34. Genetic variability and phytoplankton species.

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ABSTRACT

Molecular techniques can address many important questions concerning taxonomic affinity, genetic diversity, gene flow and dispersal. Species/genetic diversity in three ecologically important members of the marine phytoplankton (the diatom *Skeletonema costatum* and the prymnesiophytes, *Phaeocystis* and *Emiliania huxleyi*) has been characterized using a variety of molecular techniques in order to address the conservation of form in marine phytoplankton. All are widely distributed taxa. *Skeletonema* is a cosmopolitan neritic diatom, highly diverse in its ecophysiological manifestations. In this species, molecular clock calculations for 18SrDNA coding regions indicate that molecular divergence is proceeding at a very fast rate, whereas morphological divergence is not. At least one cryptic species and a cluster of isolates, which may be sibling species, are documented. Divergence among *Phaeocystis* species as measured by 18S rDNA coding regions indicates that at least four species can be recognized at this level. A warm and a cold water species complex exist, which separated approximately 25 to 30 My ago. All but one cold water species exhibit the same colony morphology. DNA content as measured by flow cytometry suggest that several cryptic species are present within the morphological species concept of the warm water taxon, *Phaeocystis globosa*. ITS sequence data can resolve species differences in the cold water complex. Sequence data from coding and non-coding regions of *Emiliania huxleyi*, a young species, show no variation among two morphotypes or any difference from its most recent ancestor, *Gephyrocapsa oceanica*. This likely reflects the recent divergence of these two species in time. Measurement of DNA content using flow cytometry indicates that the A & B morphotypes of *E. huxleyi* are genetically different. RAPD techniques reveal extreme genetic diversity within short-term spatial and temporal resolution and indicate that the isolates of *Emiliania huxleyi* taken from wide geographic areas are related by their site of isolation.

INTRODUCTION

The recognition of a species is one of the most complex, controversial topics in biology. Although many definitions (concepts) of a species exist (Cracraft, 1989; Manhart & McCourt, 1992; Gosling, 1994), the morphological species is the most widely used. The biological species is often the most difficult to document especially in marine phytoplankton where little is known of life histories and where most reproduction is asexual. The phylogenetic species concept represents monophyletic groupings and introduces an element of time into the species concept (Gosling, 1994). Molecular data can be most easily incorporated into this species concept, although they can be used to augment any concept. On one hand, species may be differentiated based on molecular divergence but may not be reproductively isolated (syngens). In addition, conflicts between phylogenetic/molecular speciation and morphological speciation may result from the different rates at which molecular and morphological changes accumulate. Thus, cryptic (sibling) species can be formed, which are morphologically identical but reproductively isolated and molecularly divergent from one another. The first step in reconciling potential conflicts in molecular and morphological speciation is to recognize that they can and do occur.

Marine phytoplankton are, by definition, taxa with large population sizes. Significant genetic diversity has been inferred to exist within and between phytoplankton populations primarily from physiological measurements (Brand, 1989). These data have been

used to speculate upon the amount of genetic diversity within phytoplankton populations but it is not known whether this physiological diversity represents inter- or intra-specific diversity. It may be that in many planktonic forms molecular speciation has proceeded, whereas morphological divergence has not. Physical forces and grazing pressures may explain some convergence of form in the marine environment (Sournia, 1988a). However, phenotypic diversity has been predicted to decline in populations where there is a cost for obtaining non-limiting nutrients (Tilman, 1982). Thus 'super species' may develop that can exploit a wide variety of environmental conditions (Kilham, 1994). These 'super species' may in fact be composed of many cryptic species whose morphological form has diverged little over time. The conservation of form resulting in similar if not identical morphotypes has analogies at all taxonomic levels in the sea (Knowlton, 1993; Sournia, 1988a) such that the abundance of cryptic (sibling) species in the marine environment is only now being revealed (Knowlton, 1993). The existence of functional morphologies within the marine phytoplankton may be one of the most intriguing questions in ecology today.

