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INTRASPECIFIC GENETIC DIVERSITY IN THE MARINE COCCOLITHOPHORE *EMILIANIA HUXLEYI* (PRYMNESIOPHYCEAE): THE USE OF MICROSATELLITE ANALYSIS IN MARINE PHYTOPLANKTON POPULATION STUDIES[1]

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Using primer pairs for seven previously described microsatellite loci and three newly characterized microsatellite loci from the coccolithophore *Emiliania huxleyi* (Lohm.) Hay and Mohler, we assessed genetic variation within this species. Analysis of microsatellite length variants (alleles) was conducted for 85 *E. huxleyi* isolates representative of different ocean basins. These results revealed high intraspecific genetic variability within the *E. huxleyi* species concept. Pairwise comparison of a 1992 Coastal Fjord group (FJ92) ($n = 41$) and a North East Atlantic (NEA) group ($n = 21$), using F_{ST} as an indicator of genetic differentiation, revealed moderate genetic differentiation ($F_{ST} = 0.09894$; $P = 0$; significance level = 0.05). Gene flow between the FJ92 and NEA groups was estimated to be low, which is in agreement with the moderate levels of genetic differentiation revealed by the microsatellite data. A genetic assignment method that uses genotype likelihoods to draw inference about the groups to which individuals belong was tested. Using FJ92 and NEA as reference groups, we observed that all the *E. huxleyi* groups tested against the two reference groups were unrelated to them. On a global biogeographical scale, *E. huxleyi* populations appear to be highly genetically diverse. Our findings raise the question of whether such a high degree of intraspecific genetic diversity in coccolithophores translates into variability in ecological function.

Key index words: **coccolithophores; *Emiliania huxleyi*; genetic diversity; microsatellites; phytoplankton**

Abbreviations: **HW, Hardy–Weinberg; T_a, annealing temperature; T_m, melting temperature**

The marine coccolithophore *Emiliania huxleyi* is the most conspicuous calcareous phytoplankton species worldwide (Berge 1962, Holligan et al. 1983, Brown and Yoder 1994). This single species forms striking, surface blooms at high latitudes with cell concentrations of up to 10^8 cells $\cdot$ L^{-1} (Berge 1962) and it is present in subsurface coccolithophorid populations in permanently oligotrophic waters of subtropical gyres with concentrations of up to 5×10^4 cells $\cdot$ L^{-1} (Cortes et al. 2001). Blooms of this organism are central to the long-term transport of carbon from surface waters to deep sea sediments via the formation of calcium carbonate (Volk and Hoffert 1985, Milliman and Droxler 1996, Lee 2001), yet we lack fundamental information about its population structure and ecology. This lack of information is important because a significant degree of morphological and physiological variability has been reported in *E. huxleyi* (Young 1994, Wolfe et al. 1994, Hiramatsu and Deckker 1996, Findlay and Giraudeau 2000), suggesting that there may be significant genetic diversity in its populations. Five *E. huxleyi* morpho-

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types have been identified, A and B being the best characterized (van Bleijswijk et al. 1991, Medlin et al. 1994, Young et al. 2003). The A morphotype is the most common in culture collections. There is little information that we are aware of regarding the numerical contribution of morphotypes to natural coccolithophore populations, except for a study of the Norwegian-Greenland Sea that showed a predominance of the A morphotype over the B morphotype (K. Baumann, personal communication), another in which the distribution of four morphotypes was correlated with different water masses off the Japanese coast (Hagino et al. 2005), and a third from Australian waters that showed a correlation of the A morphotype with the Subtropical Front (Findlay and Giraudeau 2000). There is also significant variation in coccolith morphology and physiological and immunological properties of the coccolith-associated polysaccharide among isolates of *E. huxleyi* (Paasche 2000). These morphological and physiological differences among the various morphotypes have been recognized at the varietal level (Medlin et al. 1996), but further evidence of population structure and genetic identity below this level is lacking. Such information might shed light on whether the varieties are genetically isolated and should be raised to the species level. Cryptic species have been documented in several coccolithophorid taxa, with molecular data supporting earlier morphological and geological evidence (Sáez et al. 2001), as well as in other cosmopolitan planktonic species (Sarno et al. 2005). Current classification schemes for some marine phytoplankton are inadequate to address intraspecific variation (Wood and Leatham 1992) because (1) morphological criteria do not allow differentiation between cryptic species that may be functionally and genetically distinct and (2) molecular markers, such as isozymes or nucleic acid sequences of coding regions, e.g. rDNA, *rbc*L, are often evolving so slowly that they cannot be used to distinguish between individuals of the same species.

Length variation at microsatellite loci provides a powerful analytical tool for the quantification of population differentiation at the subspecies level (Schlötterer and Pemberton 1994, Jarne and Lagoda 1996). Microsatellites are present in both coding and noncoding regions of all prokaryote or eukaryote genomes characterized to date (Zane et al. 2002). Li et al. (2002) suggest that microsatellite loci may be functional and non-randomly distributed within the genome. These markers consist of multiple repeats of simple sequence motifs from mono- to hexa-nucleotides, e.g. $(GT)_n$, $(GTC)_n$, or $(GTCA)_n$, that have high mutation rates via stepwise mechanisms (but see Li et al. 2002 for alternative models) to give multiple alternative length variants (alleles) at a given genetic site (locus) (Schlötterer and Tautz 1992, Powell et al. 1996). The allelic variants carried by an individual at a large number of microsatellite loci can be determined through the application of the PCR, which makes them powerful molecular markers for diversity studies within and between phytoplankton populations, including coccolithophores (Iglesias-Rodriguez et al. 2002), diatoms (Rynearson and Armbrust 2000, 2004, Evans and Hayes 2004, Evans et al. 2004, 2005), and dinoflagellates (Nagai et al. 2004). In the case of *E. huxleyi*, its geographic ubiquity and morphological, physiological, and ecological versatility (Brand 1994) suggest that this species may harbor significant population structure that is not detectable with highly conserved traditional markers of genetic diversity. In this study, we quantified genetic diversity within a collection of 85 clonal isolates of *E. huxleyi* established from different geographic origins between 1956 and 2000 using 10 microsatellite loci and use this information to reveal the population genetic structure of this organism.

