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ABSTRACT
The origin and evolutionary scenario of a small group of dinoflagellates with unusual chloroplasts are discussed. These dinoflagellates are known to possess an endosymbiotic alga of diatom origin. These are Durinskia baltica, Kryptoperidinium foliaceum, Peridinium quinquecorne, Durinskia sp., Gymnodinium quadrilobatum, Peridiniopsis rhomboids, Dinothrix paradoxa and a new coccoid dinoflagellate from Palau (P-18 strain). Although these eight species share a similar type of endosymbiont, morphologically they are so diverse that they may be classified as different entities, even to the ordinal level, using the current taxonomic criteria. To investigate the origin(s) and phylogenetic affinities of these dinoflagellates, the SSU rRNA and rbcL genes of D. baltica, K. foliaceum, Durinskia sp., Peridiniopsis rhomboids, Dinothrix paradoxa and P-18 strain were sequenced and analysed. Phylogenetic trees based on nuclear encoded SSU rRNA gene strongly suggested that all these endosymbiotic dinoflagellates are monophyletic. The phylogenetic analyses based on the plastid encoded rbcL gene also revealed that all the endosymbiotic algae formed a unique clade within the diatom clade. Therefore, the acquisition of the endosymbiont took place only once and species diversified later. Of the diatoms included in the alignment, a pennate diatom, Nitzschia longissima var. reversa (Bacillariaceae) was shown to be the closest relative of the dinoflagellate endosymbionts. According to the fossil record, the members of the genus Nitzschia first appeared in Late Oligocene to Early Miocene and therefore, establishment of this endosymbiotic event must have been post this era. The evolutionary scenario of these dinoflagellates after establishment of the endosymbiont has been presented.

Keywords: Chloroplast, Diatom, Dinoflagellate, Endosymbiosis, Eyespot

INTRODUCTION
The dinoflagellates are a group of protists, comprising extremely diverse (morphologically, cytologically and physiologically) assemblages. About half of the dinoflagellate species are photosynthetic and their chloroplasts are characterized by 1) being triple membrane bound and 2) containing peridinin as a major xanthophyll, in addition to chlorophylls a/c. There are, however, exceptions to this rule. Some dinoflagellates possess chloroplasts of totally different types and these different types of chloroplasts are thought to have been derived from different endosymbiotic events [1].

The origin and evolutionary scenario of one such group of dinoflagellates with unusual chloroplasts will be dealt here. It is a relatively small group of dinoflagellates known to possess an endosymbiotic alga of diatom origin. These include Durinskia baltica [2], Kryptoperidinium foliaceum [3], Peridinium quinquecorne [4], Durinskia sp.[5], Gymnodinium quadrilobatum [6], and Dinothrix paradoxa [7].
In these organisms, the dinoflagellate cytoplasm and the endosymbiont cytoplasm are separated by a single unit membrane. The endosymbiont cytoplasm is rather simple and includes only a nucleus, chloroplasts and mitochondria [2]. The host-endosymbiont relationship is a permanent one as it is no longer possible to separate these two cellular components. Although these six species share a similar type of endosymbiont, morphologically they are so diverse that they may be classified as different entities, even to the ordinal level, using the present taxonomic system. To investigate the origin(s) and phylogenetic affinities of these dinoflagellates, the SSU rRNA and rbcL genes of D. baltica, K. foliaceum, Durinskia sp. and Dinothrix paradoxa were sequenced and analysed. The same genes in a non-motile, coccoid, dinoflagellate with a similar endosymbiont from Palau (P18 strain, a new taxon) and a freshwater, bloom-forming dinoflagellate, Peridiniopsis rhomboides were also analysed.

RESULTS AND DISCUSSION

Diversity of Dinoflagellates with a Diatom Endosymbiont

The following eight species are now recognized as the dinoflagellates with a diatom endosymbiont. Although it is not the aim of this paper to go into details of each species, they are briefly described.

