containing three appressed thylakoids.

2. Many tubular invaginations are present in the pyrenoid matrix.

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1. Transmission electron microscopy of thick section showing a pyrenoid with many tubular invaginations in the matrix.

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1. TEM of longitudinal section, a spherical pyrenoid and a large nucleus are located in the epitheca and the hypotheca, respectively.

2. Many tubular invaginations are present in the pyrenoid matrix.

Plate 24. *Heterocapsa arctica* (CCMP445 strain).

1-5. Transmission electron microscopy of body scales in whole mount preparations. Note immature scale (1) and mature scale (2).

2. Thick sections of body scale.

Plate 25. *Heterocapsa circularisquama*

(1-8, HCHS95; 9-10, OK-1; 11-13, TN830 strain).

1, 2. Transmission electron microscopy of body scales in whole mount preparations. Note mature scale (1) and immature scale (2).

3-8. Thick sections of body scale.

9-13. Whole mounts.

Plate 26. *Heterocapsa circularisquama.*

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1-7. Transmission electron microscopy of body scales in whole mount preparations.

Plate 27. *Heterocapsa illdefina* (CCMP446 strain).

1-5. Transmission electron microscopy of body scales in whole mount preparations.

6-8. Thick sections of body scale.

Plate 28. *Heterocapsa niei* (NIES420 strain).

1-4. Transmission electron microscopy of body scales in whole mount preparations.

5. SEM of cell surface.

6-9. Thick sections of body scale.

Plate 29. *Heterocapsa niei* (CCMP447 strain)

1-3. Transmission electron microscopy of body scales in whole mount preparations.

Plate 30. *Heterocapsa pygmaea* (1-6, CCMP1322; 7-10, CCMP1490 strains)

1, 2. Transmission electron microscopy of body scales in whole mount preparations.

3-6. Thick sections of body scale.

7-10. Whole mounts.

Plate 31. *Heterocapsa pygmaea* (1-2, Florida; 3-5, Mersin samples; 6-8, AK23 strain).

1-8. Transmission electron microscopy of body scales in whole mount preparations.

Plate 32. *Heterocapsa rotundata* (1-3, TK12-D44; 4-5, DFLS0102; 6, Konagai; 7-9, K-483A strains).

1-9. Transmission electron microscopy of body scales in whole mount preparations.

Plate 33. *Heterocapsa triquetra* (1-9, TK12-D40; 10-12, PLY169 strains)

1-6. Transmission electron microscopy of body scales in whole mount preparations.

7-9. Thick sections of body scale.

10-12. Whole mounts.

Plate 34. *Heterocapsa lanceolata* mss. (1-7, TK6-D57; 8-12, HR7-D64 strain)

1-5. Transmission electron microscopy of body scales in whole mount preparations.

6-7. Thick sections of body scale.

8-12. Whole mounts.

Plate 35. *Heterocapsa horiguchii* mss. (1-6, FK6-D47; 7-11, HCL99706-2; NIES614 strains)

1, 2. Transmission electron microscopy of body scales in whole mount preparations.

3-6. Thick sections of body scale.

7-14. Whole mounts.

Plate 36. *Heterocapsa ovata* mss. (NIES472 strain)

1. SEM of cell surface. Many body scales surround the cell body.

2. Transmission electron microscopy of body scales in thick section.

3-10. Whole mounts.

Plate 37. *Heterocapsa pseudotriquetra* mss. (1-3, NIES473; 4-8, CCMP451 strains)

1-8. Transmission electron microscopy of body scales in whole mount preparations.

Plate 38. *Heterocapsa orientalis* mss. (D-127-C-1)

1-3. Transmission electron microscopy of body scales in whole mount preparations.

