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NEW EVIDENCE FOR MORPHOLOGICAL AND GENETIC VARIATION IN THE COSMOPOLITAN COCCOLITHOPHORE *EMILIANIA HUXLEYI* (PRYMNESIOPHYCEAE) FROM THE *COX1b-ATP4* GENES[1]

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***Emiliania huxleyi* (Lohmann) W. W. Hay et H. Mohler is a cosmopolitan coccolithophore occurring from tropical to subpolar waters and exhibiting variations in morphology of coccoliths possibly related to environmental conditions. We examined morphological characters of coccoliths and partial mitochondrial sequences of the cytochrome oxidase 1b (*cox1b*) through adenosine triphosphate synthase 4 (*atp4*) genes of 39 clonal *E. huxleyi* strains from the Atlantic and Pacific Oceans, Mediterranean Sea, and their adjacent seas. Based on the morphological study of culture strains by SEM, Type O, a new morphotype characterized by coccoliths with an open central area, was separated from existing morphotypes A, B, B/C, C, R, and var. *corona*, characterized by coccoliths with central area elements. Molecular phylogenetic studies revealed that *E. huxleyi* consists of at least two mitochondrial sequence groups with different temperature preferences/tolerances: a cool-water group occurring in subarctic North Atlantic and Pacific and a warm-water group occurring in the subtropical Atlantic and Pacific and in the Mediterranean Sea.**

***Key index words:* biogeography; coccolithophore; *Emiliania huxleyi*; mitochondrial DNA; morphotype**

***Abbreviations: atp4*, adenosine triphosphate synthase 4; *cox1*, cytochrome oxidase 1b**

Coccolithophores are single-celled marine haptophytes characterized by bearing calcareous scales called coccoliths. They play an important role as primary producers in the oceans and contribute to the global carbon cycle through photosynthesis and calcification of coccoliths (Rost and Riebesell 2004). Vast numbers of coccoliths produced in surface

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waters sink to the deep-sea floor where they constitute a significant part of deep-sea sediments. The evolutionary history of coccolithophores has been extensively studied based on the continuous fossil record of coccoliths preserved in marine sediments. Palaeontological studies have revealed that coccolithophore floras in the geological past were often dominated by a few cosmopolitan taxa (Hine and Weaver 1998, Young 1998). It is not known whether these cosmopolitan taxa were indeed single biological species or complexes of cryptic species with different environmental preferences.

E. huxleyi is the youngest coccolithophore morphospecies, appearing ~290 kiloannum (ka) (Raffi et al. 2006). It is thought that *E. huxleyi* diverged from *Gephyrocapsa oceanica*, since *E. huxleyi* and *G. oceanica* are genetically identical in SSU rDNA and RUBISCO *rbc*L sequences (Medlin et al. 1996, Fujiwara et al. 2001), and *G. oceanica* has a longer fossil record than *E. huxleyi* (Hine and Weaver 1998). In the early part of its evolutionary history, *E. huxleyi* was initially a minor species in the coccolithophore flora, but it progressively increased in relative abundance through time. The *E. huxleyi* acme, which is defined by ≥50% dominance in the total fossil coccolithophore flora, started diachronously from 85 ka in low latitudes, 73 ka in transitional latitudes, and 61 ka in high latitudes of the North Atlantic Ocean (Thierstein et al. 1977, Gard 1986, 1989, Jordan et al. 1996). In modern oceans, *E. huxleyi* is undoubtedly the most abundant and cosmopolitan coccolithophore species, occurring in almost all assemblages from tropical to subpolar waters and frequently constituting ≥50% of the coccolithophore flora (McIntyre and Bé 1967, Okada and Honjo 1973).

Morphological variation in coccoliths of *E. huxleyi* that are likely related to hydrographic conditions have been reported in various biogeographic studies, although the morphotype classification, especially for morphotypes from cold-water masses, has not always been consistent between authors. McIntyre and Bé (1967) classified *E. huxleyi* into warm- and cold-water types based on the morphology of the central area and proximal shield of coccoliths (Table 1; Fig. 1). They mentioned that the cold-water type has a central plate. Subsequent studies, however, also included specimens with open central

TABLE 1. Classification of morphotypes of *Emiliania huxleyi*.

Morphotype in this study	Morphology of distal shield	Morphology of central area	Length of distal shield	Comparable morphotypes in literature
Type A	Moderate-heavily calcified elements	Grill	<4 μm	Warm type (McIntyre and Bé 1967)
Type B	Lightly calcified elements	Solid plate	≥4 μm	Type B (Young et al. 2003)
Type B/C	Lightly calcified elements	Solid plate	<4 μm	Type B/C (Young et al. 2003)
Type C	Lightly calcified elements	Solid plate	<3.5 μm	Cold type (McIntyre and Bé 1967) Type C (Young et al. 2003)
Type O	Lightly calcified elements	Open	Varied in size	Subarctic type (Okada and Honjo 1973) Type B (Hagino et al. 2005)
Type R	*Reticulofenestra*-like heavily calcified distal shield elements	Grill	<4 μm	Type R (Young et al. 2003)
var. *corona*	Moderately calcified elements with elevated central tube	Grill	3.5–4.5 μm	var. *corona* (Okada and McIntyre 1977)

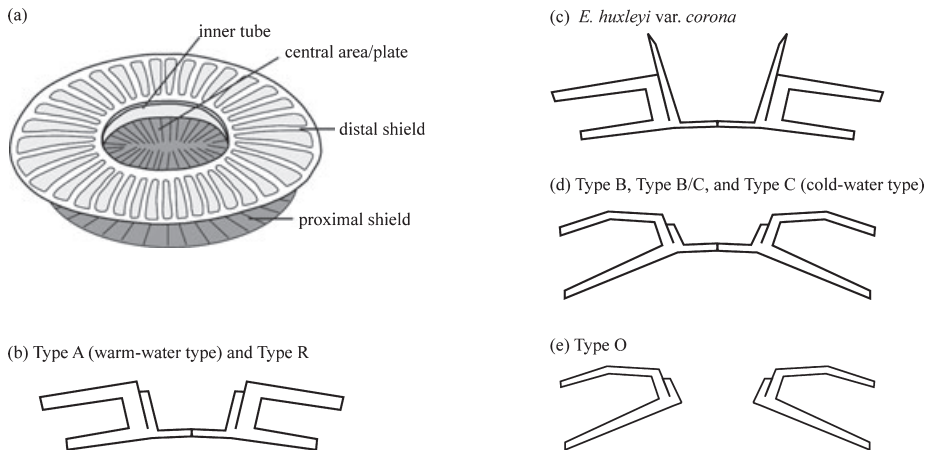

FIG. 1. (a) Schematic diagram of coccolith of *Emiliania huxleyi*. (b) Cross-section of Types A and R; (c) cross-section of *E. huxleyi* var. *corona*; (d) cross-section of Types B, B/C, and C; (e) cross-section of Type O.

area in the cold-water type (Winter 1985, Verbeek 1989) probably because the general appearance of the coccoliths with an open central area resembled the cold-water type rather than the warm-water type. Winter (1985) also mentioned that their cold-water types were not always related to low temperature. To avoid the use of morphotype names associated with temperature, Young and Westbroek (1991) renamed the warm- and cold-water types as Types A and C, respectively. They also described Type B characterized by a solid central plate and larger coccolith size than Type C (Table 1; Fig. 1). Culture strains of Type C were not available at the time, and it was not known whether the morphology of the central area was a stable character reflecting genetic differences. Therefore, Young and Westbroek (1991) included open central area morphotypes in their Type C. Later, Young et al. (2003) described Type B/C characterized by a solid/open central plate and a transitional size between that of Types B and C. Okada and Honjo (1973) described a subarctic type based on irregularly arranged distal shield elements. Hagino et al. (2005) considered this to be comparable to Type B of Young and Westbroek (1991) without consideration of central area morphology. They also reported Type B from the N.W. Pacific, illustrating specimens with an open central area. Judging from the SEM images shown in the previously published papers, all coccolithophore workers in the last three decades have classified *E. huxleyi* specimens with an open central area into either the cold-water type, Type B or Type C. Classification of *E. huxleyi* morphotypes from tropical to temperate waters has been more consistent than that of morphotypes from cold-water regimes, the following four morphotypes have been reported: *E. huxleyi* var. *corona* (Okada and McIntyre 1977), Types A and C (Young and Westbroek 1991), and Type R (Young et al. 2003) (Table 1; Fig. 1).