**Durinskia baltica** (Levander) Carty et Cox (order Peridiniales) Fig. 1a

Small motile dinoflagellate belonging to the order Peridiniales. This is a type species of the genus. Thecal plate arrangement is: Po, x, 4', 2a, 6'', 5c, 4s, 5'', 2'''. We found the species both from marine (Okinawa) and freshwater (Hokkaido) environments. This is a well known species as a diatom-harbouring dinoflagellate and extensively studied [2, 8-12].

**Durinskia sp.** (order Peridiniales) Fig. 1b

This species has been found from the tide pools in several localities in South Africa, especially along the Cape coast. Thecal plate arrangement is: Po, x, 4', 2a, 6'', 5c, 5s, 5'', 2'''. Although the thecal plate arrangement is almost same as that of the type species, gross morphology is very different from the latter and therefore, we believe it is a new species. The formal description of the species is now in preparation.

**Kryptoperidinium foliaceum** (Stein) Lindemann (order Peridiniales) Fig. 1c

A highly flattened, motile marine dinoflagellate. This is also a well known species as a diatom-harbouring dinoflagellate and extensively studied just like D. baltica. The thecal plate arrangement is: Po, x, 4', 2a, 7'', 5c, 5s, 5'', 2'''. This thecal plate arrangement is quite similar to that of Durinskia. However, the number of precingular plates is different (7 vs. 6) [3, 9-11, 13-14, 15]. The strain used in this study is from culture collection of UTEX.

**Peridinium quinquecorne** Abé (order Peridiniales) Fig. 1d

A characteristic motile dinoflagellate with prominent antapical spines. The thecal plate arrangement is: Po, x, 3', 2a, 7'', 5c, 4s, 5'', 2'''. Unfortunately, it has not been possible to sequence this species to date. This species has been found from many places along Japanese coast, including Lake Hamana.

**Peridiniopsis rhomboides** Krakhmalny (order Peridiniales) Fig. 1e

This species was found to produce blooms in a freshwater pond in Toyama Prefecture, central Japan in the spring/summer of the year 2003. The thecal plate arrangement is: Po, x, 4', 0a, 6'', ?c, ?s, 5'', 2'''. Although we have identified the species as P. rhomboides, its identity needs further study, because this species is closely related to the species such as Peridiniopsis kevei Grigorszky et al. [16] and P. corillionii Leitao et al. [17].

**Gymnodinium quadrilibatum** Horiguchi et Pienaar (order Phytodiniales?) Fig. 1f

This species has been described from beach sand from subtropical Indian Ocean coast of South Africa. This species has also been found from mud of mangrove river in Irinomote Island, Japan. It is characterized by four-leaved-clover like non-motile cell. The non-motile cell is dominant in life cycle. It reproduces asexually by forming two motile cells. The motile cell is athecate and Gymnodinium-like in morphology. This is why Horiguchi and Pienaar [5] described it as a new species of the genus Gymnodinium, rather than creating a new genus based on its unique vegetative morphology. However, now the genus Gymnodinium is strictly defined [18] and the motile cell of this species does not fit the recent definition of the genus Gymnodinium. Here I tentatively treat this species as a member of the order Phytodiniales, which is characterized by having
Dinoflagellates with a Diatom Endosymbiont

Fig. 1  Dinoflagellates with a diatom endosymbiont. a. Durinska baltica, b. Durinska sp. c. Kryptoperidinium foliaceum, d. Peridinium quinquecornes, e. Peridiniopsis rhomboids, f. Gymnodinium quadrilobatum, g. P-18 strain from Palau, h. Dinothrix paradoxa. Scale bars = 10 $\mu$m

dominant non-motile vegetative stage. Unfortunately, we have not been able to sequence this species to date.

