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Morphological and Phylogenetic Characterization of New *Gephyrocapsa* Isolates Suggests Introgressive Hybridization in the *Emiliania/Gephyrocapsa* Complex (Haptophyta)

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The coccolithophore genus *Gephyrocapsa* contains a cosmopolitan assemblage of pelagic species, including the bloom-forming *Gephyrocapsa oceanica*, and is closely related to the emblematic coccolithophore *Emiliania huxleyi* within the Noëlaerhabdaceae. These two species have been extensively studied and are well represented in culture collections, whereas cultures of other species of this family are lacking. We report on three new strains of *Gephyrocapsa* isolated into culture from samples from the Chilean coastal upwelling zone using a novel flow cytometric single-cell sorting technique. The strains were characterized by morphological analysis using scanning electron microscopy and phylogenetic analysis of 6 genes (nuclear *18S* and *28S* rDNA, plastidial *16S* and *tufA*, and mitochondrial *cox1* and *cox3* genes). Morphometric features of the coccoliths indicate that these isolates are distinct from *G. oceanica* and best correspond to *G. muellerae*. Surprisingly, both plastidial and mitochondrial gene phylogenies placed these strains within the *E. huxleyi* clade and well separated from *G. oceanica* isolates, making *Emiliania* appear polyphyletic. The only nuclear sequence difference,

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1 bp in the 28S rDNA region, also grouped *E. huxleyi* with the new *Gephyrocapsa* isolates and apart from *G. oceanica*. Specifically, the *G. muellerae* morphotype strains clustered with the mitochondrial β clade of *E. huxleyi*, which, like *G. muellerae*, has been associated with cold (temperate and sub-polar) waters. Among putative evolutionary scenarios that could explain these results we discuss the possibility that *E. huxleyi* is not a valid taxonomic unit, or, alternatively the possibility of past hybridization and introgression between each *E. huxleyi* clade and older *Gephyrocapsa* clades. In either case, the results support the transfer of *Emiliania* to *Gephyrocapsa*. These results have important implications for relating morphological species concepts to ecological and evolutionary units of diversity.

Key words: Coccolithophores; *Emiliania huxleyi*; *Gephyrocapsa muellerae*; *Gephyrocapsa oceanica*; hybridization; species concept; phylogeny.

Introduction

Emiliania huxleyi Lohmann (Hay et al. 1967) and *Gephyrocapsa oceanica* Kamptner (Kamptner 1943) are the most abundant extant coccolithophore morphospecies and thus play key roles in ocean carbon cycling due to their importance as both primary producers and calcifiers. These two morphospecies are in the family Noëlaerhabdaceae, within the Isochrysidales (Edvardsen et al. 2000), a monophyletic order within the haptophyte class Prymnesiophyceae. Along with other members of the noëlaerhabdaceaen genera *Gephyrocapsa* and *Reticulofenestra* Hay, Mohler and Wade (Hay et al. 1966), they inhabit coastal shelf and open ocean environments and are abundant in modern oceans and in fossil assemblages (Young et al. 2003). The ubiquitous *E. huxleyi* frequently forms extensive "milky water" blooms in high latitude coastal and shelf ecosystems (Winter et al. 1994). *Gephyrocapsa oceanica*, which is restricted to lower latitude waters, also occasionally blooms in transitional coastal waters in the Pacific Ocean (e.g., Blackburn and Cresswell 1993; Kai et al. 1999). Other related extant morphospecies seem to exhibit more discrete distributions, possibly restrained by more stringent environmental tolerances. For example, *Gephyrocapsa muellerae* Bréhéret (Bréhéret 1978) is associated with cool nutrient-rich waters, whereas *Gephyrocapsa ericsonii* McIntyre and Bé (McIntyre and Bé 1967) is present in lower latitude waters (Ziveri et al. 2004). The question of how the *E. huxleyi* morphospecies (or species-complex) successfully colonized very diverse surface ocean habitats while its close relatives remained more ecologically restricted has broader implications for understanding controls on phytoplankton adaptation to new and changing habitats.

The bloom-forming *E. huxleyi* has long been a model for culture-based ecophysiological studies (Paasche 2001; Westbroek et al. 1993). This status has been reinforced by establishment of genetic resources including EST libraries (von Dassow et al. 2009; Wahlund et al. 2004), mitochondrial and plastid genome sequences (Sanchez Puerta et al. 2004, 2005) and a full draft genome assembly that has been compared by genome-resequencing (Read et al. 2013) and comparative genome hybridization (Kegel et al. 2013) of 15 other strains (von Dassow et al. 2014). These latter studies notably suggested that extensive genome variability (as much as 25% variability in gene content) might occur between strains of the *E. huxleyi* morphospecies (we use the term morphospecies to highlight that it might be considered to represent a complex of cryptic species with similar morphologies). *E. huxleyi* and *G. oceanica* strains are numerically abundant in culture collections; for example, the Roscoff Culture Collection (RCC, France), the Provosoli-Guillard National Center for Marine Algae and Microbiota (NCMA, USA), and the National Institute for Environmental Studies Microbial Culture Collection (NIES, Japan), distribute, respectively 486, 23, and 12 strains of *E. huxleyi* and 85, 2, and 15 strains of *G. oceanica*. Other morphospecies of the Noelaerhabdaceae have been lacking from culture collections, limiting the ability to reconstruct the recent evolutionary history of this important group.