Interstrain genetic variation of *E. huxleyi* has been studied since the early 1990s to investigate genetic relationships among morphotypes as well as genetic diversity in natural *E. huxleyi* populations. Culture strains of Types A and B were shown to be identical in SSU rDNA sequences (Medlin et al. 1996), and therefore morphotypes of *E. huxleyi* have typically been regarded as intraspecific variants rather than discrete species. Fine-scale genetic variation within *E. huxleyi* populations has been detected by random amplification of polymorphic DNA and microsatellite analysis, although this genetic variation showed no clear relationship to morphotype (Medlin et al. 1996, Iglesias-Rodríguez et al. 2002). Schroeder et al. (2005) reported a genetic marker differentiating Type A from Type B: the gene coding for the calcium-binding protein GPA, which was isolated from coccolith-producing vesicles and shown to precipitate calcium (Corstjens et al. 1998). Subsequent studies have found variation in GPA sequences from environmental samples (Martínez-Martínez et al. 2007, Ripley et al. 2008). Iglesias-Rodríguez et al. (2006) provided evidence of low gene flow between *E. huxleyi* populations of the Bergen fjord and the N.E. Atlantic Ocean based on microsatellite analysis of multiple clonal culture strains. Strains/samples used in these genetic studies were almost exclusively collected from the North Atlantic Ocean and adjacent seas, and genetic relationships among *E. huxleyi* populations from the Atlantic and other oceans have not been revealed.

Mitochondrial DNA evolves relatively rapidly and so is often used in studies of fine-scale genetic variation and phylogeography (e.g., Schwaninger 2008). The complete mitochondrial genome sequence of *E. huxleyi* has been obtained from strain CCMP373 (Sánchez Puerta et al. 2004). In the present study, we examined, for the first time, variations in partial mitochondrial sequences of *E. huxleyi,* using multiple clonal culture strains from the Atlantic Ocean, Pacific Ocean, Mediterranean Sea, and their adjacent seas. This genetic variation was compared to coccolith morphology, biogeography, and environmental parameters. Furthermore, we reexamined the morphology of *E. huxleyi* in field samples used for previous morphological studies to refine definition of morphotypes and integrate this with our new information from culture and molecular studies.

MATERIALS AND METHODS

Morphological studies of clonal culture strains and field samples. The coccolith morhology of 39 clonal *E. huxleyi* strains (Table S1 in the supplementary material) was studied by SEM. In addition, morphological variation of *E. huxleyi* populations in 10 selected previously studied field samples was reexamined by SEM (Table 2). Clonal culture strains were maintained in MNK medium (Noël et al. 2004) at 18°C in a 18:6 light:dark regime. Ten to 100 mL of cell suspension of each strain was sampled during the exponential growth phase and filtered onto mixed cellulose ester filter HAWP04700 (Millipore, MA, USA) or polycarbonate filter 7060-4710 (Whatman, Maidstone, UK). Small pieces of dried filter samples were mounted on aluminum SEM stubs and sputter-coated with gold-palladium or platinum using an ion sputter 208HR (Cressington Scientific, Watford, England) or Hitachi E-1020 (Hitachi Corp., Hitachinaka, Japan). The morphotype of each culture strain and the morphotype composition of field samples were examined using a Phillips XL30 FEG SEM (Philips FEI, Eindhoven, the Netherlands) or a Hitachi S-3000H SEM (Hitachi Corp.).

DNA preparation, PCR, and sequencing. Thirty-nine clonal *E. huxleyi* and four clonal *G. oceanica* strains from various geographic origins were used for molecular studies (Table S1). Genomic DNA of each strain was extracted using benzyl chloride extraction (Zhu et al. 1993) or phenol-chloroform extraction (Sambrook et al. 1989) methods. The DNA extracts were then purified using Geneclean II® Kits (Bio 101 Inc., Vista, CA, USA). Purified DNA was used for PCR to amplify the region from *cox1b* through *atp4* of the mitochondrial genome. Three sets of PCR and sequencing primers listed in Table 3 were designed based on the complete mitochondrial genome sequence of *E. huxleyi* reported by Sánchez Puerta et al. (2004) (GenBank accession number AY342361). The PCR conditions were initial denaturation at 94°C for 60 s followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s,

TABLE 2. Composition of morphotypes in field samples.

Sampling station	Latitude	Longitude	Sampling date	Morphotype recognition in previous studies	Morphotype composition obtained in this study (%)		
					Type A	Type B/C	Type O
KH-30	50°00′ N	155°05′ W	1969.8.23	Subarctic form (Okada and Honjo 1973)	0	0	100
KH-31	49°12′ N	154°37′ W	1969.8.27	Subarctic form (Okada and Honjo 1973)	0	0	100
KH-32	48°02′ N	155°44′ W	1969.8.28	Subarctic form (Okada and Honjo 1973)	0	0	100
KH-33	46°49′ N	154°32′ W	1969.8.28	Subarctic form (Okada and Honjo 1973)	0	0	100
KH-34	45°04′ N	154°46′ W	1969.8.28	Subarctic form (Okada and Honjo 1973)	0	0	100
KT90-9, st. 11	42°24′ N	144°22′ E	1990.6.26	Type B (Hagino et al. 2005)	0	0	100
KT90-9, st. 35	37°49′ N	142°07′ E	1990.6.28	Types A and B (Hagino et al. 2005)	81	1	18
KT90-9, st. 38	35°58′ N	141°10′ E	1990.6.29	Types A and B/C (Hagino et al. 2005)	90	0	10
KH90-1, st. 9	33°09′ N	139°50′ E	1990.7.21	Types A and B/C (Hagino et al. 2005)	97	1	2
KH90-1, st. 18	32°47′ N	142°47′ E	1990.7.24	Types A (Hagino et al. 2005)	100	0	0

TABLE 3. Oligonucleotide primers used for amplification and sequencing.

Code of primer	Synthesis direction	Sequence (5′–3′)	Anneals to[a]
EGcox1-F2	Forward	GCTCATTCAGGAGGTTCTGT	15333–15352
EGcox1-R4	Reverse	GATAAAACAATACCTGTTAA	15978–15997
EGcox1-F3	Forward	ACTATGATTATTGCTGTTC	15846–15864
EGcox1-R5	Reverse	ACTAAGTAATCAGTTTCTGC	16401–16429
EGcox1-16274F	Forward	TGCAATTGCTTCATTTGGTAC	16274–16294
EGatp4-16959R	Reverse	TGCCGATTTCGCATCAATAAG	16959–16979

[a]Annealing site in the mtDNA of strain CCMP373 (Sánchez Puerta et al. 2004).

and extension at 72°C for 30 s. The temperature profile was completed by a final extension at 72°C for 4 min. The PCR products were sequenced directly using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) by DNA auto sequencer ABI PRISM 310 and/or 3130 Genetic Analyzers (Perkin-Elmer). Both forward and reverse strands were sequenced. GenBank accession number of each sequence is listed in Table S1.

Phylogenetic analysis. Sequences were manually aligned and based on this alignment, rooted and unrooted phylogenetic trees were inferred by maximum-likelihood (ML), neighbor-joining (NJ), and maximum-parsimony (MP) methods using PAUP version 4.0b10 (Swofford 2002). Furthermore, rooted and unrooted Bayesian trees were constructed by Mr. BAYES v3.1.2 (Ronquist and Huelsenbeck 2003). To decide which evolutionary model for ML best fit the data set, the program Modeltest V. 3.7 (Posada and Crandall 1998) was used. The model selected by the hierarchical likelihood ratio tests and by the Akaike information criterion for all data sets in rooted and unrooted ML trees was K81uf + I + G model. Base frequencies and substitution parameters were estimated by Modeltest. ML analysis was performed using the heuristic search option with a branch-swapping algorithm (tree bisection-reconnection, TBR). Starting trees were obtained by stepwise random addition of sequences (10 replicates). The distance matrix was calculated using Kimura two parameter distances (Kimura 1980), and the distance tree was constructed using the NJ method (Saitou and Nei 1987). MP was performed using the heuristic search option with random addition of sequences (1,000 replicates) and a branch-swapping algorithm (TBR). All characters were weighted equally in both rooted and unrooted trees. Bootstrap analyses with 100 replicates for rooted and unrooted ML analyses, and 1,000 replicates for rooted and unrooted NJ and MP analyses were applied to examine the robustness and statistical reliability of the topologies (Felsenstein 1985). For ML bootstrapping, the heuristic search option with a branch-swapping algorithm of nearest neighbor interchange was employed. Bayesian analyses were conducted to construct rooted and unrooted trees with two runs of four Markov chains, for at least 2 million generations, sampling every 100th generation. The burn-in option was set discarding 25% from the 20,000 trees found.