P-18 strain (order Phytodiniales) Fig. 1g

This newly isolated strain from the bottom sand of the Jellyfish Lake, Palau, possesses very unique vegetative form. It is helmet-shaped and firmly attaches to the substratum. The dominant stage in life cycle is this non-motile, attached form. It reproduces itself by means of production of two motile cells. The motile cell is athecate. The motile cell directly returns to the non-motile vegetative form. This species is probably a new genus and a species (manuscript in preparation).

Dinothrix paradoxa Pascher (order Dinotrichales, or family Dinocloniaceae, order Phytodiniales) Fig. 1h

This dinoflagellate is characteristic because it has pseudo-filamentous form, which consists of up to 10 cells of spherical shape. The pseudo-filament is
formed by successive divisions of the non-motile cells. This is the most characteristic point of this dinoflagellate, because in other dinoflagellates mentioned above the reproduction is always accompanied by formation of motile cells. The dinoflagellate also has ability to produce motile cells and its thecal plate arrangement is: Po, x, 4', 2a, 7'', 5c, 4s, 5'', 2''. This arrangement resembles that of Kryptoperidinium foliaceum. As mentioned above, these eight species are quite distinctive and different from one another both in morphology and mode of life cycles. In fact, under the present taxonomic system, some species can be classified even in the different orders. In this respect, they, at least some of them, seem to be distantly related to each other.

A Unique Type of Eyespot Shared by These Dinoflagellates

The eyespot is a structure relating to phototaxis and it is widely distributed in motile cells of many groups of algae. It is interesting to point out that all these eight species and only these 8 species in dinoflagellates possess same type of eyespot. In this type (thereafter cited as Type B eyespot), rows of red-pigmented globules are bounded by triple membrane and this is quite different from that of the typical dinoflagellate. The typical dinoflagellate eyespot is a row (or rows) of red-pigmented lipid globules located within the chloroplast, i.e. the eyespot forms a part of the chloroplast. It should be noted that the chloroplast of typical dinoflagellates is bounded also by triple membrane.

Origin of Dinoflagellates with a Diatom Endosymbiont - a Hypothesis

Because both Type B eyespot and typical dinoflagellate chloroplast are bounded by triple membrane, it has been assumed that these two structures are homologous. The following hypothesis to explain the origin of the dinoflagellates possessing a diatom endosymbiont (and origin of Type B eyespot) has been proposed (Fig. 2) [19]. Originally the ancestral dinoflagellate possessed a typical peridinin-containing dinoflagellate chloroplast with a typical eyespot. This dinoflagellate subsequently engulfed a diatom and kept it as an endosymbiont. The original dinoflagellate chloroplast is mostly replaced by that of the diatom endosymbiont and finally only the eyespot part of the original chloroplast was retained as a device for phototaxis. The Type B eyespot is, therefore, a highly reduced typical dinoflagellate chloroplast. If this hypothesis is true, and I believe it is, it is hard to believe that this kind of complicated evolutionary event took place several times independently. Alternatively, it is much simpler (and parsimonious) to think that the above mentioned cellular process took place only once and later the species with a diatom endosymbiont and Type B eyespot diversified. It was in 1994 when we [6] proposed this single origin of diatom-harboring dinoflagellate hypothesis, but at that time we did not have means to prove it. To test the hypothesis, molecular phylogenetic approach would be appropriate. For the two well known species, i.e. D. baltica and K. foliaceum, several works on molecular phylogeny have already been published [9-10, 13]. Also, a close affinity of these two species has been demonstrated by Inagaki et al. [10]. For other species, except

![Fig. 2 Hypothesis regarding the origin of a diatom-harboring dinoflagellate as well as type B eyespot.](image-url)
these two species, nothing is known as far as molecular data are concerned.