MATERIALS AND METHODS

E. huxleyi *samples.* Eighty-five clonal isolates of *E. huxleyi* from different geographic origins, and isolated between 1956 and 2000, were used in our study (Table 1). All but one of these clonal isolates were calcifying, and all but one were the A morphotype according to Young and Westbroek (1991) (Table 1). For 34 strains where genomic DNA was not available from previous studies, the cells were maintained and propagated in f/2 medium (Guillard 1975) at 15° C in a 14:10 light:dark regime with an incident irradiance of 200 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Cell suspensions were subcultured every 2 weeks with an initial inoculum of 10^4 cells $\cdot mL^{-1}$ from an early exponential phase culture growing in f/2 medium. For DNA isolation, cells were subcultured into fresh f/2 medium after five cell generations, and sampling was conducted during the exponential growth phase. Approximately 10^8 cells from exponentially growing *E. huxleyi* clonal cultures were centrifuged at 10,000*g*, frozen in liquid nitrogen, and ground to a powder. Genomic DNA was extracted using a standard phenol–chloroform extraction (Sambrook et al. 1989) followed by removal of the DNA-containing aqueous phase and ethanol precipitation as described by Iglesias-Rodríguez et al. (2002).

Microsatellite library enrichment, primer pair design, and determination of PCR conditions. Microsatellite library enrichment was performed as described in Iglesias-Rodríguez et al. (2002). In addition to the microsatellites described in Iglesias-Rodríguez et al. (2002), three additional microsatellites were developed for this study, and the minimum number needed to assess accurately the genetic diversity of *E. huxleyi* was tested from these 10 polymorphic loci. The PCR primers were designed to recognize sequences flanking microsatellites using PRIMER 3 (MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA, USA) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The following criteria were used: length of amplification product 100–400 bp, primer length 18–24 nt, GC content 20%–80%, 60–68° C melting temperature (T_m), according to Sambrook et al. (1989), and maximum of eight self-complementary bases over the whole oligonucleotide and three bases in the 3′ region. When these criteria gave no acceptable primer pairs, the allowed T_m was successively lowered and the sequence re-analyzed. Primer annealing temperatures (T_a) were optimized by starting 5° C below the T_m value calculated by PRIMER 3, and increasing/decreasing the T_a depending on the products obtained. Amplification conditions were further optimized by varying the DNA, primer and $MgCl_2$ concentrations and further adjusting the T_a (Cobb and Clarkson 1994). Microsatellite amplifications were conducted according to Iglesias-Rodríguez et al.

TABLE 1. *Emiliania huxleyi* isolates for microsatellite analysis.

Strain ID	Source	Isolation year, position	Strain ID	Source	Isolation year, position	Strain ID	Origin	Isolation year, position
1992 Bergen fjord (Norway) (FJ92)			B446[a]	AWI	1992	61/07/02[a]	AWI	1991
B11	MBA	1992	B456[a]	AWI	1992	61/12/02	MBA	1991–61°N 20°W
B13[a]	AWI	1992	B461[a]	AWI	1992	61/12/06	MBA	1991–61°N 20°W
B18[a]	AWI	1992	B472[a]	AWI	1992	61/12/07	MBA	1991–61°N 20°W
B21A[a]	AWI	1992	B500[a]	AWI	1992	61/12/09	MBA	1991–61°N 20°W
B28[a]	AWI	1992	B12[b]	AWI	1992	61/67/02[a]	AWI	1991
B43[a]	AWI	1992	B27[b]	AWI	1992	61/67/03[a]	AWI	1991
B49-1[a]	AWI	1992	B78[b]	AWI	1992	61/67/05[a]	MBA	1991–60°N 20°W
B53[a]	AWI	1992	B143[b]	AWI	1992	61/81/03	MBA	1991–56°N 10°W
B65[a]	AWI	1992	**2000 Coastal fjord Bergen (Norway) (FJ00)**			61/81/05	MBA	1991–56°N 10°W
B69[a]	AWI	1992	00–25$_a$[a]	UoB	2000	61/81/10[a]	AWI	1991
B79[a]	AWI	1992	00–26$_a$[b]	UoB	2000	61/87/17[a]	AWI	1991
B86[a]	AWI	1992	00–38[b]	UoB	2000	**Gulf of Maine, U.S.A (88E)**		
B91[a]	AWI	1992	00–71$_b$[b]	UoB	2000	88E	MBA	1988
B96[a]	AWI	1992	00–76$_b$[b]	UoB	2000	**English Channel (EC)**		
B126[a]	AWI	1992	00–184$_c$[b]	UoB	2000	92D[1]	MBA	1975–50°N 4°N
B131[a]	AWI	1992	00–188$_c$[b]	UoB	2000	92A[2]	MBA	1957
B161[a]	AWI	1992	00–189$_c$[b]	UoB	2000	182 g	AWI	
B163[a]	AWI	1992	00–203[b]	UoB	2000	**Atlantic, subtropica (STA)**		
B170[a]	AWI	1992	**NE Atlantic, Iceland Basin (60°N 20°W) (G1779)**			12–1	MBA	1987
B174[a]	AWI	1992	G1779[a]	AWI	1989	**Sargasso Sea (SS)**		
B202[a]	AWI	1992	**NE Atlantic, subtropical (24°N 20°W) (53/74/06)**			CCMP-1A1	MBA	1987–32°N 62°W
B208[a]	AWI	1992	53/74/06	MBA	1990	M-181	MBA	
B329[a]	AWI	1992	**NE Atlantic (NEA)**			MCH1[a]	MBA	1967
B333[a]	AWI	1992	60/03/08	AWI	1991	**South Pacific, Australia (SPA)**		
B340[a]	AWI	1992	60/06/01[a]	AWI	1991	EH2	MBA	1990-Gr. Barrier Reef
B349[a]	AWI	1992	60/06/02[a]	AWI	1991	**South Pacific, New Zeland (EHNZ)**		
B354[a]	AWI	1992	60/10/01[a]	AWI	1991	EHNZ	MBA	1992-S New Zealand
B366[a]	AWI	1992	61/03/02	AWI	1991–51°N 15°W	**South Pacific, tropical (SPT)**		
B370[a]	AWI	1992	61/04/06	MBA	1991–51°N 15°W	CCMP 1516	MBA	NR-2°S 82°W
B400[a]	AWI	1992	61/04/17	MBA	1991–51°N 15°W	**Indian Ocean, South Africa (IO)**		
B406[a]	AWI	1992	61/06/02[a]	AWI	1991-	South Africa	MBA	1983
B432[a]	AWI	1992	61/07/03	MBA	1991–51°N 15°W			

[a]From L. K. M. (Alfred Wegener Institute, Germany).