Comparison of environmental parameters at the sites of culture strain isolation. Annual mean environmental values at the sampling location of each strain and monthly mean environmental parameters for the sampling month of each sampling location were obtained from the *World Ocean Atlas 2005* (Antonov et al. 2006, Garcia et al. 2006a,b, Locarnini et al. 2006). Welch's *t*-test (Welch 1947) with 5% rejection rate was applied to examine whether mean values of environmental parameters (temperature, salinity, concentrations of nitrate and phosphate) between the clades obtained by molecular phylogenetic analyses were significantly different from each other.

RESULTS

Morphological studies of culture samples. Of the 39 *E. huxleyi* strains, five strains were noncalcifying and so could not be used for morphological studies of coccoliths; however, the morphotype of coccoliths in two of the five naked strains was known from previous studies (Table S1). Based on the morphology of the central area and of the distal shield of coccoliths, the 34 calcifying strains were classified into morphotypes. Thirty of the calcifying strains could be assigned to the previously established morphotypes A, B/C, and R, of Young and Westbroek (1991) and Young et al. (2003). The remaining four calcifying strains were similar in shield characteristics and in size to Types B or B/C but different in central area morphology. These four strains have

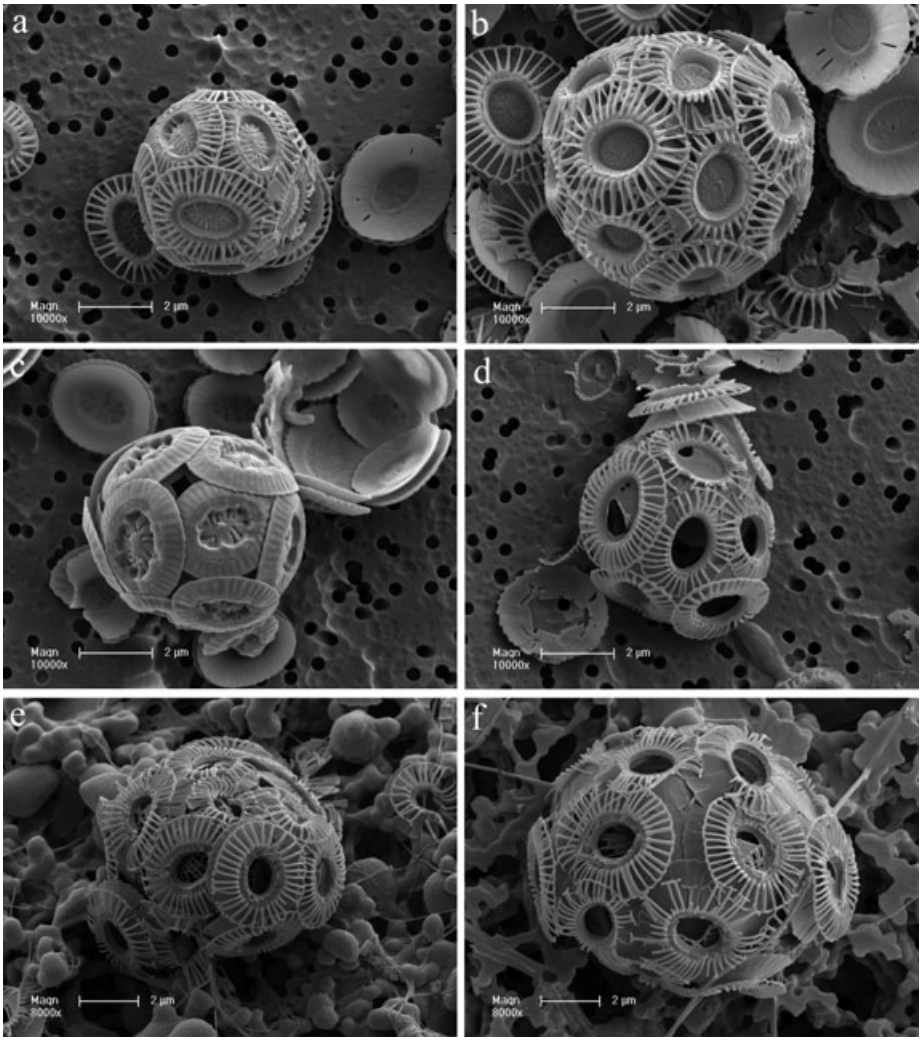

FIG. 2. SEM images of clonal culture strains and field specimens. (a) Type A strain NG-1; (b) Type B/C strain NS10Y; (c) Type R strain TQ22; (d) Type O strain NIES 1311; (e) Type O specimen from field sample KH69-4, sample KH-30; and (f) Type O specimen from field sample KT90-9, st. 11.

never calcified their central area (i.e., they have maintained an open central area) over >4 years under various laboratory culture conditions, whereas culture strains of other morphotypes have always had calcified central areas. It therefore seems clear that the morphology of the central area is stable and genetically controlled. Hereafter, we call the morphotype with an open central area ''Type O.'' The definition of morphotypes used in this study is summarized in Table 1.

Type A was the most common morphotype; 26 out of the 34 calcifying strains were classified as Type A, based on the presence of a grill in the central area and small-medium (<4 µm) distal shield length (Tables 1 and S1; Figs. 1, 2a, and 3). The degree of calcification of the distal shield and central area was stable in each strain over several years in culture but varied among strains. Variation in the degree of calcification within Type A strains, however, was not used for subdivision of this morphotype, because a classification scheme related to intensity of calcification has not yet been established. Strain NS10Y, which is characterized by a solid/plated central area, delicate distal shield elements, and medium size (3.5–4.0 µm in distal shield length), was the only Type B/C strain in this study. This Type B/C strain was isolated from the S.E. Atlantic Ocean off the coast of South Africa (Tables 1 and S1; Figs. 1, 2b, and 3). Three Type R strains with heavily calcified *Reticulofenestra*-like distal shields came from the same water sample collected from the E. Tasman Sea, off South Island, New Zealand (Tables 1 and S1; Figs. 1, 2c, and 3). Of the four Type O strains identified by having an open central area and delicate distal shield elements, two strains came from the E. Bering Sea, and two from the northern part of the Japan Sea (Tables 1 and S1; Figs. 1, 2d, and 3). The central area of the Type O coccoliths was often covered by an organic membrane that can be mistaken for the central plate, but careful observation in high magnification revealed that Type O strains never possessed a calcified central area structure (Fig. 2d).

Morphological studies of field samples. All *E. huxleyi* specimens observed from samples KH-30 through KH-34, which were used by Okada and Honjo

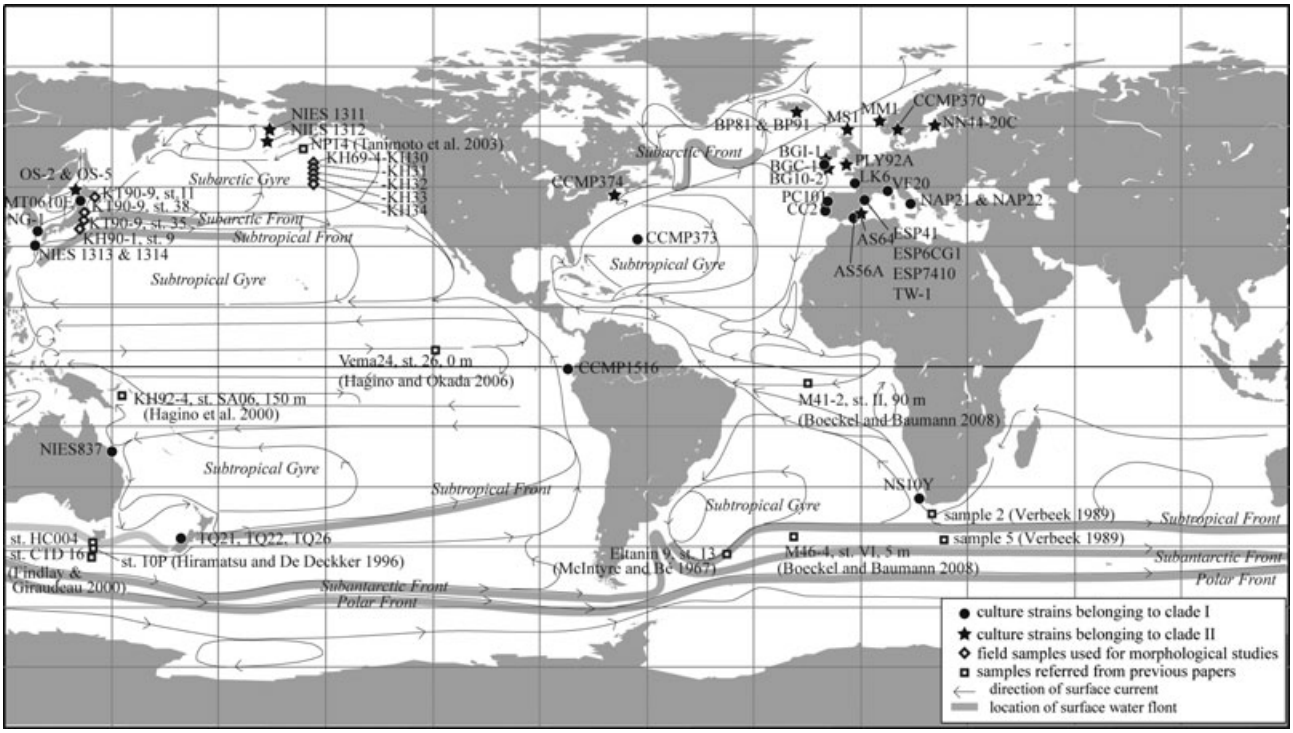

FIG. 3. Location of culture strains and field samples used or discussed in this study, and distribution of surface currents and water fronts (Tomczak and Godfrey 1994).

