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Identification of Highly Divergent Diatom-Derived Chloroplasts in Dinoflagellates, Including a Description of *Durinskia kwazulunatalensis* sp. nov. (Peridiniales, Dinophyceae)

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Abstract

Dinoflagellates are known to possess chloroplasts of multiple origins derived from a red alga, a green alga, haptophytes, or diatoms. The monophyletic "dinotoms" harbor a chloroplast of diatom origin, but their chloroplasts are polyphyletic belonging to one of four genera: *Chaetoceros*, *Cyclotella*, *Discostella*, or *Nitzschia*. It has been speculated that serial replacement of diatom-derived chloroplasts by other diatoms has caused this diversity of chloroplasts. Although previous work suggested that the endosymbionts of *Nitzschia* origin might not be monophyletic, this has not been seriously investigated. To infer the number of replacements of diatom-derived chloroplasts in dinotoms, we analyzed the phylogenetic affinities of 14 species of dinotoms based on the endosymbiotic *rbc*L gene and SSU rDNA, and the host SSU rDNA. Resultant phylogenetic trees revealed that six species of *Nitzschia* were taken up by eight marine dinoflagellate species. Our phylogenies also indicate that four separate diatom species belonging to three genera were incorporated into the five freshwater dinotoms. Particular attention was paid to two crucially closely related species, *Durinskia capensis* and a novel species, *D. kwazulunatalensis*, because they possess distantly related *Nitzschia* species. This study clarified that any of a total of at least 11 diatom species in five genera are employed as an endosymbiont by 14 dinotoms, which infers a more frequent replacement of endosymbionts in the world of dinotoms than previously envisaged.

Introduction

Recent molecular phylogenetic studies revealed that at least eight divergent eukaryotic groups, i.e., Archaeplastida (Plantae), chlorarachniophytes, euglenophytes, haptophytes, heterokonts, cryptophytes, *Paulinella chromatophora* and the Alveolata (Keeling 2010), engulfed and acquired photosynthetic endosymbionts and kept them as permanent chloroplasts. Dinoflagellates are unicellular eukaryotes, belonging to one of the three main groups in the Alveolata, and are known to possess chloroplasts of multiple origins derived from four unrelated groups of microalgae. The commonest chloroplast in dinoflagellates is derived from a certain red alga (Rhodophyta), acquired through secondary endosymbiosis (Zhang et al. 1999). These common chloroplasts, characterized as containing chlorophyll a/c_2 and peridinin, a xanthophyll unique for dinoflagellates, are therefore referred to as the "peridinin-type". The other three chloroplast-types are less common. Dinoflagellates belonging to the genus *Lepidodinium* have chloroplasts of a chlorophyte origin (Watanabe et al. 1990; Matsumoto et al. 2012), while members of the family Kareniaceae are thought to possess chloroplasts of haptophyte origin (Tengs et al. 2000). The final group, comprising 13 representatives, has chloroplasts of diatom origin (Tomas et al. 1973; Jeffrey and Vesk 1976; Horiguchi and Pienaar 1991; Horiguchi and Pienaar 1994; Tamura et al. 2005; Horiguchi and Takano 2006; Pienaar et al. 2007; Takano et al. 2008; Saburova et al. 2012; Zhang et al. 2011, 2014; Hoppenrath et al. 2014; You et al. 2015). Interestingly, multiple-gene molecular phylogenies indicate that the dinoflagellates possessing such minor three types of chloroplast (chlorophyte, haptophyte, or bacillariophyte) originally possessed peridinin-type chloroplasts (Saldarriaga et al. 2001, 2004), meaning that they have replaced the typical chloroplast with those of other microalgae.

Dinoflagellates possessing diatom-derived chloroplasts, collectively referred as "dinotoms" (Imanian et al. 2010), have the following three unique characteristics with regard to their endosymbiont diatoms (ESDs) not found in dinoflagellates with other chloroplast types. First, the ESD is separated from the cytoplasm of the host dinoflagellate by a single membrane (Tomas et al. 1973; Jeffrey and Vesk 1976; Dodge 1984), which, in addition to the chloroplasts, encapsulates the remainder of the ESDs' organelles, i.e., mitochondria, ribosomes, endoplasmic reticulum, and nucleus (Tomas et al.

1973; Jeffrey and Vesk 1976; Tamura et al. 2005). This is surprising because in other dinoflagellates that have replaced their peridinin-type chloroplasts with other microalgae (*Lepidodinium* spp. and members of the Kareniaceae) the remainder of the endosymbiont organelles are highly reduced (Watanabe et al. 1990; Schnepf and Elbrächter 1999). Second, the remnant of a peridinin-type chloroplast, in the form of a "type D eyespot" *sensu* Moestrup and Daugbjerg (2007) is found in the cytoplasm of all of the dinotom hosts (Dodge 1983; Cavalier-Smith 1993; Horiguchi and Pienaar 1994). The presence of a type D eyespot in all the dinotoms suggests that the host cells of all dinotoms are monophyletic, which has been supported by SSU rDNA molecular phylogenetic analysis (Horiguchi and Takano 2006).

Unlike the host dinoflagellates, which are monophyletic, the ESDs are polyphyletic and have been replaced by other diatoms at least three times. Early molecular phylogenetic analyses to determine the affinities of ESDs in dinotoms showed that *Durinskia baltica*, *Kryptoperidinium foliaceum* (Chesnick et al. 1996, 1997), and the coccoid dinotom *Galeidinium rugatum* (Tamura et al. 2005), all have a species of the pennate diatom, *Nitzschia*, as an endosymbiont. In Zhang et al. (2014), the phylogenetic analysis inferred that the ESDs of *D. baltica* and of *K. foliaceum* represent different species of *Nitzschia*, but this has not been seriously investigated. Subsequently, the ESD of *Peridinium quinquecorne* was found to belong to the centric genus *Chaetoceros* (Horiguchi and Takano 2006). These authors argued that the ancestral host cell must have acquired an ESD, possibly a species of *Nitzschia*, which later was replaced with a species of *Chaetoceros* in the lineage leading to *P. quinquecorne*, because the host dinoflagellates are monophyletic. A similar type of replacement seems to have taken place in freshwater dinotoms. The ESD of *Peridiniopsis* cf. *kevei*, *P. penardii*, and *P. jiulongensis* was demonstrated to be affiliated with the freshwater centric diatom, *Discostella* (Zhang et al. 2011; You et al. 2015) while, that of *Peridiniopsis niei* and *P. minima* was demonstrated to be a member of the genus *Cyclotella* (Zhang et al. 2014). Thus the ESDs in some dinotoms were obviously replaced, in a serial fashion, from the original *Nitzschia* sp. by species of one of three other genera (*Chaetoceros*, *Cyclotella* or *Discostella*).

To date, only two dinotoms, i.e., *Durinskia baltica* and *Kryptoperidinium foliaceum* have been used in both molecular and cytological studies (Figueroa et al. 2009; Imanian et al. 2010, 2012). Here we studied 12 strains representing six marine species of dinotoms, i.e., *Durinskia* cf. *baltica*, *D. capensis*, *D. kwazulunatalensis* sp. nov., *Galeidinium rugatum*, and two as yet undescribed coccoid dinotoms. Our preliminary study revealed that all these cultured dinoflagellates possess ESDs of the genus *Nitzschia*. We undertook a molecular phylogenetic analysis of these and all other dinotoms, based on two genes of the endosymbiont, the chloroplast-encoded *rbc*L gene and the nuclear-encoded SSU rDNA, and one gene of the dinoflagellate host, the SSU rDNA. This was used to determine the number of diatoms that have been incorporated by host dinotoms and to determine whether all the *Nitzschia* endosymbionts of dinotoms represent a common species. The two species collected from South Africa, *Durinskia capensis* and *D. kwazulunatalensis* sp. nov. were paid special attention because it was clarified in this study that they have distantly related *Nitzschia* species as ESDs despite their close phylogenetic relationship. This prompted a further detailed morphological study to ascertain the taxonomic positions of these two taxa.

Results

Molecular Phylogeny

We constructed four molecular phylogenetic trees (figs. 1–4) in this study. Three of which (figs. 1–3) were inferred from endosymbiotic genes. One ESD tree was based on the chloroplast-encoded *rbc*L gene (fig. 1) and the other two, based on the nuclear-encoded SSU rDNA of the ESD were split, according to the diatom type; pennate *Nitzschia* (fig. 2), or centric (fig. 3). The final tree (fig. 4) was inferred from the host SSU rDNA. *Durinskia agilis* was included in the clade of host dinoflagellates (fig. 4), but the species will not be considered further in this study because its ESD lacks any molecular characterization.

Endosymbiont Phylogenies

Phylogenetic analyses based on diatom genes clarified that the six marine dinotoms cultured in this study possessed diatoms belonging to the genus *Nitzschia* (figs. 1 and 2), and that all the nitzschioid endosymbionts of dinotoms with a molecular characterization, including those of *Durinskia baltica* (CS-38) and *Kryptoperidinium foliaceum*, were separated into six groups, groups 1–6 (figs. 1 and 2, table 1). Another marine dinoflagellate, *Peridinium quinquecorne*, formed a robust clade with marine diatoms *Chaetoceros* spp. (Group 7). The ESDs of the five freshwater dinotoms separated into four, groups 8–11 (figs. 1 and 3, table 1) and only the closest free-living diatom(s) with >50% bootstrap support (BS) to the ESD of each group was included.