Molecular techniques (nucleic acid sequence data and various types of DNA fingerprinting) can now address some of the problems surrounding these issues to augment our understanding of species concepts in the marine phytoplankton. Multi-species complexes can be identified and species limits re-evaluated. The taxonomic level to which physiological and morphological differences can be applied can be assessed within a molecular phylogenetic framework. Molecular techniques, such as sequencing, can provide insight into cryptic level diversity and differentiate it from phenotypic plasticity, which is also widely documented (Patarnello et al., 1994; Brand, 1982). The amount of nucleotide substitution between species/clones assumed to be the same taxon from a morphological concept will depend on the evolutionary age of the group and the rate of evolution in the gene/lineage selected. Also, if the reproductive isolation of the cryptic (sibling) species is too recent, then the cryptic (sibling) species will not have co-existed long enough to accumulate nucleotide substitutions in some coding regions and other, faster evolving non-coding regions must be examined before a taxonomic decision can be reached. In all cases ecophysiological data can augment the molecular data.

The potential for recognising genetic individuality is only just being realised and its use in clustering individuals into biologically meaningful groups reflecting their overall relatedness will probably increase the diversity in the marine environment by an order of magnitude (Hedgecock, 1994) and will provided insights into how genetic diversity is integrated through space and time (Brand, 1989). The purpose of this investigation is to compare the diversity within a species under three different conditions: 1) in a species/genus that is evolving very fast; 2) in a species/genus that is relatively old; 3) in a species/genus that is relatively young. We have applied various molecular techniques in each instance for the resolution needed.

Skeletonema costatum

Skeletonema costatum (Grev.) Cleve is a cosmopolitan bloom-forming diatom species in neritic waters, well-known for its extreme variation in size and shape as well as its ability to survive in different environmental conditions (Hasle, 1973). Its ability to form major blooms under a variety of water conditions implies extensive genetic variation within the taxon. Using isozyme data Gallagher (1980) first demonstrated that seasonal populations of *S. costatum* from Narragansett Bay, Rhode Island were as genetically diverse as sibling species of terrestrial plants and animals. Superimposed upon this temporal diversity were several distinct but spatially separated populations (Gallagher, 1990a) which she hypothesised to be a single interbreeding cline (Gallagher, 1990b). Sequence analysis of the 18S rRNA gene has revealed one to eleven base substitutions among five clones of *S. costatum*,

which strongly suggests that *S. costatum* is a species complex (Fig. 1A). Sufficient morphological differences from the normal morphotype of *S. costatum* have been found in two of these clones to warrant the description of a new species *Skeletonema pseudocostatum* (Medlin et al., 1991). These morphological differences, albeit small, have been used to document annual succession of *Skeletonema* species in a Mexican bay (Castillo et al., 1995). *Skeletonema* is a very rapidly evolving taxon. Rates of evolution based upon a calculation of a molecular clock for the diatoms (Kooistra & Medlin, in press) indicate that this genus is evolving at approximately 1% per 7.3 My, although the morphology of its species has changed little since its origin in the Pliocene.

In this species complex, sufficient resolution was found in the 18S rDNA gene to identify a species complex and to separate two clones as a new species; a decision supported by morphological differences. A better understanding of the relationships among clones of *Skeletonema costatatum* is likely to be obtained using non-coding regions for DNA fingerprinting methods.