(1973) for description of their subarctic type, showed type O morphology (Table 2; Figs. 2e and 3). The distal shield elements were occasionally disconnected from the neighboring elements, which resulted in the ''irregular distal shield elements'' reported by Okada and Honjo (1973). Distal and proximal shields were almost the same size and varied from 2.5 to 4.0 μm in length. Of the samples from the N.W. Pacific, sample KT90-9, station 11, which was recorded as dominated by Type B (Hagino et al. 2005), yielded exclusively specimens with an open central area and elevated delicate distal shield elements, that is, Type O. The distal shield was usually smaller than the proximal shield. The coccolith length varied greatly, ranging from 2.5 to 5.0 μm. In the five N.W. Pacific samples reexamined, the composition of *E. huxleyi* morphotypes varied latitudinally. Type O dominated the northernmost station and decreased in abundance southward (Table 2), while Type A increased in abundance southward and dominated the southernmost station (KH90-1 station 18). Type B/C with plated central area, delicate distal shield elements, and relatively small coccoliths (<4 μm in distal shield length) occurred rarely at KT90-9 station 35 and KH90-1 station 9 (Table 2).

Phylogenetic analysis of culture strains. The alignment of obtained sequences of the *cox*1b-*atp*4 region was 1,517 bp in length with no gaps. For construction of rooted ML, NJ, MP, and Bayesian trees, a total of 43 sequences, including sequences of four *G. oceanica* strains as an outgroup, were used. A likelihood score ($-\ln L$) of 2491.6389 was obtained under the K81uf + I + G model with the following parameters: assumed nucleotide frequencies A = 0.2682, C = 0.1505, G = 0.1691, and T = 0.4122; substitution-rate AC = 1, AG = 6.7470, AT = 0.0717, CG = 0.0717, CT = 6.7470, GT = 1; proportion of sites assumed to be invariable = 0.7793; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.7062, estimated by Modeltest 3.7. Parsimony analysis resulted in a single most parsimonious tree of 73 steps (CI = 0.877, RI = 0.965). ML, NJ, and MP analyses resulted in similar trees, while the topology of the Bayesian tree was different from that of ML, NJ, and MP trees. Figure 4 shows only the ML tree with bootstrap values obtained by ML, NJ, and MP methods, and Figure 5 shows the Bayesian tree with posterior probabilities.

In the rooted ML, NJ, and MP trees, *E. huxleyi* made a clade with 100% bootstrap values (Fig. 4). *E. huxleyi* consisted of two major clades, I and II, which were separated from each other by eight common substitutions and were supported by moderate to high bootstrap values (78%–91%). Clade I consisted of 26 strains including 22 Type A, one Type B/C, and three Type R strains. Type A strains in clade I had diverse mitochondrial sequences, which resulted in several small clusters with bootstrap values ≤70% and one subclade with bootstrap values 96%. This well-supported subclade comprised four

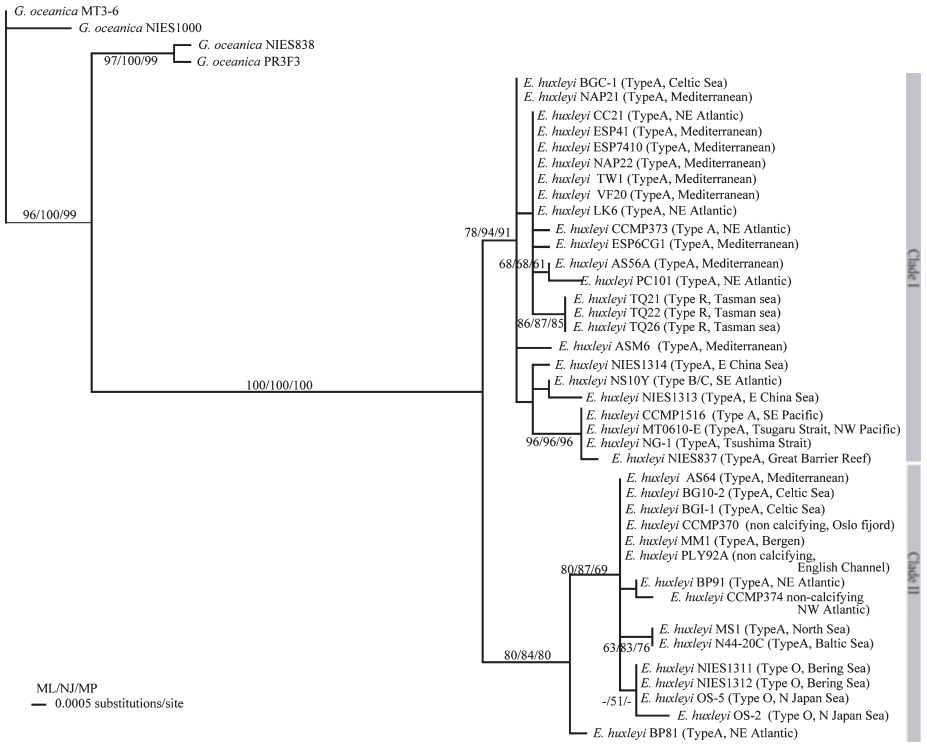

FIG. 4. Rooted ML tree (−ln L = 2491.6389) based on *cox1-atp4* sequences. Four sequences of *Gephyrocapsa oceanica* were used as an outgroup. The numbers on each node indicate the bootstrap values from ML, NJ, and MP analyses. ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining.

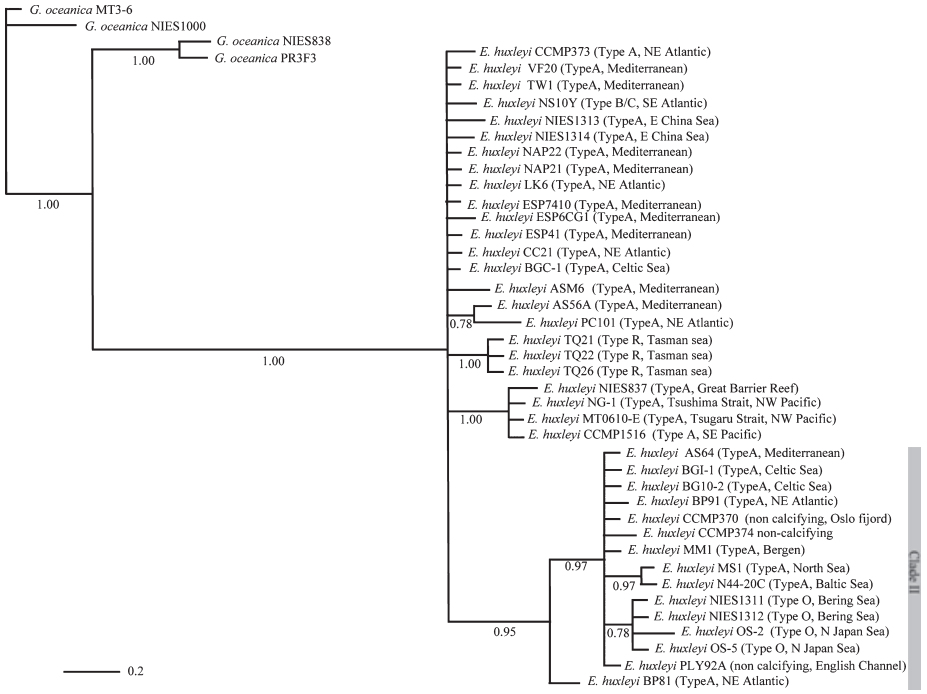

FIG. 5. Rooted Bayesian tree based on *cox1-atp 4* sequences. Four sequences of *Gephyrocapsa oceanica* were used as an outgroup. The numbers on each node indicate the Bayesian posterior probabilities.