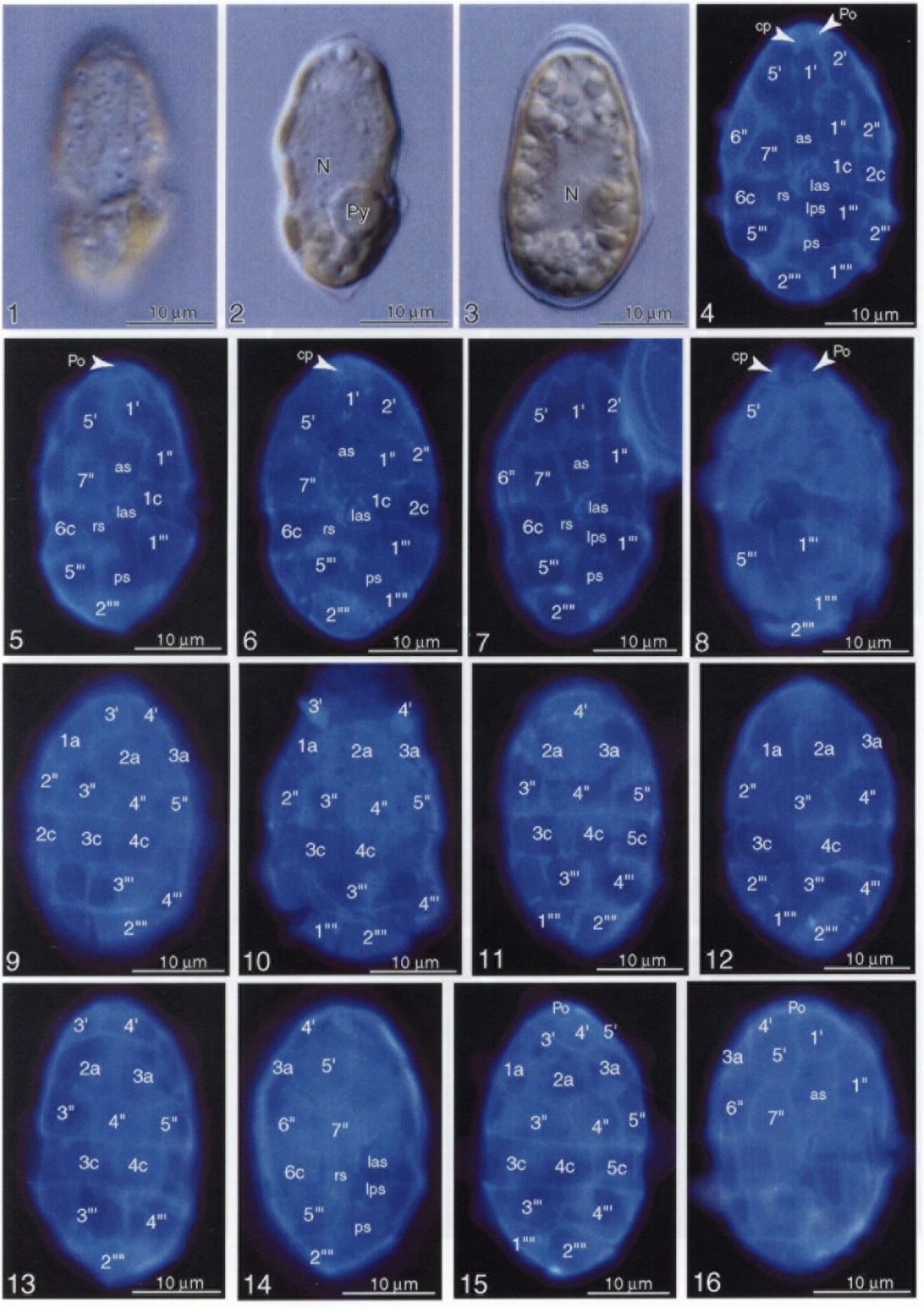
4-7. Transverse serial sections of body scale.

8-13. Longitudinal thick sections.

Plate 39. *Heterocapsa orientalis* mss. (1-4, D-87-B-2; 5-8, D-8-A-2 strain)

1-8. Transmission electron microscopy of body scales in whole mount preparations.

Plate 1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10 µm
2
N
Py
10 µm
3
N
10 µm
4
cp
Po
5'
1'
2'
6"
7"
as
1"
2"
1c
2c
6c
rs
las
lps
1'''
5'''
ps
2'''
2''''
1''''
10 µm
5
Po
5'
1'
7"
as
1"
6c
rs
las
1c
1'''
5'''
ps
2''''
10 µm
6
cp
5'
1'
2'
as
1"
2"
7"
las
1c
2c
6c
rs
1'''
5'''
ps
2''''
1''''
10 µm
7
5'
1'
2'
6"
7"
as
1"
las
6c
rs
lps
1'''
5'''
ps
2''''
10 µm
8
cp
Po
5'
5'''
1'''
1''''
2''''
10 µm
9
3'
4'
1a
2a
3a
2"
3"
4"
5"
2c
3c
4c
3'''
4'''
2''''
10 µm
10
3'
4'
1a
2a
3a
2"
3"
4"
5"
3c
4c
3'''
4'''
1''''
2''''
10 µm
11
4'
2a
3a
3"
4"
5"
3c
4c
5c
3'''
4'''
1''''
2''''
10 µm
12
1a
2a
3a
2"
3"
4"
3c
4c
2'''
3'''
4'''
1''''
2''''
10 µm
13
3'
4'
2a
3a
3"
4"
5"
3c
4c
3'''
4'''
2''''
10 µm
14
4'
3a
5'
6"
7"
las
6c
rs
lps
5'''
ps
2''''
10 µm
15
Po
3'
4'
5'
1a
2a
3a
3"
4"
5"
3c
4c
5c
3'''
4'''
1''''
2''''
10 µm
16
Po
4'
1'
3a
5'
as
1"
6"
7"
10 µm

