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The Growing Contributions of Molecular Biology and Immunology to Protistan Ecology: Molecular Signatures as Ecological Tools[1]

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ABSTRACT. Modern genetic and immunological techniques have become important tools for assessing protistan species diversity for both the identification and quantification of specific taxa in natural microbial communities. Although these methods are still gaining use among ecologists, the new approaches have already had a significant impact on our understanding of protistan diversity and biogeography. For example, genetic studies of environmental samples have uncovered many protistan phylotypes that do not match the DNA sequences of any cultured organisms, and whose morphological identities are unknown at the present time. Additionally, rapid and sensitive methods for detecting and enumerating taxa of special importance (e.g. bloom-forming algae, parasitic protists) have enabled much more detailed distributional and experimental studies than have been possible using traditional methods. Nevertheless, while the application of molecular approaches has advanced some aspects of aquatic protistan ecology, significant issues still thwart the widespread adoption of these approaches. These issues include the highly technical nature of some of the molecular methods, the reconciliation of morphology-based and sequence-based species identifications, and the species concept itself.

Key Words. Biodiversity, microbial ecology, molecular ecology, protists.

ECOLOGICAL studies of protists in aquatic ecosystems are predicated on the ability to distinguish, identify and enumerate individual species living in complex microbial assemblages. For morphologically distinctive protistan species (and some protistan groups in general) this is a reasonable undertaking. For example, planktonic foraminifera and skeleton-bearing polycystines can be adequately sampled with plankton nets, and the preservable skeletal structures produced by these species provide a sound basis for species identification and quantification, at least for adult specimens (Bé and Hamlin 1967; Bernstein, Betzer, and Takahashi 1990; Dworetzky and Morley 1987). For many species/groups of protists, however, identification and enumeration are daunting tasks. Even for 'easy' groups such as the larger sarcodine protists mentioned above, immature specimens or species that lack skeletal structures may confound our ability to properly identify and count these taxa (Dennett et al. 2002). Moreover, assaying the entire protistan biodiversity in a single study is a very difficult task at present. Our inability to properly characterize the abundance and distribution of many of these species limits our understanding of the ecology of these assemblages, their biogeochemical activities in ecosystems, and the response of microbial communities to natural or induced environmental change.

Why are species identifications and characterizing protistan community structure so difficult for ecologists? There are numerous and obvious answers to that question.

The taxonomic breadth of the protists is vast and, at present, still somewhat uncertain (Patterson 1999). Phylogenetic relationships among some of the major taxa are still unresolved (a presentation during the Symposium meeting by M.A. Farmer, "A Revised Classification of the Protists"). This situation does not facilitate the easy identification or classification of protists in ecological studies. Related to this issue, the procedures and criteria employed for identification, and thus the training of taxonomists, can differ greatly among the various protistan phyla. Together with an unfortunate reduction in the number of taxonomists in recent years, the requirement for extensive training in protistan taxonomy complicates the issue of obtaining reliable identifications that are based on traditional taxonomic schemes.

A diverse array of preservatives, preparative protocols, staining and microscopy are used to identify and count protists in natural samples. Many of these protocols are group-specific (e.g. protargol staining for determining patterns of ciliation in ciliates; specific additives for preventing the dissolution of skeletal structures in Foraminifera and Acantharia; pigment analyses for some phototrophic protists). Observation by light microscopy is sufficient to identify some species but electron microscopy may be required for others. Even more difficult for ecologists is the necessity to observe live specimens in order to identify some protists (Butler and Rogerson 1995). Collectively these preservatives, staining, and observational approaches may do a reasonable job of characterizing the diversity and abundances of some protistan taxa, but they fall far short of characterizing *all* protistan species diversity in a sample, and it is impractical to attempt to apply so many approaches in a single study.

Another practical aspect that must be considered when characterizing whole communities relates to the fact that protists vary tremendously in size, from < 3 μm microalgae and heterotrophic flagellates (e.g. *Ostreococcus*, *Bolidomonas*, *Siluania*) to species of large colony-forming polycystines whose cylindrical colonies may exceed several meters in length and a centimeter in diameter (Courties et al. 1994; Guillou et al. 1999a; Guillou et al. 1999b; Karpov, Kersanach, and Williams 1998; Swanberg and Harbison 1980). Simply collecting species of such disparate sizes can require a diverse array of sampling gear and protocols.

Finally, ecological studies of protists generally involve the collection and processing of large numbers of samples. Ecological experiments demand a high degree of replication because of the amount of natural varability that is inherent in biological systems. The difficulties noted above relating to collection, identification, and enumeration of protists are further exacerbated in ecological studies by the need for high-throughput methodology for large-scale studies.