Type A strains (NG-1, MT0610E, NIES 837, and CCMP 1516) from the Pacific Ocean and adjacent seas. The sequences of the three Type R strains (TQ21, 22, and 26) were identical to each other, and different from other morphotypes. The only Type B/C strain, NS10Y from the S.E. Atlantic, formed a subclade with NIES1313 (Type A) from the N.W. Pacific with low bootstrap support in the ML and NJ trees, but no support in the MP analysis.

Clade II included eight Type A strains, four Type O strains, and three noncalcifying strains. Strain BP81 (Type A) from northern N.E. Atlantic (Iceland) occupied a well-separated basal position within clade II. The remaining strains branched into a cluster

supported by low-to-moderate bootstrap values of 69%–87%. Within this cluster, the four Type O strains made a subclade with low bootstrap support (51%) in the NJ trees, but no support in the ML and MP analysis.

In the rooted Bayesian tree, all *E. huxleyi* strains made a clade with a posterior probability of 1.00 (Fig. 5). The *E. huxleyi* clade included three subclades supported by high posterior probabilities (0.95–1.0) and a subclade with low posterior probability (0.78) as well as 15 nonclustered strains. Of the subclades with high posterior probabilities, the largest consisted of eight Type A, four Type O, and three noncalcifying strains corresponding to clade II in the rooted ML, NJ, and MP trees (Figs. 4 and 5). Hereafter, we refer to this largest subclade as clade II following the classification in the rooted ML, NJ, and MP trees. In clade II, Type A strain BP81 occupied a basal position. The remaining strains formed an internal subclade with 0.97 posterior probability. Within this subclade, two Type A strains clustered together with 0.97 posterior probability, and four Type O strains clustered with low posterior probability (0.78). The *E. huxleyi* strains outside clade II varied in sequences, and two Type A strains from N.E. Atlantic and Mediterranean Sea, four Type A strains from the Pacific and its adjacent seas, and three Type R strains from the Tasman Sea made subclades with 0.78, 1.0, 1.0 posterior probability, respectively.

A total of 39 *E. huxleyi* sequences were used for constructing unrooted ML, NJ, MP, and Bayesian trees. A likelihood score (−ln *L*) of 2491.6389 was obtained under the K81uf + I + G model with the following parameters: assumed nucleotide frequencies A = 0.2682, C = 0.1505, G = 0.1691, and T = 0.4122; substitution-rate AC = 1, AG = 6.7471, AT = 0.0717, CG = 0.0717, CT = 6.7471, GT = 1; proportion of sites assumed to be invariable = 0.7793; rates for variable sites assumed to follow a gamma distribution with shape parameter = 0.7062, estimated by Modeltest 3.7. Parsimony analysis resulted in a single most parsimonious tree of 73 steps (CI = 0.877, RI = 0.965). ML, NJ, MP, and Bayesian analyses without outgroup sequences resulted in similar trees, and here we show only the ML tree with bootstrap values obtained by ML, NJ, and MP methods and Bayesian posterior probabilities (Fig. 6).

In unrooted ML, NJ, MP, and Bayesian trees, the 15 *E. huxleyi* strains that constituted clade II in the rooted ML, NJ, and MP trees (Fig. 4) made a clade with very high bootstrap values from 99% to 100% and 1.0 Bayesian posterior probability (Fig. 6). Hereafter, we refer to this clade as clade II following the classification in the rooted trees. In this clade, strain BP81 occupied a well-separated basal position, and other strains formed an internal subclade with moderate to high bootstrap values (from 84% to 91%) and high Bayesian posterior probability (1.0). Type O strains formed a subclade within clade II with very low bootstrap support (52%) in the NJ tree and with low posterior probability (0.69) in the Bayesian tree, but no support in the ML and MP analyses. Type A strains were distributed throughout the phylogenetic trees, although four Type A strains from the Pacific Ocean and adjacent seas (NG-1, MT0610E, NIES 837, and CCMP 1516) formed a clade with high bootstrap values (96%–97%) and 1.0 posterior probability.

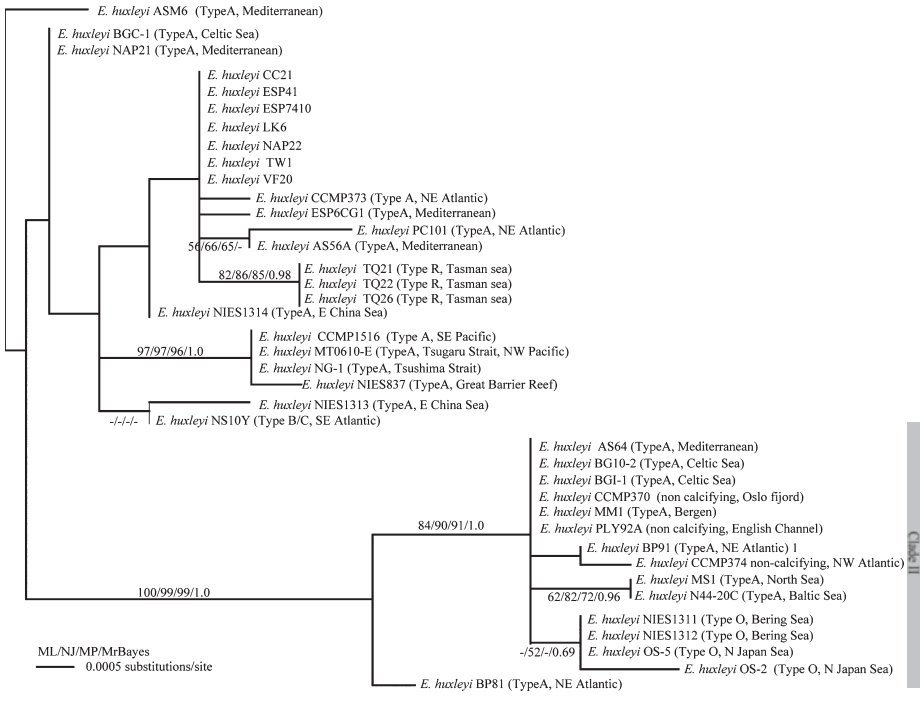

FIG. 6. Unrooted ML tree (−ln *L* = 2491.6389) based on *cox1-atp4* sequences. The numbers on each node indicate the bootstrap values from ML, NJ, and MP analyses and Bayesian posterior probabilities. ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining.

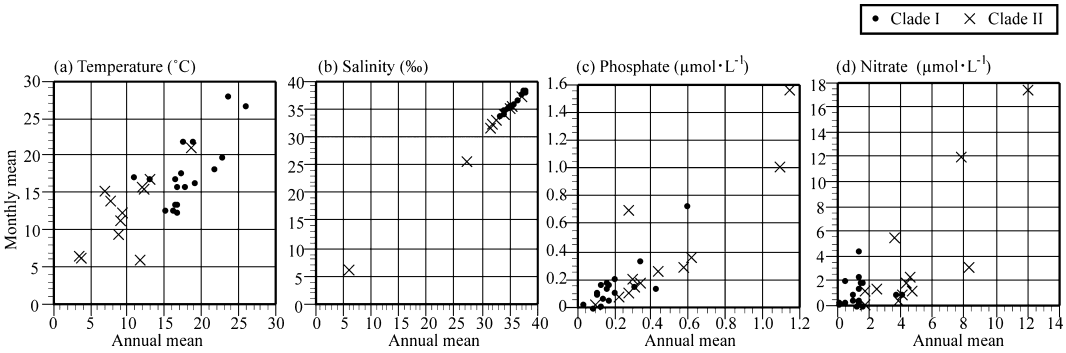

FIG. 7. Plots showing the annual mean and monthly mean (for the month in which they were collected) of various environmental parameters for the sampling locations from which the strains were collected. These plots show the degree to which the two clades occur under different conditions.

TABLE 4. Results of Welch's *t*-test.

	t-Stat	*t*-Critical (two-tail)	*P*-value (two-tail)	Assumption by Welch's *t*-test
Temperature (annual mean)	6.962476846	1.699126996	0.0000001182	Not equal variance
Temperature (monthly mean)	3.576598296	1.701130908	0.0012909848	Not equal variance
Salinity (annual mean)	2.043838627	2.131449536	0.0589416046	Equal variance
Salinity (monthly mean)	2.058859027	2.144786681	0.0586170981	Equal variance
Phosphate (annual mean)	−2.766845052	2.09302405	0.0122778891	Not equal variance
Phosphate (monthly mean)	−2.136999339	2.131449536	0.0494780018	Not equal variance
Nitrate (annual mean)	−4.572295083	2.109815559	0.0002705760	Not equal variance
Nitrate (monthly mean)	−1.942597981	2.144786681	0.0724553362	Equal variance

Geographic and hydrographic range of mitochondrial clades I and II. The culture strains included in clades I and II of rooted ML, NJ, and MP trees showed different biogeographic distributions (Fig. 3). Clade I strains were isolated from tropical to temperate waters on the equatorial side of subarctic fronts. By contrast, clade II strains were mainly collected from boreal subarctic waters, except for one clade II strain, AS64, from the western Mediterranean Sea (Fig. 3). Identical sequences were found between N. Atlantic and Mediterranean strains, but not between ''N Atlantic + Mediterranean'' and Pacific strains (Fig. 4).