Plate 2

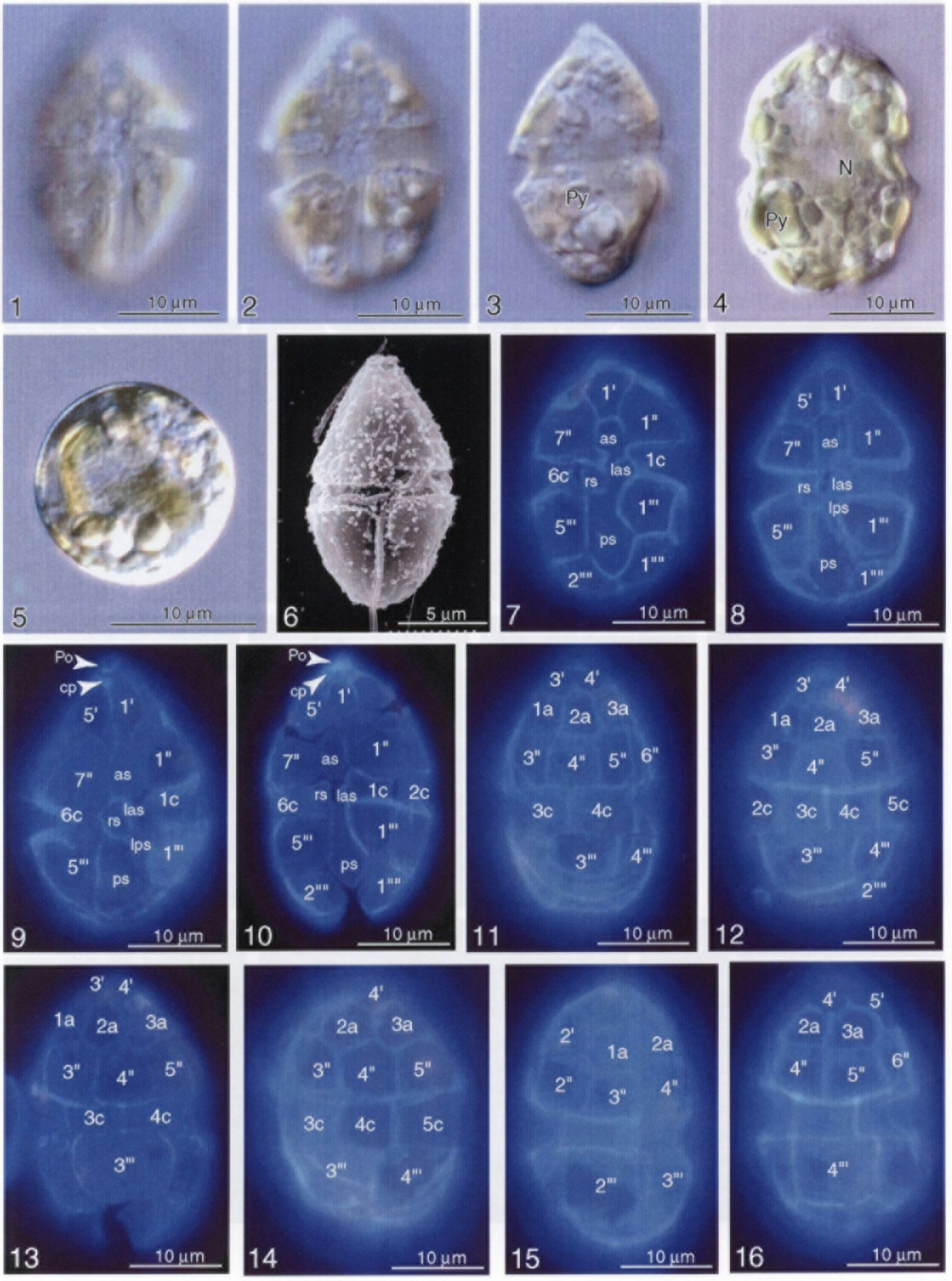

1
10 µm
2
10 µm
3
Py
10 µm
4
N
Py
10 µm
5
10 µm
6
5 µm
7
1'
1"
7"
as
6c
rs
las
1c
1'''
5'''
ps
1''''
2''''
10 µm
8
5'
1'
7"
as
1"
rs
las
lps
5'''
1'''
ps
1''''
10 µm
9
Po
cp
5'
1'
1"
7"
as
6c
rs
las
1c
lps
1'''
5'''
ps
10 µm
10
Po
cp
5'
1'
7"
as
1"
6c
rs
las
1c
2c
1'''
5'''
ps
2''''
1''''
10 µm
11
3'
4'
1a
2a
3a
3"
4"
5"
6"
3c
4c
3'''
4'''
10 µm
12
3'
4'
1a
2a
3a
3"
4"
5"
2c
3c
4c
5c
3'''
4'''
2''''
10 µm
13
3'
4'
1a
2a
3a
3"
4"
5"
3c
4c
3'''
10 µm
14
4'
2a
3a
3"
4"
5"
3c
4c
5c
3'''
4'''
10 µm
15
2'
1a
2a
2"
3"
4"
2'''
3'''
10 µm
16
4'
5'
2a
3a
4"
5"
6"
4'''
10 µm