The resulting effect of these various complexities for identifying and counting species in natural water samples has resulted in two general approaches in ecological studies of protists: (1) Restricting the study to a taxon/taxa that can be adequately characterized using technology and taxonomic expertise available at hand (e.g. ciliates or dinoflagellates). (2) Grouping protistan species into size categories, or trophic 'guilds' that disregard taxonomic distinctions but attempt to describe broad ecological roles of protistan species in natural communities. The latter approach is a common one that has given rise to a variety of models which reduce the diversity and activity of all protists in a community to a relatively small number (generally 2–9) of size and trophic categories (Azam et al. 1983; Caron and Finlay 1994; Ducklow 1994; Fenchel 1987; Sherr and Sherr

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1994). These depictions are useful exercises for assessing energy production and elemental flow in food webs but they lack the biological detail that might be fundamental for accurate prediction of community function.

Alternative strategies for ecologists. Environmental microbiologists have long recognized the limitations of morphology and other traditional taxonomic characters (e.g. physiological ability of cultured species) for studying the ecology of prokaryotes in natural waters. It was suspected for many years that only a fraction of the naturally occurring bacteria in plankton communities were amenable to laboratory culture, and thus much of the diversity of prokaryote assemblages might be poorly characterized. Accordingly, attempts to examine prokaryote diversity in marine ecosystems more than a decade ago via the sequencing of ribosomal RNA genes led to the revolutionary insight that many (perhaps most) of the prokaryote species in surface waters of the ocean had not yet been cultured (Giovannoni et al. 1990). Subsequent studies have revealed the presence of large numbers of previously-uncharacterized bacterial phylotypes, as well as high abundances of archaea, prokaryotes that were formally believed to have very limited distributions and ecological roles (DeLong 1992; Fuhrman, McCallum, and Davis 1992). These novel approaches have dramatically changed our view of community structure and function in aquatic microbial ecology (Fuhrman 2002; Pace 1997), and have provided insights into how to culture these 'unculturable' strains of prokaryotes (Connon and Giovannoni 2002).

These 'early' findings also led to methodologies (primarily in-situ hybridization with fluorescently labeled oligonucleotides for genetic approaches, and immunofluorescent-labeling for immunological approaches) for documenting the presence and abundance in natural samples of prokaryotes of ecological interest without the need for cultivation (Amann, Ludwig, and Schleifer 1995; Ward 1990). Additionally, a number of approaches were developed that allowed, to some degree, an assessment of microbial community diversity with a single method. These approaches included denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphisms (T-RFLP), and other methods relying on DNA fragment length heterogeneities to provide information on community diversity (Fisher and Triplett 1999; Liu et al. 1997; Muyzer, de Waal, and Uitterlinden 1993). Most recently, the application of microarray technology has been adapted to begin to address questions of prokaryote biodiversity and gene function in environmental samples (El Fantroussi et al. 2003; Loy et al. 2002; Stine et al. 2003; Wu et al. 2001).

These milestones in the ecology of aquatic prokaryotes have not gone unnoticed by protistan ecologists, although progress has not proceeded as rapidly for eukaryotic microbial communities. This shallower trajectory for protistan molecular ecology has been due, in part, to a greater dependence within protistology on morphology-based species identifications. Thus, gene sequences for protists in public databases have accumulated more slowly (relative to prokaryotes), slowing the exploitation of these genetic 'signatures' as tools for ecological studies. Nevertheless, molecular biological and immunological methods have begun to contribute significantly to our understanding of the ecology of aquatic protists, and these approaches hold tremendous promise for ecological studies in the future.

Assaying biodiversity of protistan assemblages of diverse ecosystems: is the age of discovery over? Biological oceanographic research has often been described as passing through three distinct periods in its relatively young history. The global expedition of the H.M.S. *Challenger* (1872–1876) heralded an 'age of discovery' during which vast numbers of new taxa, including many protists, were described from marine environments (Haeckel 1887). An 'age of description' followed during which the basic geographical distributions of many macroscopic marine species were established, leading to the present 'age of quantification' in which accurate estimates of abundance, biomass, and life histories have been defined for many taxa.

The recent advances described above for prokaryote ecology clearly do not indicate an end to the 'age of discovery' for aquatic microbiologists. A similar (though perhaps not as extreme) scenario appears to be unfolding for marine protistologists as well, fostered by the application of genetic approaches to studies of protistan biodiversity. Most recent studies of protistan diversity have involved phylogenetic analyses of eukaryotic ribosomal RNA genes that have been PCR-amplified and cloned from natural water samples. This culture-independent approach has demonstrated the presence of eukaryotic DNA sequences from a variety of marine environments that are unique from all sequences entered into public databases. Moreover, these sequences are sufficiently unique that they appear to indicate previously undescribed lineages that may represent new phyla of protists and possibly even new kingdoms that, based on morphological criteria, remain undescribed at this time (Dawson and Pace 2002; Massana et al. 2002).