Noëlaerhabdaceaen coccolithophores share a distinctive coccolith structure, with R-unit crystals forming the grill, both shields and the two-layered tube, while the V-units are vestigial (Hoffmann et al. 2014; Young et al. 1992). *Gephyrocapsa* morphospecies are distinguished from other noelaerhabdaceaen genera by the extension of a few of the inner tube crystals on opposite sides of the

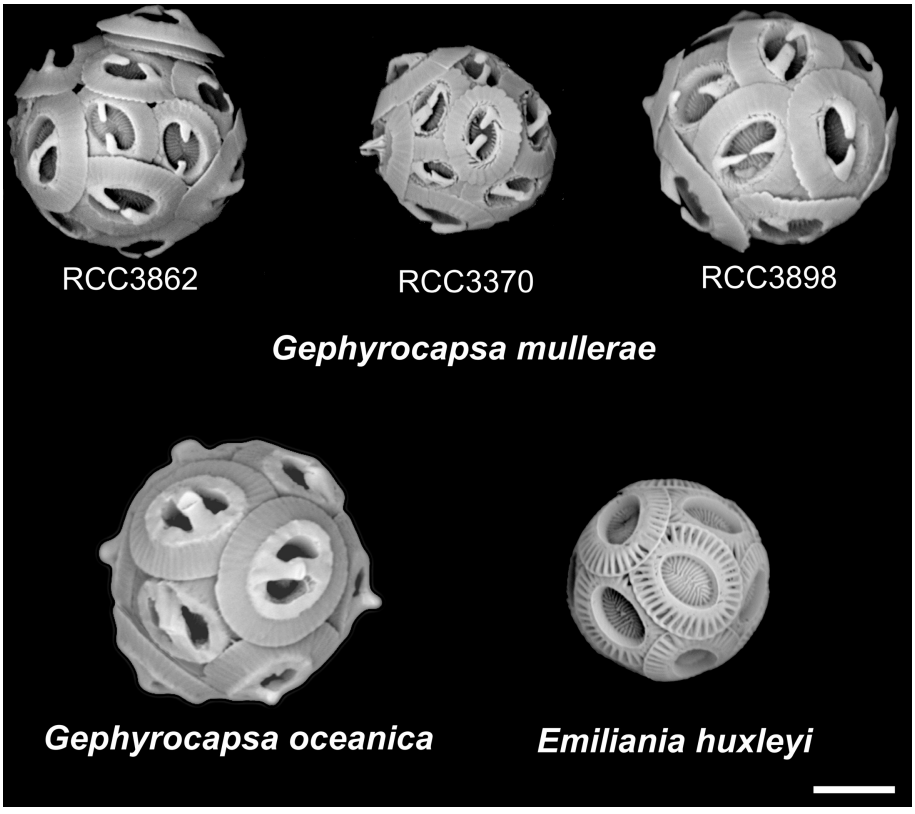

Figure 1. SEM images of the three new isolates of *Gephyrocapsa muellerae* and of representative strains of *Gephyrocapsa oceanica* and *Emiliania huxleyi*. Scale = 2 μm.

coccolith to form a conjunct bridge over the central area of the coccolith. From paleontological evidence, it has been hypothesized that *Emiliania* evolved from the *Gephyrocapsa* complex via *Gephyrocapsa protohuxleyi* McIntyre (McIntyre 1970), a taxon often considered to be a conspecific variant of the extant morphospecies *G. ericsonii* (e.g. Cros and Fortuño 2002; Young et al. 2004). The first appearance of *E. huxleyi* in the fossil record was relatively recent (290 Ka; Raffi et al. 2006), while *Gephyrocapsa* were first unambiguously present in the Pliocene, around 3.5 Ma (Samtleben 1980). There are a few older records (e.g., Jiang and Gartner 1984; Pujos 1987) but these are not well-documented. *Gephyrocapsa* became abundant from around 1.7 Ma (Raffi et al. 2006) with a succession of different morphospecies occurring (Matsuoka and Okada 1990). The dominant noëlaerhabdaceaen taxon shifted to *G. muellerae* around 110 Ka and then to *E. huxleyi* around 87 Ka (Hine and Weaver 1998; Thierstein et al. 1977).

Different morphometric concepts have been used to distinguish and describe *Gephyrocapsa* morphospecies or morphotypes for taxonomic and stratigraphical use, especially in the fossil record. The simplest concept for distinguishing *Gephyrocapsa* morphospecies was proposed by McIntyre et al. (1970) using only coccolith size and bridge-angle. He defined three main morphospecies occurring in the Holocene, *G. oceanica* (bridge angle >45°), *Gephyrocapsa caribbeanica* Boudreaux and Hay (Hay et al. 1967); bridge angle <45°), and *G. ericsonii* (small coccoliths, <2.2 μm in length). The name *G. caribbeanica* has since been shown to be properly applied to a rather different fossil morphospecies and the extant morphotype is now termed *G. muellerae* (Young et al. 2003). Adopting a similar concept, Bollmann (1997) conducted an extensive study of Holocene (sediment) assemblages of *Gephyrocapsa*, leading to definition of 6 informally named types that were related to environmental conditions and biogeography. The six types (Fig. 2) are: *Gephyrocapsa* Equatorial (GE; equatorial type having a mean bridge angle >56° and mean coccolith length between 3.1 and 3.9 μm), *Gephyrocapsa* Oligotrophic (GO; subtropical central gyre, bridge angle 27-56°, coccolith length > 3.1 μm), *Gephyrocapsa* Transitional (GT; transitional zones