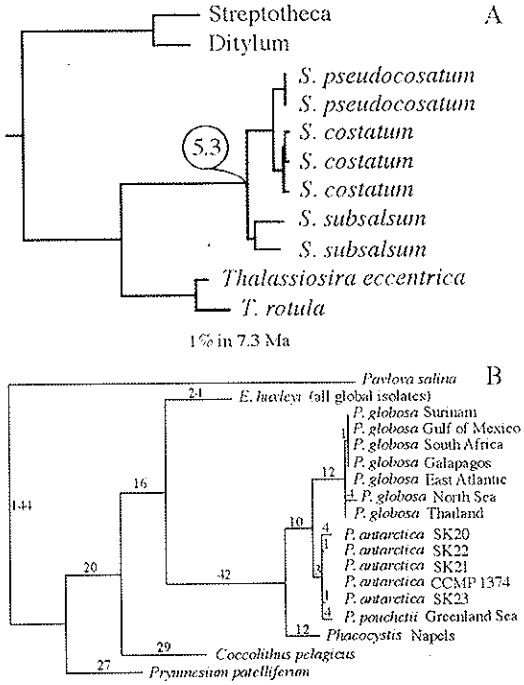

Fig. 1. A. Phylogeny of the genus *Skeletonema* inferred from ssu rRNA sequence comparisons with the neighbour - joining method. The encircled figure indicates the date in the fossil record (Ma) at which the genus first appears. The rate of evolution calculated below the figure is based on the number of base substitutions in that lineage since its divergence.
B. Phylogeny of the genus *Phaeocystis* inferred with the maximum parsimony method. Branch lengths represent the numbers of base substitutions along that lineage (seen above the lineage).

Phaeocystis

Phaeocystis is recognised both as a nuisance and as an ecologically important phytoplankton taxon (see review in Davidson & Marchant, 1993). Its polymorphic life cycle with both colonial and flagellated cells (Kornmann, 1955) continues to cause many taxonomic problems. *Phaeocystis pouchetii* (Pouch.) Lagerheim occurs in cold waters and forms globular, cloud-like colonies with cells arranged in packets of four (see for illustrations Jahnke & Baumann, 1987). *Phaeocystis globosa* Scherffel, a warm water species, forms spherical colonies with cells arranged homogeneously within the gelatinous matrix (Jahnke & Baumann, 1987). Recently, colony morphology has been judged an unreliable species-level character and only two of its nine species are recognised by Sournia (1988b) as nomenclaturally valid: *Phaeocystis scrobiculata* Moestrup, (known only from the flagellated state) and *P. pouchetii*, (which includes *P. globosa* as a later synonym).

Recent studies based on ultrastructural and physiological data dispute Sournia's circumscription of the genus (Baumann & Jahnke, 1986; Jahnke & Baumann, 1986; 1987; Jahnke, 1989; Larsen & Moestrup, 1989; Moestrup & Larsen, 1992; Baumann et al., 1994). Our analysis of 18S sequence data among *Phaeocystis* species supports the separation of the colony forming taxa into three species (Fig. 1B) (Medlin et al., 1994a; 1994b). Two complexes can be recognized. The cold water complex contains the Antarctic *Phaeocystis antarctica* Karsten and the Arctic *Phaeocystis pouchetii*. The warmwater complex contains isolates of *Phaeocystis globosa*. This separation of the cold and warm water taxa, which we have calculated to be approximately 25-30 My ago, is further supported by

Table 1. Comparison of DNA content in Phaeocystis strains determined by flow cytometry with strains whose 18S rRNA gene sequence has been determined (Vaulot et al., 1994; Vaulot, unpubl.). Each DNA cluster likely represents a different species..

Strain	DNA content pg/cell	Ploidy	DNA Cluster
Naples (new species)	0.12 (0.24)	1	1
P. antarctica SK 22	0.37	2	2
P. globosa			
Gulf of Mexico	0.34	2	3
South Africa	0.32	2	3
Thailand	0.35	2	3
P. globosa			
Surinam	0.38	2	4
Galapagos	0.38	2	4
P. globosa			
North Sea	0.21 (0.42)	1	5
Eastern Atlantic	0.44	2	5

differences in DNA content, morphology and pigment spectra (Table 1, Medlin et al., 1994a; 1994b; Vaulot et al., 1994). Using a 1% diverg.ence in 20 My, we have estimated that the warm and cold water taxa separated ca. 25-30 My ago, whereas the separation of the two polar species is calculated to be ca. 8-12 My ago. We can only resolve differences in the warm water complex using the DNA content per cell (Table 1). The different DNA clusters are probably different species. In the cold water complex, resolution among the strains could be achieved with the internal transcribed spacer region in the ribosomal operon (Fig. 2). *Phaeocystis pouchetii*, is closely related