Discoveries of novel protistan phylotypes do not appear to be limited in spatial distribution. Studies at various locations in the ocean (from equatorial to polar environments, from deep-sea plankton and at hydrothermal vents, in activated sludge, anoxic waters, and sediments, and in extremely low pH environments) have reported a sizeable number of new phylotypes that are apparently widely distributed and which have not yet been linked to morphological types (Amaral Zettler et al. 2002; Dawson and Pace 2002; Díez, Pedrós-Alió, and Massana 2001; Edgcomb et al. 2002; López-Garcia et al. 2001; Marsh et al. 1998; Massana et al. 2002; Moon-van der Staay, De Wachter, and Vaulot 2001; Moon-van der Staay et al. 2000; Staeck and Epstein 2003). One study has even indicated the presence of large numbers of novel phylotypes in benthic freshwater environments whose closest affinities appear to be the marine foraminifera (Holzmann et al. 2003).

Molecular tools in action. We have been examining protistan community structure using culture-independent molecular approaches at a study site in the Pacific Ocean (33° 33′ North, 118° 24′ West) off the coast of Southern California. Preliminary analysis of a subset of the data (four monthly samples collected throughout 2001 at the depth of the subsurface chlorophyll maximum) has indicated the presence of a number of protistan rDNA sequences that cannot be assigned to well-established protistan phyla (Fig. 1). Clone libraries obtained from four seasons all contained substantial numbers of unclassified eukaryotic sequences (7–14% of clones over all four seasons) that could not be readily assigned to existing phyla based on their primary BLAST affinity (Altschul et al. 1997). The closest matches for these sequences were phylotypes reported from other environmental samples (López-Garcia et al. 2001; Massana et al. 2002; Moon-van der Staay, De Wachter, and Vaulot 2001) that do not share strong sequence similarity with any known, sequenced protists. Similarly, approximately 14% of the sequences in two of the four libraries (January, October) were sequences that most closely matched uncultivated alveolate lineages from other oceanic ecosystems (Massana et al. 2002). These findings are consistent with reports cited above regarding the presence of previously undescribed protistan taxa in aquatic ecosystems.

The strategy described above for obtaining protistan 18S rDNA from environmental samples has been effective at retrieving a diverse array of species from numerous phyla in this and other studies (see references above). Applicability to all

