Application of Molecular Techniques for Genetic Differentiation of Microalgae

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Introduction

The advent of molecular biological techniques has greatly enhanced our ability to analyse all organisms, not just the microalgae. However, the small size and paucity of morphological markers common to many microalgae, the inability to bring many into culture, and the difficulty of obtaining samples for long term seasonal studies in aquatic environments has hampered our knowledge of microalgal diversity and of population structure. Despite this, physiological/ biochemical measurements have been used to infer the existence of significant genetic diversity within and between microalgal populations (Waterbury et al., 1986, Brand, 1989, Partensky et al., 1993). With these data researchers have speculated on hidden biodiversity and temporal and spatial structuring of genetic diversity or gene flow. Now molecular techniques can present a quantitative framework through which the diversity, structure and evolution of microalgal populations can be analysed, predictive models of the dynamics of aquatic ecosystems formulated, and the idea of functional groups in the plankton proven.

Molecular analysis of microalgal population structure is behind other groups and has been usually inferred from physiological data determined from relatively few clones. This unfortunately is a very naive approach because nearly every physiological measurement has shown that no single clone of any microalgal species can be considered truly representative of that species (Wood & Leatham, 1992). The need to establish clonal cultures prior to genetic analysis and the inability to perform fine-scale sampling under most conditions are probably the overlying reasons why studies of microalgal population structure are perhaps twenty or more years behind those of other organisms. Isozyme analysis, performed for a few species, has revealed heterozygosity between some populations. In addition, fingerprinting analyses, such as RAPDs or multi locus probes, have shown that phytoplankton blooms are not clonal but are highly diverse with isolates being related by geographic origin.

The interaction of a species with environmental parameters is influenced by the genetic diversity at the population level of a species. Spatial and temporal partitioning of genetic diversity will occur as these interactions structure the ecosystem. Such structuring has seldom been measured in the microalgal community and studies of genetic diversity are virtually non-existent in pelagic ecosystems. All evidence of geographically isolated populations would be erased if we continue to assume that microalgae with high dispersal capacities are genetically homogeneous over their entire range. Support for this assumption has come mainly from phenotypic comparisons based initially on net phytoplankton biogeographic studies and later on isozyme studies. It is clear that the same morphotype/species may be endemic or cosmopolitan

(Kristiansen, 2001) but it more likely that cosmopolitan species will exhibit regional differentiation when examined with molecular techniques (Medlin *et al.*, 2000b). Some of the reasons why studies of microalgal diversity and their population structure have lagged behind those of other organisms are because of their small size and the lack of morphological markers, and the ability to bring into culture only a small part the known biodiversity. The lack of knowledge of their breeding systems makes genetic or demographic studies difficult. Logistical problems of collecting samples for long term seasonal studies in aquatic environments or for doing fine-scale sampling are additional reasons.

In 1975, Doyle hypothesized that microalgae must consist of a multitude of competing genotypes, but this study has been largely ignored because it has been assumed that microalgal taxa may have little genetic structure over very large geographic areas. It has been assumed that highly dispersed organisms at the mercy of the currents have no trace of genetic structure and so we find the microalgal organisms living in an ever-changing three-dimensional environment and it follows that they must be homogeneous. Speciation and dispersal mechanisms in microalgae may be very different from those on land as suggested by recent evidence (Palumbi, 1992) making our knowledge of microalgal genetic diversity becomes even less certain because generalizations about terrestrial plant diversity and population structure may not apply to aquatic ecosystems.

With the advent of nucleic acid methods, however, these views on the absence of genetic structure in the microalgae have been seriously challenged. It is now known that genetic structure and physical, spatial partitioning occur within biogeographic regions. The idea of a single globally distributed species or of temporal stasis is no longer believed. Temporal genetic change may often be greater than spatial change or change between species (Brand, 1982, Gallagher, 1980, Hedgecock, 1994) and may very well apply to bloom populations. The rate of genetic change can and does occur on ecological time scales (Palumbi, 1992). Why? We don't know, but such changes may play a role in determining how local adaptations and speciation can occur in apparently homogeneous populations. The groundwork for temporal genetic change may lie with the idea of a 'super species' with the ability to exploit a wide spectrum of environmental conditions.

Karp *et al.* (1998) provide an excellent introduction into the variety of molecular techniques available for use in studying biodiversity at all taxonomic levels, and a review of biodiversity in the marine environment can be found in Ormond *et al.* (1998) and in the marine phytoplankton (Medlin *et al.*, 2000c). The difficulty of finding polymorphic markers for ecological genetic studies created much of our limited knowledge about microalgal genetic diversity. Isozymes, the molecular genetic markers used in early studies, evolve so slowly that closely related populations appear identical. The early ideas of the absence of genetic diversity in microalgae have undoubtedly been propagated from these studies. The use of high resolution DNA fingerprinting techniques *sensu lato* circumvents these problems and has thus opened areas previously considered unreachable for the microalgae.

Recent developments

What questions can molecular techniques answer?

Taxonomic affinities - Plastid and flagellar apparatus characteristics are the features that define most microalgal classes, making them are monophyletic taxa. Although some surprises have been revealed by molecular analyses. For example, the Euglenophyceae, are shown to be a very early eukaryotic radiation and not related to the Chlorophyceae, which is part of the major eukaryotic radiation, the so-called crown group radiation. The Kingdom Chromista did contain the bulk of eukaryotic microalgal taxa, i.e., the Heterokonta, Haptophyta, and Cryptophyta. But this kingdom is now recognized as a polyphyletic taxon (Bhattacharya *et al.*, 1992, Medlin *et al.*, 1997), although a view difficult to relinquish (Cavalier-Smith *et al.*, 1994). Molecular analyses based on total evidence, which includes both morphological and molecular data from the rDNA data set, continue to reinforce the clear separation of the Haptophyta from the Heterokonta (Medlin *et al.*, 1997), whereas those based on many other genes have distanced the cryptophytes from both the heterokonts and the haptophytes (Bhattacharya & Medlin, 1995,

McFadden *et al.*, 1995, Rensing *et al.*, 1996). A fourth group, the Chlorarachniophytes are now shown to be clearly related to the foliose ameba (Bhattacharya *et al.*, 1995) and cannot be placed in the Chromista where it was originally. Clearly the Kingdom Chromista is an idea whose time has past. In some cases commonly used external morphological features found in the microalgae have supported the molecular clades, e.g., in the Haptophyta (Edvardsen *et al.*, 2000) but in others, e.g., the diatoms, the internal structure of the cell has best supported the deeper branches in the molecular tree, whereas the more commonly taxonomically used feature of the siliceous cell wall support the younger branches (Medlin *et al.*, 2000a). The dinoflagellates have probably proven the most difficult to analyze. The phylogenetic outcome of the analyses is strongly dependent on the algorithm used (Medlin *et al.*, unpubl.)