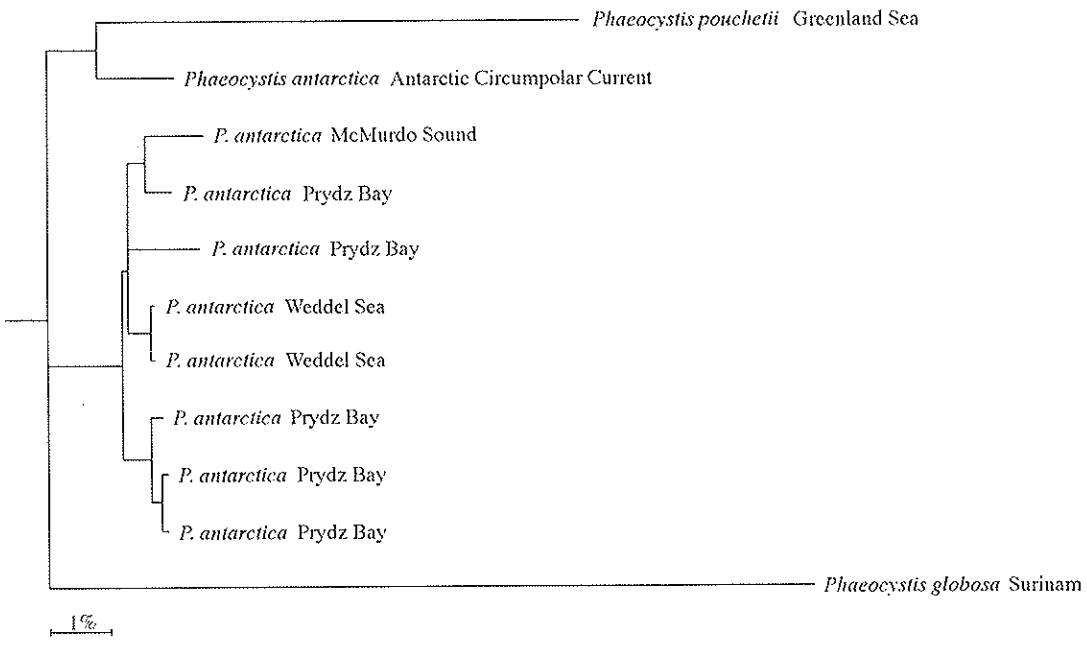

Fig. 2. Neighbor-joining distance tree of ITS1 sequences from *Phaeocystis globosa*, *P. pouchetii* and *P. antarctica* . The distance corresponding to one change per 100 nucleotide positions is placed below the tree. All but one strain of *Ph. antarctica* were isolated from Antarctic continental water masses, indicating that there is little population structure within *Phaeocystis antarctica* within this water mass as detected with ITS1 sequence data. *P. pouchetii* is most closely related to the *P. antarctica* isolate from the Antarctic circumpolar current.

to one isolate of *P. antarctica* taken from the Antarctic circumpolar current, whereas all other isolates of *P. antarctica* fall into the same clade and are distributed around Antarctica in the continental water masses. Except for *Phaeocystis pouchetii*, similar colony morphologies are found in all of the other isolates regardless of the amount of genetic difference among them. In this species complex, relatively old divergences between taxa can be resolved with the 18S gene but phylogenetic relationships in recently-evolved species complexes can only be detected using non-coding regions and genome size.