In the molecular phylogeny of the *rbc*L gene (fig. 1), the ESD of *D. baltica* (GU591327, strain CS-38) was recovered in a strongly supported clade (98% BS value) with *Nitzschia palea* from Spain (FN557025, strain Spain C), from Brazil (FN557017, strain Brazil) and Sri Lanka (HF675121, strain SriLanka 1). This clade, in turn, formed a sister to *Nitzschia capitellata* from Spain (FN557032, strain Spain) with weaker but still relatively strong support (70% BS value) (Group 1). The three ESDs of *D.* cf. *baltica*, including that of a strain registered as *D. baltica* (AB195670), all collected from Japan, formed a clade (Group 2), with 100% BS, removed from that of *D. baltica* strain CS-38. The five currently characterized ESDs of *D. capensis* and of the type *D. capensis* (AB271108) made a clade with *Nitzschia draveillensis* (KC736605, strain TCC700) with an 82% BS value (Group 3). The ESDs of the three *D. kwazulunatalensis* strains also formed an independent clade (Clade 4) with *Simonsenia aveniformis* (KR048205) although with low (51%) BS. The ESDs of *Galeidinium rugatum* and of the two unidentified coccoid dinotoms formed a clade with an 87% BS value (Group 5), which is indicative of a common species of *Nitzschia*. The endosymbiotic *Nitzschia* of

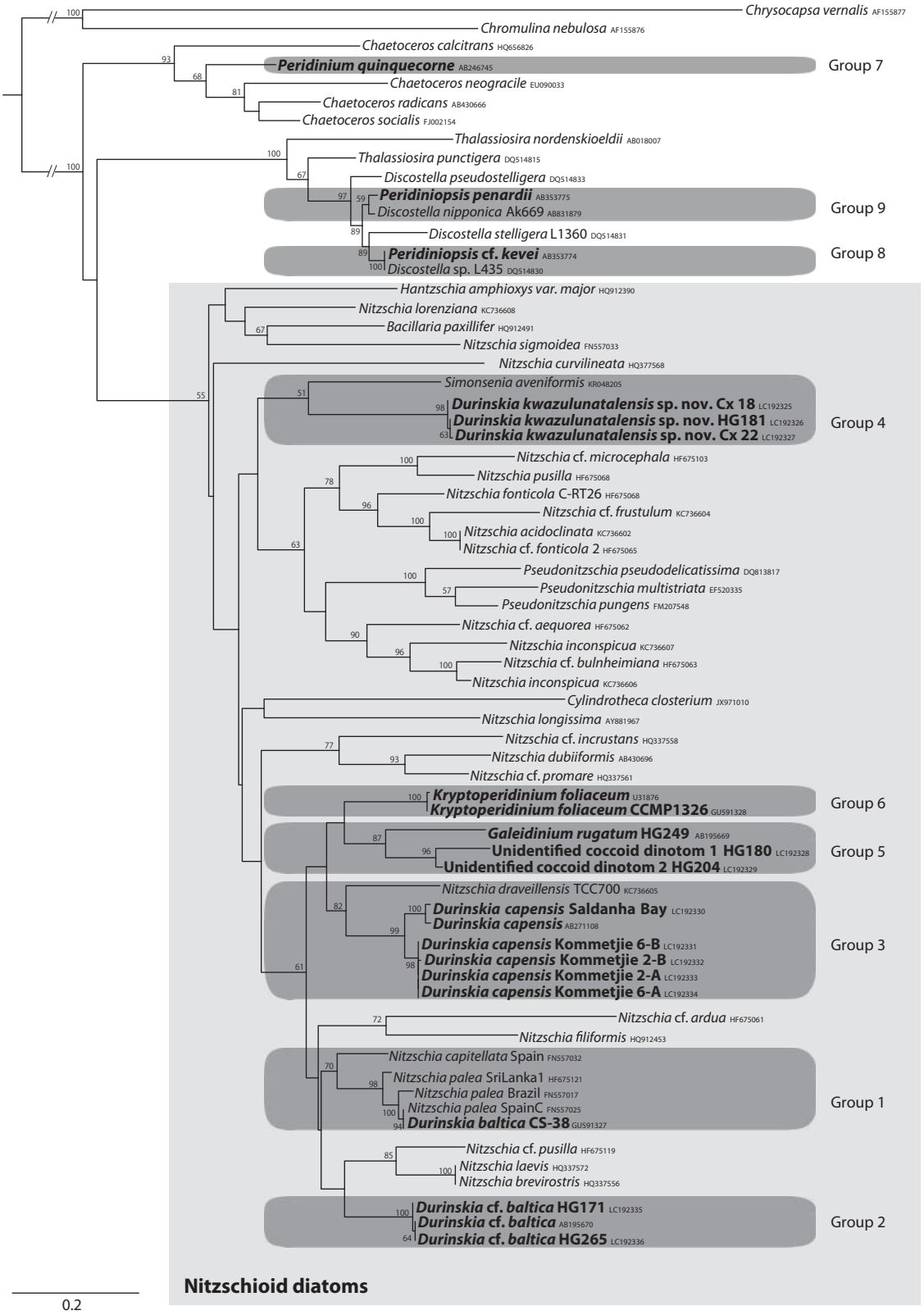

Fig. 1. Diatom ML tree based on 1383 aligned positions, including gaps, of the *rbc*L gene of free-living and endosymbiotic diatoms with *Chromulina nebulosa and Chrysocapsa vernalis* as outgroups. ESDs and, where appropriate, their closely affiliated free-living diatoms comprise groups 1–9. Bold type indicates ESDs of dinotoms. Numbers on the major nodes represent ML (100 pseudoreplicates) BS values. Only bootstrap values >50% are shown. GenBank accession numbers follow taxon names.

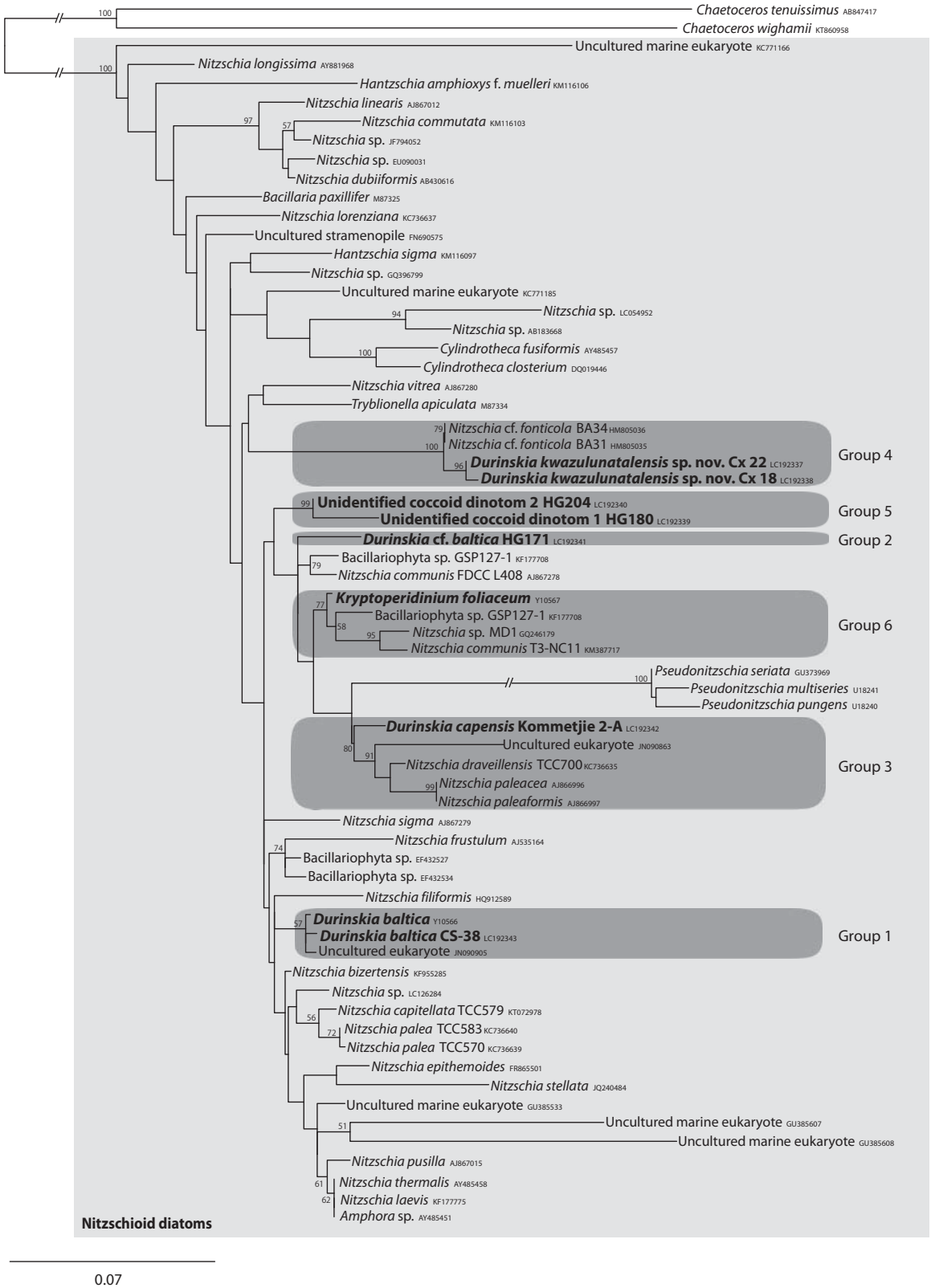

Fig. 2. Pennate diatom ML tree focused on *Nitzschia*-type marine dinotoms and based on 1611 aligned positions, including gaps, of SSU rDNA of free-living and endosymbiotic diatoms with *Chaetoceros tenuissimus* and *Chaetoceros wighamii* as outgroups. Each group number for ESDs follow those recovered in the *rbc*L phylogeny of ESDs. Bold type indicates ESDs of dinotoms. Numbers on the major nodes represent ML (100 pseudoreplicates) BS values. Only bootstrap values >50% are shown. GenBank accession numbers follow taxon names.