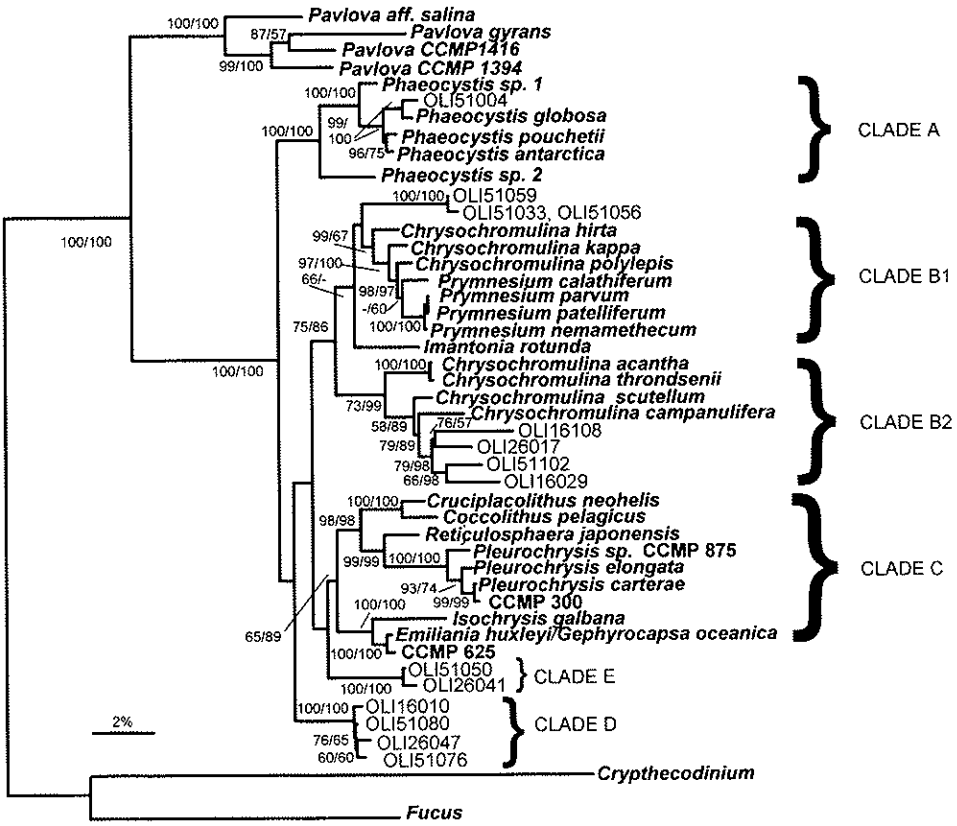

Figure 1. Phylogenetic tree based upon a maximum likelihood analysis showing the relationship of haptophyte taxa. The tree is root on the branch leading to *Fucus* and *Crypthecodinium*. Clades A to C are presented by taxa in culture whereas Clades D &E represent novel taxa with no representatives in culture collections. Bootstrap values (500 replications). represented at internal nodes for values more than 50% for neighbour/joining and maximum parsimony analyses, respectively. Figure redrawn from Edvardsen *et al.*, (2000).

Molecular techniques have wreaked the most havoc upon systematics, at the genus and species level, showing polyphyletic and paraphyletic lineages across many algal groups (Fig. 1), not just the microalgae. Groups with few morphological markers, and where morphological species definitions have been too broad have seen the most changes. Polyphyletic taxa include such taxa as the prochlorophytes, *Chlorella*, *Chlamydomonas* and *Chrysochromulina* (Fujiwara *et al.*, 1994, Edvardsen *et al.*, 2000). But even in groups with good morphological markers, e.g., *Skeletonema* and *Crypthecodinium*, (cryptic) sibling species; have been found and in others not so easy to differentiate e.g., *Phaeocystis* may also contain cryptic species (Lange et al., 2003, Vaulot *et al.*, 1994).

Population structure - Population structure in many microalgal species has been reconstructed from molecular data. Most molecular studies have shown that multiple isolates of a single species are related by geographic but sometimes the geographic groupings reveal polyphyletic or paraphyletic taxa (Scholin *et al.,* 1994, 1995, Bakker *et al.,* 1995, Medlin *et al.,* 1996). Recognizing and separating polyphyletic or paraphyletic taxa can be controversial and are difficult taxonomic decisions to rectify. Often our species concept must be re-evaluated (Cracraft, 1989; Wood & Leatham, 1992; Gosling 1994, Medlin *et al.,* 1995a,b) along with a re-evaluation of the combined resolution that both molecular and morphological data sets can have (Costas *et al.,* 1995, Friedl, 1995, Edvardsen *et al.,* 2000). More cryptic (sibling) species are being reocognised by molecular data (Nanney *et al.,* 1989; Medlin *et al.,* 1991; Beam *et al.,* 1993; Knowlton, 1993) and with these data biologists can better determine the taxonomic affinities of taxa with few or controversial morphological characters (Andersen *et al.,* 1993, DeYoe *et al.,* 1995, Potter *et al.,* 1996). Our knowledge of microalgal biodiversity is likely to increase by an order of magnitude when we have true estimate of all sibling species and an identification of all the „little brown/green balls" and the „very small red fluorescing bodies" (Hedgecock, 1994).
Genetic diversity - Molecular tools provide the only means to estimate genetic diversity and gene flow, besides addressing the obvious questions about phylogenetic relationships and taxonomic affinity. The interaction of a species with environmental parameters is greatly influenced by genetic diversity at the population level of a species. Spatial and temporal partitioning of genetic diversity can occur because interactions structure the ecosystem, It has long been assumed that marine organisms with high dispersal rates will be genetically homogeneous over their entire range, thus erasing traces of geographically isolated populations. Thus, such structuring has seldom been measured in the marine planktonic community. Allozyme (allelic variants of enzymes) analyses have generally shown the planktonic community to be homogeneous, other molecular techniques have identified genetic structure within geographical regions. Physiological measurements detect ecological adaptations. The grouping of isolates from the same geographic region by using molecular markers that are amenable to tree building algorithms can provide even strong evidence for population structure.