Emiliania huxleyi

Emiliania huxleyi (Lohm.) Hay et Mohler, the most abundant representative of the Haptophyta, can be found in oceanic and neritic waters from subpolar to tropical latitudes. Annual north Atlantic blooms may cover up to half a million square kilometres (Brown & Yoder, 1994), with cell densities of up to 10^5 ml^{-1} and have a major impact on both carbon and sulphur cycles. It is a very young genus, having separated from *Gephyrocapsa* ca. 268,000 years ago.

Physiological, morphological, and biochemical evidence suggests that *E. huxleyi* may be a multi-species complex. Three morphotypes, designated *A, B* and *C,* are recognized and can be sorted using standard light microscopical techniques (Young & Westbroek, 1991; van Bleijswijk et al., 1991) with significant, but overlapping ultra-structural differences between the coccoliths of the three morphotypes (Young & Westbroek, 1991). Of the three, the *C* morphotype has never been isolated into culture. Antisera raised against coccolith-associated polysaccharides can differentiate the three morphotypes (van Bleijswijk et al., 1991; Young & Westbroek, 1991; van der Wahl, pers. comm.); however, antisera raised against naked cells can not (Campbell et al., 1989; and pers. comm.). Growth rates of *A* and *B* morphotype cells differ significantly (van Bleijswijk et al., 1994), as do acclimatised growth rates for individual clones of a single morphotype from the same water sample (Brand, 1982). Different key biomarker compounds (Conte et al., 1995) and different pigment compositions (Mantoura, unpubl.) can be used to discriminate oceanic from neritic strains of *E. huxleyi* but not *A* from *B* morphotypes. On the basis of the above evi-

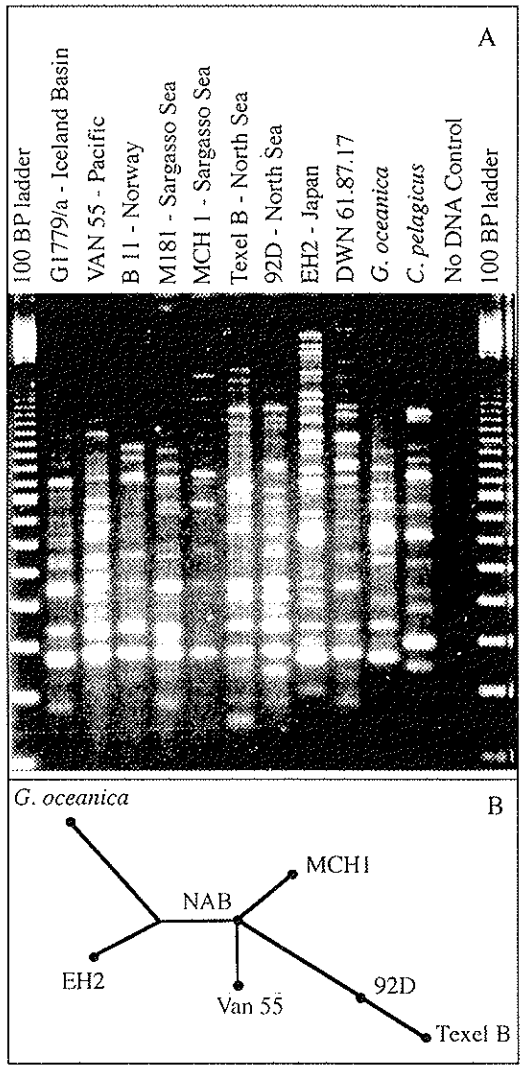

Fig. 3. A. Agarose gel showing banding patterns produced with the RAPD-PCR technique using the 10-mer oligonucleotide primer OPE-02 from globally distributed *E. huxleyi* isolates. 100 base pair ladder (Pharmacia) used as a size marker.
B. Representation of the genetic distance among global strains of *E. huxleyi* from the agarose gel shown (A) using the split-decomposition evolutionary network model with the outgroup *Coccolithus pelagicus* excluded. Length of all lines proportional to the isolation index or the split between the taxa. *E. huxleyi* strains belonging to the A morphotype are G1779/a, Van 55, B11, M181, DWN 61.87.17; those belonging to the B morphotype are MCH 1, Texel B and 92D. *Gephyrocapsa oceanica* isolates are EH2 and *G. oceanica*. *E. huxleyi* isolates belonging to the North Atlantic bloom are G1779/a and DWN 61.87.17.

dence it seems likely that at least two species may exist within the general concept of *E. huxleyi*, each exhibiting considerable genetic variability at the intraspecific level.