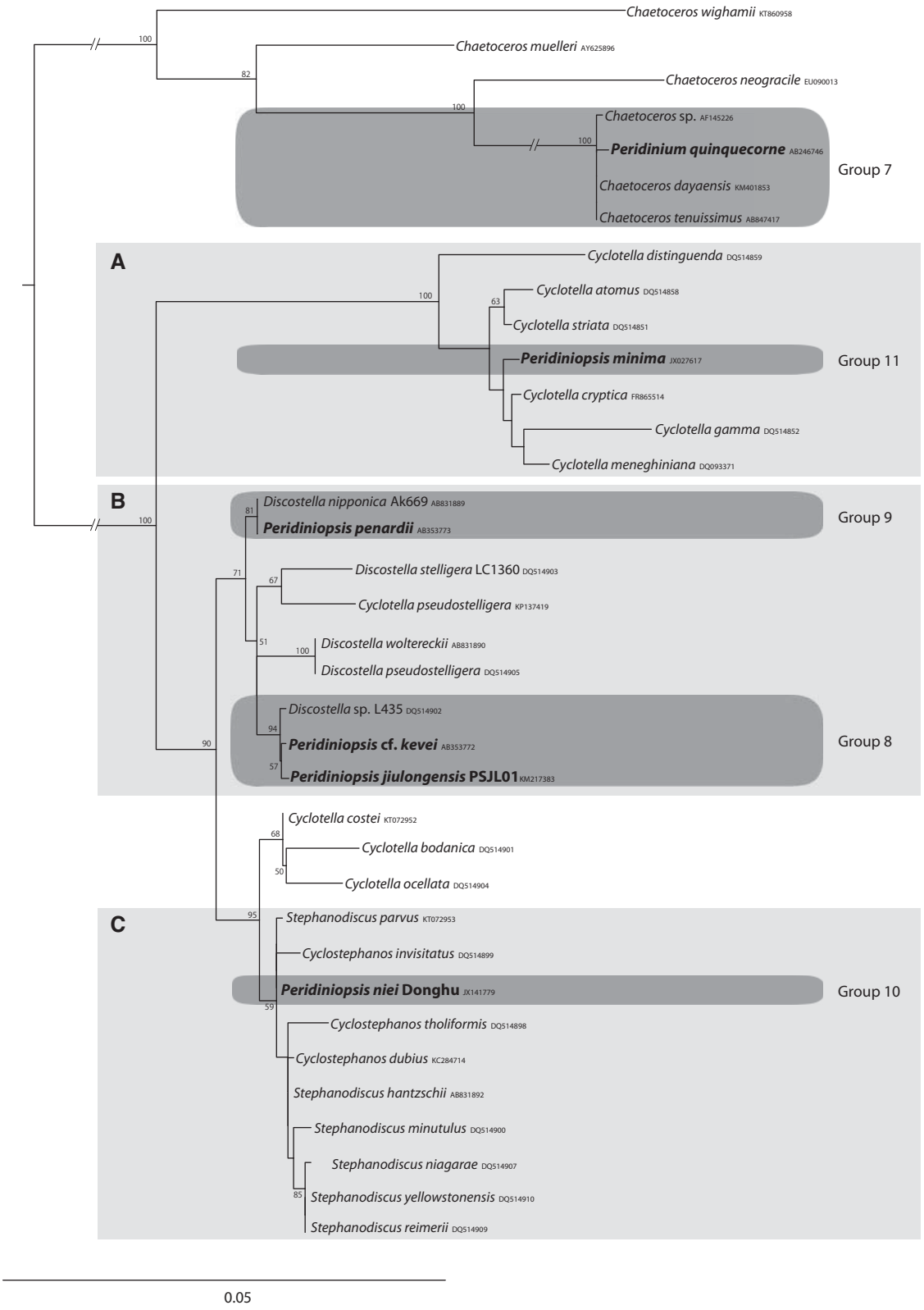

Fig. 3. Centric diatom ML tree focused on freshwater dinotoms and a marine *Chaetoceros*-type dinotom based on 1604 aligned positions, including gaps, of SSU rDNA of free-living and endosymbiotic diatoms. Each group number for ESDs follow those recovered in the *rbc*L gene phylogeny of ESDs. Bold type indicates ESDs of dinotoms. (*A*) *Cyclotella* clade, (*B*) *Discostella* clade, and (*C*) *Cyclostephanos* and *Stephanodiscus* clade. Numbers on the major nodes represent ML (100 pseudoreplicates) BS values. Only bootstrap values >50% are shown. GenBank accession numbers follow taxon names.

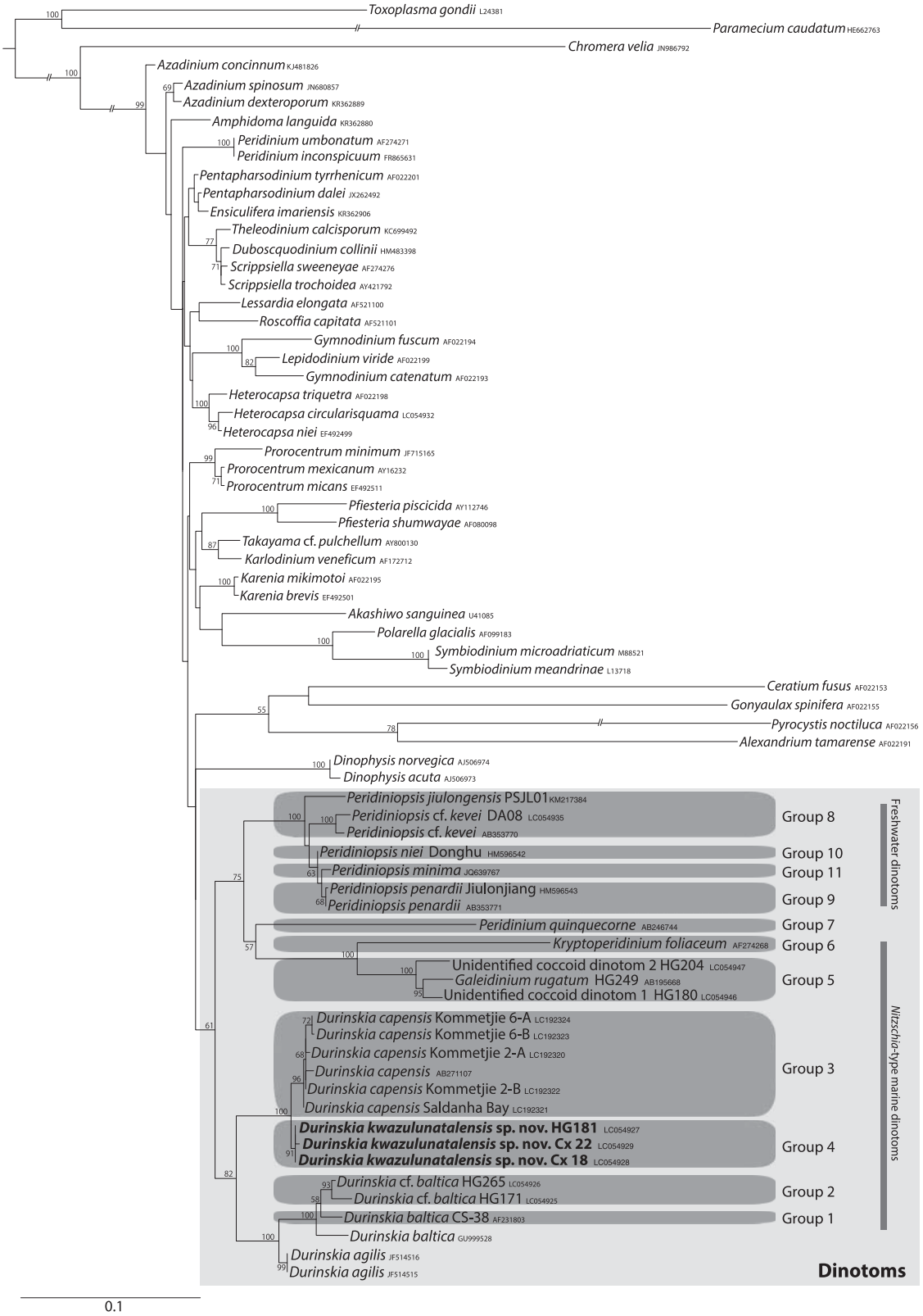

Fig. 4. Dinoflagellate ML tree based on 1759 aligned positions, including gaps, of the SSU rDNA of selected dinoflagellates. *Toxoplasma gondii*, *Chromera velia*, and *Paramecium caudatum* were used as outgroups. Each group number for these host dinoflagellates follows that of their ESD recovered from the *rbc*L gene and SSU rDNA phylogenies, and is not dependent on clades recovered from analysis of this data. Numbers at the major nodes represent ML (100 pseudoreplicates) BS values. Only bootstrap values >50% are shown. GenBank accession numbers follow taxon names. Bold type indicates the novel dinotom, *Durinskia kwazulunatalensis* sp. nov.

Table 1. Dinotoms Described or Cultured in this Study.

Group Number	Strain Name (Accession number)	Species Name[a]	Closest Affiliation to ESD
Marine *Nitzschia*-type dinotoms			
Group 1	**CS-38 (AF231803, GU591327, LC192343)**	***Durinskia baltica***	***Nitzschia palea* (strain SpainC)**
	Unknown (Y10566)	***Durinskia baltica***	
Group 2	**HG171 (LC054925, LC192335, LC192341)**	***Durinskia* cf. *baltica***	**Unknown *Nitzschia* species**
	HG265 (LC054926, LC192336)	***Durinskia* cf. *baltica***	
	Uncultured (AB195670)	***Durinskia baltica***	
Group 3	**Kommetjie 2 (LC192320, LC192322, LC192332, LC192333, LC192342)**	***Durinskia capensis***	***Nitzschia draveillensis* (strain TCC700)**
	Kommetjie 6 (LC192323, LC192324, LC192331, LC192334)	***Durinskia capensis***	
	Saldanha Bay (LC192321, LC192330)	***Durinskia capensis***	
Group 4	**Cx18 (LC054928, LC192325, LC192338,)**	***Durinskia kwazulunatalensis* sp. nov.**	***Nitzschia* cf. *fonticola* (strain BA31 and BA34)**
	Cx22 (LC054929, LC192327, LC192337)	***Durinskia kwazulunatalensis* sp. nov.**	
	HG181 (LC054927, LC192326)	***Durinskia kwazulunatalensis* sp. nov.**	
Group 5	**HG249 (AB195668, AB195669)**	***Galeidinium rugatum***	**Unknown *Nitzschia* species**
	HG180 (LC054946, LC192328, LC192339)	**Unidentified coccoid dinotom 1**	
	HG204 (LC054947, LC192329, LC192340)	**Unidentified coccoid dinotom 2**	
Group 6	**UTEX LB1688 (AF274268, EF492508, DQ847436, AF231804)**	***Kryptoperidinium foliaceum***	***Nitzschia communis* (strain T3-NC11)**
	CCMP1326 (GU591328)	***Kryptoperidinium foliaceum***	
	Unknown (Y10567, U31876)	***Kryptoperidinium foliaceum***	
Marine *Chaetoceros*-type dinotom			
Group 7	**Uncultured (AB246744, AB246745, AB246746)**	***Peridinium quinquecorne***	***Chaetoceros* sp.**
Freshwater dinotoms			
Group 8	**PSJL01 (KM217384, KM217383)**	***Peridiniopsis jiulongensis***	***Discostella* sp. (strain L435)**
	Uncultured (AB353770, AB353772, AB353774)	***Peridiniopsis* cf. *kevei***	
	DA08 (LC054935)	***Peridiniopsis* cf. *kevei***	
	HG327 (LC054936)	***Peridiniopsis* cf. *kevei***	
Group 9	**Uncultured (AB353771, AB353773)**	***Peridiniopsis penardii***	***Discostella nipponica* (strain Ak699)**
	Jiulonjiang (HM596543, HM596547)	***Peridiniopsis penardii***	
	Suizhou (HM596548)	***Peridiniopsis penardii***	
	Manwan (HM596549)	***Peridiniopsis penardii* var. *robusta***	
Group 10	**Donghu (HM596542, JX141779)**	***Peridiniopsis niei***	***Cyclostephanos* sp.**
Group 11	**Uncultured (JX027617, JQ639767)**	***Peridiniopsis minima***	***Cyclotella* sp.**
Other dinotoms			
–	**Uncultured (no molecular data)**	***Dinothrix paradoxa***	**Unknown**
–	**Uncultured (JF514515, JF514516)**	***Durinskia agilis***	**Unknown**
–	**Uncultured (no molecular data)**	***Gymnodinium quadrilobatum***	**Unknown**

[a]Species in bold face are cultured in this study.

Kryptoperidinium foliaceum (U31876 and GU591328, the latter representing strain CCMP1326) formed an independent clade (Group 6) with a 100% BS value. Group 6 ESDs formed a clade with Group 5, but without high (>50%) BS value. The ESD of another marine dinotom, of *Peridinium quinquecorne*, was characteristic (Group 7). It located in marine centric diatom clade, *Chaetoceros* spp. group as indicated in Horiguchi and Takano (2006).