Two standard population genetic measurements for estimating genetic variation and gene flow are the Fst and Nm statistic (Wright, 1969). The Fst statistic measures the observed genetic variation that can be partitioned between populations, whereas the Nm statistic measures the numbers of individuals exchanged between two populations per generation. A high or low gene flow species can be identified by the distribution of genetic variation within and between populations. A high gene flow species is homogenous over its entire range; the low gene flow one not. There are few estimates of the genetic diversity and amount of gene flow among microalgal populations (Gallagher, 1980, Rynearson & Armburst, 2000).

Phylogeography - An objective framework with which to reconstruct the historical biogeographic distribution of taxa as well as recover recent dispersal events can be obtained by using molecular data (Lange & Medlin, 2002) and to estimate divergence times (Medlin *et al.,* 1997). It is possible to correlate present-day distributions of taxa with their biogeographic history by using the fossil record or geological events to date divergences between species. It is possible to correlate the divergence of taxa with palaeo-oceanographic and palaeo-climatic events to explain their present-day biogeographic distribution if there is no fossil record, and if the phylogenetic reconstruction of a group is congruent with a biogeographic history of the study area (Bakker *et al.,* 1992, 1995, Kooistra *et al.,* 1993, Medlin *et al.,* 2000, Lange & Medlin, 2002).
Phylogeography, a new field, has been termed for such studies reconstructing the biogeographic history of plants (Avise *et al.,* 1987). Dates for the opening and closing of oceans, for the movements of the continents relative to the water masses and for climate changes resulting in fluctuations in sea-level can be used for similar studies in microalgae communities (Scholin *et al.,* 1995, Medlin *et al.,* 1994b, Patarnello,, pers comm., Lange et al., 2003). But if the group has a detailed fossil record, e.g., the diatoms, coccolithophorids and the dinoflagellates, then these can be used for dating divergences (Costas and Løpez-Rodas, 1995, Medlin *et al.,* 1997). Relative rates of evolution should be calculated (Wu and Li, 1985, Kooistra & Medlin, 1996) prior

to estimating divergence times (Ochmann & Wilson, 1987, Olsen *et al.,* 1994) to ensure that fast-evolving species, which bias the determination of divergence times, are eliminated from such interpretations.

How does one choose the correct molecular marker for the question asked?
Biases and resolution limits are present in all molecular techniques, but one must be aware of them in any analysis. Photosynthetic organisms have a different array of genomes/genes to access than heterotrophic organisms that researchers can use to reconstruct phylogenies. However, to carry out very broad comparisons one should use one of the many genes that are shared by all organisms irrespective of their nutritional class.

Several questions should be considered when selecting a molecular marker for phylogenetic or population structure analyses. Answers to these will strongly influence the molecular markers selected for a study. These questions include among others: (1). To what taxonomic level is the question addressed? (2) Is the rate of evolution in the chosen molecular marker appropriate for the taxonomic level addressed? (3) What is the geological age of the species or group of organisms investigated? (4) Can adequate sampling strategies can be employed for microalgal populations to address spatial and/or temporal genetic variation questions?

Methods
Sequence data - Sequence data for both coding and non-coding regions of the genome can be used to reconstruct the evolutionary history of organisms and to examine relationships at all taxonomic levels. The ribosomal RNA genes are usually used for phylogenetic analyses, although many genes are potentially available. They have special attributes that make them ideally suited as molecular markers (Woese, 1987). Ribosomal RNA genes are of a relatively large size. Both variable and highly conserved regions are present within the molecule to address both close and distant evolutionary relationships, respectively. They are of a universally conserved function with no evidence to suggest that they are laterally transferred (Woese, 1987). Because a large rRNA sequence database exists, members of all major phyla can be included for any analysis. The ribosomal database project (RDP, http://rdp.life.uiuc.edu) contains over 436 eukaryotic small subunit (SSU) rDNA and 28 large subunit (LSU) gene sequences to provide representatives of nearly all major organismal groups. More extensive compilations of ribosomal sequences have been made available by Van de Peer (http://rna.uia.ac.be). Alignments can be downloaded from both databases so that the individual nucleotides in each species/sequence are arranged so that only positions assumed to be homologous are compared between taxa.

Nuclear rDNA genes evolve at a different rate than organelle rDNA genes in most algal groups, and are best used for phylogenetic questions at higher taxonomic levels. These genes often reach a limit to their level of resolution at the species level for many protists and probably at higher taxonomic levels in animals and in higher plants because they are more recently evolved. The phylogenetic history reconstructed from their analysis genes from organelles can reflect only the history of the organisms since endosymbiosis occurred because organelle genomes (plastid and mitochondria) are the results of endosymbiotic events. Since their reduction from a free-living organism to a captive endosymbiont, a different mutation rate may have occurred.

Sequences of the large subunit of RUBISCO are also used for phylogenetic analyses at the genus level or higher, (Clegg, 1993, Fujiwara *et al.,* 1994, Daugbjerg *et al.,* 1995). Little or no alignment problems are encountered with this gene in contrast to the problems with aligning the secondary structure of the rRNA molecule, thus they have an advantage over rDNA genes. The question of lateral transfer of these genes has been repeatedly discussed (Palmer, 1995), although their inheritance appears vertical since their original lateral transfer into the eukaryotic host from their bacterial ancestor.

Non-coding regions separating genes are termed spacer regions. In some operons, such as in the ribosomal operon, they function in the final processing of the mature rRNA molecular but in most other genes their function is not well understood. They can evolve at a faster rate because they are not subjected to the same evolutionary constraints as coding regions. However, a very rapid fixation of mutations in coding regions can be seen within a