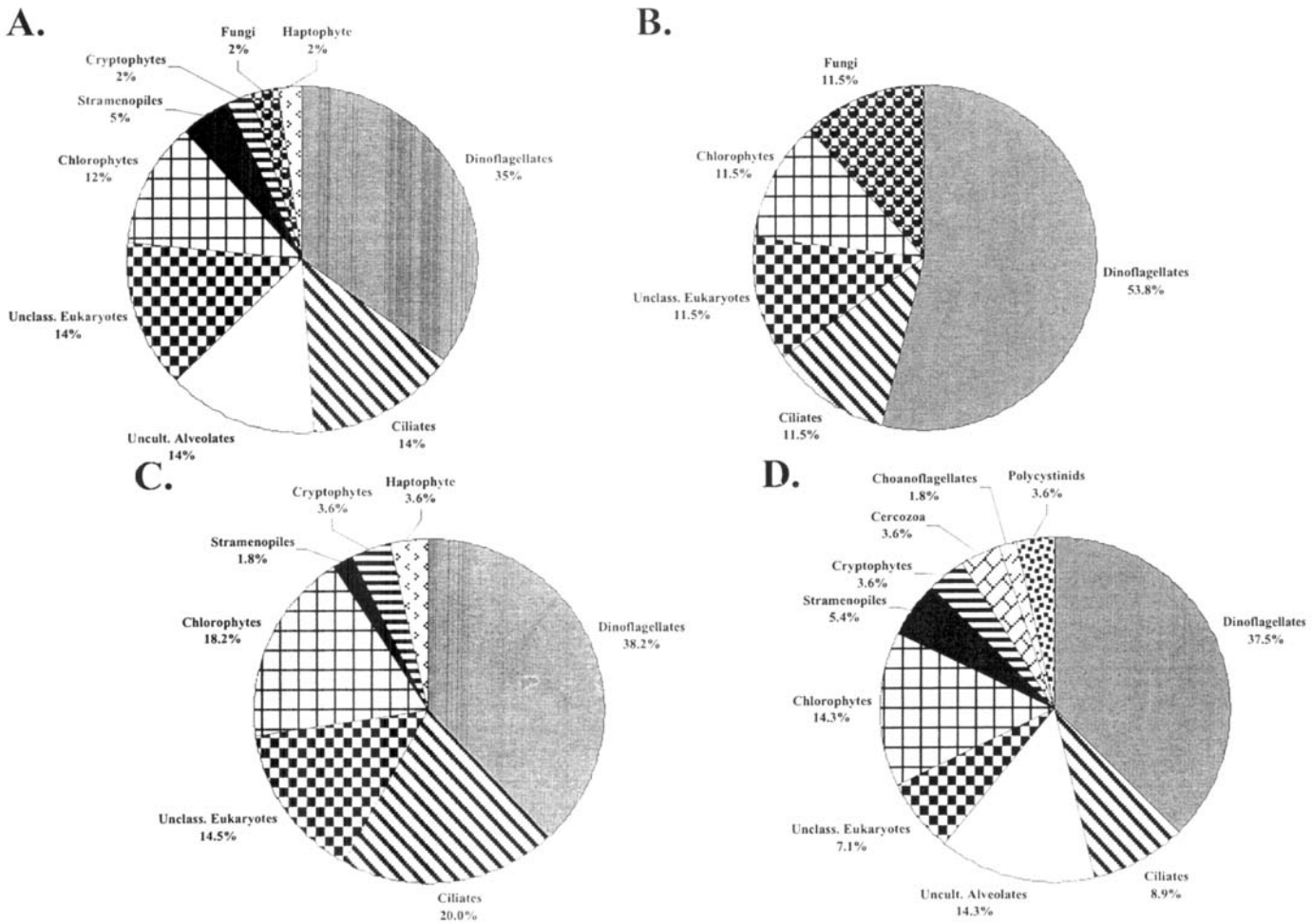

Fig. 1. Phylogenetic distribution of 18S rDNA sequences from the subsurface chlorophyll *a* maximum layer in the central San Pedro Channel (U.S.C. Microbial Observatory Time-Series Station) in 2001 based on the primary BLAST match of each sequence. (A) January, (B) April, (C) July, (D) October. Samples were gravity-filtered through 200-μm mesh to remove large metazoa, collected onto Whatman® GF/F filters and placed in lysis buffer (40 mM Tris, pH 8; 100 mM EDTA, pH 8; 100 mM NaCl, 1% SDS). Samples were heated (70 °C) and disrupted by bead-beating with 0.5 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK). Polysaccharides were precipitated by the addition of 10% CTAB (Stewart and Via 1993) to a final concentration of 1% along with 2.5 M NaCl to a final concentration of 0.7 M. DNA was purified from the lysate by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1), followed by two extractions with chloroform:isoamyl alcohol (24:1) to remove residual phenol. DNA was precipitated overnight at −20 °C with 1× vol. of 95% ice-cold ethanol and 0.1× vol. of 10.5 M ammonium acetate, and centrifuged to pellet the DNA (20,000 *g*, 4 °C for 30 min). DNA pellets were cleaned with two washes of 500 μl of 70% ethanol. Dried pellets were dissolved in sterile TE (10 mM Tris, 1 mM EDTA, pH 7.5). Full-length eukaryotic 18S rRNA genes were amplified from genomic environmental DNA extracts using universal eukaryote primers (Medlin et al. 1988), band-isolated and purified from agarose gel slices using Zymoclean gel DNA recovery kits (Zymo Research, Orange, CA), and eluted from the associated spin columns with sterile water for cloning and sequencing following standard procedures. Sequencing reactions were conducted with Euk-570F (5′-GTAATTCCAGCTCCAATAGC-3′) of n = 43, 26, 55 and 56 clones, respectively, and sequenced on an automated capillary sequencer (Azadan, Fogleman, and Danielson 2002). Distribution of the phylotypes among the taxa is based on approximately 500-bp sequences. The resulting sequences were submitted to the BLAST sequence matching algorithm (Altschul et al. 1997) on the NCBI website for preliminary identification. Sequence data were aligned with ClustalX (Thompson et al. 1997) and de-replicated at the 95% similarity level using the Java program FastGroup (Seguritan and Rohwer 2001) over the maximum region of sequence coverage common to all samples.

species is a requirement if these methods are going to prove useful as a means of describing the diversity within a sample. Nevertheless, not all protistan taxa amplify equally well with a single set of primers, so some degree of bias is presently inherent in PCR-based approaches. For example, amplification of the full-length 18S rRNA genes of planktonic foraminifera is ineffective by commonly used approaches because of the exceptionally long sequence inserts in the genes of these species (Darling et al. 1996). Amplification protocols and priming strategies will undoubtedly evolve to accommodate these idiosyncrasies of the target nucleic acids.

Another striking result of the preliminary analysis presented in Fig. 1 was the relatively low redundancy of phylotypes obtained among the four clone libraries (Fig. 2). Only eleven of the 72 phylotypes observed among the 180 clones were recovered in more than one clone library even when phylotypes were defined using a conservative sequence similarity of 95%. Moreover, most of the phylotypes (53 out of 72) were observed only once. This low level of redundancy of phylotypes in our clone libraries implies a highly diverse protistan assemblage at this coastal ocean station.