The two ESDs from the freshwater *Peridiniopsis* spp. were resolved as two independent lineages within the diatom genus *Discostella* (fig. 1); that of *P.* cf. *kevei* affiliating with an unidentified species of *Discostella*. (DQ514830, strain L435) with a 100% BS value (Group 8), and that of *P. penardii* affiliating with *Discostella nipponica* (AB831879, strain Ak669) with 59% BS value (Group 9). The phylogenetic positioning of the ESDs of *P. jiulongensis*, *P. minima* and *P. niei* could not be interrogated because there are no *rbc*L data available for them.

This polyphyly of the endosymbiotic diatoms was reinforced by the ESD SSU rDNA trees (figs. 2 and 3) as they were separable into 11 groups. Group 1, with 57% BS (fig. 2), consisted of the ESDs of two *Durinskia baltica* isolates (Y10566 and a currently sequenced strain, CS-38) and an environmental sequence (JN090905), while the position of the nitzschioid ESD of *D.* cf. *baltica* (strain HG171) was remote from it (Group 2). The ESD of *D. capensis* (strain Kommejie 2-A) formed a further independent clade (Group 3), with 80% BS, with three *Nitzschia* species including *N. draveillensis*, as was found in the *rbc*L phylogeny (fig. 1). The ESDs of the two *D. kwazulunatalensis* strains grouped with two isolates of *N.* cf. *fonticola* (HM805035, strain BA31 and HM805036, strain BA34) with 100% BS (Group 4, fig. 2). The ESDs of the two coccoid dinotoms grouped (Group 5) with 99% BS. The ESD of *Kryptoperidinium foliaceum* made yet another independent clade (Group 6) with *N. communis* (KM387717, strain T3-NC11), *Nitzschia* sp. (GQ246179, strain MD1), and an unidentified diatom (KF177708, strain GSP127-1) with 77% BS. As found in the *rbc*L tree (fig. 1), the ESD of the marine *P. quinquecorne* was recovered within the *Chaetoceros*-clade (Group 7), with *C. dayaensis*

(KM401853)/*C. tenuissimus* (AB847417)/*Chaetoceros* sp. (AF145226) as the closest diatoms (fig. 3). The affiliation of the ESDs to known species of *Nitzschia* in each of Groups 2 and 5 could not be resolved using either of these *rbc*L or SSU rDNA phylogenies. Regrettably, we were unable to recover the SSU rDNA sequences of any of the ESDs of *G. rugatum* (strain HG249), of *D.* cf. *baltica* (strain HG265), of *D. capensis* (strains Kommetjie-6 and Saldanha Bay), and of *D. kwazulunatalensis* (strain HG181).

With regard to the ESDs of the freshwater *Peridiniopsis* spp. (fig. 3), those of *P. jiulongensis* and *P.* cf. *kevei* made a clade (Group 8) with an unidentified species of *Discostella* sp. (DQ514902, strain 435) with 94% BS, The ESD of *P. penardii* made a clade (Group 9) with *Discostella nipponica* (AB831889, strain Ak699) with 81% BS. Both clades reflect similar groupings recovered using *rbc*L data (fig. 1). The ESDs of the remaining freshwater dinotoms segregated, corresponding with the species of the host. The ESD of *P. niei* affiliated with the *Cyclostephanos-Stephanodiscus* group (Group 10), while the ESD of *P. minima* shared identity with the genus *Cyclotella* (Group 11). Based on the taxonomic treatment of thalassiosiroids (Alverson et al. 2007), we identified the genus of the affiliated ESD of Group 10 as genus *Cyclostephanos*. The affiliation of the ESDs, based on their SSU rDNA data, with known free-living species, could not be resolved for either of Groups 10 or 11.

Host Phylogeny

The molecular phylogeny of dinoflagellates based on the host SSU rDNA showed that the dinotoms are monophyletic with 61% BS value (fig. 4). Although there was not always sound cladistic support for the various groups recovered by phylogenetic analysis of the ESD genes, the topology of the host tree still provided a reasonable basis for understanding independent ESD acquisitions (fig. 4).

Marine *Nitzschia*-type dinotoms could be separated into six 'groups'. All species of the genus *Durinskia* formed a clade with 82% BS. *Durinskia baltica* CS-38 (Group 1) and two *D.* cf. *baltica* (Group 2) were sister with a 58% BS value. The nucleotide gene differences in host SSU rDNA between *D. baltica* (AF231803, strain CS-38, collected in US) and the two strains of *D.* cf. *baltica* (strains HG171 and HG265, collected from Japan) ranged from 1.13% to 1.53% (over 1766 bp). Because its ESD is unknown, *D. baltica* (GU999528, collected from China) was not included in Group 1. In Group 3, the three *D. capensis* strains (Kommetjie-2, Kommetjie-6, and Saldanha Bay, collected from the Western Cape, South Africa) formed a clade with the type sequence of *D. capensis* (AB271107, also collected from the Western Cape, South Africa) with 96% BS value. The three *D. kwazulunatalensis* strains (HG181, Cx18, and Cx22, collected from KwaZulu-Natal, South Africa; Group 4) positioned as a sister to Group 3 with 100% BS value. The nucleotide differences between *D. capensis* and *D. kwazulunatalensis* ranged from 0.58% to 1.21% (over 1739 bp). *Galeidinium rugatum* (AB195668, strain HG249, collected in Palau) and the two unidentified coccoid dinotoms (one species, strain HG180, collected from Japan, and another, strain HG204, from South Africa) formed a single clade (Group 5) with 100% BS value. Although closely related, the two coccoid forms are considered to represent independent species from each other because of their distinguishable morphologies (Yamada et al. 2015). The nucleotide divergence of the two coccoid species relative to *G. rugatum* (over 1726 bp) was 1.27% (for unidentified coccoid dinotom 1) and 1.85% (for unidentified coccoid dinotom 2). A sequence of *Kryptoperidinium foliaceum* (from the strain UTEX LB1688) was located as a sister to Group 5 (Group 6). The marine planktonic dinoflagellate, *Peridiniopsis quinquecorne* (Group 7) was resolved as sister to the combined Group 5/6-clade, although BS was low (57%).

The five freshwater dinotoms formed a robust (100% BS value) *Peridiniopsis* clade. In Group 8, *Peridiniopsis jiulongensis* (KM217384) positioned at the base of the genus. Two sequences of *P.* cf. *kevei* (AB353770 and LC054935, the strain of the latter is DA08) formed a maximally supported clade which was also rooted deeply in the genus. The other three species also could be separated to three groups, Group 9 (*Peridiniopsis penardii*), Group 10 (*Peridiniopsis niei*), or Group 11 (*Peridinio*psis *minima*).

The Modified Endosymbiont SSU rDNA in *Durinskia kwazulunatalensis* sp. nov.

The SSU rRNA gene of the ESDs in two strains of *Durinskia kwazulunatalensis* has been modified with specific insert regions at four sites. Inserts of 89 bp, 86 bp, 86 bp, and 95 bp were inserted at positions of 347, 1019, 1318, 1549, respectively, of the corresponding SSU rDNA sequence of *N.* cf. *fonticola* (strain BA34, HM805036). Such insert regions have never been detected in free-living diatoms and other ESDs. In addition, a Blast search for these four insert regions recovered no homologous sequences.

Morphological Comparison of *Durinskia kwazulunatalensis* sp. nov. with *D. capensis*

D. capensis and *D. kwazulunatalensis* are phylogenetically closely related (fig. 4), but they harbor different species of ESD, which are distantly related (figs. 1 and 2). Therefore, a morphological comparison between the two is desirable.

Durinskia capensis

The morphology of currently investigated material corresponds to the original description of *D. capensis* (Pienaar et al. 2007) except for the cell size. All three strains of *D. capensis* showed two cell size classes; 22.6–28.4 μm long (mean = 24.7 μm, $n = 10$) and 20.0–27.3μm wide (mean = 22.7 μm, $n = 10$) or 11.6–16.3 μm long (mean = 14.2 μm, $n = 5$) and 13.2–14.2 μm wide (mean = 13.6 μm, $n = 5$). The cells of *D. capensis* are dorsoventrally flattened and are roughly rhombic in ventral view because each of the epitheca and the hypotheca form rounded triangles (figs. 5A1–A3 and 6*A*, *B*). The cingulum is median, left-handed and displaced by a distance roughly equal to its own width (fig. 6*A*). The sulcus is shallow, wider posteriorly and reaches the antapex of the