[b]Isolated and maintained by M.D.I.-R. (University of Southampton, U.K.); the remaining samples were maintained by M.D.I.-R and obtained from Dr. John Greens collection (Marine Biological Association, Plymouth, U.K.). Isolates were grouped in 13 populations/groups represented in bold. a, b and c subscripts represent multiple cell isolation events from the same water sample. All strains were calcifying except 92A that did not produce coccoliths. All the *E. huxleyi* strains were A morphotype except 92D that has morphotype B. NR, not reported. Information on year of isolation and position (latitude/longitude) was not available for all clonal isolates.

(2002). Genotypes were determined by PCR amplification for 85 clonal isolates of *E. huxleyi* of different geographical origin. Microsatellite amplification products were initially visualized by electrophoretic separation in 2% (w/v) agarose gels in 1×Tris-borate/EDTA (TBE) buffer, and subsequently staining in ethidium bromide (0.5 μg/mL). The size of the amplified products was determined using a standardized 100 bp DNA ladder (Promega, Southampton, UK). All PCR products that appeared to indicate homozygous loci were reanalyzed in 20% (w/v) acrylamide gels to check for the presence of alleles of similar, but non-identical length. The allele sizes for the 10 loci were accurately determined on a MegaBACE 1000 using genetic profiler software (Amersham Pharmacia, Chalfont St. Gres, UK). For the MegaBACE anal-

yses, the forward primers, of those primer pairs that amplified successfully, were end-labeled with one of three different fluorescent phosphoramidite dyes (6-FAM, HEX, or TET) (Applied Biosystems, Foster City, CA, USA). Microsatellite scoring artifacts, i.e. "stutter bands," resulting from the imperfect amplification of repeats by *Taq* polymerase (Luty et al. 1990), were associated with some loci. However, these additional products of smaller size and low intensity were easily distinguished as artifacts and did not compromise the identification of alleles for genotypic analysis.

Statistical analysis. General measures of genetic variation, including allele frequencies, observed genotype frequencies and observed (H_o) and expected (H_e) heterozygosity were calculated using the GENEPOP 3.2 software package (Raymond and Rousset 1995, http://www.cefe.cnrs-mop.fr/). Tests for departure from Hardy–Weinberg (HW) proportions for each locus were also performed using GENEPOP 3.2 (Table 2) with an exact test where the *P* values were estimated using a Markov chain method following the algorithm of Guo and Thompson (1992). The dememorization number used for the tests was 1000 with 200 batches and 1000 iterations per batch.

Population subdivision was measured as F_{ST} using *F* statistics based upon a weighted analysis of variance fixation (Weir and Cockerham 1984). Wright's (1951) original definition is based on the inbreeding coefficient: the probability of alleles that are identical-by-descent (from an ancestral population/group) being combined in zygotes. Later definitions are based explicitly on parameters of allele frequency distributions, such as the proportion of variance in allele frequencies among groups. We made the parsimonious assumption that the population is in equilibrium, the loci under study are neutral (not under selection, but see Li et al. 2002), and that any "new" alleles in the groups are a result of migration and not mutation. The *F*-statistics were computed using GENEPOP 3.2 for all populations/groups and for all pairs of groups. The significances of the F_{ST} values were estimated using the permutation test option of Arlequin (Schneider et al. 2000), using 1023 permutations, under the null hypothesis of no genetic differentiation (Schneider et al. 2000) (Table 3). According to Wright (1978) F_{ST} values range between 0 (lack of genetic differentiation) and 1 (identical groups), with values of 0–0.05 implying little genetic differentiation, 0.05–0.15 moderate genetic differentiation, 0.15–0.25 great genetic differentiation, and >0.25 very great genetic differentiation. The FJ00 group was omitted from the analysis because nine individuals were too few to estimate genetic distances at microsatellite loci with high levels of polymorphism (Kalinowski 2005).

Assignment of individual genotypes to populations/groups. A genetic assignment method that uses genotype likelihoods was tested to draw inferences about the groups to which individuals belong. Using two reference populations (FJ92 and North East Atlantic [NEA], both comprising a sufficient number of individuals to conduct these tests), with a clear separation between clusters, should provide the ability to identify individuals originating in one of these populations and captured in the other.

The log-likelihood of each individual multilocus genotype was calculated for the two reference groups using GeneClass2 (Piry et al. 2004). GeneClass2 automatically excludes loci that do not show detectable alleles. Therefore, one strain from FJ92, FJ00, EC, and SPA, three strains from the NEA group, and the SPT strain were excluded from the analysis (see Table 1 for groups). This analysis calculates the likelihood of identifying genotypes in each group and assigns each genotype to the group for which it has the highest likelihood. For computing the likelihood, the allele frequencies estimated in each sample were used and it was assumed that loci were independent (Paetkau et al. 1995, 1997).