population that may be comparable to the rate of evolution in non-coding regions if selection pressure pulls coding sequences in a particular direction. To resolve closely related species or population level genetic structure, faster-evolving non coding regions are best used but even these regions can be conserved at the genus level or higher in some algae (see review in Medlin *et al.*, 2000b). Also, the geological age of the group and the rate of evolution in the spacer region may play a role in the lack of resolution seen within these regions. Rapid morphological changes can influence the way one perceives species and generic level differences, and this may influence the interpretation of the resolving power achieved with any genomic region. Sufficient differences were detected in the RUBISCO spacer from eastern and western Atlantic populations of the red alga, *Gymnogongrus devoniensis,* believed to be conspecific to warrant the recognition of a cryptic species (Maggs *et al.,* 1992), whereas no differences were detected among global isolates of *Emiliania huxleyi*, a very young taxon (< 270,000 years old) (Medlin *et al.,* 1996). Among distantly related diatom genera, RUBISCO spacer regions were nearly identical (Chesnick *et al.,* 1995, Lange et al., 2003), whereas only differences among distantly related species level could be resolved in *Phaeocystis* (Lange et al., 2003). The ITS spacer in the ribosomal cistron can distinguish closely related species/genera in the green algal orders, Cladophorales and in the diatoms (Scholin *et al.,* 1994, Bakker *et al.,* 1995, Medlin *et al.,* 1996). Both inter and intra-specific variation in *Ulva* species around Roscoff, France could be resolved with ITS regions (S. Loiseaux-de-Goër, pers. comm.). The biogeographic distribution of *Phaeocystis antarctica* populations in Antarctic continental waters could be delineated with the ITS (Lange et al., 2003), but diatoms speciated less than 12,000 years from nearby lakes could not be differentiated from one another (Zechmann *et al.,* 1994).

Analysis can be performed on the sequences obtained from mixed natural samples/ communities. With these methods novel insights into diversity can be obtained, which are unobtainable using more traditional means of community analyses. This is especially true for unculturable groups. Random clones from a PCR-bank of clones can be screened for genetic diversity. To provide a representation of the phylogenetic/haplotype diversity in complex microbial communities, PCR products that differ by only a few nucleotides can be identified through DGGE, denaturing gradient gel electrophoresis (Muyzer *et al.,* 1993), separated and sequenced. The first documented evidence that bacterial diversity in the world's oceans was severely underestimated came from the random clone approach (DeLong, 1992, Giovannoni *et al.,* 1995, Pace *et al.,* 1986). To describe distributional patterns and diversity for oceanic bacterial populations, this type of analysis has been most frequently used (DeLong *et al.,* 1994; Giovannoni *et al.,* 1996). Although, it has been recently applied to microalgal populations in the oceans (Rappé *et al.,* 1995, Van der Staay, *et al.,* 2000, Medlin & Valentin 2001). It appears that in some algal groups novel taxa abound and our estimates of eukaryotic biodiversity may be severely underestimated, as was the prokaryotic community ten years ago.

Taxon sampling will affect interpretations. Very different conclusions can be reached if too few taxa/isolates or if different taxa/isolates are taken as representatives of the group being studied. A better taxon representation for analysis can be obtained today because, with increased ease of molecular data acquisition, molecular systematists can devote more time to improving their sampling strategy.

Oligonucleotide probes. - Oligonucleotide sequences of varying specificity can be easily found within the vast amount of rapidly accumulating sequence data. Oligonucleotide probes or signature sequences have been developed as a phylogenetic determinative tool in environmental microbiology. Although these techniques have been largely used for Bacteria (Stahl & Amann, 1991, Amann, 1995), work is beginning for pico- and nanophytoplankton (0.2-2 and 2-20 ?m, Knauber *et al.,* 1996; Simon *et al.,* 1995, 2000), which, like most marine heterotrophic bacteria, often lack taxonomically useful morphological features and for larger plankton because of their importance as harmful algae (e.g., *Pseudo-nitzschia* and *Alexandrium* (Scholin *et al.,* 1994, 1995, Miller & Scholin, 1996, Bakker *et al.,* 1995, Medlin *et al.,* 1996, Medlin *et al.,* 2000b.

A homologous strand of DNA that has been radioactively-, fluorescently- or enzyme-labeled binds to the target nucleic acid sequences for detection of the target DNA/RNA. Only when there is perfect or nearly perfect complementarity in the high-fidelity base pairing between

target sequence and probe can sufficient sensitivity and specificity be obtained. The same features that make rRNA sequences so useful for taxonomic purposes (see above) also account for their popularity as targets for taxon-specific oligonucleotide probes. Fluorescent probes to identify individual cells recognize the ribosomal RNAs, the transcripts of these genes, which are abundant with several thousand copies per cell. Although SSU-rRNAs are most often chosen as targets for taxonomic probes, but other genes (nuclear encoded LSU-rRNA, chloroplast or mitochondrial LSU and SSU rRNAs) will also be available for probe design as databases grow (Scholin *et al.,* 1994). Among the prokaryotic community, ribosomal RNA content increases with growth rate; therefore hybridizations should primarily detect the active part of the community, which is often the target of choice in environmental studies of microbial communities (Kramer & Singleton, 1993). In some eukaryotic cells, there is, however, some evidence that rRNA content may be constant throughout the growth cycle, making rRNA measurements good proxies for biomass measurements (Parrott & Slater, 1980; Laws *et al.,* 1983; Thomas & Carr, 1985). In others where this is not the case, the lysis of the cell to release the nucleic acids from cytoplasmic influence may actually overcome the low fluorescence of the probes when they must bind to the native ribosomes (Scholin, pers. comm.).

To design successfully a rRNA-targeted probes for groups or species one must follow a few simple rules: First, probes can only target monophyletic groups. No probe exists for polyphyletic taxa, such as *Chlorella* or *Chlamydomonas*. Thus, a thorough knowledge of the phylogeny of the organisms of interest is required. Second, sequence databases upon which the probes are designed should be representative of the biodiversity of the community to be investigated to insure probe specificity. Only then can the specificity of the probe be tested on cultures. However, for many algal groups, the true extent of their biodiversity is unknown, and therefore rRNA probes should be regarded as tools subject to refinement until we learn more about the group's biodiversity.

Once a monophyletic group is defined, probe design is performed using software packages, such as the ARB program package (http://www.biol.chemie.tu-muechen.de). Taxonomic „signatures" are rapidly found by these packages that allow rapid screening of databases. Although longer probes have been made for the identification of bacterial species, oligonucleotide probes usually consisting of 15 to 20 bases are designed for taxonomic purposes, (Amann, 1995). By using the „PROBE_MATCH" function of ARB or the „CHECK _PROBE" function of RDP and by BLAST searches of Genbank, localization of potential target sites in other sequences may be checked. Target site suitability should then be checked by eye from partial sequence alignments provided by the output from these programs: the number, quality and localization of mismatches between the target and non-target sequences are of prime importance. For best discrimination, probes should be 100% complementary to sites in the target species with mismatches placed ideally in the center of the probe. To discriminate best target from non target sequences at least 2 mismatches are necessary for the probe (Amann *et al.,* 1995), but a single mismatch can be sufficient in some cases with the use of competitor probe to block the target signature in the non-target species, thus preventing false positives. Because the number of sequences in databases is increasing rapidly, the specificity of the probes should be routinely re-assessed for specificity.