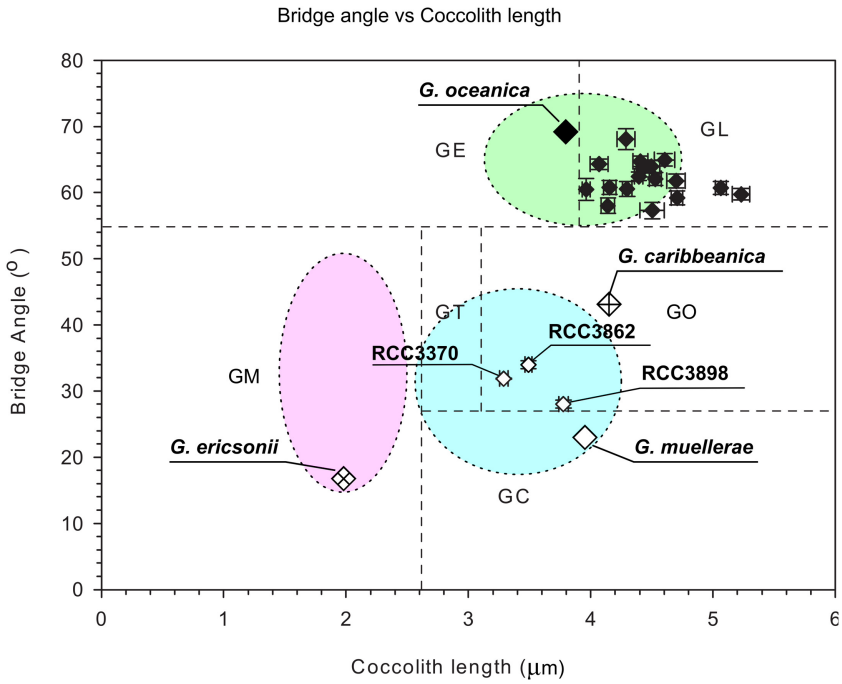

Figure 2. Scatter plots of mean coccolith length versus mean bridge angle of morphotypes defined in Holocene sediment samples with plotted original descriptions of *Gephyrocapsa caribbeanica, Gephyrocapsa ericsonii, Gephyrocapsa muellerae* and *Gephyrocapsa oceanica* and applied concept (circle for *G. ericsonii, G. muellerae* and *G. oceanica*; after Bollmann 1997 and Young et al. 2003): *Gephyrocapsa* Equatorial (**GE**), *Gephyrocapsa* Oligotrophic (**GO**), *Gephyrocapsa* Transitional (**GT**), *Gephyrocapsa* Cold (**GC**), *Gephyrocapsa* Larger (**GL**), *Gephyrocapsa* Minute (**GM**), compared to the 3 *Gephyrocapsa muellerae* isolates and 16 *Gephyrocapsa oceanica* culture strains. Error bars represent the standard error.

(mean sea surface temperature between 19 and 20 °C), bridge angle 27-56°, coccolith length 2.4 - 3.1 µm), *Gephyrocapsa* Cold (GC; transitional to subarctic cold waters, bridge angle <27°, coccolith length > 2.4 µm), *Gephyrocapsa* Larger (GL; high productivity temperate zones, bridge angle >56°, coccolith length > 3.9 µm), and *Gephyrocapsa* Minute (GM; no clear environmental preference, bridge angle 20-50°, coccolith length < 2.4 µm). These designations were tentatively related by Bollmann (1997) to previously described mosphospecies (GE = *G. oceanica*, GC = *G. muellerae*, GO = *caribbeanica*, GM = *G. aperta* Kamptner (Kamptner 1956), GT = *G. margereli* Bréhéret (Bréhéret 1978), GL = *G. oceanica rodela* Samtleben (Samtleben 1980), but their genetic relatedness and biological significance are not known. Bollmann and Klaas (2008) showed that in the plankton there was continuous variation between the morphotypes GE and GL (I.e. within *G. oceanica*) but that GM (i.e. *G. ericsonii*) and GC (i.e. *G. muellerae*) were morphologically discrete, they did not find the morphotypes GT or GO in the plankton.

Molecular phylogenetic studies using marker genes have proven useful for resolving the taxonomic pertinence of phenotypic microdiversity. For the Noëlaerhabdaceae, the use of classical markers revealed a close genetic relationship between *E. huxleyi* and *G. oceanica*: the two morphospecies have identical sequences of nuclear 18S rDNA (Edvardsen et al. 2000; Medlin et al. 1996) and plastidial rbcL (Fujiwara et al. 2001), probably due to the recentness of their diversification. More recent studies were able to separate these morphospecies, partially with analysis of the plastidial tufA gene (Cook et al. 2011; Medlin et al. 2008), and completely with the nuclear 28S rDNA (Liu et al. 2009) and various mitochondrial genes (Bendif et al. 2014; Hagino et al. 2011). The variability of the plastidial tufA gene and the mitochondrial cox1 and cox3 genes among *G. oceanica* strains suggested potential cryptic differentiation within this morphospecies, while the inability of tufA to completely separate *E. huxleyi* and *G. oceanica* strains suggested that this gene may have been subject to incomplete lineage sorting or past and/or recent introgressive hybridization events (Bendif et al.

2014). At the species level, the phylogenetic discordance between the plastidial and mitochondrial gene sequences could be interpreted by different evolutionary hypotheses in attempting to resolve the morphological concept of *E. huxleyi* and *G. oceanica*. At the genus level, it has been suggested that *E. huxleyi* and *G. oceanica* could be considered con-generic (Bendif et al. 2014), a status that can be resolved by the genetic characterization of other *Gephyrocapsa* morphospecies for which phylogenetic positions remain unknown.

In this study, we conducted morphological and phylogenetic characterization of 3 new monoclonal strains of *Gephyrocapsa*. These strains were isolated by single cell sorting of calcified cells using a novel flow cytometric methodology (von Dassow et al. 2012) from samples from a coastal upwelling zone in the Eastern South Pacific Ocean (near Coquimbo, Chile). Initial Scanning Electron Microscopy (SEM) observations indicated that these strains were morphologically distinct from *G. oceanica* and had affinity to *G. muellerae*, which suggested that further analysis might be able to reveal important new information about the evolution of the Noelaerhabdaceae. Here we report the results of quantitative morphological analyses by SEM and molecular phylogenetic analyses based on sequences of the nuclear 18S and 28S rDNA, the plastidial 16S rDNA and encoded elongation factor tufA gene, and the mitochondrial cytochrome c oxidase 1 and 3 (cox1 and cox3) genes.

Results

Description and Identification

The three new culture isolates were identified as *Gephyrocapsa* due to the presence of a bipartite bridge over the central area of the coccoliths (Fig. 1). Coccospheres of the three strains varied in size from 3.9 to 7.2 μm, coccoliths from 2.8 to 4.5 μm (mean 3.5 μm), and bridge angle from 15.4 to 41.1° (mean 31.3°; Table 1). In the classification scheme of Bollmann (1997), the three strains corresponded to morphotype GO, whereas *G. oceanica* strains clustered in the GL morphotype (Fig. 2). Bollmann (1997) associated the GO morphotype with the morphospecies *G. caribbeanica*, but the mean morphometric measurements of the 3 new strains more closely corresponded to those of the holotype description of *G. muellerae* that has mean coccolith length of 3.9 μm and bridge angle of 23°, compared to 4.1 μm and 45° respectively for *G. caribbeanica*. Finally *G. caribbeanica* as originally described by Hay et al. (1967) and as used by most authors is a form with a narrow central area almost filled by a wide bridge, this form is very abundant prior to the first occurrence of *E. huxleyi* (e.g. Hine and Weaver 1998) but is absent from the modern plankton (Young et al. 2003).