Plate 3

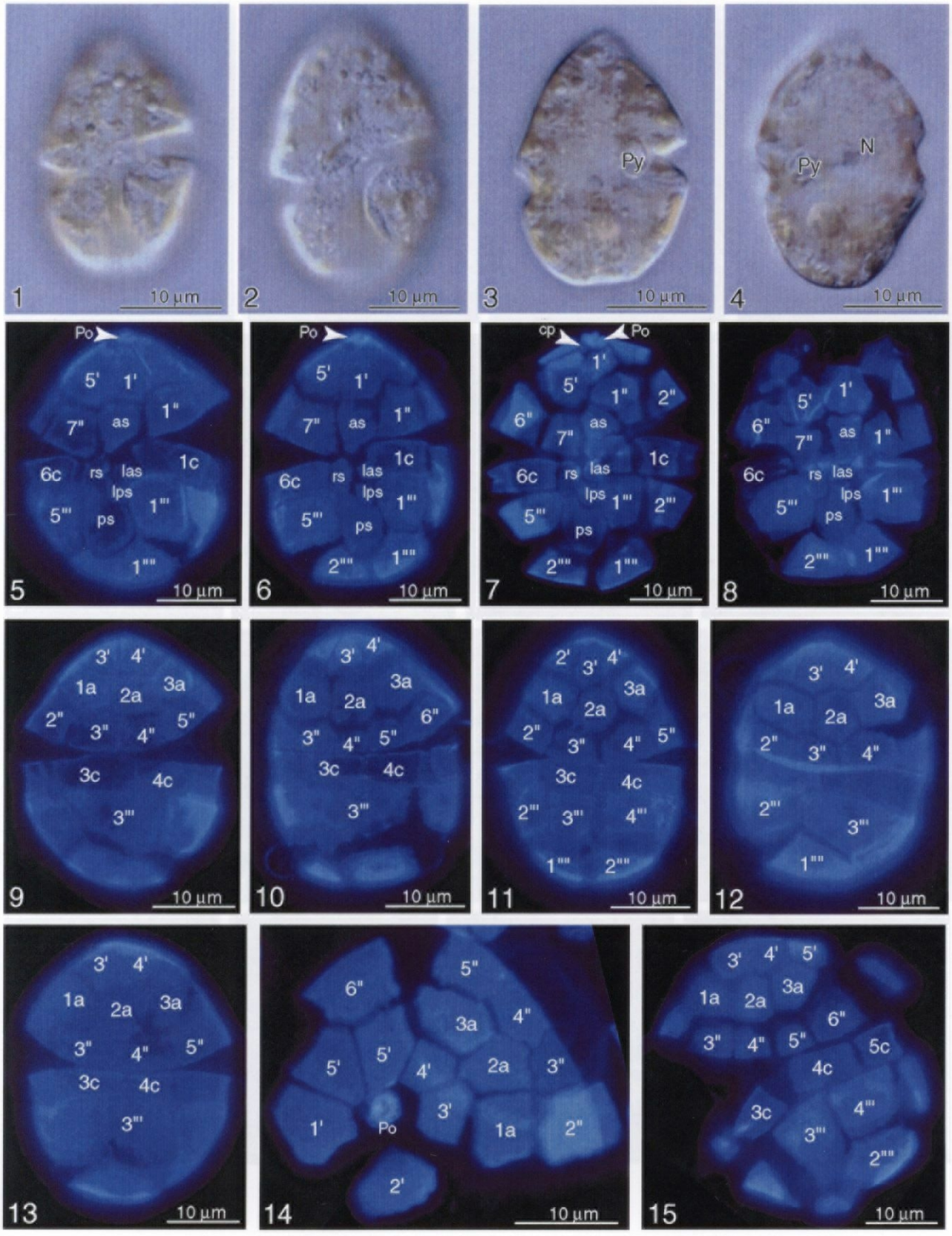