RESULTS

Microsatellite description. Our microsatellite-enriched library of *E. huxleyi* was enriched for GA and GT repeats and included (described according to Weber 1990): a pure (perfect) GT repeat, an imperfect (discontinuous, i.e. sequence repeat with an interruption of non-repeat nucleotides) GT repeat, and an imperfect (discontinuous) GA repeat (Table 2). Among all the sequences containing microsatellite repeats, 60% comprised GT repeats (65% of which were imperfect); 19% comprised GA repeats (half of which were imperfect); 25% of all the inserts analyzed had long $(GT)_n$ and $(GA)_n$ stretches. Among 123 loci sequenced, 63 contained microsatellite repeats, of which 33 were not suitable for primer design because they lacked sufficient flanking sequence. Of the 30 suitable microsatellite sequences, only 10 were selected for further analysis based upon their reproducibility in amplification reactions and because they generated only one or two amplification products (seven of these loci are described by Iglesias-Rodriguez et al. (2002); Table 2). The BLAST searches using forward and reverse microsatellite sequences against all sequences available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) showed that the loci tested were not part of coding regions for any fully or partially characterized gene.

Successful amplification of alleles at each locus was achieved for between 41% and 89% of the *E. huxleyi* isolates (Table 2); the inability to generate amplification products in some clonal isolates at some loci may indicate null alleles, i.e. microsatellite alleles that fail to amplify because of deletion or point mutations in one of the priming sites at these loci. Seven (all from the North Atlantic) out of the 85 isolates tested gave products for all the 10 loci (Fig. 1A). The B11 isolate used in the construction of the microsatellite-enriched library did not, surprisingly, generate detectable alleles using primer pairs for the P02A08 and P01E05 loci. It is unlikely that changes occurred in the B11 genome between library construction and the subsequent PCR characterization of microsatellites, therefore this finding probably indicates that some of the "null alleles" in the analyses may in fact represent PCR failures, despite the fact that great care was taken to optimize all reaction conditions. More than half of the *E. huxleyi* clonal cultures showed products for at least half of the loci tested (Fig. 1A). Among the 10 microsatellite loci used, P01F08, EHMS37, and P01E05 were amplifiable only in 58, 34, and 62 northern hemisphere strains, respectively; the remaining loci were amplifiable in both northern and southern hemisphere strains. Detectable alleles in the northern hemisphere were observed for loci EHMS15 (54), A01A08 (37), P02E11 (56), P02B12 (46), P02E10 (36), P02F11 (52), and P02E09 (71). In southern hemisphere populations, three detectable alleles were observed for loci EHMS15, A01A08, P02E11, P02B12, and P02F11; two detectable alleles were observed for loci P02E10 and P02E09.

TABLE 2. Attributes of 10 microsatellite loci isolated from *Emiliania huxleyi* B11.

Locus	Primer sequence	EMBL Acces. No	Sequenced repeat motif	T_a	Allele size (bp)	No. alleles	No. genotypes	% Un. Gen.	H_O	H_E	P-val ± SE
EHMS15[b] $N = 61$	F: TCGAGGCGCGTCACACAC	AJ487304	$(GT)_{27}GC$	54	72–149	26	38	71.05	0.70[a]	0.93	0.0068 ± 0.00440
	R: GCGAGCGGTGGGCAATGT	AJ487305	$(GT)_9$								
EHMS37 $N = 35$	F: TGTGAGAGTGAGCACGCA	AJ494737	$(GT)_{23}$	60	203–240	11	17	70.27	0.58[a]	0.78	0.0109 ± 0.0021
	R: TTGAGGAGGATTACGAGGTC	AJ494738									
P01F08[b] $N = 60$	F: CGGAGCAGTCCAGTACACAA	AJ487306	$(GT)_{14}$	60	144–198	18	38	61.11	0.75[a]	0.88	0.0000 ± 0.0000
	R: CGCATCTCAGTCGTTCTTCA	AJ487307									
P02A08[b] $N = 41$	F: CCCCGTGTTTGAGAGAGAGA	AJ487308	$(GA)_6GG\ (GA)_{23}$	58	281–331	15	18	70.73	0.34[a]	0.84	0.0000 ± 0.0000
	R: TCGGAGATCAGGGAGTTGTC	AJ487309									
P02E11[b] $N = 58$	F: CGGTGCTACGAGGTGTGTAA	AJ487312	$(GA)_7TA\ (GA)_7$	58	206–251	21	41	63.63	0.74[a]	0.92	0.0000 ± 0.0000
	R: CACGCGCTTCACAATGTAAT	AJ487313									
P02B12[b] $N = 50$	F: GGTTAATCGCAGCAAAGAGC	AJ487309	$(GT)_{10}$	58	204–220	7	12	60.00	0.40	0.47	0.7479 ± 0.0039
	R: CAGTCTTGATCGGGAACGA	AJ487311									
P02E10[b] $N = 40$	F: CTCGTGTAGTCGGGGAGTGT	AJ487314	$(GT)_{11}GCAA(GT)_{11}$	58	164–180	8	11	70.58	0.28[a]	0.52	0.0104 ± 0.0007
	R: CACGCGGTCCAATATTACCT	AJ487315									
P02F11[b] $N = 57$	F: CTCGTGTGGCTATGCCTATG	AJ487316	$(GT)_{11}$	58	100–149	16	18	28.57	0.58	0.67	0.1071 ± 0.0075
	R: TCCAAGAGCAAAGTGCAAAA	AJ487317									
P01E05 $N = 66$	F: GTGTGCCCTTTTGTGCTTTT	AJ494739	$(GA)_{14}GG\ (GA)_{21}$	57	120–190	26	39	58.82	0.70[a]	0.92	0.0018 ± 0.0018
	R: GTGATGGTGTGCCTGTGTTC	AJ494740									
P02E09 $N = 76$	F: ACTCGGACTGGACGCACA	AJ494741	$(GT)_9$	59	82–104	9	14	66.66	0.68	0.69	0.3452 ± 0.0131
	R: GGCTGCTCTTCCCCTCTCTA	AJ494742									

[a]Heterozygote deficiency.

[b]Loci attributes described in Iglesias-Rodríguez et al. (2002).

Acces No, Accession Number; T_a, annealing temperature (° C); observed (H_o) and expected (H_e) heterozygosity (calculated as $1\text{-}\Sigma P_i^2$, where P_i is the frequency of each allele). Un. Gen., unique genotypes. A collection of 85 *E. huxleyi* clonal isolates was used in this study. *N*, number of isolates with detectable alleles; *P*-val, significant exact probability (*P*) for Hardy–Weinberg departure proportions. SE, standard error.