These initial efforts have raised questions regarding the ef-

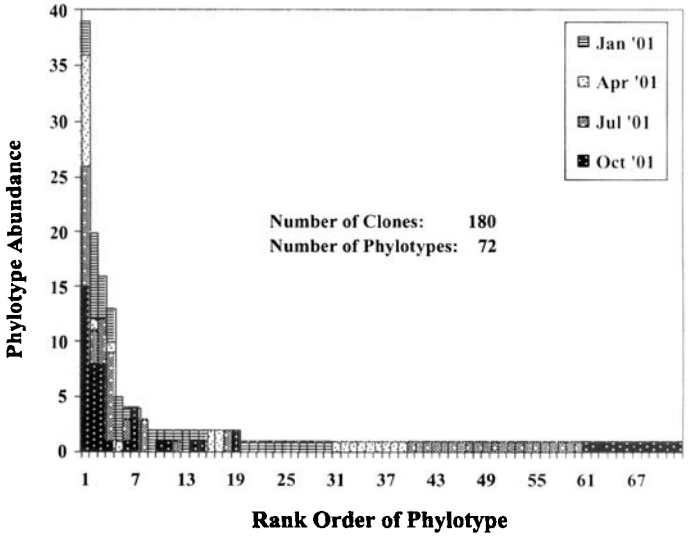

Fig. 2. Phylotype rank abundance curve showing 180 protistan 18S rDNA sequences (from Fig. 1) distributed among 72 phylotypes. Sequences were aligned with ClustalX (Thompson et al. 1997) to find the greatest homologous region among all sequences and de-replicated at a similarity value of 95% with FastGroup (Seguritan and Rohwer 2001).

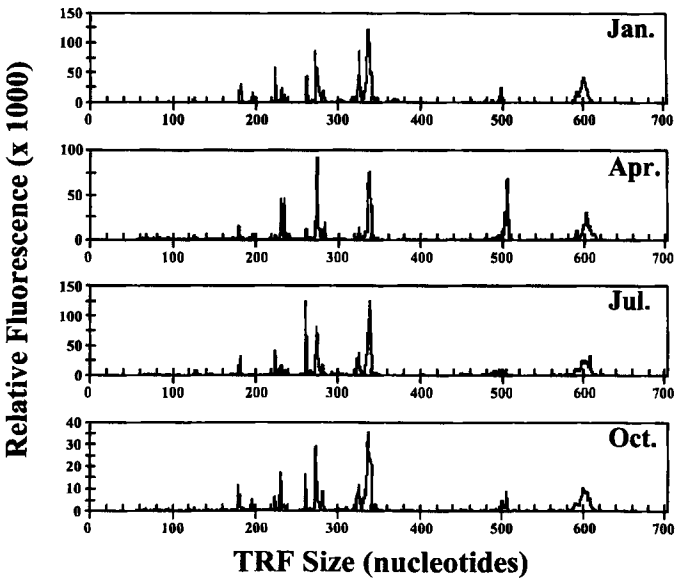

Fig. 3. Terminal restriction fragment length polymorphism (T-RFLP) profiles of four environmental samples collected from the subsurface chlorophyll *a* maximum layer in the central San Pedro Channel. Purified D4-labeled PCR products were digested with the restriction enzyme *HaeIII* (New England Biolabs, Beverley, MA) and analyzed on a Beckman-Coulter CEQ8000 8-capillary DNA sequencer (Trotha et al. 2002). DNA restriction fragments (20 μl) were purified by precipitation with ice-cold 95% ethanol (50 μl), glycogen (1 μl of 20 mg/ml) and 3 M sodium acetate (2 μl). Digests were vortexed, centrifuged (15 min at 4 °C, 20,000 *g*), washed (100 μl ice-cold 70% ethanol) and centrifuged again. The pellets were air-dried and then resuspended in 20 μl of Beckman-Coulter SLS (formamide) solution. Samples were mixed with Beckman-Coulter 600 size-standard (fluorescently labeled with Beckman D1) before injection, which permitted the sizing of D4-labled fragments over the range of 60–640 nucleotides. Data were analyzed with the CEQ8000 software package.

ficacy of traditional microscopical approaches for fully documenting the diversity of natural assemblages of protists, and the degree to which extant culture collections are representative of the most numerically dominant and ecologically relevant protistan species. The answers to these questions are not clear at present, and further work is needed to describe the morphologies of these unknown species, bring them into culture in the laboratory, and establish both their physiological abilities and environmental requirements. The precise nature of these novel phylotypes, and the extent to which they are ecologically important members of marine ecosystems is unresolved at this time, but protistan biodiversity in the ocean clearly has not yet been sampled exhaustively. These pioneering genetic studies argue convincingly for the presence of a significant 'undiscovered/undescribed' eukaryotic microbiota in aquatic ecosystems.