The very different biogeographic distributions suggest that the culture strains of clades I and II have different environmental preferences; this was tested by comparing environmental data from the locations at which the strains were isolated. Comparison of annual and monthly mean environmental parameters showed that the variation range of clades I and II differed in annual and monthly mean temperature, and annual mean nitrate and phosphate concentration, but overlapped greatly in annual and monthly mean salinity and in monthly mean nitrate and phosphate concentration (Fig. 7). Table 4 shows the results of the Welch's *t*-test. When *P*-values are <0.05 or absolute *t* values are less than absolute *t*-Stat values, the two groups are significantly different. The results indicate that clades I and II are statistically different from each other in annual and monthly mean temperature and phosphate concentration and in annual mean nitrate concentration, but equal in variance in annual and monthly salinity and in monthly mean nitrate concentration (Table 4). These results suggest that the habitat separation of clades I and II strains is primarily related to differences in temperature and/or phosphate and not to salinity.

DISCUSSION

Geographic distribution of morphotypes. Based on the morphological stability of the central area of the coccoliths reported here, we propose to distinguish coccoliths with an open central area as Type O from Types B, B/C, and C, which are characterized by the presence of a solid plate in the central area of coccoliths. This new subdivision means that the geographic distribution of Types B, B/C, and C reported in previous studies could be biased by inclusion of Type O. Distribution of Types B, B/C, C, and O, however, can be reevaluated from SEM images shown in previous studies and from the geographic origin of culture strains.

Morphotypes B, B/C, and C (with a central plate) were separated from each other based on the size of the distal shield (Table 1; Young et al. 2003). Van Bleijswijk et al. (1991) reported Type B with plated central area from the North Sea and English Channel, and the only three Type B strains reported to

date (92D, Ch25, and MCH) were isolated from the North Sea and English Channel (Young and Westbroek 1991). Type B is evidently distributed in the N.E. Atlantic, especially in the seas surrounding the UK, but it is currently not clear whether this type is restricted to this area.

In this study, Type B/C specimens (<4 μm in distal shield length) were found rarely from surface waters of the temperate N.W. Pacific (Table 2). In the literature, Types B/C and C have mainly been reported from temperate surface waters and from the lower photic zone of stratified tropical waters. McIntyre and Bé (1967) described the cold-water type (=Type C) with solid central plate from the S.W. Atlantic, close to Subtropical Front (Eltanin 9 station 13 of Fig. 3). Hagino and Okada (2006) reported the common occurrence of Type C in surface samples from the equatorial upwelling zone and showed an image of an *E. huxleyi* specimen with a central plate as an example of their Type C (Vema24, station 26, 0 m). Hagino et al. (2000) and Boeckel and Baumann (2008) showed SEM images of *E. huxleyi* with plated central area as examples of Type C (KH92-4, SA06, 150 m) and Type B/C (M41-2, station II, 90 m) from the lower photic zone of stratified tropical water masses (Fig. 3). From these observations, it is thought that Types B/C and C with plated central area change their depth habitat depending on the intensity of stratification, temperature, and/or nutrient level (Hagino et al. 2000).

Reobservation of the samples used in previous studies revealed that the subarctic type of Okada and Honjo (1973) from the northern central N. Pacific and Type B of Hagino et al. (2005) from the N.W. Pacific correspond to our Type O. Tanimoto et al. (2003) displayed an SEM image of an *E. huxleyi* specimen with an open central area from the northern N.E. Pacific Ocean as an example of their *E. huxleyi* var. *kleijniae* (st. NP14 of Fig. 3). Furthermore, all four culture strains isolated from adjacent seas of the northern N. Pacific were Type O. Therefore, it appears that the *E. huxleyi* population of the North Pacific Subarctic Gyre and its adjacent seas is dominated by Type O.

Biogeographic studies from the Southern Ocean have also shown SEM images of *E. huxleyi* with an open central area (Type O). Verbeek (1989) showed SEM images of their cold-water and malformed forms with open central area from subtropical and polar stations of the Southern Ocean (samples 2 and 15 of Fig. 3). Hiramatsu and De Deckker (1996) showed two *E. huxleyi* specimens with open central area as their Type K (station 10P, Fig. 2) and mentioned that the coccolithophore flora south of the Subtropical Front was dominated by Type K. Findlay and Giraudeau (2000) showed SEM images of their Types C and D with open central area, which were collected from transitional water between the Subtropical and Subantarctic Fronts (stations HC004 and CTD16 of Fig. 3). Boeckel and Baumann (2008) showed an SEM image of an *E. huxleyi* specimen with an open central area as an example of their Type B from south of the Subtropical Front of the S. Atlantic Ocean (M46-4, st. VI, 5 m of Fig. 3). From these reports, it is evident that Type O is extensively distributed in the Southern Ocean. Type O is therefore a dominant morphotype in the northern North Pacific and in the Southern Ocean, but rare/absent in tropical surface waters of the Pacific Ocean. These results suggest that Type O is a cold-water dweller with bipolar geographic distribution.

Diversity of mitochondrial sequences of E. huxleyi *and relationships to environmental conditions and morphotypes.* In the rooted phylogenetic trees, *E. huxleyi* strains were divided into two main clades with relatively minor but highly consistent differences in *cox*1b-*atp*4 sequences. The differences in nucleotide sequences, do not affect amino acid sequences and at this stage, it is unclear whether the two clades correspond to discrete biological entities (cryptic species) or intraspecific lineages. The topology of ML and Bayesian trees differed in the relationships of the two main groups; in the ML tree, clades I and II are sister groups, whereas in the Bayesian analysis, clade II is derived from ''clade'' I, which is thus a paraphyletic group. Nonetheless, the separation of the strains into these two groups was a consistent feature of all the unrooted trees and was supported by high bootstrap values and posterior probability. In this context, it can be postulated that gene flow may well be limited between the two clades given the spatial separation (in surface/upper-subsurface waters at least), clade I strains originating from warm tropical/temperate water masses and clade II strains mainly from colder subarctic water masses (Figs. 3–5). The occurrence of a single clade II strain (AS64) in warmer water (Mediterranean Sea), however, may indicate that separation is not so clear-cut.

Similarity in mitochondrial sequences between the northern N. Atlantic and northern N. Pacific clade II populations indicates that these two boreal populations have the same genetic origin even though they are separated from each other by the polar ice cap in the north and warm-water masses in the south. How did the clade II population migrate between the northern N. Atlantic and N. Pacific Oceans? Reid et al. (2007) reported that a Pacific cold-water diatom *Neodenticula seminae* appeared in the N. Atlantic in May 1999 for the first time in the past 800 kiloyears, and concluded that it had probably migrated from the N. Pacific to N. Atlantic through the Arctic pathway opened by melting of Arctic ice in 1998/early1999. Surface water of the Arctic pathway flows from the Pacific to the Atlantic, so migration of planktonic microalgae is likely to occur in this direction. The presence of strain PLY92A (clade II), isolated from the English

Channel in 1957, indicates that the clade II population has been in the Atlantic before opening of the Arctic pathway in 1998/1999. Migration of *E. huxleyi* populations through the Arctic pathway might have occurred in the geological past, particularly during the last interglacial MIS5e. However, the absence of evidence for similar migration in other plankton groups at this time makes this unlikely. An alternative route for clade II migration is within the lower photic zone of equatorial waters. Morphological studies of *E. huxleyi* have reported that morphotype composition is different between the upper and lower photic zone in tropical waters, and Type C changes its depth habitat from warm oligotrophic surface water to relatively cool eutrophic lower photic zone water (below the thermocline from 50 to 150 m) according to water stratification, as opposed to Type A that remains in the upper photic zone regardless of water stratification (Hagino et al. 2000). Therefore, there is a possibility that clade II populations cross the equator in the lower photic zone. All culture strains used in this study were isolated from surface water or upper-subsurface water samples. To assess the possibility of clade II migration through the lower photic zone, mitochondrial DNA of *E. huxleyi* strains from the lower photic zone of tropical waters should be studied. A final possible cause of migration of the clade II population is mixing by human activity (e.g., transportation by ship ballast water). This possibility cannot be ruled out completely, but geological core studies have shown that *E. huxleyi* has been distributed in both the northern N. Atlantic and northern N. Pacific Oceans since ~250–260 ka (Worsley 1973, Thierstein et al. 1977, Sato et al. 2002), so migration of cold-adapted populations into the subarctic was unlikely to have been a result of human activity.