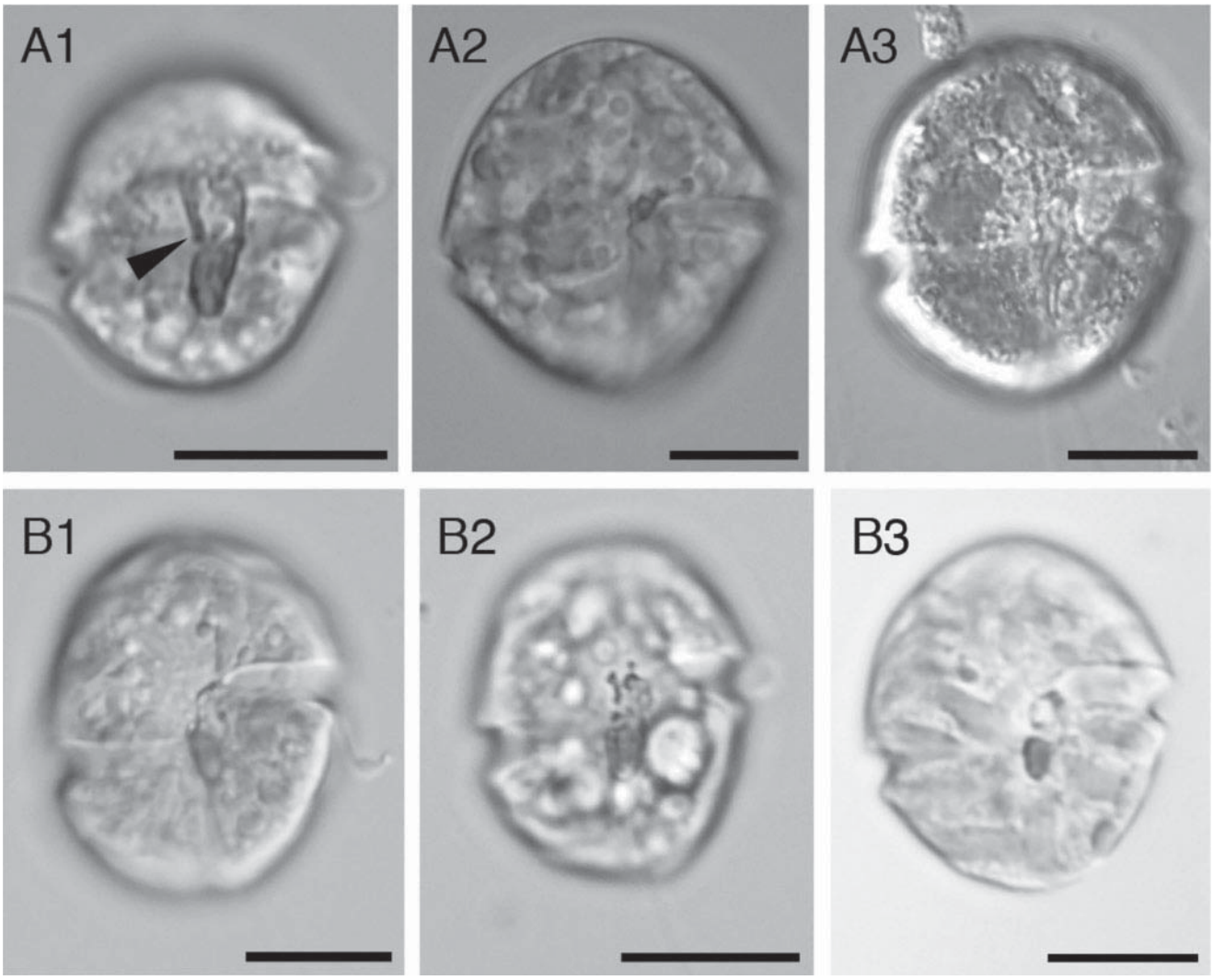

Fig. 5. Light micrographs of cells of two species of *Durinskia* from South Africa in ventral view. (*A*) *Durinskia capensis* and (*B*) *Durinskia kwazulunatalensis* N. Yamada, Sym et Horiguchi sp. nov. (A1) Kommetjie-2 strain. The eyespot is mid-ventral (arrow). (A2) Kommetjie-6 strain. (A3) Saldanha Bay strain (B1) Cx18 strain. (B2) Cx22 strain. (B3) HG181 strain. Scale Bar = 10 μm.

cell (fig. 6*A*). The chloroplasts are yellowish-brown, irregular in shape from disc-like to strap-like, and peripherally located. The characteristic red eyespot with a hook-like extension is located in the sulcal region (fig. 5*A*1–*A*3). The thecal plates are smooth with an identical arrangement to that of the original description (Pienaar et al. 2007): Po, x, 4′, 2a, 6″, 5c, 4s, 5‴, 2‴′ (fig. 6*A* and *B*). Pienaar et al. (2007) reported two variations in the shape of the 2a plate, pentagonal or hexagonal, but all samples we observed in this study have the hexagonal 2a plate.

Durinskia kwazulunatalensis

The cells of *Durinskia kwazulunatalensis* (fig. 5*B*1–*B*3) are dorsoventrally flattened and almost ovoid in ventral view, consisting of hemispherical epi- and hypothecae (figs. 5*B*1–*B*3 and 6*C*, *D*). The cingulum is median, left-handed and displaced by a distance approximating its own width (fig. 6*C*). The sulcus is shallow, wider posteriorly and reaches the antapex (fig. 6*C*). The cell is 18.4–27.4 μm long (mean = 21.6 μm, $n = 10$) and 15.8–20.0 μm wide (mean = 18.2 μm, $n = 10$). Small cells were never observed in any strains of this species. The peripheral discoidal or tube-like chloroplasts are brown to dark yellow (fig. 5*B*3). A conspicuous red eyespot with a hook-like extension is located in the sulcal region (fig. 5*B*1–*B*3).

The thecal plates are smooth. The plate tabulation is the same as that of *D. capensis*: Po, x, 4′, 2a, 6″, 5c, 4s, 5‴, 2‴′ (fig. 6*C*, *D*). The apical pore plate (Po) is small and circular to rectangular (fig. 6*E*). The canal plate (x) is small, rectangular, and situated between plates 1′, 2′, and 4′. The arrangement of the epitheca is asymmetrical. The size of the 1a and 2a plates is almost the same (fig. 6*D*). The subtle difference between *D. capensis* and *D. kwazulunatalensis* lies in the shape of the thecal plates in the epitheca; in *D. capensis*, 3″ plate is pentagonal and the 4″ plate is square (trapezoidal) (fig. 6*B*), whereas *D. kwazulunatalensis* has a 3″ plate that is square (trapezoidal) and a 4″ plate that is pentagonal (fig. 6*D*). These differences in precingular plates cause the difference in the relative positions and shapes of the 1a and 2a plates, in the two species. The 2a plate of *D. capensis* is hexagonal and

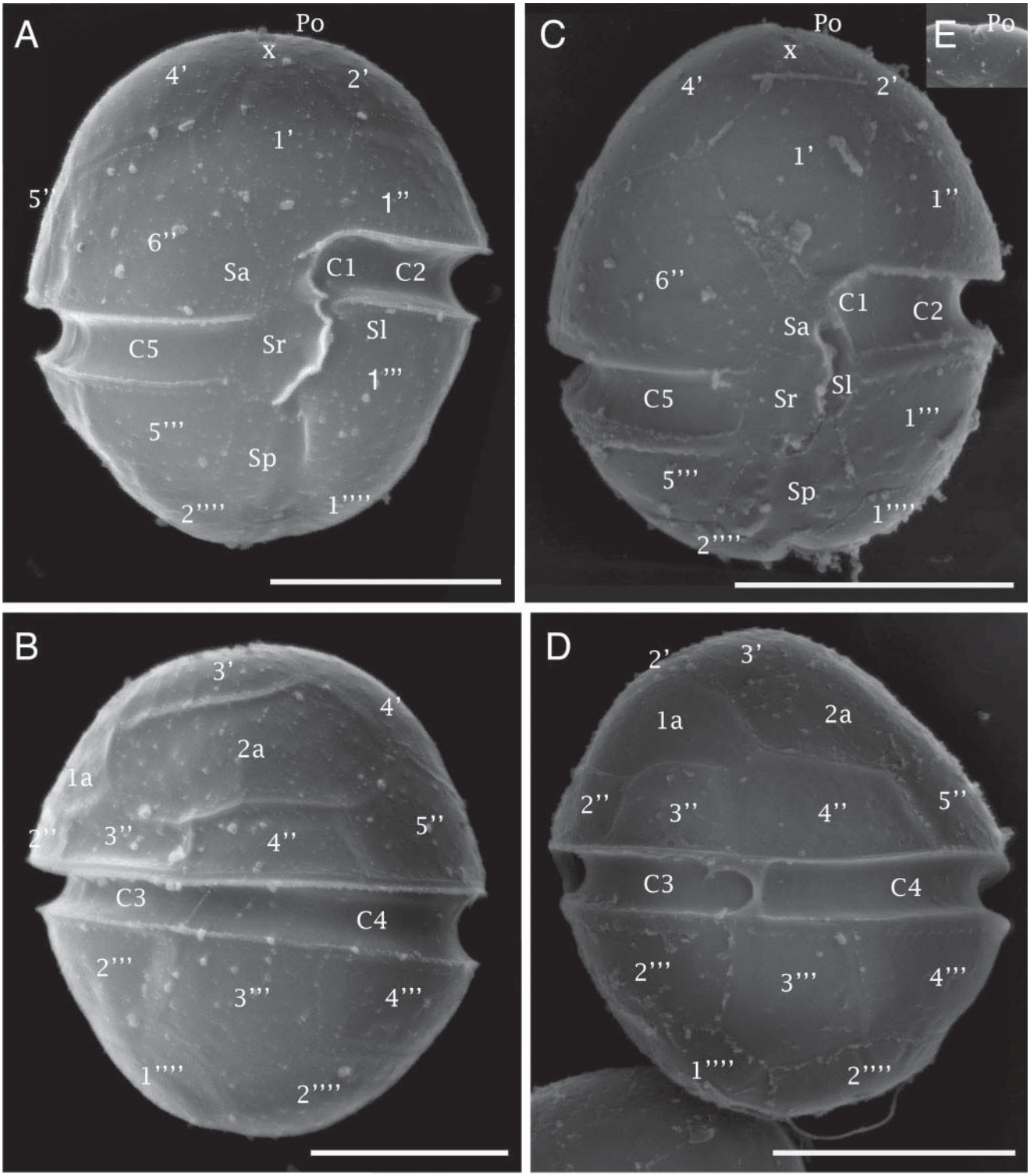

Fig. 6. Scanning electron micrographs of *Durinskia capensis* (strain Kommetjie-6, *A* and *B*) and *Durinskia kwazulunatalensis* N. Yamada, Sym et Horiguchi sp. nov. (strain Cx22, C and D). (*A* and *C*) ventral views and (*B* and *D*) dorsal views. (*E*) Enlarged view of apical pore plate (Po). Scale Bar = 10 μm.

located in the center of the dorsal side of the epitheca (fig. 6*B*). It is larger than the 1a plate and the suture it shares with this plate is located just above the 3″ plate. On the other hand, in *D. kwazulunatalensis*, the 1a and 2a plates are almost of equal size and the suture between them is located above the 4″ plate. The shape of 2a plate in *D. kwazulunatalensis* is pentagonal. The number and arrangement of plates in the cingulum, the sulcus and the hypotheca are the same as those of *D. capensis*. The cingulum consists of five plates (fig. 6*C and D*) and the sulcus consists of four plates. The left edge of the right sulcal plate (sr) extends toward the left and covers the flagellar pores (fig. 6*C*).