Phylogenetic Analyses

The 3 strains were examined by sequencing partial fragments of nuclear 18S and 28S rDNA, plastidial 16S rDNA and tufA, and mitochondrial cox1 genes, and the complete mitochondrial cox3 gene. Comparison with haptophyte nuclear 18S and 28S rDNA sequences retrieved from Genbank confirmed the phylogenetic position of the *G. muellerae* strains within the Noelaerhabdaceae, clustering with the *E. huxleyi* - *G. oceanica* complex (Fig. 3). 18S rDNA sequences were identical for all of the strains in this complex (Fig. 3A), whereas the 28S rDNA exhibited a difference of 1 nucleotidic substitution between *G. oceanica* and *G. muellerae* sequences, the latter being identical to all *E. huxleyi* sequences (Fig. 3B).

The plastidial 16S rDNA sequences of the 3 *G. muellerae* strains also confirmed the position of these strains within the Noelaerhabdaceae (Fig. 3C). The 16S rDNA sequences from *G. muellerae* were identical to those of *E. huxleyi* and *G. oceanica* retrieved from Genbank. Plastidial tufA sequence phylogenies (Fig. 3D, Supplementary Material Fig. S1) clustered *G. muellerae* within the tufAII haplogroup defined by Bendif et al. (2014), previously composed exclusively of *E. huxleyi* strains. The three *G. muellerae* sequences were identical to other *E. huxleyi* strains within the tufAII haplogroup (Supplementary Material Fig. S1), and differed by 15 substitutions from the tufAI group that includes both *E. huxleyi* and *G. oceanica* strains, and by the same number of substitutions from the tufAGO group comprised exclusively of *G. oceanica* strains.

Unrooted cox1 and cox3 phylogenies including *E. huxleyi* and *G. oceanica* sequences retrieved from Genbank, together with sequences from the three new *G. muellerae* strains produced topologies that were similar to those reported by Bendif et al. (2014) (Supplementary Material Fig. S2A,B). The *G. muellerae* sequences clustered within the β haplogroup of *E. huxleyi* for both genes. For cox1, the three sequences were identical to other sequences from the β haplogroup except that of the *E. huxleyi* strain RCC174, which exhibited 1 substitution. The *G. muellerae* cox3 sequences differed by only 3 substitutions from *E. huxleyi* sequences in the β haplogroup, differences that were not greater than

Table 1. Average characteristics of measured isolates in this study. In situ temperatures are mentioned in bracket for the *Gephyrocapsa muellerae* strains.

Morphospecies	RCC#	Strain name	Locality	Isolator	Date of Isolation	Latitude	Longitude	Coccosphere Diameter	StDev	StError	Coccolith length	StDev	StError	Bridge Angle ()	StDev	StError	Morphotype
Gephyrocapsa muellerae	3862	CHC126	Tongoy	P. von Dassow	2011	-30.25	-71.70	5.63	0.58	0.05	3.49	0.34	0.03	34.01	6.85	0.60	GO
Gephyrocapsa muellerae	3898	CHC194b	Tongoy	P. von Dassow	2011	-30.25	-71.70	6.30	0.93	0.11	3.78	0.31	0.04	28.03	4.90	0.62	GO
Gephyrocapsa muellerae	3370	CHC184	Tongoy	P. von Dassow	2011	-30.25	-71.70	5.49	0.52	0.05	3.29	0.38	0.03	31.89	7.09	0.08	GO
Gephyrocapsa oceanica	1281	THAU1	Indian Ocean	I. Probert	2000	-31.93	115.73	7.81	0.82	0.16	4.29	0.49	0.07	68.10	8.53	1.58	GL
Gephyrocapsa oceanica	1282	ESP6M11	Mediterranean Sea	I. Probert	1999	41.47	2.32	8.13	1.00	0.20	5.23	0.52	0.07	59.77	6.96	0.90	GL
Gephyrocapsa oceanica	1284	ESP6M3	Mediterranean Sea	I. Probert	1999	41.47	2.32	7.11									
Gephyrocapsa oceanica	1286	AS62E	Mediterranean Sea	I. Probert	1999	37.28	-0.80	7.77									
Gephyrocapsa oceanica	1292	PR3F1	Atlantic Ocean	I. Probert	2001	14.82	-67.05	7.18									
Gephyrocapsa oceanica	1300	PZ3-1	Pacific Ocean	I. Probert	2001			7.81									
Gephyrocapsa oceanica	1305	PC65	Atlantic Ocean	I. Probert	1998	38.23	-9.72	7.34									
Gephyrocapsa oceanica	1306	PC64	Atlantic Ocean	I. Probert	1998	38.23	-9.72	7.78									
Gephyrocapsa oceanica	1307	PC51	Atlantic Ocean	I. Probert	1998	38.20	-9.63	7.61									
Gephyrocapsa oceanica	1316	LK9	Atlantic Ocean	I. Probert	1999	45.00	-1.08	7.78									
Gephyrocapsa oceanica	1317	JS10	Mediterranean Sea	I. Probert	1998	36.25	-1.58	6.98									
Gephyrocapsa oceanica	1318	THAU4	Indian Ocean	I. Probert	2000	-31.93	115.73	6.71									
Gephyrocapsa oceanica	1319	NS6-2	Atlantic Ocean	I. Probert	2002	-36.67	16.77	7.22									
Gephyrocapsa oceanica	1320	ESP6M6	Mediterranean Sea	I. Probert	1999	41.47	2.32	7.12									
Gephyrocapsa oceanica	1562	NIES1000	Pacific Ocean	M. Kawachi	1999	34.08	139.57	7.77									
Gephyrocapsa oceanica	1839	B50	Mediterranean Sea	I. Probert	2008	39.10	5.35	7.27									