The Celtic Sea and English Channel appear to be the boundary between the habitats of clade I and II strains in the eastern N.E. Atlantic. Three strains (BGC-1, BGI-1, and BG10-2) isolated from an *E. huxleyi* bloom in the Celtic Sea in 2007 varied in mitochondrial sequence (Figs. 3 and 4). Strain BGC-1 was genetically identical to strain NAP 21 (Mediterranean Sea) and belonged to clade I. Strains BGI-1 and BG10-2 were identical to the four clade II strains CCMP370 (North Sea), MM1 (Bergen), PLY92A (English Channel), and AS64 (Mediterranean Sea) (Fig. 4). Previous studies reported that multiple GPA genotypes can be found in *E. huxleyi* blooms (Martínez-Martínez et al. 2007, Ripley et al. 2008). Genetic diversity in mitochondrial sequences in bloom-forming *E. huxleyi* strains suggests that *E. huxleyi* populations forming blooms can have diverse genetic origins, with similar environmental preferences.

Clades I and II were rather well separated by correlation with both monthly and annual mean temperature and annual nutrient concentrations, but were less well separated by monthly nutrient levels and were not separated by monthly or annual salinity (Table 4; Fig. 5). Weaker correlations of clades I and II with monthly mean nutrient concentration suggest that culture strains of clades I and II came from similar (overlapping) nutrient ranges, and the better correlation shown in annual mean nutrient values is probably a misleading result caused by including environmental data from winter, which is an unsuitable period for growth of *E. huxleyi* in high latitudes. Differences in the range of sampling months between clades I and II also suggest that including winter environmental parameters is unsuitable for the discussion of environmental preferences of clade II strains, since the sampling month of clade II is limited from March to October (spring–autumn), whereas the clade I strains were collected from January to December. Therefore, it is thought that genetic separation between clades I and II likely stem from difference in temperature preference/tolerance rather than nutrient limitation. We have observed that culture strains collected from warm-water masses usually do not grow in low-temperature culture conditions (<13°C), although the temperature preference/tolerance of most culture strains has not yet been systematically studied. For a better understanding of the relationship between geographic diversification in mitochondrial DNA of *E. huxleyi* and its range of temperature preference/tolerance, more culture studies using strains from various regions are required.

The phylogenetic diversification observed from mitochondrial sequences of *E. huxleyi* was not consistent with the genetic variation reported in the GPA gene in previous research. Schroeder et al. (2005) sequenced the GPA region of 13 clonal *E. huxleyi* strains and classified the strains into four coccolith morphology motif (CMM) groups. Our mitochondrial analysis included four strains (CCMP370, CCMP 373, CCMP 374, and CCMP1516) used by Schroeder et al. (2005). In our results, CCMP373 and CCMP1516 belonged to clade I, and CCMP370 and CCMP374 belonged to clade II (Fig. 4). In the CMM grouping of Schroeder et al. (2005), however, CCMP 370 and CCMP373 belonged to CMM group I. Schroeder et al. (2005) also showed that strains CCMP374 and CCMP 1516 exhibited interclonal variation in CMM sequences, with CCMP 374 having both CMM I and IV sequences, and CCMP 1516 having both CMM III and IV sequences. To clarify population structure of *E. huxleyi*, further studies based on multiple genetic regions are needed.

From our data set, we cannot draw strong conclusions as to potential relationships between mitochondrial genotypes and coccolith morphotypes since our molecular analyses included limited numbers of Types B/C, C, R, and O strains relative to Type A strains (and no Type B strains). Type A strains occurred in both clades I and II and occupied basal positions in both clades. Type O and

Type R strains formed discrete subclades within clades II and I, respectively. This may suggest that Type A is the primitive morphology of *E. huxleyi*, and that other morphotypes have evolved from Type A within one or other of the clades. Type O may well be mitochondrial genotype specific, since in surface waters it is evidently restricted to higher latitudes like clade II. The known distributions of Types B, B/C, C, and R, however, do not so clearly correspond with the biogeography of *cox*1b-*atp*4 clades, and the possibility clearly exists that morphotypes diversified before separation of the two clades, in which case morphotype would be an inherited polymorphism present in each clade.

CONCLUDING REMARKS

Variability in the *cox*1b-*atp*4 region of the *E. huxleyi* mitochondrial genome appears to correspond to geographic origin and to some extent to environmental conditions at the site of origin of the strain, and this genetic region is potentially a useful marker either for intraspecific diversity within this taxon or for species-level diversity between cryptic taxa within the morphospecies. From the data currently available, however, the diversity revealed by this marker does not clearly relate to morphological diversity of coccoliths, which itself seems to be a relatively stable, genetically controlled character. Further comparison of morphological and molecular characters of *E. huxleyi*, in environmental studies, may shed light on the biological, ecological, and evolutionary implications of microdiversity of these important microalgae. However, ultimate proof of whether speciation has already occurred within this relatively young lineage and whether biological diversification has any link to coccolith morphology is likely to require either extensive comparative genomic information from multiple *E. huxleyi* strains and/or evidence from mating experiments between haploid culture strains. In either case, culture-based studies are likely to provide key information.

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Antonov, J., Locarnini, I. R. A., Boyer, T. P., Mishonov, A. V. & Garcia, H. E. 2006. World Ocean Atlas 2005, Volume 2: Salinity. *In* Levitus, S. [Ed.] *NOAA Atlas NESDIS 62*. U.S. Government Printing Office, Washington, D.C., 182 pp.

Boeckel, B. & Baumann, K.-H. 2008. Vertical and lateral variations in coccolithophore community structure across the subtropical frontal zone in the South Atlantic Ocean. *Mar. Micropaleontol.* 67:255–73.

Corstjens, P. L. A. M., van der Kooij, A., Linschooten, C., Brouwers, G.-J., Westbroek, P. & de Vrind-de Jong, E. W. 1998. GPA, a calcium-binding protein in the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae). *J. Phycol.* 34:622–30.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–91.

Findlay, C. S. & Giraudeau, J. 2000. Extant calcareous nannoplankton in the Australian Sector of the Southern Ocean (austral summers 1994 and 1995). *Mar. Micropaleontol.* 40:417–39.

Fujiwara, S., Tsuzuki, M., Kawachi, M., Minaka, N. & Inouye, I. 2001. Molecular phylogeny of the Haptophyta based on the *rbc*L gene and sequence variation in the spacer region of the RUBISCO operon. *J. Phycol.* 37:121–9.

Garcia, H. E., Locarnini, R. A., Boyer, T. P. & Antonov, J. I. 2006a. World Ocean Atlas 2005, Volume 3: Dissolved Oxygen, Apparent Oxygen Utilization, and Oxygen Saturation. *In* Levitus, S. [Ed.] *NOAA Atlas NESDIS 63*. U.S. Government Printing Office, Washington, D.C., 342 pp.

Garcia, H. E., Locarnini, R. A., Boyer, T. P. & Antonov, J. I. 2006b. World Ocean Atlas 2005, Volume 4: Nutrients (phosphate, nitrate, silicate). *In* Levitus, S. [Ed.] *NOAA Atlas NESDIS 64*. U.S. Government Printing Office, Washington, D.C., 396 pp.

Gard, G. 1986. Calcareous nannofossil biostratigraphy of Late Quaternary Arctic sediments. *Boreas* 15:217–29.

Gard, G. 1989. Variations in coccolith assemblages during the last glacial cycle in the high and mid-latitude Atlantic and Indian Oceans. *In* Crux, J. A. & van Heck, S. E. [Eds.] *Nannofossils and Their Applications*. Ellis Horwood, Chichester, UK, pp. 108–21.

Hagino, K. & Okada, H. 2006. Intra- and infra-specific morphological variation in selected coccolithophore species in the equatorial and subequatorial Pacific Ocean. *Mar. Micropaleontol.* 58:184–206.

Hagino, K., Okada, H. & Matsuoka, H. 2000. Spatial dynamics of coccolithophore assemblages in the equatorial western-central Pacific Ocean. *Mar. Micropaleontol.* 39:53–72.

Hagino, K., Okada, H. & Matsuoka, H. 2005. Coccolithophore assemblages and morphotypes of *Emiliania huxleyi* in the boundary zone between the cold Oyashio and warm Kuroshio currents off the coast of Japan. *Mar. Micropaleontol.* 55:19–47.