TABLE 3. Observed (H_o) and expected (H_e) heterozygosities for the 1992 coastal fjord (FJ92) and the North East Atlantic (NEA) groups at each locus.

	FJ92				NEA				*F* statistics	
Locus	H_o	H_e	*P* (SE)	Genotypes	H_o	H_e	*P* (SE)	Genotypes	$F_{ST\ (FJ92\text{-}NEA)}$	N_m
EHMS15	0.70732	0.94128	0.00000 (0.00000)	24 (20)	0.33333	0.76539	0.00000 (0.00000)	9 (8)	0.09894 ($P<0.05$)	2.27
P01F08	0.73171	0.89672	0.00000 (0.00000)	29 (20)	0.38095	0.84088	0.00000 (0.00000)	11 (6)		
P02A08	0.26829	0.84312	0.00000 (0.00000)	14 (10)	0.14286	0.45877	0.00000 (0.00000)	5 (5)		
P02E11	0.70732	0.90545	0.00000 (0.00000)	27 (25)	0.42857	0.80952	0.00000 (0.00000)	10 (9)		
P02B12	0.19512	0.50888	0.00000 (0.00000)	5 (3)	0.28571	0.63879	0.00000 (0.00000)	4 (1)		
P02E10	0.04878	0.55224	0.00000 (0.00000)	2 (0)	0.23810	0.68757	0.00000 (0.00000)	5 (2)		
P02F11	0.48780	0.73984	0.00000 (0.00000)	8 (4)	0.28571	0.74216	0.00000 (0.00000)	7 (3)		
P02E09	0.73171	0.71515	0.02579 (0.00033)	11 (6)	0.52381	0.80372	0.00084 (0.00009)	7 (5)		
EHMS37	0.39024	0.77838	0.00000 (0.00000)	11 (8)	0.14286	0.52381	0.00000 (0.00000)	6 (3)		
P01E05	0.63415	0.93646	0.00000 (0.00000)	24 (18)	0.61905	0.89663	0.00000 (0.00000)	24 (8)		

Number of unique genotypes are indicated in parentheses next to the genotype numbers. The last two columns indicate pairwise comparisons of F_{ST} and gene flow (N_m) between the FJ92 and the NEA groups. Number of permutations: 1023; number of steps in Markov chain: 10,000; number of dememorization steps:1000.

Microsatellite polymorphisms demonstrate that populations of E. huxleyi *are not clonal.* The properties of the 10 loci tested in the *E. huxleyi* isolates are listed in Table 2. The percentage of unique genotypes per locus varied between 29% and 71% (Table 2). Across all 10 microsatellite loci, each of the 85 DNA samples examined

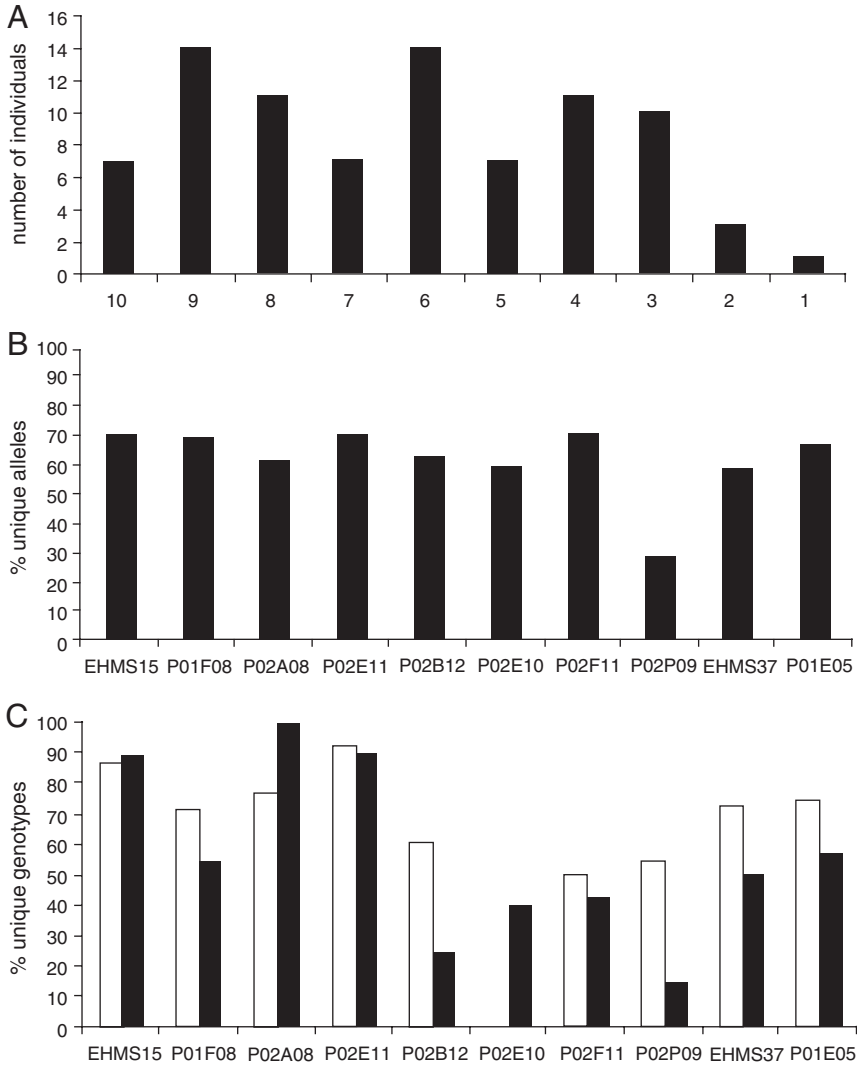

FIG. 1. (A) Number of individuals producing amplication products at different numbers of loci, from a maximum of 10 to a minimum of one. (B) Percentages of unique alleles in all individuals tested out of the total of detectable allelic products for the 10 loci tested. (C) Percentages of genotypes unique to the FJ92 (white) and the NEA (black) groups for the 10 loci tested.

exhibited a distinct multilocus genotype, including strains isolated on the same day from the same water sample (Table 1). H_o were between 28% and 75% and suggest a high degree of genetic diversity. A global test of HW proportions (all loci and all groups) revealed significant heterozygote deficiencies (Markov chain method, $P<0.05$) at seven of the 10 loci tested (Table 2). In nine of the 10 loci, the largest percentage of alleles at each locus was represented by a single individual (Fig. 1B), indicating that the loci used were highly polymorphic and that *E. huxleyi* has a significant degree of intraspecific variability. Additionally, the likely presence of null alleles most likely leads to an underestimation of the diversity present.