Table 1 (*Continued*)

StDev	StError	Coccolith length	StDev	StError	Bridge Angle ()	StDev	StError	Morphotype
0.79	0.13	4.07	0.54	0.07	64.31	6.26	0.81	GL
0.74	0.13	4.70	0.46	0.05	59.22	7.60	1.03	GL
0.62	0.10	4.39	0.46	0.04	62.47	7.22	0.65	GL
0.75	0.14	5.06	0.56	0.04	60.71	7.59	0.98	GL
0.70	0.13	3.96	0.32	0.03	60.48	9.17	1.65	GL
0.79	0.14	4.50	0.58	0.07	63.93	7.30	0.94	GL
0.36	0.15	4.70	0.52	0.08	61.78	7.08	1.04	GL
0.79	0.14	4.60	0.61	0.08	64.96	7.27	0.94	GL
0.69	0.13	4.15	0.41	0.05	60.79	8.23	1.06	GL
0.66	0.12	4.14	0.47	0.06	58.02	9.11	1.16	GL
0.73	0.08	4.53	0.39	0.05	62.08	7.36	0.97	GL
0.74	0.14	4.50	0.75	0.10	57.30	9.44	1.26	GL
0.08	0.04	4.43	0.58	0.05	63.63	9.32	1.29	GL
0.74	0.09	4.41	0.48	0.06	64.73	6.31	0.80	GL

the variability among *E. huxleyi* sequences within this haplogroup. Interestingly, some environmental sequences previously reported from waters near the site of isolation of the *G. muellerae* isolates (Beaufort et al. 2011) clustered together with the *G. muellerae* sequences, varying by only 1 bp (Supplementary Material Fig. S2B).

Comparison of mitochondrial genes and plastidial gene phylogenies (e.g, between cox3 and tufA) revealed some topological incongruency in grouping the different strains, depicting different phylogenetic signals (Fig. 4). Topology tests rejected significantly any congruence (P values below 0.05) between tufA and cox3 phylogenies (Supplementary Material Table S1). In total, 9 of the 13 identified cases of incongruency involved *G. oceanica* strains. All *G. oceanica* strains grouped in the cox3 γ clade, but these 9 strains grouped in the chloroplastidial tufAI clade, which is shared among *G. oceanica* and *E. huxleyi* strains originating from warmer waters. Within the mitochondrial compartment, congruence was also rejected (except by the most conservative S-H criteria) between cox1 and cox3 phylogenies (Supplementary Material Table S1). However, incongruence between cox1 and cox3 was all at only fine-scale tree topologies, with the separation of the α, β, and γ clades being completely congruent between cox1 and cox3 (Supplementary Material Fig. S2A, B).

Discussion

The identification of the new culture strains reported here as corresponding most closely to *G. muellerae* was based on coccolith morphometry and fine structure (specifically the fact that the open central area and raised bridge of their coccoliths are distinct from the closest fossil alternative *G. caribbeanica*). This identification is also consistent with their origin from cooler waters, typical of the environmental distribution of *G. muellerae* and different from those of *G. oceanica*: *G. muellerae* is found in productive regions with mean sea surface temperatures (SST) <21 °C (Young et al. 2003), whereas *G. oceanica*, with a much higher bridge angle, is typically found in waters of mean SST of 18-30 °C. The three strains isolated here were isolated from samples collected from the Humboldt Current System, where highly productive cool water flows northward with coastal SSTs in the range of 12-19 °C (Hormazabal et al. 2001; Thiel et al. 2007). More specifically, they were isolated from a strong upwelling center when SST was $\leq$ 13 °C.