Hine, N. & Weaver, P. P. E. 1998. Quaternary. *In* Bown, P. [Ed.] *Calcareous Nannofossil Biostratigraphy*. Chapman & Hall, Cambridge, pp. 266–83.

Hiramatsu, C. & De Deckker, P. 1996. Distribution of calcareous nannoplankton near the subtropical convergence, south of Tasmania, Australia. *Mar. Freshw. Res.* 47:707–13.

Iglesias-Rodríguez, M. D., Sáez, A., Groben, R., Edwards, K. J., Batley, J., Medlin, L. & Hayes, P. K. 2002. Polymorphic microsatellite loci in global populations of the marine coccolithophorid *Emiliania huxleyi*. *Mol. Ecol. Notes* 2:495–7.

Iglesias-Rodríguez, M. D., Schfield, O. M., Batley, J., Medlin, L. K. & Hayes, P. K. 2006. Intraspecific genetic diversity in the marine coccolithophore *Emiliania huxleyi* (Prymnesiophyceae): the use of microsatellite analysis in marine phytoplankton population studies. *J. Phycol.* 42:526–36.

Jordan, R. W., Zhao, M., Eglinton, G. & Weaver, P. P. E. 1996. Coccolith and alkenone stratigraphy and palaeoceanography at an upwelling site off NW Africa (ODP 658C) during the last 130,000 years. *In* Moguilevsky, A. & Whatley, R. [Eds.] *Microfossils and Oceanic Environments*. University of Wales, Aberystwyth – Press, Aberystwyth, UK, pp. 111–30.

Kimura, M. 1980. A simple method for estimating rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111–20.

Locarnini, R. A., Mishonov, A. V., Antonov, J. I., Boyer, T. P. & Garcia, H. E. 2006. World Ocean Atlas 2005, Volume 1: Temperature. *In* Levitus, S. [Ed.] *NOAA Atlas NESDIS 61*, U.S. Government Printing Office, Washington, D.C., 182 pp.

Martínez-Martínez, J., Schroeder, D. C., Larsen, A., Bratbak, G. & Wilson, W. H. 2007. Molecular dynamics of *Emiliania huxleyi*

and cooccurring viruses during two separate mesocosm studies. *Appl. Environ. Microbiol.* 73:554–62.
McIntyre, A. & Bé, A. 1967. Modern Coccolithophoridae of the Atlantic Ocean-I. Placoliths and cyrtoliths. *Deep-Sea Res.* 14:561–97.
Medlin, L. K., Barker, G. L. A., Campbell, L., Green, J. C., Hayes, P. K., Marie, D., Wrieden, S. & Vaulot, D. 1996. Genetic characterization of *Emiliania huxleyi* (Haptophyta). *J. Mar. Syst.* 9:13–31.
Noël, M.-H., Kawachi, M. & Inouye, I. 2004. Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). *J. Phycol.* 40:112–29.
Okada, H. & Honjo, S. 1973. The distribution of oceanic coccolithophorids in the Pacific. *Deep-Sea Res.* 20:355–74.
Okada, H. & McIntyre, A. 1977. Modern coccolithophores of the Pacific and North Atlantic Oceans. *Micropaleontology* 23:1–55.
Posada, M. A. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–8.
Raffi, I., Backman, J., Fornaciari, E., Pälike, H., Rio, D., Lourens, L. & Hilgen, F. 2006. A review of calcareous nannofossil astrobiochronology encompassing the past 25 million years. *Quat. Sci. Rev.* 25:3113–37.
Reid, P. C., Johns, D. G., Edwards, M., Starr, M., Poulin, M. & Snoeijs, P. 2007. A biological consequence of reducing Arctic ice cover: arrival of the Pacific diatom *Neodenticula seminae* in the North Atlantic for the first time in 800,000 years. *Glob. Change Biol.* 13:1910–21.
Ripley, S. J., Baker, A. C., Miller, P. I., Walne, A. W. & Schroeder, D. C. 2008. Development and validation of a molecular technique for the analysis of archived formalin-preserved phytoplankton samples permits retrospective assessment of *Emiliania huxleyi* communities. *J. Microbiol. Methods* 73:118–24.
Ronquist, F. & Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–4.
Rost, B. & Riebesell, U. 2004. Coccolithophores and the biological pump: responses to environmental changes. *In* Thierstein, H. R. & Young, J. R. [Eds.] *Coccolithophores From Molecular Processes to Global Impact.* Springer-Verlag, Berlin Heidelberg, Germany, pp. 99–125.
Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–25.
Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York, 1659 pp.
Sánchez Puerta, V., Bachvaroff, T. R. & Delwiche, C. F. 2004. The complete mitochondrial genome sequence of the haptophyte *Emiliania huxleyi* and its relation to heterokonts. *DNA Res.* 11:1–10.
Sato, T., Saito, T., Yuguchi, S., Nakagawa, H., Kameo, K. & Takayama, T. 2002. Late Pliocene calcareous nannofossil palaeobiogeography of the Pacific Ocean: evidence for glaciation at 2.75 Ma. *Rev. Mexicana Cienc. Geol.* 19:175–89.
Schroeder, D. C., Biggi, G. F., Hall, M., Davy, J., Martínez-Martínez, J., Richardson, A. J., Malin, G. & Wilson, W. H. 2005. Note: a genetic marker to separate *Emiliania huxleyi* (Prymnesiophyceae) morphotypes. *J. Phycol.* 41:874–9.
Schwaninger, H. R. 2008. Global mitochondrial DNA phylogeography and biogeographic history of the antitropically and longitudinally disjunct marine bryozoan *Membranipora membranacea* L. (Cheilostomata): another cryptic marine sibling species complex? *Mol. Phylogenet. Evol.* 49:893–908.
Swofford, D. L. 2002. *PAUP*—Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4.* Sinauer Associates, Sunderland, Massachusetts.
Tanimoto, M., Aizawa, C. & Jordan, R. W. 2003. Assemblages of living microplankton from the subarctic North Pacific and Bering Sea during July–August 1999. *Cour. Forschungsinst. Senckenb.* 244:83–103.
Thierstein, H. R., Geitzenauer, K. R. & Molfino, B. 1977. Global synchroneity of Late Quaternary coccolith datum levels: validation by oxygen isotope. *Geology* 5:400–4.
Tomczak, M. & Godfrey, J. S. 1994. *Regional Oceanography: An Introduction.* Pergamon, London, 422 pp.
Van Bleijswijk, J., van der Wal, P., Kempers, R., Veldhuis, M., Young, J. R., Muyzer, G., de Vrind-de Jong, E. & Westbroek, P. 1991. Distribution of two types of *Emiliania huxleyi* (Prymnesiophyceae) in the northeast Atlantic region as determined by immunofluorescence and coccolith morphology. *J. Phycol.* 27:566–70.
Verbeek, J. W. 1989. Recent calcareous nannoplankton in the southernmost Atlantic. *Polarforschung* 59:45–60.
Welch, B. L. 1947. The generalization of ''Student's'' problem when several different population variances are involved. *Biometrika* 34:28–35.
Winter, A. 1985. Distribution of living coccolithophore in the California Current system, southern California Borderland. *Mar. Micropaleontol.* 9:385–93.
Worsley, T. R. 1973. Calcareous nannofossils: Leg 19 of the Deep Sea Drilling Project. *In* Creager, J. S., Scholl, D. W., Boyce, R. E., Echols, R. J., Fullam, T. J., Grow, J. A., Koizumi, I., et al. [Eds.] *Init. Repts. Deep Sea Drilling Project, 19.* U.S. Govt. Printing Office, Washington, D.C., pp. 741–50.
Young, J. R. 1998. Neogene. *In* Bown, P. [Ed.] *Calcareous Nannofossil Biostratigraphy.* Champman & Hall, Cambridge, pp. 225–65.
Young, J. R., Geisen, M., Cros, L., Kleijne, A., Sprengel, C., Probert, I. & Ostergaard, J. 2003. A guide to extant coccolithophore taxonomy. *J. Nannoplankton Res. Spec. Issue* 1:1–125.
Young, J. R. & Westbroek, P. 1991. Genotypic variation in the coccolithophorid species *Emiliania huxleyi. Mar. Micropaleontol.* 18:5–23.
Zhu, H., Qu, F. & Zhu, L. H. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Res.* 21:5279–80.

Supplementary Material

The following supplementary material is available for this article:

Table S1. List of clonal culture strains, with information of morphotype, sampling month and location, and GenBank accession number of their SSU rDNA sequences.

This material is available as part of the online article.

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