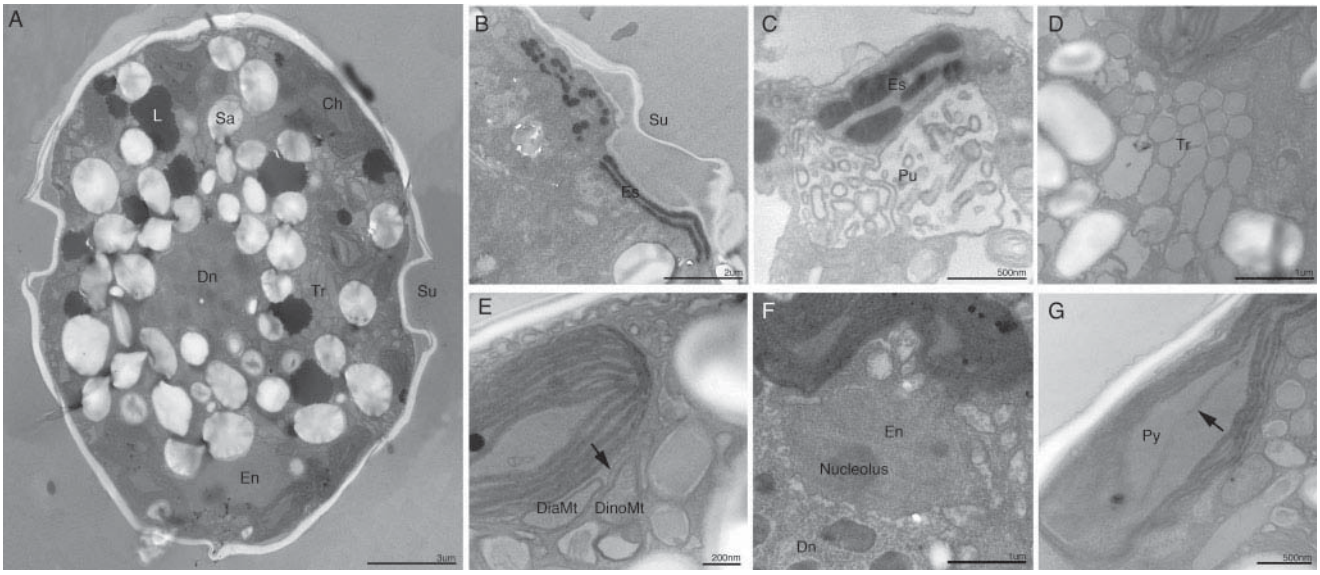

Fig. 7. *Durinskia kwazulunatalensis* N. Yamada, Sym et Horiguchi sp. nov. TEM. (*A*) Longitudinal section of Cx22 strain. (*B*) Eyespot located in the sulcal region. (*C*) Pusule underlies the sulcus near the eyespot. (*D*) Grouped trichocysts. (*E*) Cytoplasm of ESD separated from that of dinoflagellate by a single membrane (arrow). (*F*) Nucleus of ESD with nucleolus. (*G*) Pyrenoid invaded by thylakoid membranes. Ch = chloroplast, DiaMt = mitochondrion of ESD, DinoMt = mitochondrion of host dinoflagellate, Dn = nucleus of host dinoflagellate, En = nucleus of ESD, Es = eyespot, L = lipid, Py = pyrenoid, Pu = pusule, Sa = starch, Su = sulcus, Tr = trichocyst.

Because the ultrastructure of *Durinskia capensis* has been published (Pienaar et al. 2007), we restricted transmission electron microscopy observation to the ultrastructure of *Durinskia kwazulunatalensis* (fig. 7). The nucleus of the host is a typical dinokaryon and is located in the center of the cell. It is spherical and, contains granular chromosomes (fig. 7*A*). The eyespot is located near the sulcus and, in longitudinal section, is composed of two rows of spherical osmiophilic granules (fig. 7*B*). The pusule is also located in the sulcal region close to the eyespot (fig. 7*C*). *D. kwazulunatalensis* contains a number of grouped trichocysts (fig. 7*D*) and a large number of starch granules (fig. 7*A*). The ESD cytoplasm is separated from the dinoflagellate cytoplasm by a single membrane (fig. 7*E*). The nucleus contains a nucleolus (fig. 7*F*) and the chloroplasts are peripheral (fig. 7*A*). Each lamella is composed of three thylakoid bands (fig. 7*E*). The elongated pyrenoids are of the internal type, surrounded by lamellae, and are lens-shaped or rhomboidal (fig. 7*G*). Each pyrenoid matrix is usually thylakoid-free, but in some cases, it is partially invaded by longitudinally traversing thylakoid-like membranes (fig. 7*G*).

Discussion

Various Diatom Endosymbionts Exist in Dinotoms

Four molecular phylogenetic analyses, based on two endosymbiont-genes, the plastidial *rbc*L gene and the nuclear-encoded SSU rDNA, and on one host gene, the SSU rDNA, highlighted five issues: (1) the various ESDs of *Durinskia baltica*, *Kryptoperidinium foliaceum* and the six marine dinotoms cultured in this study were affiliated with *Nitzschia* and separated into six groups, each representing a different species; (2) each of the endosymbiont *Nitzschia* groups correspond to a separate species of host, with the exception of the *Galeidinium*/coccoid dinotoms clade (Group 5) and the *Peridiniopsis jiulongensis*/*P.* cf. *kevei* (Group 8), where two or three very closely related hosts share the same ESD (discussed in the next section). (3) Similarly, the five ESDs of the freshwater *Peridiniopsis* spp. were found to represent four species of freshwater diatoms belonging to three genera. (4) The ESD of marine *P. quinquecorne* formed a strongly supported clade with members of the marine centric diatom, *Chaetoceros* spp., as previously reported (Horiguchi and Takano 2006). (5) The SSU rDNA sequences of *D. baltica* strain CS-38 (CSIRO, as *Peridinium balticum* Levander) diverge from those of the two strains (HG171 and HG265) of *D. baltica*-like species by 1.13–1.53%, although their plate tabulations are identical (data not shown). In addition, their ESDs are derived from independent species of *Nitzschia*. We therefore opine that the *Durinskia*-like strains are independent species from *D. baltica* CS-38, and therefore refer to them as *Durinskia* cf. *baltica*. This study therefore revealed that different free-living diatoms were independently acquired as tertiary chloroplasts by different species of host dinoflagellates.

The affiliation of the ESDs of *Durinskia baltica* CS-38 and *D. kwazulunatalensis* were not clearly resolved as they changed considerably depending on the data set used. In *D. baltica*, this was either *Nitzschia palea* (*rbc*L) or an unidentified *Nitzschia* somewhat removed from *N. palea* (SSU), while, in *D. kwazulunatalensis*, this changed from a weak association with *Simonsenia aveniformis* (*rbc*L) to a fully supported link with *N.*cf. *fonticola* (SSU). It is possible that at least one of the affiliated diatom strains of each ESD was misidentified because the affiliation of the ESD of other hosts was reinforced by both data sets (e.g., *Durinskia capensis-Nitzschia draveillensis*). We provisionally identified the ESDs by selecting those

species with high support from either of the ESD phylogenies (table 1). Intriguingly, it was clarified that half of all (18, including the undescribed species) dinotoms, and nearly all marine dinotoms, have nitzschioid diatoms as endosymbionts (table 1). Of the marine dinotoms, only *Peridinium quinquecorne* has an ESD belonging to another genus, *Chaetoceros*. With the inclusion of *P. quinquecorne*, a total of 11 ESDs belonging to five genera (*Chaetoceros*, *Cyclostephanos*, *Cyclotella*, *Discostella* and *Nitzschia*), are incorporated in 14 dinotoms as their photosynthetic apparatus.

What Factors May Have Caused the Replacements of ESDs in Dinotoms?

The hosts of all dinotoms belong to a monophyletic group. Our results indicate that the ancestral *Nitzschia* ESD was not only independently replaced with species belonging to other genera, but also with other species of *Nitzschia*, in various host lineages (table 1). In the *Nitzschia*-type dinotoms alone, the replacement with other diatoms happened at least five times. Although we cannot directly define the benefits to the dinotom of replacing their established ESDs with others, we suggest that two factors have promoted this phenomenon in dinotoms.

First, we speculate that the maintained autonomy of ESDs in dinotoms facilitated the serial diatom replacements. The analyses of the nuclear transcriptome of ESDs in *D. baltica* (strain CS-38) and *K. foliaceum* (strain CCMP1326), indicated that a significant amount of the DNA resident in the ESD nucleus was transcribed (Burki et al. 2014; Hehenberger et al. 2016). Burki et al. (2014) also compared the numbers of plastidial genes that were horizontally transferred to the host nucleus in dinotoms to those in the Kareniaceae. Ninety haptophyte nuclear genes were transferred to the host dinoflagellate nucleus in the Kareniaceae, while a much more restricted number (nine) was transferred in dinotoms. Imanian et al. (2010, 2012) also sequenced the plastidial and mitochondrial genomes of the ESDs of the same strains of *D. baltica* and *K. foliaceum* mentioned above, and concluded that both genomes are highly conserved relative to those of the free-living diatom, *Phaeodactylum tricornutum*, with almost no gene loss. Intriguingly, the host dinoflagellates are able to retain the ESDs permanently and control their cell division (Figueroa et al. 2009) even though horizontal gene transfer is very limited in dinotom lineages. Dorrell and Howe (2015) noted a lessened integration of ESDs as reflected by a limited gene transfer, and proposed that this reduction in gene transfer from ESDs was possible because the host dinoflagellates retain the horizontally accessed pathways already derived from their previous red algal, peridinin-type chloroplast. Diatoms themselves possess a red alga-derived chloroplast, therefore, if the host nucleus retains the peridinin-type pathways, these could contribute to retention of the ESDs even though gene transfer from these ESDs is very limited (Larkum et al. 2007). Such historically acquired genomic factors in the host nucleus would promote the maintenance of ESD autonomy, and facilitate the serial replacement of ESDs.

Second, the acquisition of various taxa of ESDs might be a consequence of the diversity of habitats or life forms of the host dinoflagellates. Unlike members of the Kareniaceae or the two known species of *Lepidodinium*, the dinotoms inhabit diverse habitats. Marine species have been reported from sandy beaches (Tamura et al. 2005), seafloors (this study), tidal pools (Pienaar et al. 2007) as benthos, or marine surfaces as plankton (Horiguchi and Pienaar 1991), while, species of *Peridiniopsis* inhabit lakes (Takano et al. 2008) or rivers (Zhang et al. 2011) as freshwater plankton. These distributions correspond well with those of the nearest free-living relatives of their ESDs. Many *Nitzschia* spp. are benthic and psammophilic and *Nitzschia*-type dinotoms, with the exception of the planktonic *Durinskia baltica* (CS-38) and *Kryptoperidinium foliaceum*, are similarly benthic, being found in the sand of beaches, seafloors or tidal pools (supplementary table S1, Supplementary Material online). Such correlation between the habitats of endosymbionts and their hosts is evidenced in other dinotoms. The coastal planktonic dinotom *Peridinium quinquecorne* possesses the marine planktonic genus *Chaetoceros* (Takano et al. 2008), which often dominates the planktonic community of coastal waters (Chamnansinp et al. 2013). Thus, *P. quinquecorne* would commonly encounter and ultimately acquire a species of *Chaetoceros*. The freshwater planktonic dinotoms (*Peridiniopsis* spp.) have been shown to have freshwater planktonic diatoms as endosymbionts (Zhang et al. 2011, 2014; this study). Such correlations suggest that the acquisition of various diatoms happened subsequent to the diversification of the dinotom host and its migration into a novel habitat.

One example is to be found in the *Galeidinium rugatum*—coccoid dinoflagellates group (Group 5). The three strains here represent independent species based on their genetic and morphological features (Yamada et al. 2015) and their sample locations are widely separated (Palau, South Africa and Japan, supplementary table S1, Supplementary Material online). Although they have diverged in terms of their morphology, genetic make-up, and geographical locations, they share the same species of ESD. We suggest that their similar life styles might result in them sharing the same ESD. All three spend most of their life cycle as a benthic, non-motile stage (temporary cysts) in the sands, with an extremely short motile phase, probably representing the feeding stage (Tamura et al. 2005; Yamada et al., unpublished data). In *Galeidinium rugatum*, swimming is restricted to only 0.5–1 h per day (Tamura et al. 2005), making it difficult for them to feed on an organism and ultimately acquire it through co-evolution as a new endosymbiont. Therefore, it is speculated that the ESD in these forms was acquired before the development of the dominant sessile stage of their life history and when they probably still had a dominant motile (feeding) stage.