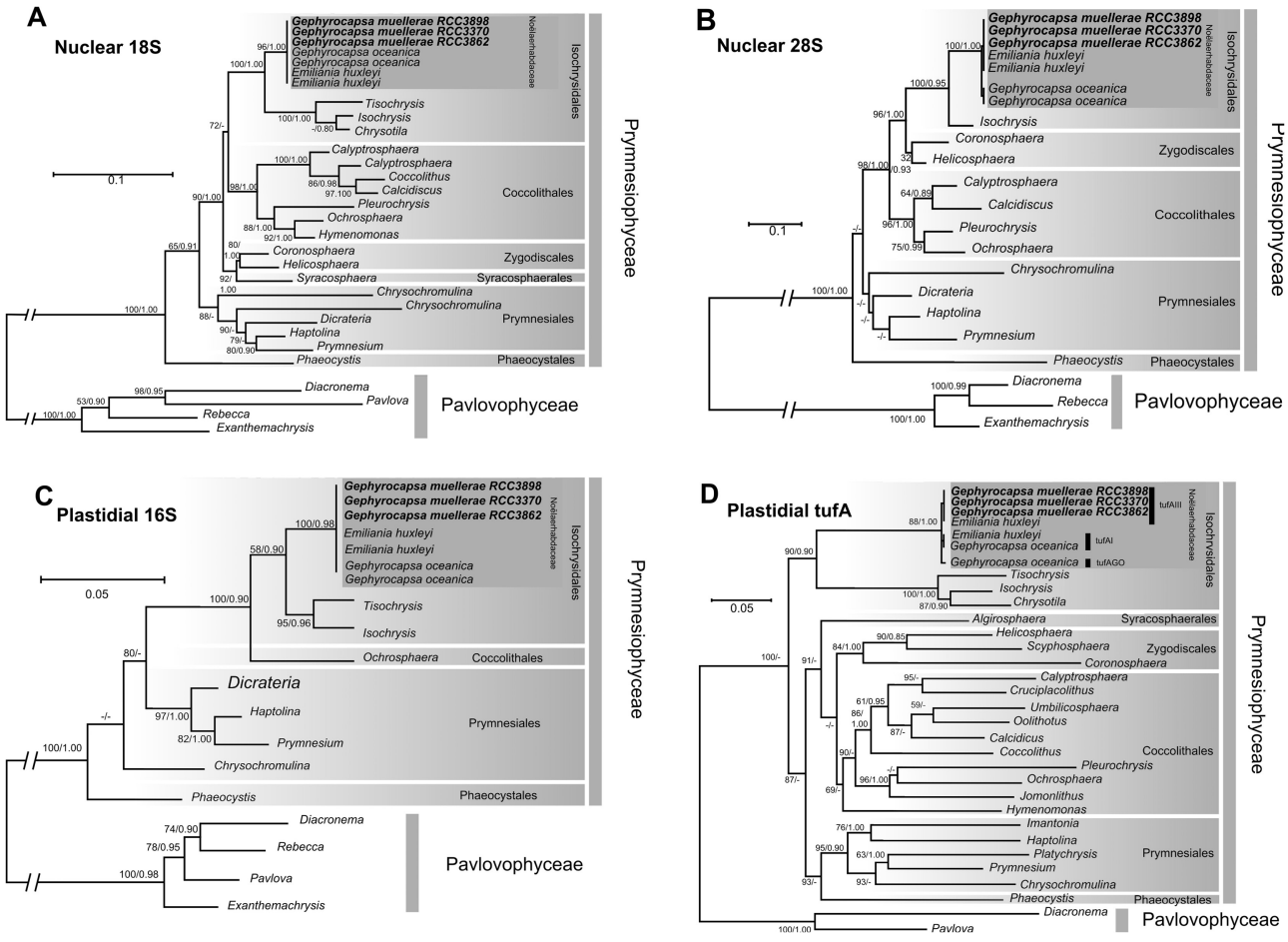

Figure 3. Molecular phylogenies of the Haptophyta inferred from comparisons of nuclear 18S rDNA sequences (**A**), of nuclear 28S rDNA sequences (**B**), of plastidial 16S rDNA sequences (**C**) and from comparison of plastidial tufA gene sequences (**D**). Support values at each node are presented for ML/Bayes analyses. Bootstrap values larger than 50 and posterior probabilities larger than 0.80 are shown. Lesser values are represented by "–.".

For the rest of the discussion we therefore refer to the new strains as *G. muellerae*.

To our knowledge, no Noëlaerhabdacean taxon other than *E. huxleyi* or *G. oceanica* has ever previously been successfully isolated and maintained in culture. This highlights the potential of single-cell sorting by flow cytometry for initiation of cultures of species that may be less amenable to isolation by traditional methods. The new strains characterized here specifically represent an important source of information for understanding evolutionary succession in the plankton, as *G. muellerae* was the numerically dominant coccolithophore morphotype prior to being replaced by *E. huxleyi* in the fossil record.

The close relationship between *E. huxleyi* and *G. oceanica* was first highlighted by Kamptner (1956) who noted the high degree of homology of the structure of coccolith elements between these morphospecies. Reinhardt (1972) first formally proposed the combination *Gephyrocapsa huxleyi* based on this similarity in coccolith structure, but this proposal has not been widely followed in subsequent literature. The phylogenetic position of *G. muellerae*, assessed here using gene markers from 3 genomic compartments, further challenges the classical taxonomic separation of the genera *Gephyrocapsa* and *Emiliania*. In both independent (Figs 3-4) and concatenated phylogenies (not shown), the nuclear 28S rDNA, the plastidial tufA, and the mitochondrial cox1 and cox3 genes all unambiguously classified the *G. muellerae* isolates as distinct from *G. oceanica* and closely associated with *E. huxleyi*.

Much of the high genomic variability exhibited among strains classified as *E. huxleyi* has been shown to be related to changes in the life cycle and biotic pressure (von Dassow et al. 2014), implying