There are many hybridization techniques for these probes: the choice will depend on the type of information or resolution required and the number and type of samples to be analyzed. To provide distributional patterns of species, hybridizations can be performed on extracted nucleic acids (from cultures or natural communities). Quantification of a given group's SSU-rRNA relative to total SSU-rRNA can be obtained by dot blot hybridization with specific and universal oligonucleotide probes in a complex community. Target molecules can be either RNA or DNA (cDNA derived from RNA by reverse transcription or amplified DNA). This technique has been successfully applied to marine bacterial assemblages (Rehnstam *et al.,* 1993). Because rRNA content per cell varies between different physiological states (but see exceptions for eukaryotes listed above), absolute cell numbers cannot be derived from these measurements. Information on the composition and dynamics of bacterioplankton can be gained from dot blot hybridization with oligonucleotide probes (Giovanonni *et al.,* 1990; DeLong, 1992; Rehnstam *et al.,* 1993).

Whole cell hybridization of probes to targets directly within whole fixed cells is another

way of detecting, identifying and quantifying small algal cells. Labeling with FITC or CY3 at the 5' end of the oligonucleotide probe is the standard way of labeling. Protocols similar to those used for immobilized bacterial cells (Amann, 1995) or cells in suspensions (Wallner *et al.,* 1993) can be used for whole cell hybridization of algae (Simon *et al.,* 2000). Algae, either immobilized on a slide or in suspension, are preserved and permeabilized for oligonucleotide probes by fixation with aldehydes and/or alcohols. Temperature and monovalent cation concentration are used to control hybridization stringency (Stahl & Amann, 1991). Formamide can be added up to 50% if higher stringency is required (Stahl & Amann, 1991; Amann, 1995). Epi-fluorescent microscopy or flow cytometry are the usual means to visualize whole cell hybridization. The problem of autofluorescence of photosynthetic cells and background fluorescence can be overcome with flow cytometry or with the use of fluorochromes outside the fluorescent range of chlorophyll or with chemical solvents to remove the chlorophylls. The most important problem to be addressed in future research on natural populations is likely to be the low signal from natural populations and this problem cannot be ignored. For the eukaryotic cells (see above), the low ribosome number may not be always related to the physiological state of the organisms. Enhancement of the signal can be achieved with some of the new enhancement kits commercially available (Schonuber *et al.,* 1997).

There are several advantages of whole cell methods over dot blot hybridizations. These include: (1) retention of cell features, such as morphology and chlorophyll fluorescence, to separate photosynthetic from heterotrophic organisms, which can be closely related to some algal taxa, and (2) evaluation of a cell's physiology on a cell-by-cell basis (Kemp, 1993, DeLong *et al.,* 1989). The first advantage is particularly useful for analysis by flow cytometry. Bulk dot blot analyses only provide bulk measurements of a population's rRNA content, which cannot be converted to cell numbers (but see above).

Molecular markers - As mentioned earlier, studies on the population structure of microalgae have seriously lagged behind those of other organisms. Microalgologists cannot walk down to the shore and pick up 100 or so individuals for analysis. At present most genetic studies for microalgae must rely on clonal cultures for their analyses. At sea these single-cell isolations, made from natural populations, can be difficult to perform. If only 10 - 30% of clonal isolates from natural populations survive, then this may mean that the range of genetic diversity determined from a bank of clonal isolates may not be a true reflection of the genetic diversity in the original population and may not be adequate for the level of genetic diversity being addressed (see Hillis *et al.,* 1996, Table 2.1 for a detailed description of how many samples need to be taken to estimate genetic diversity given certain confidence intervals). Life histories in most algal groups are incomplete or unknown, and if during culturing the algae undergo sexual reproduction, this may also alter the type of genetic analysis performed or the interpretation of the data. Also, ploidy level of the clonal isolates may be unknown, especially in the haptophytes, which have a haplo-diploid life cycle, and in many heterokonts, where the life is completely unknown. Sampling at depth along a transect, rather than in a grid-like fashion as is usually done for hierarchical population studies, may be dictated by pre-established cruise tracks. Even when research programs, such as the Arabian Sea project, are able to provide fine-scale sampling in a grid like fashion, no genetic studies were included as part of the research program. If current regimes in the study area are not well known, then samples may be unknowingly taken from separate water masses, which may also bias sampling strategies and data interpretation. Despite these many problems, significant genetic diversity has been inferred to exist within and between microalgal populations primarily from physiological/biochemical measurements (Brand, 1989, Wood & Leatham, 1992). Hidden biodiversity and temporal and spatial structuring of genetic diversity or gene flow have been speculated upon from physiological data.

DNA fingerprinting (Fp) relies on the existence of contain tandemly arrayed, highly repetitive DNA sequences called VNTRs (variable number tandem repeats) in the genomes of most eukaryotes. These multi-locus fingerprints (MLP's) are literally individual-specific but ironically can provide too much variation in the case of population studies because of the high variability at each locus and the large number of loci. Banding patterns can quickly become so complex that they cannot be analyzed in terms of allele frequencies (the data of population

genetic measures). Four methods (RFLPs, RAPDs, AFLPs, VNTRs) produce a type of fingerprint that can be considered unique for an individual.

RFLPs (Restriction Fragment Length Polymorphisms) are the pattern of fragments of purified DNA resulting from its digestion by restriction enzymes. Restriction enzymes recognize and cleave DNA molecules at specific nucleotide sequences, resulting in a non-random, reproducible fragmentation of the DNA. RFLPs can also be revealed by Southern Blot Hybridization (Sambrook *et al.,* 1989) of digested total DNA to either single-locus or multi-locus probes (Burke *et al.,* 1991). RFLPs of chloroplast DNA (cp DNA) or digestions of total DNA probed for chloroplast fragments have been used in algal studies to delimit populations This method one of the least convenient means of population analysis because good quality and high yields of cp DNA can be difficult to obtain. However, a quick screening tool to identify algae can be performed by the digestion of amplified genes (e.g. rDNA) to reveal fragment lengths diagnostic of a particular taxon. To distinguish toxic from non-toxic strains of the toxic dinoflagellate, *Alexandrium*, such techniques were developed (Scholin and Anderson, 1994).