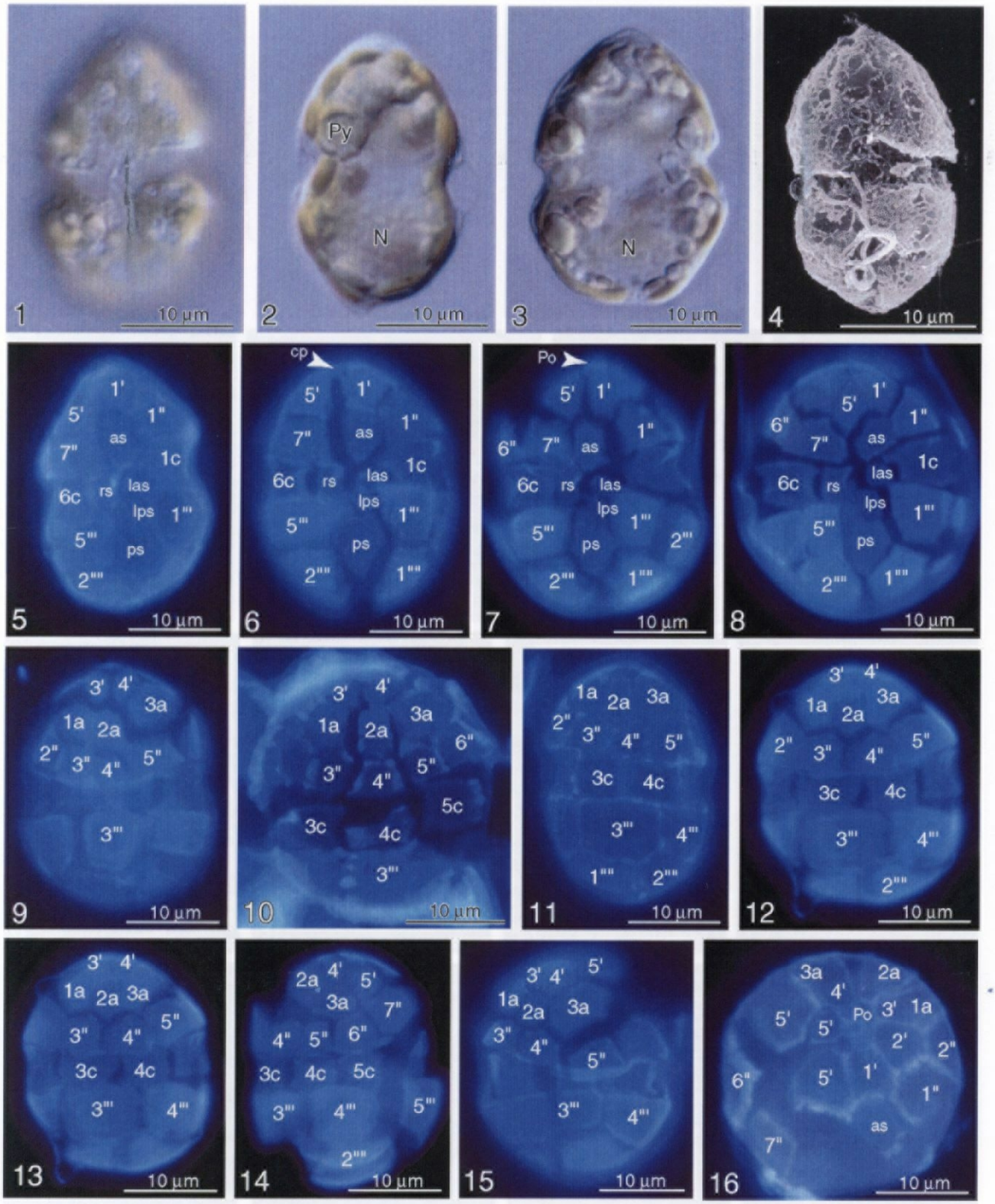