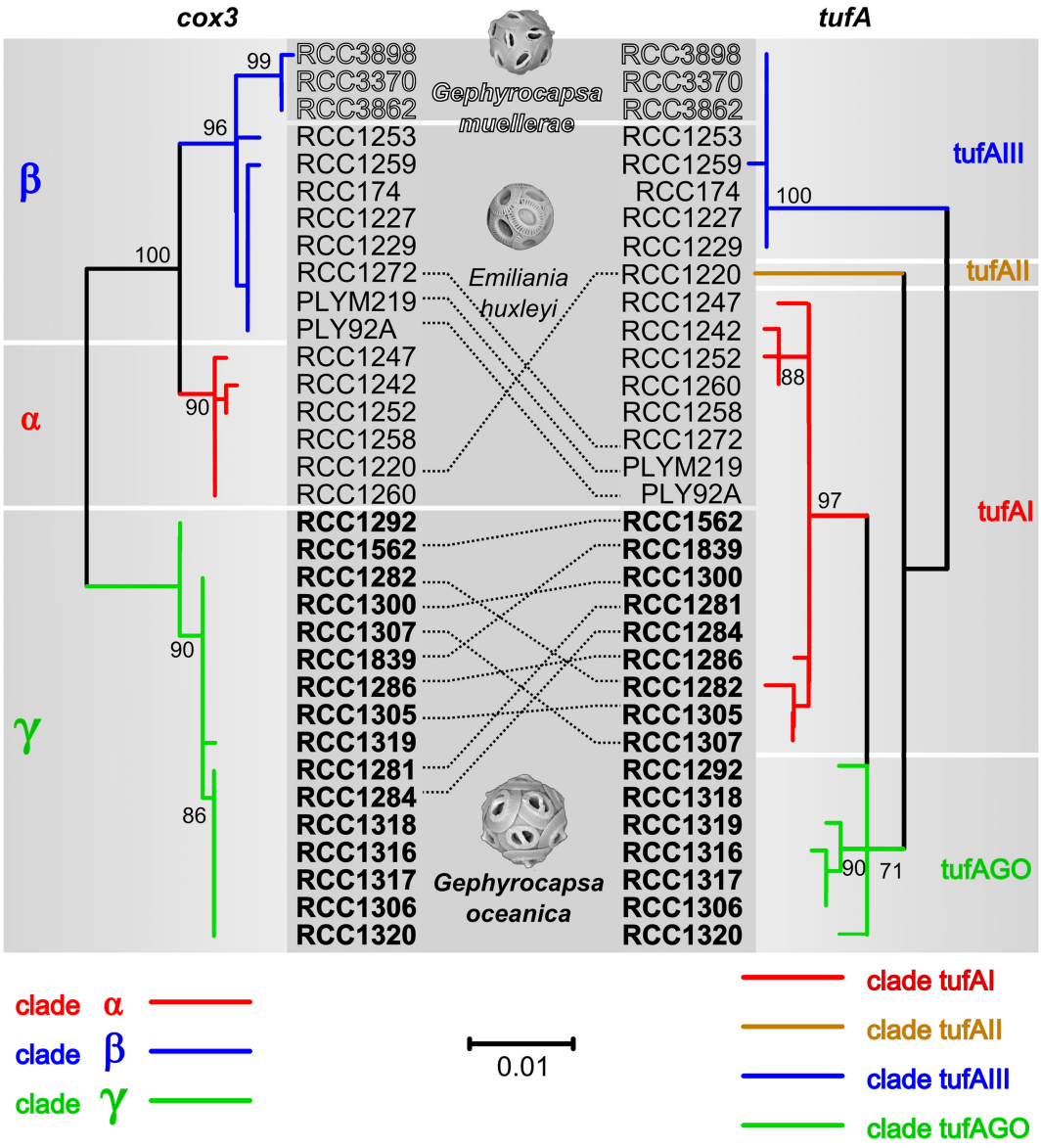

Figure 4. Comparison of cox3 and tufA phylogenies. Incongruent positions are indicated with dashlines. Support value for each nodes are presented for ML. Bootstrap values larger than 50 are shown.

that some *E. huxleyi* genotypes cannot interbreed. This already suggests that *E. huxleyi* might be considered to include a complex of cryptic species with differing ecologies. Consistent with this, the plastidial (tufA) and mitochondrial (cox1 and cox3) genes exhibited different phylogenetic structures, but in both cases *Emiliania* and *Gephyrocapsa* appeared polyphyletic, with *G. muellerae* clustering within the *E. huxleyi* mitochondrial clade β and plastidial clade tufAII. This might suggest that different *E. huxleyi* lineages evolved from different *Gephyrocapsa* lineages which each experienced the loss of the central bridge and decrease in the degree of calcification of distal shield elements (i.e. convergent evolution). If this were the case, the different lineages classified by morphology as *E. huxleyi* should not be considered as a species or a species-complex, but rather as non-sister lineages of a larger *Gephyrocapsa* species complex.

An intriguing alternative hypothesis is that the *E. huxleyi* morphospecies did arise once (from an unspecified *Gephyrocapsa* lineage), but that some populations colonizing low-latitude waters might have hybridized with *G. oceanica* populations, whereas *E. huxleyi* that colonized colder waters might have hybridized with *G. muellerae* populations (the *E. huxleyi* lineages may have subsequently diverged into more than one cryptic

species). Hybridization and introgression have been estimated to be important in the speciation of up to 25% of terrestrial plant species (Baack and Rieseberg 2007) and have been extensively documented in zooplankton, molluscs, fish, and birds (Baack and Rieseberg 2007). To date, hybridization and introgression have been poorly documented in planktonic protists, but have been reported between cryptic sub-species of the diatoms *Pseudonitzschia multistriata* and *P. pungens* (respectively Casteleyn et al. 2009 and D'Alelio et al. 2009). The hybridization/introgression hypothesis could explain the incongruences between phylogenies and morphology, and would be consistent with ecological associations: *G. muellerae* and *E. huxleyi* mitochondrial clade β are both associated with cold (temperate and sub-polar) waters, whereas both *G. oceanica* and the *E. huxleyi* mitochondrial clade α strains that group with *G. oceanica* by tufA are associated with warmer waters (Hagino et al. 2011).

Kamptner (1943) first suggested that *Gephyrocapsa* and *Emiliania* might be capable of hybridizing due to observation of confusing combinations of both coccolith types on single coccospheres occurring in some sediment samples (as documented in, e.g., Clocchiatti 1971). However, such anomalous coccospheres containing coccoliths normally regarded as forming on discrete morphospecies have been termed "xenospheres", on the basis that they are considered to be artefactually-formed coccospheres, for example by attachment of loose coccoliths to a coccosphere *post-mortem*, and have never been observed in water column samples (discussed in detail in Young and Geisen 2002). Hybrid noëlaerhabdaceans might occur in nature, but it is very unlikely that it would be possible to conclusively identify them based on morphological evidence.

Finally, incomplete lineage sorting of plastidial and mitochondrial genomes as distinct *Gephyrocapsa* lineages diverged, with a single lineage leading to the *E. huxleyi* morphospecies, cannot be ruled out as a potential explanation for our results. It is generally challenging to resolve the roles of incomplete lineage sorting versus hybridization and introgression (reviewed in Twyford and Ennos 2012). Potential approaches include application of microsatellite markers (that exist for *E. huxleyi*: Iglesias-Rodriguez et al. 2006; Krueger-Hadfield et al. 2014) or next-generation sequencing technologies (Twyford and Ennos 2012). However, during our sampling effort, *G. muellerae* represented <2% of the culture strains obtained, the rest corresponding to *E. huxleyi*. Thus a key challenge to applying either population genetics or population genomics tests will be to increase the number of clonal isolates of *G. mullerae* and other Noëlaerhabdacean morphospecies successfully cultured, which will also improve phylogenetic resolution.

Concluding Remarks

Regardless of which of the alternative hypotheses presented here is supported or rejected by future studies, our results conclusively show that *Emiliania* should be transferred to *Gephyrocapsa*, because generic-level separation reflects neither the structural similarity, the evolutionarily history, nor the ecology of these important organisms. However, whether or not *G. huxleyi* makes sense as a single taxonomic unit is still unclear.