**Molecular Phylogenetic Studies**

To test whether all these dinoflagellates are monophyletic (single origin hypothesis) or polyphyletic (multiple origin hypothesis), we have sequenced nuclear encoded small subunit ribosomal RNA genes (SSU rDNA) as well as plastid encoded Ribulose-1, 5-bisphosphat-carboxylase/oxygenase (rbcL). Because the latter gene is included in the chloroplasts of diatom origin, the gene should reflect phylogenetic affinities of the endosymbionts. The molecular phylogenetic tree deduced from rbcL (Fig. 3) revealed that all the endosymbionts are monophyletic and the clade sits in the terminal position of the free-living diatom clade. Therefore, it is no doubt that the endosymbionts are of diatom origin and as the tree suggests, all of them had a single ancestor.

The nuclear encoded SSU rDNA tree is expected to show phylogenetic affinities of the dinoflagellates (hosts) and the tree clearly reveals that all the diatom-harboring dinoflagellates are monophyletic (data not shown), thus supporting the idea that these dinoflagellates with a diatom endosymbiont originated from a single ancestor.

**Evolutionary Scenario**

Figure 4 shows evolutionary scenario of this small group of dinoflagellates based on the SSU rDNA tree. As mentioned earlier, the acquisition of a diatom endosymbiont (we do not know how it hap-

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**Fig. 3** A simplified phylogenetic tree (ML tree) based on rbcL to show phylogenetic position of the endosymbiotic algae within the heterokontphyte clade. The endosymbionts formed a clade, which is embedded in the Diatom-clade. Scale bar = 0.05 substitutions/site
Evolutionary scenario

Common ancestor!

Gain of ability to divide in non-motile stage

Loss of thecal plates

Fig. 4 Evolutionary scenario of the diatom-harboring dinoflagellates based on SSU rDNA tree. For detail, see text.

pened!), subsequent loss of original dinoflagellate chloroplast and gain of the Type B eyespot took place in one dinoflagellate lineage only once.

According to the SSU rDNA tree, the clade which includes all these diatom-harboring dinoflagellates is divided into two major sub-clades. One clade contains all the *Durinskia* species and *Peridiniopsis rhomboïdes*. Therefore, this clade is characterized by having 6 precingular plates (6-precingular lineage). The other clade contains species with 7 precingular plates, i.e. *Kryptoperidinium foliaceum* and *Dinothrix paradoxa* (motile cell). Therefore, this clade can be designated as 7-precingular lineage. Although it was not possible to sequence *P. quinquecorne*, because it possesses 7 precingulars, I tentatively placed this species in this lineage. After the establishment of a common ancestor, there was a split between the 6-precingular and 7-precingular lineages. We do not know whether the ancestor possessed 6-precingular or 7-precingular plates. In the 6-precingular lineage, the speciation took place and species of *Durinskia* spp. and *Peridiniopsis rhomboïdes* have evolved. It is highly likely that *P. rhomboïdes* is also a member of the genus *Durinskia* rather than *Peridiniopsis*, because of the similarity of cellular structure. In the 7-precingular lineage, the acquisition of dominant non-motile stage has taken place and species such as P-18 strain has evolved. It was not possible to sequence *G. quadrilobatum*, but I tentatively placed it near P-18 because of similar mode of life cycle. Also in these two non-motile dominant forms, loss of thecal plates in motile stage has taken place. In this lineage, acquisition of novel ability to continuously divide in non-motile stage let to the evolution of species like *Dinothrix paradoxa*.

Origin of a Diatom Endosymbiont

Which genus of diatom serves as a potential progenitor of the endosymbiont is an interesting question to ask. Of the diatoms included in our alignment of *rbcL* gene, a pennate diatom, *Nitzschia longissima* var. *reversa* (Bacillariaceae) was shown to be the closest relative of the dinoflagellate endosymbionts. The results suggest that the common ancestor of these dinoflagellates had acquired a bacillariaceous diatom, most likely a member of the genus *Nitzschia*, with which it had established an endosymbiotic relationship. According to the fossil re-
cord, the members of the genus *Nitzschia* first appeared sometimes during Late Oligocene to Early Miocene [20] and therefore, this endosymbiotic event must have been established after that time.

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