The genetic relatedness among geographically isolated clones of both *A* and *B* morphotypes of *E. huxleyi* as well as *Gephyrocapsa*, its closest ancestor, was compared using the nuclear (18S) and plastid (16S) rDNA gene and the non-coding region separating the plastid-borne genes encoding the two subunits of ribulose 1,5 bisphosphate carboxylase (rbcL-rbcS spacer). The nuclear and plastid-encoded ssu-rRNA genes from both morphotypes of *E. huxleyi* (Fig. 1B) as well as from *Gephyrocapsa oceanica* are identical. The faster evolving rbcL-rbcS spacer region in the two *E. huxleyi* morphotypes is also identical. The lack of variation in these coding and non-coding regions strongly suggests that the genetic separation of the *A* and *B* morphotypes of *E. huxleyi* from each other or from their last common ancestor shared with *G. oceanica* is too recent to be detected by base substitutions in the regions we have selected.

Thus, the more sensitive PCR-based fingerprinting technique of Randomly Amplified Polymorphic DNA (RAPD, Williams et al., 1990) was used to determine relationships among the isolates of *E. huxleyi.* In Fig. 3A RAPD bands of the global isolates amplified with OPERON primer OPE-02 (Fig. 3A) indicate the high level of diversity among all of the isolates taken from various ocean basins. RAPD data were converted to binary data and analysed with the split decomposition program (Fig. 3B, Bandelt & Dress, 1992). The analysis was performed with and without the outgroup taxon, *Coccolithus pelagicus* (Fig. 3A, B). When *C. pelagicus* is included, homoplasy is recovered in the split-diagram (data not shown), which is likely due to a comparison of nonhomologous alleles/bands because *C. pelagicus* is too distantly related to *Gephyrocapsa* and *Emiliania* (see phylogenetic tree in Fig. 1B). If *C. pelagicus* is omitted from the RAPD analysis, then a phylogenetic signal in the data set can be interpreted (Fig. 3B). Both isolates of *G. oceanica* are distinct from all *E. huxleyi* isolates. None of the North Atlantic bloom isolates (NAB) can be differentiated; however, both the Pacific isolate (Van 55) and the *B* morphotype (MCH-1) from the Sargasso Sea are distinct. Both *B* morphotypes from North Sea/English Channel are closely related and well removed from the remaining *A* morphotypes.

Relative genome size of four *A* and two *B* morphotypes of *E. huxleyi* were compared using flow cytometry, with *E. huxleyi* clones BT6 (morphotype *A*) and Texel B (morphotype *B*) as internal standards (Table 2). At least two different genome sizes are present within the *E. huxleyi* complex and these appear to segregate the *A* and *B* morphotypes. Among the *B* morphotypes measured, both a haploid genotype (Texel *B*) and a diploid genotype (92D) were identified. Among the four *A* morphotypes tested, BT6 has half the DNA of the other three *A* morphotypes and has also been designated a haploid genotype.

Table 2. Flow cytometric determination of relative amounts of DNA in A & B morphotypes of *Emiliania huxleyi* using chromomycin A3 on methanol fixed cells. Texel B (morphotype B) and BT6 (morphotype A) used as internal standards. (D. Marie & D. Vaulot, unpubl.). Both A and B morphotypes contain haploid and diploid components. The A and B morphotypes are likely different taxa.