RAPD (Randomly Amplified Polymorphic DNA) markers are obtained from the PCR amplification of the sequence between inverted repeats using one short primer, 10-12 bp in length and high in G-C content and can be used to provide a clone/individual specific genetic fingerprint (Hadrys *et al.,* 1992). The peculiarities of RAPD PCR kinetics combined with unique/ unusual properties of genomic organization of the organisms of interest can cause problems when applying RAPDs at narrow population levels where the signal to noise ratio will/can be too high. This technique is inexpensive, requires small amounts of DNA, and is besieged with many problems. The fingerprints may not be reproducible from one laboratory to another or from one person to another, and are sensitive to [Mg++], enzyme, template concentrations and to the type of thermal cycle used. Other frequent problems included the issues of non-amplifiable products, codominance and comigration of alleles, and scoring of weak bands. For these reasons RAPDs must be coupled with other data measurements in an investigation of population level genetic diversity. Despite these problems, RAPDs can be used as a quick screening tool to assess initial levels of genetic variation and perhaps to pre-select clones for further analysis. The presence of a band can represent either the AA or Aa genotype because RAPDs are inherited as dominant Mendelian markers. As a result, allele frequencies cannot be observed directly and must be calculated from the frequency of the recessive homozygotes, aa (no band, Lynch & Milligan, 1994), under the assumption that the population is in Hardy-Weinberg equilibrium. Statistical measures to analyze RAPD markers are based on these features and suggest that over 100 loci should be sampled to ensure that adequate population sampling has been met (Lynch & Milligan, 1994). Representation of relationships inferred from RAPDs can be displayed with distance matrix methods, multivariate analyses, or split decomposition methods (Medlin *et al.,* 1995). Homoplasies in the data or insights into the phylogenetic signal in the data set can be detected and displayed with the latter method.

AFLPs (Amplified Fragment Length Polymorphisms) are similar to RAPDs, except that the DNA is digested by a pair of both rare and frequent cutter restriction enzymes, such as msel an ecoRI (Vos *et al.,* 1995). The resulting fragments are ligated to an adapter. PCR primers are designed to anneal to the adapter plus one or more extra nucleotides. In this manner subsets of the digested DNA can be amplified of varying resolution and complexity in the banding patterns. Bands are scored as plus or minus as in RAPDs and are analyzed similarly (Fig. 2). They have several advantages over RAPDs in that they are more easily reproduced and do not produce artifacts, such as nested alleles because the DNA has been digested. However, the purity of the DNA can affect enzyme digestion (John, pers. comm.).

The shortcomings of MLPs have prompted development of methods that offer the same high resolution but with electrophoretic patterns that are easier to interpret in natural population level studies and that lend themselves to standard population genetic methods of analysis. These are the so-called single-locus fingerprinting probes (SLPs). An example is the VNTRs, which are regions of DNA that consist of Variable Numbers of Tandem Repeats of short nucleotide sequences; the number of repeats at a given locus can be used to distinguish between individuals within a population (Wright & Bentzen, 1994). The length of the repeat classifies the type of VNTR, i.e. 1-2 nt (microsatellites), 3-6 nt (short tandem repeats), > 10 nt

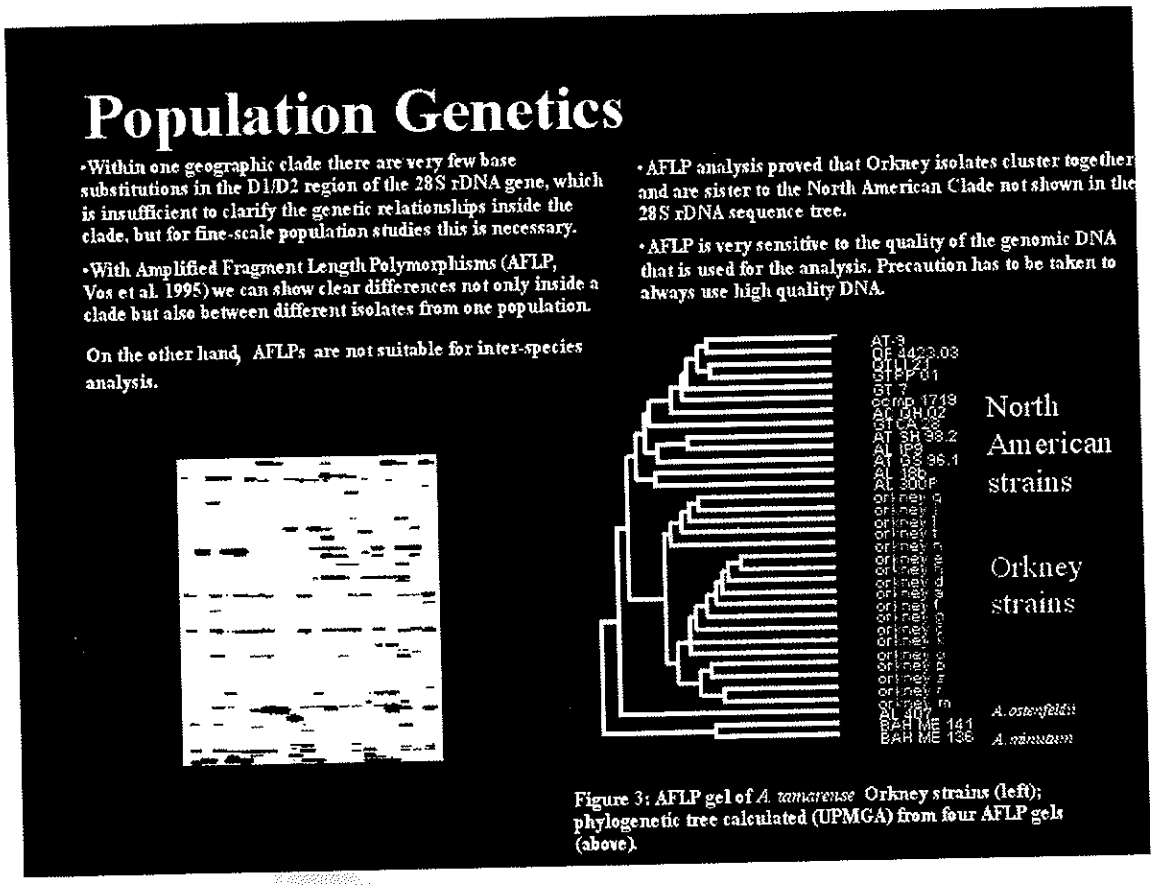

Fig 2. Representative gel from an AFLP analysis using one additional nucleotide added to theadapter sequence ligated to the restriction site. The corresponding tree is constructed with the UPGMA algorithm building methods. Each lane is a different strain of *Alexandrium tamarense* isolated from the Orkney Islands. Data courtesy of U. Jon.