Cloning and sequencing of environmental DNA are clearly yielding new insights into protistan species diversity as briefly outlined above, but these endeavors are still rather labor-intensive. Thus, they do not yet represent a viable means of *routinely* assessing protistan species diversity in nature that can be readily applied in large ecological studies. A growing array of alternative approaches designed to reduce the time and effort required to obtain an overall assessment of protistan biodiversity attempt to address this shortcoming. These approaches, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphisms (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), and amplicon length heterogeneity (ALH, LH-PCR), involve a variety of manipulations that exploit small heterogeneities in DNA sequences to produce patterns of DNA fragments that can be used to estimate the number (and in some cases relative abundances) of phylotypes in a sample (Liu et al. 1997; Muyzer, de Waal and Uitterlinden 1993; Rosenbaum and Riesner 1987; Suzuki, Rappé, and Giovannoni 1998; Vaneechoutte et al. 1995). The main advantage of these approaches is that they allow a 'snapshot' of total community diversity in a single, relatively rapid procedure. This capability facilitates comparative studies of species richness and relative abundances in multiple samples (e.g. different locations, seasons, or experimental treatments), and thus fulfills a central requirement for the application of molecular methods to ecological studies.

T-RFLP analysis of the protistan community at our study site in the Pacific Ocean off Southern California indicates the nature and potential usefulness of these approaches (Table 1 and Fig. 3). Each T-RFLP pattern provides a 'fingerprint' of the eukaryotic assemblage with (in theory) each species represented by a unique terminal restriction fragment (TRF) length, as indicated by a peak on an electropherogram. Peak heights indicate the relative abundance of target molecules, assuming that all target DNA has amplified with equal efficiency within a particular sample. Thus, DNA fragment lengths serve as proxies for spe-

Table 1. Results of a similarity matrix analysis of the four samples whose T-RFLP patterns are shown in Fig. 3. Electropherograms were analyzed with the T-RFLP Profile tool on the RDP II website (Cole et al., 2003) to generate similarity matrices from a comparison of T-RFLP patterns.

Number of unique fragment sizes	Date	Date			
		January	April	July	October
74	January	1	0.53	0.67	0.55
69	April	0.53	1	0.58	0.50
89	July	0.67	0.58	1	0.56
44	October	0.55	0.50	0.56	1

cies in this approach, and changes in fragment patterns indicate shifts in the contribution of species to the assemblage. Comparative studies of eukaryotic diversity in a community are greatly simplified via this approach relative to traditional approaches (and relative to cloning/sequencing). For example, a visual inspection of the four samples taken at different times of year in our study reveals that there was considerable variability in the number and relative abundance of species producing fragment lengths between 480 and 520 nucleotides (Fig. 3). In contrast, strong signals are present in all four samples at approximately 275 and 335 nucleotides. Thus, differences (or similarities) in the relative and absolute abundance of specific taxa within the assemblage can be observed readily using this approach. Combined with information on the species represented by these fragments (see below), one can infer a considerable amount of information regarding shifts in community structure based on differences in the patterns of the DNA fragments in the electropherograms. Normalization or transformation of TRF peak heights within an electropherogram may improve sample to sample comparisons (Blackwood et al. 2003).

Fragment patterns generated by techniques such as T-RFLP also allow profile analyses as available through the online T-RFLP tool (Cole et al. 2003). These analyses can indicate relationships between samples from disparate locations, times, or experimental manipulations. In the case of our four seasonal samples off Southern California, similarities ranged from 0.50 to 0.67 among the four samples (Table 1). Interestingly, the highest similarity among these samples was observed between the samples from January and July. The high similarity of these 'winter' and 'summer' samples may be a consequence of the low seasonal amplitude of the climatic forcing at this coastal station, and the importance of small-scale upwelling and eddy formation (Bray, Keyes, and Morawitz 1999).

Can we hope to develop a single, rapid technique that will assess protistan community structure in natural samples and thus facilitate ecological studies? That long-term goal is a lofty one for molecular ecologists at present given the high diversity of protistan assemblages. However, the continued development of T-RFLP and similar approaches holds great promise, and one can envision a suite of methods in coming years that could provide an accurate 'snapshot' of species presence and abundance. Microarray development and application to ecological studies of prokaryotes is moving in that direction (El Fantroussi et al. 2003; Loy et al. 2002). Rapid methodological improvements and automation in molecular biology make this an increasingly realistic hope. As with cloning and sequencing efforts, however, the effectiveness of genetic approaches such as T-RFLP is presently dependent upon amplification of the target gene from all species in a sample. Additionally, the latter methods assume sufficient sequence heterogeneity among the amplicons to differentiate all of the species in the sample (e.g. in T-RFLP analysis the ideal scenario would be one in which each species produces a fragment of unique length). The efficacy of these methods is therefore dependent on optimizing conditions that will maximize resolution. For fragment analysis this is often done by performing multiple restriction digests on a single sample to generate multiple estimates of community diversity.