We speculate that the shared ESD in *Peridiniopsis jiulongensis* and *P.* cf. *kevei* (Group 8) represents the original endosymbiont for this genus. Both host species are rooted deep in the genus in the SSU rDNA phylogeny, which indicates that both are early species. We suggest that the ancestor of *Peridiniopsis* (probably a near-relative of *P. jiulongensis*)

initially replaced its *Nitzschia*-type ESD with a *Discostella*-type, and subsequent radiation of this resulted in other *Peridiniopsis* species (*P. minima*, *P. niei*, and *P. penardii*) that independently replaced the *Discostella*-type ESD with other freshwater diatoms.

Further to such genetic and ecological considerations, what is required is an investigation of the physiological mechanisms that dinotoms employed to maintain a newly acquired ESD. Such physiological, together with genomic, studies could explain the reason why all dinotoms have restricted their selection of endosymbionts to diatoms, and why these ESDs needed to be replaced. In addition, the ESDs of dinotoms might prove to be more diverse than we have yet shown because the endosymbionts of three dinotoms, *Dinothrix paradoxa*, *Durinskia agilis*, and *Gymnodinium quadrilobatum* remain unsequenced. We suggest there might be heterotrophic forms of the dinotom host or dinotoms that possess temporary ESDs.

Durinskia kwazulunatalensis sp. nov. from Marina Beach, South Africa

Durinskia capensis and *D. kwazulunatalensis* are superficially very similar in morphology, but we regard them as independent species for the following reasons.

First, they possess different species of ESD, which are distantly related (figs. 1 and 2). *D. capensis* possesses a diatom closely related to *Nitzschia draveillensis*, while the ESD of *D. kwazulunatalensis* is more like *Nitzschia* cf. *fonticola*. Moreover, the three strains of *D. kwazulunatalensis* have four novel inserts to their endosymbiotic SSU rDNA not found in the ESDs of any other dinotoms, which preserve the majority of their SSU rDNA relative to those of their free-living counterparts. Second, it is sufficient to resolve the hosts of *D. capensis* and *D. kwazulunatalensis* are resolved as independent phylogenetic clades with 96% or 91% BS value, respectively. This difference is considered enough to regard them as discrete taxonomic entities.

This interpretation is reinforced by morphological data. *D. kwazulunatalensis* can be distinguished from *D. capensis* by the shape and arrangement of the thecal plates in the epitheca, i.e. the position of the 1a and 2a plates and the shape of the 3″, 4″, and 2a plates. In the original description of *D. capensis* (Pienaar et al. 2007), two variations of the shape of thecal plates in *D. capensis* were reported. One of these variations, where the shape of the 3″, 4″, and 2a plates are pentagonal, square, and hexagonal, respectively (fig. 4*b* in Pienaar et al. 2007), was consistently found in all strains of *D. kwazulunatalensis*, while, those of all samples of *D. capensis* currently investigated were consistently found to be square, pentagonal and pentagonal respectively (fig. 4*c* in Pienaar et al. 2007). Therefore, we concluded that the two species could be distinguished by the shape and positions of these key plates (fig. 8) and that the first variant of *D. capensis* (fig. 4*b* in Pienaar et al. 2007) is probably synonymous with *D. kwazulunatalensis*. The ultrastructure of pyrenoid invasion by thylakoids is also distinguishable; in *D. kwazulunatalensis* they are invaded by few string-like thylakoids, while those of *D. capensis* are invaded by many circular thylakoids (Pienaar et al. 2007). The sum total of all these genetic and morphological differences in the hosts, together with the fact that their ESDs are distantly related, led to the conclusion that *D. capensis* and *D. kwazulunatalensis* are sufficiently divergent to warrant their separation at the species level. Here we describe the strains collected from Marina Beach, South Africa, as a new species of the genus, *Durinskia*, *D. kwazulunatalensis* sp. nov.

Durinskia kwazulunatalensis N. Yamada, Sym et Horiguchi sp. nov.

Description

Cells ovoidal in ventral view, dorsiventrally flattened, both epitheca and hypotheca almost hemi-circular; 18.4–27.4 μm long and 15.8–20.0 μm wide; dinokaryon typical, spherical, and central; chloroplasts brownish yellow, discoidal, and peripheral; red eyespot with hook-like extension (type D *sensu* Moestrup and Daugbjerg 2007) on the left of the sulcus; thecal plate smooth, plate formula: Po, x, 4′, 2a, 6″, 5c, 4s, 5‴, 2⁗, the suture between 1a and 2a approximately central on the dorsal side of epitheca. Endosymbiont affiliated with *Nitzschia* cf. *fonticola* like diatom.

Pigment Composition

The pigment composition of the three strains of *Durinskia kwazulunatalensis* was investigated in Yamada et al. (2015) as *Durinskia* sp. The three strains mainly contain chlorophyll c_2, chlorophyll c_1, a fucoxanthin-like carotenoid, fucoxanthin, diadinoxanthin, diatoxanthin, zeaxanthin, chlorophyll *a*, β-ψ carotene, pheophytin *a*, and β-carotene (in order of retention time). Because no reports of the pigment composition for *D. capensis* exist, we could not compare pigment compositions between them.

Holotype: The SEM stub used for figure 6 has been deposited in the herbarium of the Faculty of Science, Hokkaido University (SAP No. 115074).

Type locality: Marina Beach, City of Margate, KwaZulu-Natal Province, South Africa (30°56′13.9″S, 30°18′26.8″E).

Habitat: Tidal pools in rocky shore.

Etymology: *kwazulunatalensis* named after their sampling location, KwaZulu-Natal Province in South Africa.

Materials and Methods

Sample Collections and Cultures

Seawater and sand samples were collected from tidal pools or sandy beaches (supplementary table S1, Supplementary Material online). Each sand sample was placed in a plastic cup and enriched with Daigo's IMK medium (Nihon Pharmaceutical Co., Ltd., Tokyo). The samples were cultured at 20 or 25 °C, temperatures approximating those of the water at the time of sampling of each species, with an illumination of 60 μmol·photons·m^{-2}·s^{-1} under a 16:8 h light:-dark cycle (supplementary table S1, Supplementary Material online). The tidal pool water samples were mixed with an equivalent amount of Daigo's IMK medium, and cultured in the same conditions as those outlined above. These crude

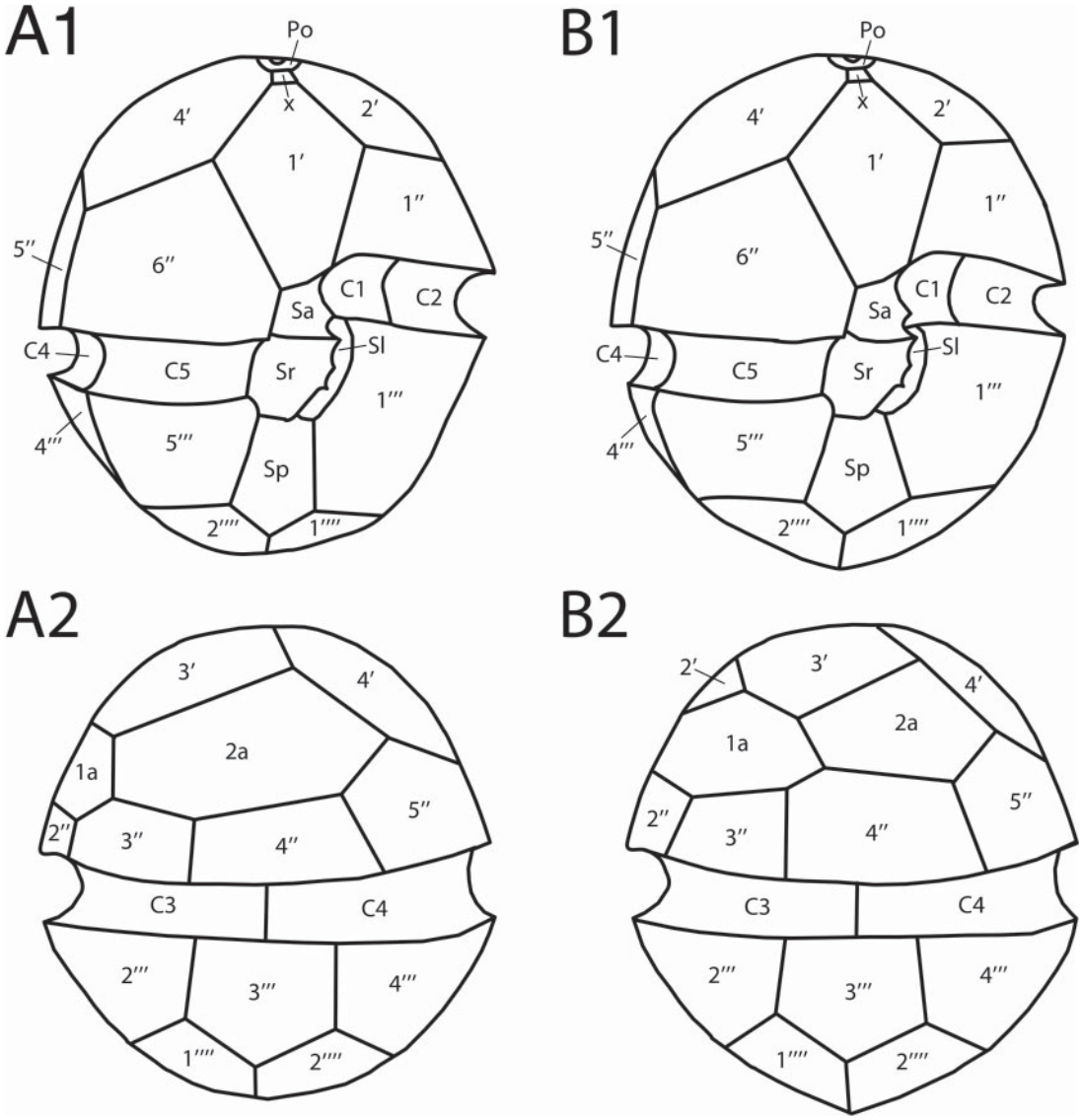

Fig. 8. Comparative drawing of new species, *Durinskia kwazulunatalensis* N. Yamada, Sym et Horiguchi sp. nov. and its closely related species, *D. capensis*. (*A*) *Durinskia capensis* (*B*) *Durinskia kwazulunatalensis* sp. nov.

cultures of water/sand samples were checked daily using an inverted microscope (Olympus CKX41, Tokyo) for two weeks for the development of any dinoflagellate populations. Clonal cultures of cells were isolated using capillary pipettes with several rinses in drops of sterilized medium. Initially, *Durinskia capensis* clonal cultures could not be established, but a supplementation of the medium with a small dried piece of the brown alga *Ecklonia maxima*, sampled from same location as *D. capensis*, allowed for the maintenance of cultures up to 7 months.