Examples of allelic geographic specificity per locus are illustrated in Figure 2. This figure provides a graphical representation of genetic diversity at the EHMS15 (Fig. 2A) and P01E05 (Fig. 2B) loci using unitless axes; these loci were selected because they have the highest number of detectable alleles and genotypes, but the pattern obtained was representative of the remaining loci (the more polymorphic the higher the percentage of unique alleles). The results showed that the majority of the genotypes (89% in EHMS15% and 82% in P01E05) were unique (Fig. 2). Four distinct groups were identified in the northern hemisphere for locus EHMS15 (Fig. 2A) and three for locus P01E05 (Fig. 2B). Three distinct southern hemisphere groups represented by a single individual were identified for EHMS15 (Fig. 2A) whereas no detectable alleles were found in southern hemisphere individuals for locus P01E05 (Fig. 2B).

Intra and interpopulation genetic structure. We selected the two largest groups of isolates, a 1992 coastal fjord group (FJ92), and a pelagic group from the northeast Atlantic (NEA) sampled in 1991 (41 and 21 clonal isolates, respectively), for more detailed analysis of allele frequencies. Results showed that the majority of the loci gave at least 50% genotypes (per locus) unique to the FJ92 and NEA groups (Fig. 1C); the loci EHMS15, P02A08, and PO2E11 were found to be highly polymorphic.

Kalinowski (2005) suggests that when F_{ST} is greater than 0.05, sampling fewer than 20 individuals (per group/population) is sufficient. Therefore, given the small sample size of most *E. huxleyi* groups (Table 1) we only conducted pairwise comparisons for the FJ92 and NEA groups with 41 and 21 individuals, respectively. Pair-wise comparisons of FJ92 and NEA gave an F_{ST} value of 0.09894 ($P = 0$; significance level = 0.05) (Table 3), which falls within "moderate genetic differentiation" according to Wright's definition of F_{ST} values (Wright 1978).

Table 3 also reports on H_o and H_e heterozygosities for the FJ92 and the NEA groups at each locus. In the FJ92 group, H_o varied between 5% and 73% and a global test of HW proportions (all loci and all groups) revealed significant heterozygote deficiencies (Markov chain method, $P<0.05$) at nine of the 10 loci tested (Table 3), and one case of slight heterozygote excess. In the NEA group, H_o varied between 14% and 62% and

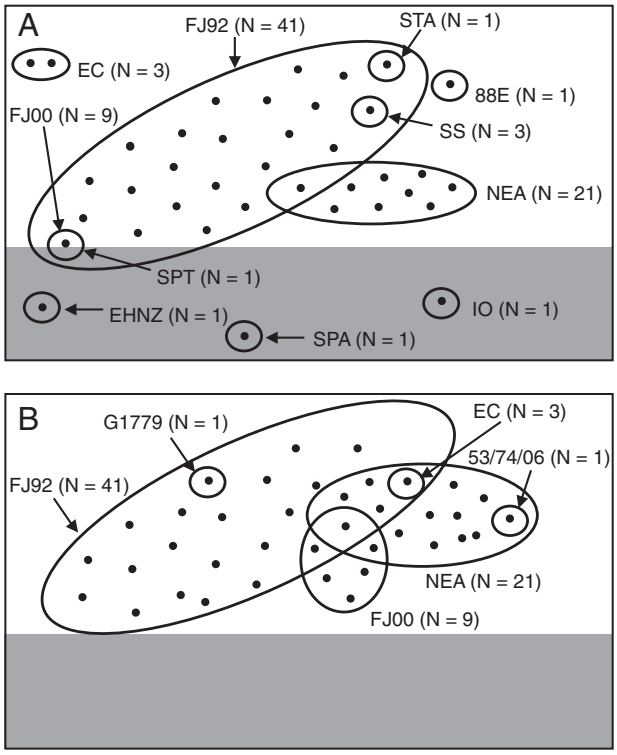

FIG. 2. Geographic distribution of genotypes based upon the microsatellite loci EHMS15 (A) and P01E05 (B). Black spots within the enclosed areas represent the genotypes of *Emiliania huxleyi* isolates grouped according to the geographic area from where the strains were isolated. The delimited areas represent groups/populations made according to geographic origin. FJ92 (1992 coastal fjord); FJ00 (2000 coastal fjord); G1779 (1989 North East Atlantic (NEA), Iceland Basin); 53/74/06 (NEA, subtropical); NEA; 88E (Gulf of Maine, USA); EC (English Channel); STA (sup-tropical Atlantic); SS (Sargasso Sea); SPA (South Pacific Australia); EHNZ (South Pacific New Zealand); SPT (South Pacific tropical); IO (Indian Ocean). Dots enclosed in more than one area represent genotypes shared by the groups represented by those delimited areas. The shaded area represents Southern Hemisphere genotypes; the clear area represents Northern Hemisphere genotypes.

heterozygote deficiency was observed at all loci tested (Table 3). The number of genotypes varied between 2 and 29 in the FJ92 group and between 4 and 24 in the NEA group.