At a broader level, this study highlights the challenge of reconciling morphological and genetic species concepts for microbial eukaryotes. Species- and genus-level classifications of mineralizing eukaryotic microbes (such as coccolithophores, foraminifera, diatoms, and certain dinoflagellates), and the association of morphological classifications with distinct ecological conditions, are crucial in studies of aquatic environments, both as indicators for present-day ecological status and of paleo-climates. It has so far been possible to manipulate the life cycle and breeding in culture of very few representatives of microbial eukaryote plankton (discussed in von Dassow and Montresor 2011) to directly investigate breeding barriers and biological species concepts. Further efforts to isolate representatives of new lineages of "model" groups into culture, combined with molecular, genomic, and morphometric approaches, may provide a clearer picture of the evolution of morphological characters and how they can be reliably used in plankton ecology and paleo-oceanography.

Methods

Origin, isolation, culture and morphological characterization of analyzed strains: The new *G. muellerae* strains were isolated as follows: seawater was collected in Niskin bottles from 5 m and 30 m depth at three sites of strong coastal upwelling in front of Punta Lengua de Vaca and Tongoy Bay along the Chilean coast (Lat/Long: -30.15°/-71.60°; -30.20/-71.59; -30.25°/-71.65°) from the *R/V Stella Maris II* on 12 and 13 October 2011. Surface water temperature at these stations was 12.5 °C, 12.4 °C, and 13.0 °C, respectively. Water samples were initially filtered through 20 μm or 40 μm nitex, and stored dark and cool (<15°) for transport to the laboratory in Concepción, Chile for flow cytometric sorting within 48 hours of collection. Prior to sorting, samples were filtered through

12 μm pore-size polycarbonate membrane filters using gravity alone to remove microplankton. To concentrate heavy (mineralized) nannoplankton cells, 2 subsamples of 50 ml were concentrated by centrifugation for 10 min at 500x g + 3 min at 1000x g, 45 ml of supernatant was discarded, and the two subsamples were combined and centrifuged again, to concentrate 100 ml to approximately 2 ml. Calcified phytoplankton (chlorophyll-fluorescent particles depolarizing forward scatter light) were detected with an InFlux flow cytometer equipped with a small particle detector and Brewster's Angle polarization detector for FSC optics (von Dassow et al. 2012) and individual cells were sorted in purity mode into 100 μl of K/5 medium. After 1 month of incubation in the same conditions as described above, individual colonies were harvested and transferred to the Roscoff Culture Collection (RCC) for culturing.

All *Gephyrocapsa* strains (Table 1) from the RCC were maintained in K/2(-Si,-Tris,-Cu) medium (Keller et al. 2000) at 17 °C with 50 μmol-photons·m^{-2}·s^{-1} illumination provided by daylight neon tubes with a 14:10 h L:D cycle.

Living cells were observed with an Olympus BX51 light microscope equipped with differential interference contrast (DIC) optics. For scanning electron microscopy (SEM), cells were grown until early exponential phase and then filtered onto polycarbonate filters that were dried in a vacuum desiccator before being sputter coated with a thin layer of Au/Pd. Observations were made with a Phenom ProX Desktop SEM (Phenom-World, Eindhoven, Netherlands) and measured using ImageJ software (http://imagej.nih.gov/ij/). Morphometric measurements were carried out according to Bollmann (1997) with a minimum of 60 isolated coccoliths and coccospheres analysed per sample.

DNA extraction, amplifications and molecular analysis: Total DNA was isolated from all of the strains using a DNA purification kit (Macherey Nagel). Nuclear 18S and 28S rDNA, plastidial 16S rDNA and tufA, mitochondrial cox1 and cox3 genes were amplified using the primers used in Bendif et al. (2014) using the GoTaq Polymerase kit (Promega). A standard PCR protocol was used with a thermal cycler T1 (Biometra): 2 min initial denaturation at 95 °C, followed then by 35 cycles of 30s at 95 °C, 30s annealing at 55 °C and 1 min extension at 72 °C. A final 5 min extension step at 72 °C was conducted to complete the amplification. Amplification products were analyzed by electrophoresis on a 1% agarose gel to control amplification success. Amplifications were sequenced on an ABI PRISM 3100xl DNA auto sequencer (Perkin-Elmer) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer). Sequences chromatograms were checked using FinchTV (http://www.geospiza.com/Products/finchtv.shtml). Accession numbers of new or updated sequences are provided in Supplementary Table 2.

For each gene, an alignment was preformed adding a large set of sequences from other Noelaerhabdaceae and haptophytes with the online version of the multiple alignment program MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/software, Katoh and Standley 2013) and then manually checked using SEAVIEW (Gouy et al. 2010) as a sequence editor. Final sequence lengths and completion were as in Bendif et al. (2014). Appropriate models for DNA substitution were estimated with JModeltest2 (Darriba et al. 2012) which selected the same models as in Bendif et al. (2014) for each gene. Phylogenetic trees were constructed using two phylogenetic methods: maximum likelihood (ML) using TREEFINDER (Jobb et al. 2004) and Bayesian analysis with Mr. BAYES v3.1.2 (Huelsenbeck and Ronquist 2001). The robustness of the branching of trees was tested by bootstrapping for the ML inference where bootstrap values were based on 1000 replicates. Bayesian analysis was conducted with two runs of four Markov chains, for at least 5 000 000 generations, sampling every 100th generation to reach minimum likelihood convergence. The burn-in option was set discarding 25% from the 50 000 trees found. We note that we show unrooted trees for *cox* and *tufA* to avoid phylogenetic bias because the nearest potential outgroup for rooting, *Isochrysis*, is too divergent, with mutations potentially occurring at different rates, and would introduce a bias in phylogenetic interpretation.

Tree incongruences were assessed using most used topology tests, the K-H test (Kishino and Hasegawa 1989), the S-H test (Shimodaira and Hasegawa 1999), and the AU test (Shimodaira 2002). The null distribution was generated by non-parametric boostrapping and log likelihood scores of trees constrained by topological conflicts and test values including Pvalues were calculated using the program TREEFINDER.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2015.05.003.

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