Strain	Morphotype	Stage	DNA/Texel B	DNA/BT6	Interpretation Group
Texel B	B	Naked	1.00	1.31	Haploid group 1
92D	B	Naked	1.97	2.57	Diploid group 1
BT6/CCMP 373	A	Naked	0.73	1.00	Haploid group 2
B11	A	Naked & Coccoliths	0.73 + 1.34	1.0 + 2.0	Haploid+Diploid group 2
DWN61/81/5	A	Naked	1.30	1.69	Diploid group 2 or ?3
DWN61/87/17	A	Naked	1.30	1.71	Diploid group 2 or ?3

Isolate B11 from Norway contains both haploid and diploid components. A comparison of a mixture of strains B11 (morphotype *A*) and Texel B (morphotype *B*) is shown in Fig. 4 to illustrate the relative difference in the DNA content of the two morphotypes. The determination of genome size strongly supports the distinction of the *A* and *B* morphotypes and also suggests that both morphotypes may exhibit an alteration of generation.

Emiliana huxleyi represents an unusual case because phenotypic data suggest that it is a species complex. However, it is so recent that no differences could be found in either coding or non-coding regions among eleven global isolates to warrant separation of the two morphotypes based on molecular divergence. Only DNA fingerprinting could provide any resolution in our global isolates. The determination of genome size enabled us to make a taxonomic decision to separate the morphotypes at the varietal level based on phenotypic divergence (Medlin et al., in press).

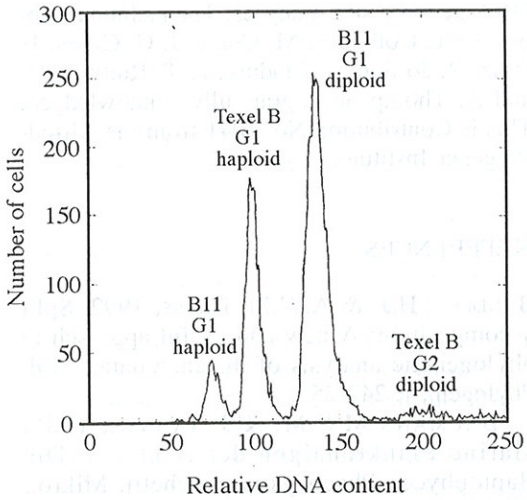

Fig. 4. Mixture of strains B11 (morphotype A) and Texel B (morphotypes B) stained with the DNA specific dye chromomycin A3 and analyzed by flow cytometry. The first and third peaks correspond respectively to the haploid and diploid B11 cells in the G1 phase of the cell cycle. Few G2 haploid cells are also probably included in the third peak. The second peak correspond to haploid G1 Texel B cells. The fourth minor peak is likely to be constituted of haploid G2 Texel B cells, although the presence of diploid G1 Texel B cells cannot be ruled out. (Marie and Vaulot, pers. comm.).

Conclusions

Molecular biology provides tools that can identify multi-species complexes and help clarify our definition of a species. Often the taxonomic level to which physiological and morphological differences should be applied can be better interpreted through a molecular framework. The molecular technique and the region of the genome should be carefully selected in order to provide the resolution needed to answer the taxonomic questions posed. If the separation of sibling species is too recent in geological time, then a variety of molecular techniques may be needed to resolve the problem.

The examples presented here illustrate how molecular techniques have been specifically used to enhance our understanding of a "species" in certain ecologically important marine phytoplankton taxa cosmopolitan in their distribution. The amount of genetic divergence separating the isolates into different species or varieties increases with time and may also be influenced by the rate of evolution in the region of the genome analysed. Species complexes have been identified in each taxon; in the youngest species, *Emiliania huxleyi,* separation at the varietal level seems appropriate (Medlin et al., in press). These data also suggest that, despite differences in genetic divergence in these cosmopolitan taxa, a conservation of form may have been achieved because each has retained a specific morphology. Molecular divergence has continued, which separates the taxa in time and space and likely provides the ability for these taxa to bloom under a multitude of environmental conditions.

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