(minisatellites). VNTR loci can be relatively easily scored using a combination of PCR amplification followed by electrophoresis to separate the different alleles. Microsatellites can be selected that provide very high levels of heterozygosity so that the resolution obtainable with a given probe can be selected to match the population question. This is why microsatellites offer such enormous promise for natural population studies. Moreover, microsatellite fingerprinting can be converted into a PCR-based assay. Finding microsatellites is labor intensive and involves the establishment of a genomic library for each target species, screening the library with oligonucleotide probes, sequencing the positive clones, and using these as probes.

At the population level, all **VNTR** loci are genetic markers used because of their high heterozygosities and large numbers of alleles resulting from high mutation rates (Schriver *et al.*, 1995). High amounts of genetic variation in populations deemed irresolvable by allozyme markers and even by mitochondrial DNA have been resolved with these markers (Paetkau *et al.*, 1995). Because VNTR loci mutate via stepwise mechanisms, i.e. size changes occur in small increments, usually one repeat unit, a high genetic variation occurs among individuals. Thus, these loci can not be evaluated with specific distance measures used to reconstruct relatedness and gene flow between populations, such as Nei's distance (Nei, 1972). Many of the allele-sharing measures, become non-linear with increasing time since divergence or with high mutation rates (Shriver *et al.*, 1995). To address the special requirements of the VNTRs new distance measures have been developed (Goldstein *et al.*, 1995, Shriver *et al.*, 1995). Computer simulated data sets reveal their efficiencies in recovering the correct tree (Takezaki & Nei, 1996).

At the population level, genetic variation can be measured by allozymes or by fingerprinting techniques, e.g. restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNA (RAPDs) and variable numbers of tandem repeats (VNTRs). The allelic variants of enzymes (allozymes) or

the fragments of DNA obtained by the fingerprinting techniques can be separated electrophoretically and the frequency of the alleles of each used to analyze the distribution of genetic variation within and between populations. Both the Fst and the Nm statistics (Wright, 1969) are commonly used to assay population structure and analyze gene flow from all of these methods.

Review of microalgal genetic diversity studies

The goal of most early molecular studies concerning microalgae using isozyme analysis was to resolve species-level issues among species with conflicting or little morphological resolution rather than to study genetic structure within bloom populations. The recognition of cryptic species or of previously discounted morphological markers that can be used for separation of a species complex was the most common results of early isozyme studies. For example, different isozyme banding patterns in neretic, shelf and oceanic populations of *Thalassiosira pseudonana* prompted Murphy & Guillard 1976,) and Brand *et al.*, 1981) initially to suggest that this species was composed of clinal populations but later detailed morphological investigations separated each ecological population into a different species (Hasle, 1978, 1983 for *Thalassiosira guillardii, oceanica* and *pseudonana*). In the PSP-toxin producing dinoflagellate *Alexandrium tamarense/fundyense/catenella*, the ciguatera-toxin producing dinoflagellate *Gambierdiscus toxicus* and the freshwater dinoflagellate *Peridinium volzii*, light was shed on the complex nature of these microalgal species complexes using isozyme studies (Cembella & Taylor, 1986, Hayhome *et al.*, 1987, 1989, Chinain *et al.*, 1997).

In the first study of *Alexandrium*, isozyme analyses showed a high degree of enzymatic heterogeneity among isolates from the West Coast of the United States, with isolates from the same locality being most similar (Cembella & Taylor, 1986). A relative lack of enzymatic heterogeneity was revealed by a similar analysis of East Coast *Alexandrium* populations (Hayhome *et al.*, 1989). From isoenzyme data a common origin for the East Coast populations was hypothesised together with a dispersal hypothesis along the east coast of the United States from Canada down to Massachusetts that has related to hydrographic events dissipating a massive red tide that occurred in 1972. *Alexandrium* species have been studied in more detail using sequence analysis of rapidly evolving genomic regions, such as the ITS and the D1/D2 region of the LSU rRNA gene. Using these regions, isolates of the *Alexandrium tamarense/ fundyense /catenella* species complex were shown to be related by geographic origin rather than by morphological affinities (Scholin *et al.*, 1995), which was originally indicated by the isozyme analysis. The world-wide biogeographic dispersal of an ancestral population from the Pacific into the Atlantic has been hypothesized from these data. Furthermore, *Alexandrium* isolates will interbreed more successfully if they have similar isozyme patterns from two different locations than will isolates from the same locations but with different isozyme patterns (Sako *et al.*, 1990). *Peridinium volzii* isolates from the same location were also found to be more closely related, although quite distinct between locations (Hayhome *et al.*, 1987). In contrast, other dinoflagellates, such as isolates of *Gambierdiscus toxicus* from similar geographical regions were not shown to be closely related, which suggested a multiclonal origin (Chiniain *et al.*, 1997). Populations of the green freshwater alga, *Gonium pectorale*, also appear to be multiclonal from several locations (Sako *et al.*, 1991).

Within microalgal bloom populations genetic structure using isozymes has only been studied in the marine planktonic diatom *Skeletonema costatum* (Gallagher, 1980, 1982) and the freshwater planktonic diatom *Asterionella formosa* (Soudek & Robinson, 1983). Both species are initiators of the spring bloom in their respective environments. Gallagher (1980, 1982) suggested strong temporal distinctions in populations of *Skeletonema costatum* from the same locality because she found that spring and fall populations were more genetically distinct than sibling species of higher plants. Neither population of *Skeletonema* was clonal. In contrast, populations of *Asterionella formosa* from different lakes were different, but there were no detectable differences between isolates of *Asterionella* taken from a single location, separated both in time and space, suggesting that clonal populations from a single location had developed. Clonal populations could evolve within this time frame given the 20-40 years time lag before populations of certain diatoms become sexual, (Mann, 1988). All isolates taken from a bloom of

Pseudo-nitzschia pseudodelicatissima were unique (Skov *et al.,* 1997). Similarly, all strains of the zooxanthellae algae *Symbiodinium microadriaticum* inhabiting a variety of hosts were unique (Schoenberg & Trench, 1980). This suggests that cryptic species were likely to be present.