1
10 µm
2
10 µm
3
Py
10 µm
4
Py
N
10 µm
5
Po
5' 1'
7" as 1"
6c rs las 1c
lps
5''' ps 1'''
1''''
10 µm
6
Po
5' 1'
7" as 1"
6c rs las 1c
lps
5''' ps 1'''
2'''' 1''''
10 µm
7
cp
Po
1'
5' 1" 2"
6" 7" as
6c rs las 1c
lps 1''' 2'''
5'''
ps
2'''' 1''''
10 µm
8
5' 1'
6" 7" as 1"
6c rs las
lps 1'''
5''' ps
2'''' 1''''
10 µm
9
3' 4'
1a 2a 3a
2" 3" 4" 5"
3c 4c
3'''
10 µm
10
3' 4'
1a 2a 3a 6"
3" 4" 5"
3c 4c
3'''
10 µm
11
2' 3' 4'
1a 2a 3a
2" 3" 4" 5"
3c 4c
2''' 3'' 4''
1'''' 2''''
10 µm
12
3' 4'
1a 2a 3a
2" 3" 4"
2''
3'''
1''''
10 µm
13
3' 4'
1a 2a 3a
3" 4" 5"
3c 4c
3'''
10 µm
14
5"
6"
3a 4"
5' 5' 4' 2a 3"
3'
1' Po 1a 2"
2'
10 µm
15
3' 4' 5'
1a 2a 3a
6"
3" 4" 5" 5c
4c
3c 4'''
3'''
2''''
10 µm

Plate 4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10 µm
2
Py
N
10 µm
3
N
10 µm
4
10 µm
5
1'
5'
1"
as
7"
1c
6c
rs
las
lps
1'''
5'''
ps
2''''
10 µm
6
cp
5'
1'
7"
as
1"
6c
rs
las
1c
lps
5'''
1'''
ps
2''''
1''''
10 µm
7
Po
5'
1'
1"
6"
7"
as
6c
rs
las
lps
1'''
5'''
ps
2'''
2''''
1''''
10 µm
8
5'
1'
6"
1"
7"
as
1c
6c
rs
las
lps
1'''
5'''
ps
2''''
1''''
10 µm
9
3'
4'
3a
1a
2a
2"
3"
4"
5"
3'''
10 µm
10
3'
4'
3a
1a
2a
6"
3"
5"
4"
5c
3c
4c
3'''
10 µm
11
1a
2a
3a
2"
3"
4"
5"
3c
4c
3'''
4'''
1''''
2''''
10 µm
12
3'
4'
1a
2a
3a
2"
3"
4"
5"
3c
4c
3'''
4'''
2''''
10 µm
13
3'
4'
1a
2a
3a
5"
3"
4"
3c
4c
3'''
4'''
10 µm
14
2a
4'
5'
3a
7"
4"
5"
6"
3c
4c
5c
3'''
4'''
5'''
2''''
10 µm
15
3'
4'
5'
1a
2a
3a
3"
4"
5"
3'''
4'''
10 µm
16
3a
2a
4'
5'
5'
Po
3'
1a
2'
2"
6"
5'
1'
1"
as
7"
10 µm