Gene Selection for Molecular Phylogenies

The phylogenetic positions of the hosts and ESDs were inferred using the SSU rDNA of the host, and the plastidial *rbc*L gene and the nuclear SSU rDNA of the ESDs independently. It is acknowledged that these genes are problematic for phylogenetic analyses because they have different evolutionary rates relative to those of other genes and, in particular, morphological variation does not correspond with change in the SSU rRNA gene, resulting in intra-individual variations (Delwiche and Palmer 1996; Evans et al. 2007). However, to date, only three genes of hosts (SSU rDNA, LSU rDNA, and ITS regions), and two genes of ESDs (*rbc*L gene and SSU rDNA), are available to allow for the comprehensive phylogenetic analysis of dinotoms. Currently 25 sequences of SSU rDNA (from 15 species), 10 of LSU rDNA (from five species), or 10 of ITS regions (from five species) sequences for host dinotoms are registered in NCBI (not including our data in this study). For ESDs of dinotoms, 11 sequences of SSU rDNA (from eight species), nine of the *rbc*L gene (from eight species), three of ITS regions (from one species), and three of the cytb gene (from one species) are registered in NCBI (again, excluding our current data). *D. baltica* CS-38 and *K. foliaceum* CCMP1326 are the only species which could be analyzed using additional genes, because the whole mitochondrial and chloroplast genomes of their ESDs have been sequenced. Therefore, our only option, to be as inclusive of all characterized dinotoms as possible,

was to restrict our analyses to the SSU rDNA (host and ESD) and *rbc*L gene (ESD).

DNA Extraction and PCR Amplification

DNA extractions were performed using the QuickExtract FFPE RNA Extraction Kit (Epicentre, Wisconsin). A single dinoflagellate cell, observed with an inverted microscope, was isolated by capillary pipettes, rinsed several times in serial drops of sterilized culture medium and transferred into 10 μl of QuickExtract FFPE solution. The solution was heated to and maintained at 56 °C for 1 hour, followed by 98 °C for 2 min was after which it used as template DNA without dilution. For PCR amplification of two genes, the nuclear-encoded SSU rDNA of the host dinoflagellates and the chloroplast-encoded *rbc*L gene of the ESD, 25 cycles of the following steps were used after an initial cycle of denaturation at 94 °C for 1 min: denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s. The final extension cycle was at 72 °C for 7 min. To amplify the SSU rDNA, the following universal primers were used; SR1b and SR3, SR2spin and SR7, SR4 and SR9p, and SR6 and SR11 (Nakayama et al. 1996; Yamada et al. 2014). For the *rbc*L gene of the ESD, the following sets of primers were used; DiatrbcL1- DiatrbcL3, DiatrbcL2–DiatrbcL5, and DiatrbcL4–DiatrbcL6 (Tamura et al. 2005).

The method used above for the SSU rDNA always recovered the sequence from the host dinoflagellates. To amplify the SSU rDNA of the ESDs, a preceding PCR product was obtained using the primers SR1 (F: Nakayama et al. 1996) and DiaSR12 (R: TAGACAAGTTCTCGCRA). The latter primer was specially designed for diatoms to recover almost the full length of the diatom SSU rDNA without contamination from the corresponding gene of the host dinoflagellates. The preceding PCR consisted of 1 initial cycle of denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min, and the final extension cycle was at 72 °C for 7 min. A 100-fold dilution of the resultant PCR product was used as a template of diatom SSU rDNA and was amplified using the same method for SSU rDNA of the host dinoflagellates.

The PCR products of the three genes were purified and sequenced using an ABI PRISM Big Dye Terminator (Applied Biosystems, Foster City). The sequence reactions were run on a DNA autosequencer ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City). Both forward and reverse strands were sequenced.

OTU Sequence Selection

To include a fair selection of OTUs, all sequences of dinotoms (25 registered and five currently characterized sequences of host SSU rDNA, nine registered and 12 new sequences of *rbc*L gene of ESDs, and 11 registered and seven new sequences of ESD SSU rDNA) were blasted in NCBI database. The top 100 highly similar sequences (in order of total score) in each sequence were selected as potential OTUs. We also added to the sequences by searching the NCBI taxonomy database by name for target genera (*Nitzschia*, *Cyclotella*, *Cyclostephanos*, *Discostella*, and *Stephanodiscus*) as potential OTUs for ESDs. These sequences were aligned by ClustalW in MEGA 5.2.2 (Tamura et al. 2011), and their inferred phylogenetic positioning for each data set was determined using ML trees created by MEGA 5.2.2. Taxa, identified on these ML trees as being distantly related from the dinotoms, were excluded from further analysis. Nuclear sequences of all dinoflagellates, even if unrelated to dinotoms, but with the exception of environmental sequences, were retained as potential OTUs to position the host dinotoms. Two or three representative sequences from each clade were then ultimately selected as determinate OTUs. After a Blast search, three SSU ESD sequences of *Peridiniopsis penardii* (HM596547, HM596548, and HM596549) and one of *Peridiniopsis* cf. *kevei* (LC054936, strain HG327) were excluded from the phylogenetic analyses because they were too short (those of the *P. penardii* sequences were only 13.1–13.6% of that of *P. penardii* AB353773, 1738 bp; and that of *P.* cf. *kevei* was 87.9% of that of *P.* cf. *kevei* LC054935, 1745 bp). We randomly selected a single sequence of the host SSU rDNA of *K. foliaceum* for phylogenetic analysis because all the sequences of this gene available were determined from the same strain, UTEX LB1688. As a consequence of all these conditions, 43 OTU sequences were used for the host SSU rDNA analysis and 46 sequences for the *rbc*L gene analysis. The phylogenetic tree based on the diatom nuclear-encoded SSU rDNA was split in two based on diatom classification (pennate vs. centric) because the number of sequences exceeded 100, making it difficult to recognize individuals on the tree. For the phylogenetic tree of pennate diatoms, 56 OTU sequences were used, while 30 sequences were adopted in the centric diatom tree.

Molecular Phylogeny Analysis

The accession numbers of the species used in the various analyses are indicated in figures 1–4. The OTU sequences, selected as outlined in the former section, were aligned by ClustalW in MEGA 5.2.2 (Tamura et al. 2011). Before aligning the ESD SSU rDNA sequences, the four novel small inserts in *D. kwazulunatalensis* sp. nov. were excluded. The following were used as outgroups for each phylogenetic analysis: For host dinoflagellate phylogeny, *Toxoplasma gondii* (L24381), *Chromera velia* (JN986792) *and Paramecium caudatum* (HE662763); for the *rbc*L gene analysis, *Chromulina nebulosa* (AF155876) and *Chrysocapsa vernalis* (AF155877), and for the ESD SSU rDNA phylogeny, *Chaetoceros* spp. and *Peridinium quinquecorne* (AB246746). The aligned sequences were analyzed by the maximum likelihood (ML) method using PhyML 3.0 beta version (Guindon et al. 2010). An additional bootstrap analysis (100 replicates) was also performed. The selected model for ML analysis by the Akaike Information Criterion was the GTR + G+I + F (for SSU rDNA of host dinoflagellates, and the *rbc*L gene of the ESDs), or TN93 + G + I+F (for both trees based on the ESD SSU rDNA). A heuristic search was performed using a SPR algorithm and the BIONJ tree (Gascuel 1997) as the starting tree.

Light and Scanning Electron Microscopy

All cultured species were observed using a ZEISS Axioskop2 Plus light microscope (Carl Zeiss Japan, Tokyo) and photographs were taken with a ZEISS AxioCam ERc 5 s to identify species. In addition, we observed the detailed surface morphology of *Durinskia kwazulunatalensis* Cx22 strain and *Durinskia capensis* Kommetjie-6 strain by scanning electron microscope (SEM). The contrast of LM photo was modified using Adobe Photoshop CS6 (Adobe Systems Inc., California). For SEM observation, the dinoflagellate cells were collected by centrifugation at 2000 rpm for 5 min and the cell pellet was fixed in 0.4% Lugol's solution (KI 100 g, I_2 50 g and glacial acetic acid 100 ml in 1 l DW) diluted using PES culture medium (Provasoli 1968) for 1 h at room temperature. After rinsing once in sterilized culture medium and then twice in distilled water for 10 min in each wash, a drop of water containing cells was placed on a poly-L-lysine coated SEM glass plate and the cells were allowed to settle for 10 min. The cells attached to the SEM plate were then gradually dehydrated by introducing the SEM plate to each of an increasing series of ethanol concentrations (25%, 30%, 50%, 70%, 80%, 90%, and 95%) for 10 min each, and thoroughly dehydrating in 100% ethanol twice, each for 30 min. Finally, the cells were critically point dried (HITACHI HCP-2, Tokyo), sputter-coated with gold for 120 s at 15 mA (HITACHI E-1045, Tokyo) and viewed with a SEM (S-3000N, HITACHI, Tokyo).

Transmission Electron Microscopy

The ultrastructure of *Durinskia capensis* was already published (Pienaar et al. 2007) and hence, only *Durinskia kwazulunatalensis* was examined in this study for the morphological comparison. Cells were harvested by centrifugation at 2000 rpm. After removing the supernatant, the sample was fixed for 30 min at room temperature in a cocktail of 1% (w/v) OsO_4 and 2.5% (v/v) glutaraldehyde in 0.5 M sucrose made up in 0.5 M phosphate buffer (pH 7.1). The cells were rinsed once in the same buffer with 0.5 M sucrose and subsequently twice in buffer alone. All rinses were allowed to stand for 10 min. Washed cells were fixed again using 2% OsO_4 made up in 0.5 M phosphate buffer for 1 hour at room temperature. After fixation, the cell pellet was dehydrated for 10 min in each of the following concentrations of acetone, 30%, 50%, 70%, 80%, 90%, and 95%, finally, dehydrated completely by washing twice in 100% acetone each time for 30 min. Infiltration of samples was then carried out with an acetone–resin mixture. 100% acetone and resin (Agar Low Viscosity Resin, Agar Scientific Limited, Essex) were mixed in a 3:1, a 1:1, and a 1:3 ratio and the sample was introduced to each higher resin concentration sequentially for 15 min. Finally, the cells were embedded in 100% resin and, after 30 min, polymerized at 65 °C for 20 h and sectioned using a diamond knife on an ultramicrotome (LEICA EM UC6, Germany). Sections were picked up on formvar coated one-slot grids. Because the transmission electron microscope used (H-7650, HITACHI, Tokyo) is equipped with a high contrast mode, the sections were viewed without staining.

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