Fingerprinting techniques are only just beginning to be used in microalgal studies. RAPDs and AFLPs have been used to analyze bloom populations of the prymnesiophyte *Emiliania huxleyi* (Medlin *et al.,* 1996), of the dinoflagellate *Alexandrium tamarense* (John *et al.,* 2001) and of the diatom *Fragilaria capucina* (Lewis *et al.,* 1996). Highly diverse populations within temporal and spatial scales were found in each study. Extreme genetic variation among isolates in the diatom *Skeletonema costatum* using random priming of the M13 universal primer as a multi-locus fingerprinting marker (Croyer *et al.,* 1996.), but reassuringly nearly identical banding patterns in the same strain separated in culture for more than twenty years and maintained apart in two laboratories. Both spatial and temporal differences were found in populations of the dinoflagellate *Gyrodinium catenatum* among Australian and global populations using RAPD fingerprinting data (Boalch *et al.,* 1999). Despite this, it was not possible to define the route of introduction into Australian waters, although the introduction is quite recent as judged from sediment records. AFLP banding patterns in isolates of the dinoflagellate *Alexandrium tamarense* from the Orkney Islands were correlated with toxin patterns as determined by HPLC analysis (John *et al.,* 2001). AFLP banding patterns in isolates of the diatom *Pseudo-nitzschia pungens* were so complicated that the addition of 3 nucleotides to the primers could not reduce the complexity of the banding pattern to permit analysis (Lundholm, pers. comm.).

Although microsatellites have been found in all species examined for these molecular makers, they have only been documented in a few studies involving marine microalgae. Microsatellites have only been found in the large planktonic diatom *Ditylum brightwellii* (Rynearson & Armburst, 2000) and both spring and fall bloom populations in Puget Sound, Washington have been analyzed. The genetic diversity within these populations was three times more diverse than that found with isozyme or RAPD methods for other microalgae. Microsatellites have also been found in the coccolithophorid *Emiliania huxleyi* (Rodriguez *et al.,* unpubl.). Most microsatellites were predominately (GT)n repeat units and appeared as multiple bands in PCR reactions, indicating that there were probably multiple alleles or nested microsatellites were involved. Simpler banding patterns were generally monomorphic, although now at least three polymorphic loci have been found (Rodriguez *et al.,* unpubl.).

The use of microsatellites will probably be the method of choice for microalgal analyses. However, microalgal population studies depend on the success of obtaining a representative number of clones in culture. Empirical testing of sample (clones or isolates) and loci sizes for microsatellite analysis have shown that sample sizes greater than 25 clones or isolates does not significantly reduce the variance, whereas an increase of loci from 25 to 50 will decrease the variance by nearly 50% (Shriver *et al.,* 1995). The possibility that some algal clones may undergo sexual reproduction while in culture will always be a concern, thus making the sampling of more loci essential if the variance is to be reduced.

The future

The advent of molecular biological techniques has greatly enhanced our ability to analyze microalgae and can present a quantitative framework through which we can evaluate the diversity, structure and evolution of microalgal populations. Molecular techniques provide the only means to access temporal and spatial structuring of biodiversity below the species level. Thus, predictive models of the dynamics of aquatic ecosystems can be formulated, and the idea of functional groups in the microalgae tested. Many important questions concerning population structure in the microalgae can be addressed, such as: How much genetic diversity exists and over what spatial scale should we be investigating? Do neretic vs. oceanic or eutrophic vs. oligotrophic environments correspondingly support differing amount of diversity? Do spatial and temporal genetic changes balance one another? Can ecological change be mirrored in the distribution of genetic diversity? Are bloom populations clonal or highly polymorphic? Does the amount of genetic diversity observed depend on the species or are all bloom species naturally

highly diverse? How does the ploidy level of the species influence the structure of genetic diversity? How do phytoplankton with different reproductive strategies or different ploidy levels affect the genetic diversity of the ecosystem?

It is unknown how diminished genetic diversity at lower trophic levels will impact on higher trophic levels. We need to establish baseline values for a range of microalgal diversities before we can make predictions about the role of anthropogenic imput into the system because little or no baseline information exists for the assessment of genetic diversity in microalgae with modern molecular techniques. Predictive models cannot be constructed without baseline values. We are only just realizing the potential for recognizing genetic individuality and its use in clustering individuals into biologically meaningful groups reflecting their overall relatedness will probably increase the diversity in the aquatic environment by an order of magnitude (Hedgecock, 1994) and will provide insights into how genetic diversity is integrated though space and time (Brand, 1989).

An individual's adaptive advantage will vary over time because population structure and genetic divergence are tightly coupled and because microalgal organisms are subjected to ever changing environmental conditions. It is thus important to know how genetic diversity is distributed and dynamically maintained in an ecosystem. Having even partial answers to these questions is relevant to (1) the establishment of new population genetic models for marine organisms rather that to try to fit them into terrestrial models; (2) the building of better ecosystem models to accommodate the existence of genetically diverse populations that behave in ecophysiologically different ways, thus contributing to different predictions and different outcomes in the model; (3) an understanding of the role of pelagic population dynamics from a genetic perspective; and (4) the exploration of genetic diversity in phytoplankton in the context of biodiversity, i.e., we need to know how different levels of genetic diversity affect or actually drive changes in biodiversity at higher levels - including baseline values, which are entirely unknown.

Despite the paucity of population structure and genetic diversity studies in microalgae, considerable genetic diversity has been inferred to exist on both spatial and temporal scales in the microalgae. The uncovering of cryptic species certainly contributes to some of the increased diversity. By discovering different life cycle strategies of the various classes of microalgae previously assigned to different species, we will revise biodiversity downward, e.g., in the coccolithophorids. We can only begin to develop some estimate of the range of diversity that we can expect by investigating several different species in several different settings. The use of population-based molecular methods for single cell analysis of microalgae must be developed to enhance our ability to analyze microalgae in time and space and to zoom in with more detail within populations. Flow cytometry and immunobeads are sorting methods that offer great potential for obtaining clonal isolates. We can analyze multiple banding patterns within a natural population obtained from multiplexing PCR reactions of molecular markers with quantitative PCR, making it possible to avoid the need to rely totally on clonal isolates.

New research into genomics and proteomics will undoubtedly open many new avenues in algal research and many new gene products will likely be found among the algal for they are experimental organisms in the tree of life.

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