Sexual reproduction and geographical dispersal of eukaryotic phytoplankton are key in determining gene flow between groups. The parameter N_m (Slatkin 1995) represents the number of effective migrants per generation and can be used as a reference estimate of gene flow between groups of individuals. The parameter N represents the number of individuals in a given population and m is the percentage of those individuals resulting from immigration (Wright 1969). As a reference, N_m values between 0 and 1 are indicative of strong population differentiation, whereas groups/populations with N_m values >4 are interpreted as single randomly breeding units (Kimura and Maruyama 1971). However, the accuracy of N_m estimates depends on many assumptions that probably are

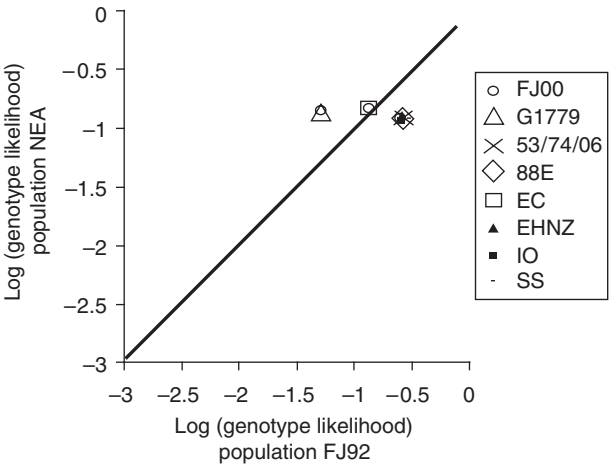

FIG. 3. Assignment plot for a pair of populations (FJ92 and NEA) where the power to identify F_0 migrants is high (Number of migrants [N_m] after correction for size = 0.9496113). The plot shows genotypes corresponding to the eight groups tested and the area where they "fall" in relation to the two reference populations tested. The distance from the diagonal center line can be used as a reference (Rannala and Mountain, 1997).

not met in the case of the present study: populations in equilibrium with respect to genetic drift and migration, and an island model of migration. Taking these considerations into account, a N_m value of 2.27 in our analysis suggests low gene flow between the FJ92 and NEA groups (Table 3).

Assignment tests (Paetkau et al. 2004) revealed that three individuals, one from the subtropical NEA (53/74/06), one from the Gulf of Maine (88E), and one from the Sargasso Sea (SS) group, showed a probability below 0.01 (not shown) and the potential for being F_0 migrants. Some of the groups tested showed overlap in their distribution suggesting that there is no clear separation between these groups. The log-likelihood values indicate that all the groups tested were unrelated to the reference FJ92 and NEA groups (Fig. 3).

DISCUSSION

Microsatellite analysis. Our data validate the suitability of microsatellites for the study of phytoplankton genetic diversity, demonstrate that the diversity detected with these neutral markers exceeds that of other slower-evolving DNA markers (Gallagher 1982, Scholin et al. 1994, Miller and Scholin 2000) and are consistent with DNA fingerprinting techniques, including microsatellites, used for the analysis of marine planktonic diatoms (Rynearson and Armbrust 2000, 2004, Evans et al. 2004, 2005, Evans and Hayes 2004).

The effect of the number of microsatellite loci on the identification of unique genotypes was assessed using all combinations of one through 10 loci (Fig. 4). Analysis of the possible numbers of combinations of all the microsatellite loci used in this study indicated that five polymorphic microsatellite loci is the minimum number to provide a representative estimate of the genetic diversity using 85 *E. huxleyi* isolates of the 13 groups of isolates tested (Fig. 4). Considering the observed number of alleles (AL) per locus, for N loci the minimum potential number of genotypes (G_N) can be calculated as:

$$G_N = \{[AL_1 \times (AL_1 - 1)/2] + AL_1\} \times \{[AL_2 \times (AL_2 - 1)/2] + AL_2\} \times \ldots \times \{[AL_N \times (AL_N - 1)/2] + AL_N\} \quad (1)$$

Our estimations suggest a minimum of 2.4×10^{20} potential different genotypes for our 10 loci, although this number is likely an underestimate because extended sampling of *E. huxleyi* strains would result in a higher number of alleles. The possible effect of homoplasy (alleles that are identical in size but that differ in ancestry) adds to the underestimated potential minimum genotypic differentiation, and it poses a problem for the use of microsatellites as indicators of evolutionary relationships. Therefore, our results are only interpreted in terms of genetic differentiation, and no attempt has been made to estimate the *tempo* of evolution or to quantify phylogenetic relationships between strains or groups.

Genetic differentiation. The moderate genetic differentiation observed between the FJ92 and the NEA populations may indicate a selection of different clones as a result of differences in the environment, or physical separation of distinct strains as a consequence of prevailing currents, also reported by Evans et al. (2004) and Rynearson and Armbrust (2004). Our results might also be affected by an insufficient number of loci or low sample size (Kalinowski 2005). The low number of individuals isolated at the Southern Hemisphere locations and the time separation between the strain isolation dates made it impossible to establish additional groups for further pairwise comparison of genetic relatedness.

Our results indicate that the individuals tested against the two reference populations are unrelated to them. Significant differences in allele frequency distributions among samples may also result from non-ran-

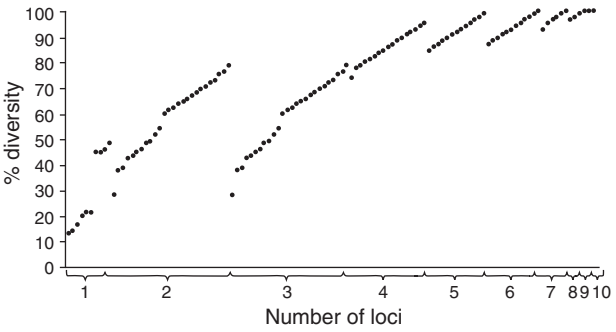

FIG. 4. Degree of genetic diversity obtained using combinations of the 10 microsatellite loci used in this study. Dots represent percentage of genotypes identified out of the 85 genotypes established in our study, using loci in combinations of up to 10 loci.

dom sampling of groups, for example when samples differ in age (Allendorf and Phelps 1981). The low gene flow between the FJ92 and NEA groups is in agreement with the moderate levels of genetic differentiation revealed by the microsatellite data. The relatively rapid mutation rate of microsatellite loci (Primmer et al. 1996, Schlotterer et al. 1998), combined with low gene flow, may explain the moderate levels of population differentiation and the presence of relatively high percentages of group-specific microsatellite alleles, as shown by the dominance of genotypes unique to the two groups tested (Fig. 1C). However, it should be noted that the high mutation rate of microsatellite loci could generate a large number of distinct alleles over a relatively short time period. Our limited sample sizes mean that a number of these alleles would remain undetected, potentially leading us to underestimate the numbers of shared alleles among populations.