Plate 5

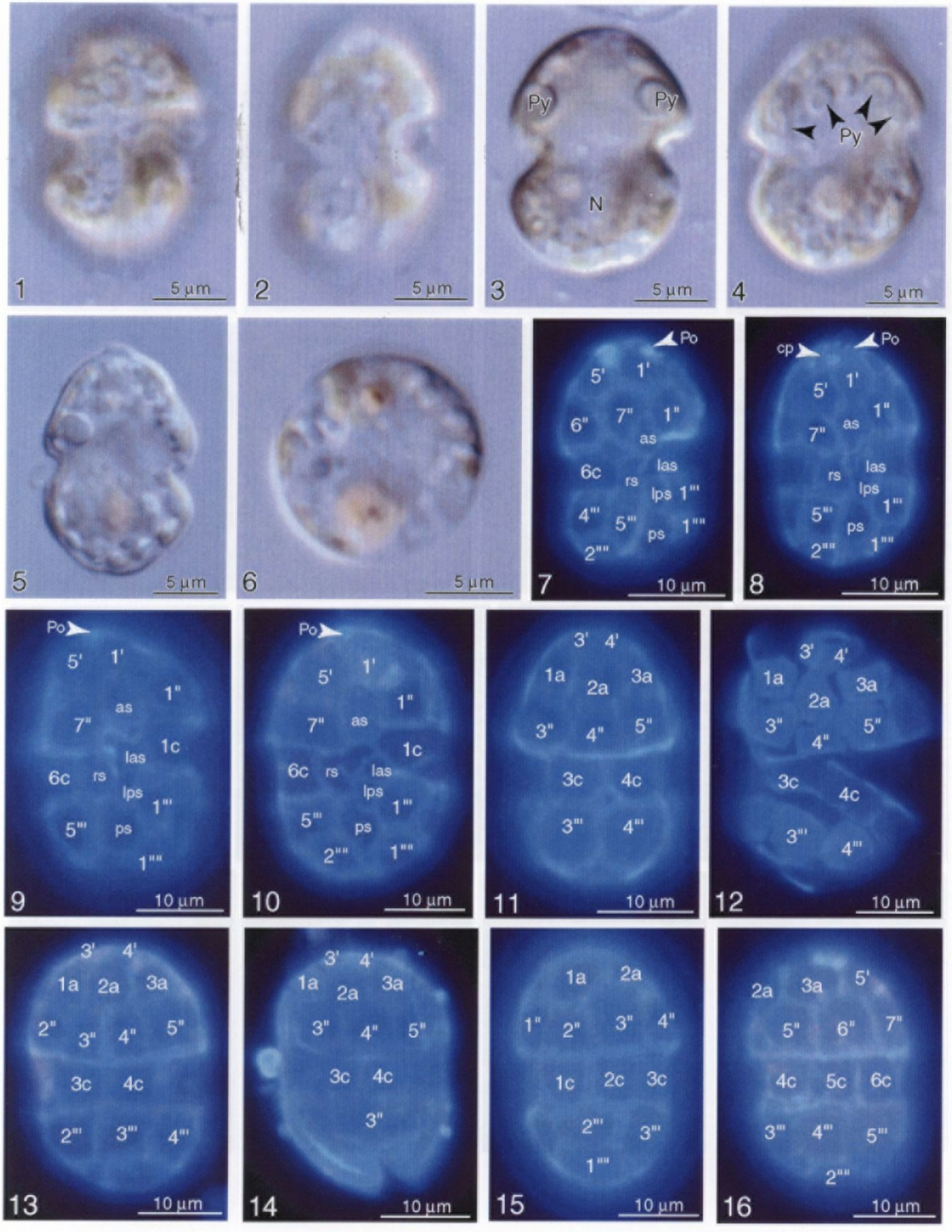
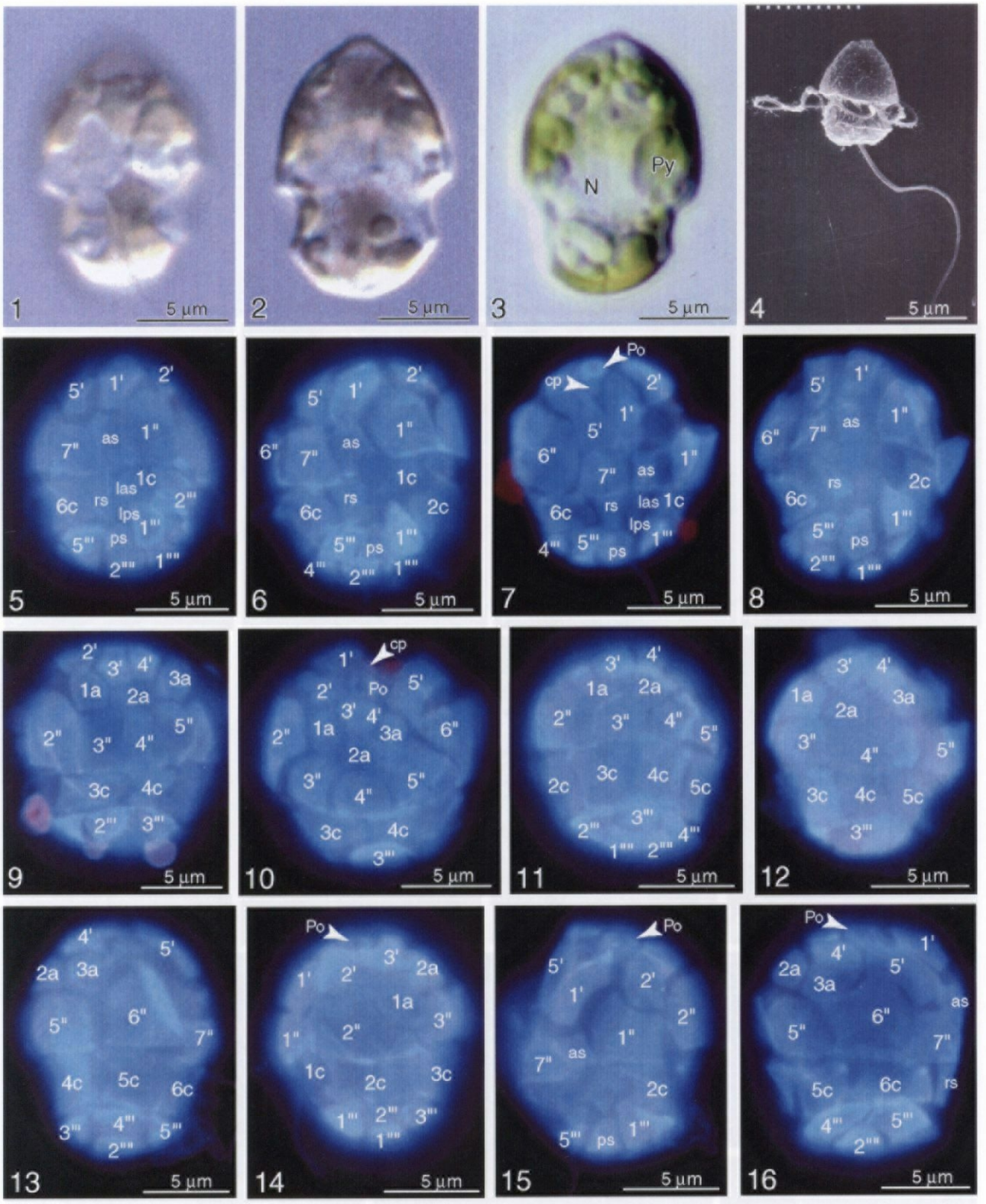