Identifying and enumerating individual taxa in natural assemblages—finding (and counting) the needle in the haystack. On the other end of the scale from total community analysis is the desire by many ecologists to follow the population dynamics of individual species within natural microbial communities. Autecological studies of many protists are straightforward using traditional approaches if the target species can be readily identified by simple techniques. However, for species that possess few distinguishable morphological features (e.g. many small protists, most amoebae), traditional methods of identifying and counting these species in natural samples are tedious at best, and often ineffective. For the latter species, genetic and immunological approaches offer an alternative means of accomplishing this task that may provide both improved accuracy and speed relative to traditional investigations using microscopy.

Much of the work to date has focused on the application of fluorescently-labeled in-situ hybridization (FISH) or sandwich hybridization (SHA) approaches to visualize and count target species in natural assemblages (Anderson et al. 1999; Gunderson and Goss 1997; Knauber, Berry, and Fawley 1996; Lange et al. 1996; Lim, Caron, and Dennett 1999; Miller and Scholin 1996; Ragan et al. 1996; Rice et al. 1997; Scholin et al. 1996; Simon et al. 1997; Simon et al. 1995; Tyrell et al. 1997). The application of these methods has enabled studies on the distribution of these species that were not possible previously. These tools also have proven useful for the detection and identification of protistan species with multiple life stages. Examples include studies of the symbiotic dinoflagellates of foraminifera, radiolaria, and cnidaria which do not produce some important, taxonomically-informative, morphological features *in hospice* (Gast and Caron 1996; Rowan and Powers 1991), and the detection and identification of parasitic protists whose morphologies may differ between their free-living and parasitic life stages (Ey et al. 1993; Laberge et al. 1996; Leng, Mosier, and Oberst 1996; McLaughlin et al. 1992; Watt, Jogsakul, and Long 1992). Genetic approaches may also help resolve controversies regarding the life stages of protists with uncertain life histories (Burkholder, Glasgow, and Steidinger 1995).

Fragment analysis also has the potential to identify specific taxa in mixed assemblages. This method is especially useful when trying to distinguish between a small number of taxa that are closely related or morphologically similar. RFLP has been employed to distinguish between cultured species of dinoflagellates, small heterokont flagellates and isolates of *Giardia* (Lim, Dennett, and Caron 2001; Scholin and Anderson 1996; Upcroft and Upcroft 1994).

Fragment analysis can also be insightful for natural samples when information is known on the fragment size that will be produced by a particular restriction enzyme acting on a particular DNA sequence. For example, 18S rDNA sequences obtained from our environmental samples collected off Southern California indicated the presence of the minute chlorophyte *Ostreococcus* sp. in our clone libraries. We examined full-length 18S sequences of *Ostreococcus* for the presence of cut sights for restriction enzymes that we commonly employ, and then used that information to predict the length of terminally-labeled DNA fragments expected in our T-RFLP analysis. Subsequent T-RFLP analysis of the sample demonstrated strong peaks on electropherograms for the fragment lengths predicted for three enzymes (Fig. 4). Moreover, the signal intensities were quite similar for all three enzymes employed (approximately 10–11% of the total DNA amplified during the T-RFLP analysis). Chlorophyte sequences in the clone library obtained from the same sample constituted approximately 18% of the total number of clones (Fig. 1). Of the ten clones determined to be chlorophytes, five clone sequences displayed a primary BLAST affinity to *Ostreococcus* sequences in the NCBI database. Thus, approximately 9% of the total community 18S rDNA sequences and half of the chlorophyte sequences were attributable to this taxon. It is noteworthy that, until this analysis, *Ostrecoccus* had gone undetected in these waters because of its minute size and lack of distinctive morphological features (Courties et al. 1994). Molecular approaches are presently the only way in which