Role of sexual reproduction in maintaining population resilience. One of the most challenging puzzles in biology is to understand the mechanisms controlling large-scale patterns of spatial and temporal population distribution, abundance, and diversity. Genetic diversity is key in maintaining functional resilience, which guarantees the persistence of ecological functions (Grimm and Wissel 1997). Our study reveals a high degree of intraspecific genetic diversity within the species concept of *E. huxleyi*, which may suggest a role for sexual reproduction in maintaining this high degree of genetic diversity. Using random amplified polymorphic DNA data, Barker et al. (1994) and Medlin et al. (1994) showed extremely high genetic diversity in *E. huxleyi* and suggested that sexual reproduction must be widespread to maintain such diversity. During non-limiting light and nutrient conditions, such as those inducing bloom formation, *E. huxleyi* cells can divide at a rate of two generations per day (Brand and Guillard 1981); therefore it would be expected that those clones with the highest growth rates would become dominant in the population. Given this, the probability of detecting genetic polymorphisms should be low in a small population sample unless the organisms undergo sexual reproduction. Knowledge of the life cycle of coccolithophores during bloom development is still in its infancy, and the role of sexual reproduction in the population dynamics of *E. huxleyi* remains an open question. Also, if there is a genetically diverse seed population, then any differences in growth rate may not be sufficient (Brand 1994) to allow single genotypes to become dominant within the period of population development; more extensive characterization of individual population samples is required to address such issues.

In our study, the most remarkable example of microsatellite polymorphism within a population is that observed in individuals of the 2000 coastal fjord group collected from the same body of water. All of these individuals were genetically distinct even though seven were obtained from water samples where at least one additional clonal isolation was made (Table 1). Similarly, Evans et al. (2005) and Rynearson and Armbrust (2005) using much larger sample sizes observed that 98% and 87% (respectively) of the cells sampled were genetically distinct. This is surprising in bloom events, where populations are expected to develop predominantly by binary cell division. For *E. huxleyi*, however, increasing evidence from flow cytometric analysis has revealed that the coccolith-bearing cells have double the amount of DNA compared with motile scale-bearing cells, suggesting diploid ploidy state and a sexual/asexual life-cycle (van Bleijswijk et al. 1994). Indeed, coccolithophores can be haploid (individuals possess one copy of each chromosome) or diploid (individuals possess two copies of each chromosome, and therefore two alleles at each locus that are either the same [homozygous] or different [heterozygous]) (van Bleijvist et al. 1994). In our observations, all *E. huxleyi* isolates were heterozygous at atleast one locus, except isolate 00-71, which appeared to be homozygous at all loci. This strain should be targeted for an examination of its ploidy state. Heterozygosity at one or more loci provides convincing evidence for a diploid ploidy state. Generally a population of diploid individuals is reproducing sexually in HW equilibrium (HWE), if the observed (H_o) and expected (H_e) heterozygosities are not significantly different. A departure from HWE can be used to estimate indirectly the extent of sexual reproduction in a population. The 10 loci showed considerable polymorphism across the groups tested with a mean of 15.7 alleles per locus (ranging from 7 to 26, Table 2). Microsatellite analysis of single loci revealed relatively low numbers of genotypes (between 11 and 41) (Table 2). The observed deviations from HWE may indicate the operation of selection pressure or nonrandom mating, or they may reflect the presence of null alleles, or the Wahlund effect (reduction in heterozygosity as populations diverge) (Lehmann et al. 1996). In our study, the heterozygote deficit is most likely because of null alleles.

A number of many different combinations of cells (Cros et al. 2000, Geisen et al. 2002) have been reported among many species, in which the holococcolith stage as the female gametangium is partially open with a heterococcolith cell inside it. These reports imply that sexual reproduction as part of the life cycle of coccolithophores is widespread. There is also substantial evidence that strongly suggests that the life cycle of *E. huxleyi* involves several cell types including non-motile coccolith-bearing cells, naked non-motile cells, and motile scale-bearing cell types (Klaveness 1972) that can maintain themselves by vegetative reproduction. From an evolutionary perspective, sexual reproduction represents a great advantage because it enables the organism to adapt to changing environmental conditions by producing a great deal of genetic variability through recombination. In eukaryotic organisms, sexual reproduction requires diploid organisms (two sets of chromosomes), which confers greater genetic variability than does haploid organisms. Given the

high degree of environmental variability in the oceans, a diploid state may confer a selective advantage in maintaining population resilience. Noel et al. (2004) have speculated on the environmental conditions necessary to transform diploid coccolithophore stages into haploid holococcolith stages. In their study of the media composition that induces life stage changes in coccolithophores, they hypothesized that as holococcolith-bearing cells are swept into coastal areas they may be induced to undergo sexual reproduction and form heterococcolith stages. The heterococcolith stages may settle on benthic surfaces where the stresses that they encounter likely induce them to undergo meiosis and form holococcolith stages, which are then swept back out to sea where the motile cells encounter a more oceanic ecosystem. If this scenario, which has been inferred only from media composition, is true, then the heterococcolith stage encounters a much more varied and harsh environment than the holococcolith stage, and the diploid stage, being very diverse, has a selective advantage.

Our results strongly suggest that sexual reproduction plays an important role during coccolithophore bloom events, and the largely monospecific bloom forming *E. huxleyi* may indeed be composed of an array of coexisting genotypes. We argue that blooms are composed of a finite genetic pool of individuals with high rates of sexual reproduction such that the population achieves a high degree of intraspecific genetic variability. We propose that, on a global biogeographical scale, *E. huxleyi* bloom populations are composed of an array of coexisting clones (strains of *E. huxleyi* with distinct microsatellite genotypes), and that these genotypes may coexist such that the population can adapt to changing environmental conditions.

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