1
5 µm
2
5 µm
3
Py
Py
N
5 µm
4
Py
5 µm
5
5 µm
6
5 µm
7
Po
5' 1'
6" 7" 1"
as
6c las
rs lps 1'''
4''' 5''' ps 1''''
2''''
10 µm
8
cp
Po
5' 1'
7" as 1"
rs las
lps 1'''
5''' ps
2'''' 1''''
10 µm
9
Po
5' 1'
1"
as
7"
1c
las
6c rs
lps
1'''
5''' ps
1''''
10 µm
10
Po
5' 1'
1"
7" as
1c
6c rs las
lps
1'''
5''' ps
2'''' 1''''
10 µm
11
3' 4'
1a 2a 3a
3" 4" 5"
3c 4c
3''' 4'''
10 µm
12
3' 4'
1a 3a
2a
3" 5"
4"
3c 4c
3''' 4'''
10 µm
13
3' 4'
1a 2a 3a
2" 3" 4" 5"
3c 4c
2''' 3''' 4'''
10 µm
14
3' 4'
1a 2a 3a
3" 4" 5"
3c 4c
3"
10 µm
15
1a 2a
1" 2" 3" 4"
1c 2c 3c
2''' 3'''
1''''
10 µm
16
2a 3a 5'
5" 6" 7"
4c 5c 6c
3''' 4''' 5'''
2''''
10 µm

Plate 6


1
5 µm
2
5 µm
3
N
Py
5 µm
4
5 µm
5
5' 1' 2' 7" as 1" las 1c 6c rs lps 2''' 1'' 5''' ps 2'''' 1''''
5 µm
6
5' 1' 2' 6" 7" as 1" 1c 6c rs 2c 5''' ps 1''' 4''' 2'''' 1''''
5 µm
7
Po
cp
2' 1' 5' 6" 7" as 1" 6c rs las 1c lps 4''' 5''' ps 1'''
5 µm
8
5' 1' 6" 7" as 1" rs 2c 6c 1'' 5''' ps 2'''' 1''''
5 µm
9
2' 3' 4' 3a 1a 2a 5" 2" 3" 4" 3c 4c 2''' 3'''
5 µm
10
1' cp 2' Po 5' 3' 4' 2" 1a 3a 6" 2a 3" 5" 4" 3c 4c 3'''
5 µm
11
3' 4' 1a 2a 2" 3" 4" 5" 2c 3c 4c 5c 3''' 2''' 4''' 1'''' 2''''
5 µm
12
3' 4' 1a 3a 2a 3" 4" 5" 3c 4c 5c 3'''
5 µm
13
4' 5' 2a 3a 5" 6" 7" 4c 5c 6c 3'' 4''' 5''' 2''''
5 µm
14
Po
3' 1' 2' 2a 1a 3" 1" 2" 1c 2c 3c 1''' 2''' 3''' 1''''
5 µm
15
Po
5' 1' 2' 2" 1" 7" as 2c 5''' ps 1'''
5 µm
16
Po
4' 1' 2a 5' 3a as 6" 5" 7" 5c 6c rs 4''' 5''' 2''''
5 µm