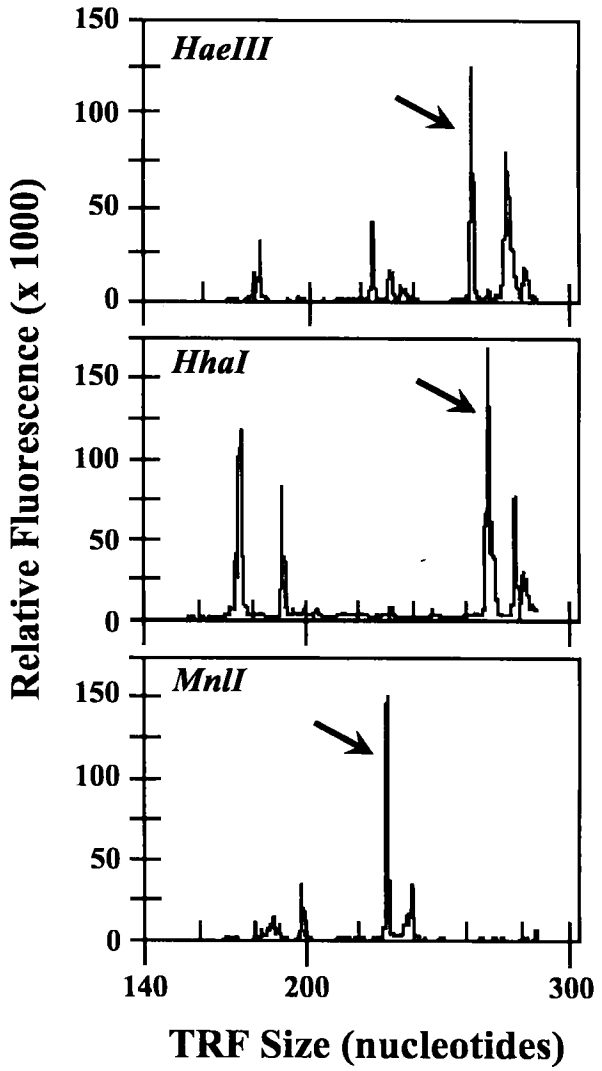

Fig. 4. Use of T-RFLP to identify the contribution of *Ostreococcus* sp., a minute chlorophyte, to the protistan assemblage in a sample collected in July, 2001, from the subsurface chlorophyll *a* maximum layer in the central San Pedro Channel. Amplicons of approximately 600 base-pairs in length were individually digested with three different restriction enzymes and analyzed on a Beckman-Coulter CEQ8000 8-capillary DNA sequencer. *Ostrecoccus*-specific fragments were predicted for the enzymes *HaeIII* (A), *HhaI* (B), and *MnlI* (C) based on the location of the three restriction enzyme cut sites determined from analysis of the full-length sequence of the *Ostreococcus* phylotype observed in our clone libraries. Peaks indicated by arrows show the location of putative *Ostreococcus* sp. DNA fragments (i.e. these peaks occupy the predicted location of *Ostreococcus* sp fragments). Each peak comprised between 9–11% of the total amplified DNA in the T-RFLP profile. The contribution of all chlorophyte phylotypes (18.2%) in the clone library associated with this sample is indicated in Fig. 1C. Half of these chlorophyte clones were identified as *Ostreococcus* based on the similarity of sequences to those in the NCBI database (i.e. 9.1% of the total number of clones in the library).

abundances of this common, previously undetected species can be established.

Quantitative real-time PCR is another genetic approach that is rapidly becoming common in microbial ecology for identifying and enumerating individual species. This method uses the rate of accumulation of amplified target DNA during the polymerase chain reaction to estimate the copy number of the target in the original sample. Copy number is then related empirically to the abundance of the target species in the sample. The advantages of the method include high throughout, rapid processing time, and exceptionally high sensitivity with a large dynamic range (several orders of magnitude) over which accurate estimates of target number can be determined (Fig. 5). Recent applications for protistan species have involved distributional studies of parasitic species and harmful bloom-forming algae (Bowers et al. 2000; Coss et al. 1997; Coyne et al. 2001; Hermsen et al. 2001; Lin et al. 2000), but a wider array of applications for ecologists in the near future is certain (Wawrik, Paul, and Tabita 2002).

Immunological approaches have also proven useful in ecological studies for the detection and enumeration of specific protistan taxa, although their use to date has not been as extensive as genetic approaches (Lin and Carpenter 1996; Vrieling and Anderson 1996). 'Early' studies largely examined the use of polyclonal antisera towards specific types of phytoplankton (Anderson, Kulis, and Cosper 1989; Campbell, Shapiro, and Haugen 1994; Mendoza et al. 1995; Shapiro, Campbell, and Haugen 1989), but more recent applications have employed monoclonal antibodies as well (Anderson et al. 1993; Caron et al. 2003; Okazaki et al. 2001; Romestand, Torreilles, and Roch 2001; Sako, Adachi, and Ishida 1993; Uchida et al. 1989). These latter approaches have proven effective for increasing the sensitivity of immunological detection of protists because of the potential for higher specificity of monoclonal antibodies relative to polyclonal antisera.

Equally important to high specificity and sensitivity for the genetic or immunological detection of protistan species is the potential for automation of these approaches. For genetic approaches, improvements in amplification, cloning, and sequencing technologies are rapidly reducing the time required to analyze large numbers of samples. Similarly, for immunological approaches the Enzyme Linked ImmunoSorbent Assay (ELISA) format allows the processing of samples in 96-well microtiter plates, greatly increasing the rate at which samples can be processed (Caron et al. 2003). For protistologists, the adaptation of molecular methods to high throughput protocols is fundamental for handling the large number of samples often generated in ecological studies. The potential for continual improvements in the rate of sample processing, sensitivity, and miniaturization are primary reasons why molecular approaches hold great promise as tools for these studies.

Enabling experimental studies, now and in the future. Many long-standing ecological questions (and some new ones) are becoming tractable to protistan ecologists as a consequence of the application of modern genetic and immunological approaches to the study of these populations. As described above, reassessment of the species diversity of natural protistan assemblages is presently underway due to the discovery of a considerable number of uncultured/undetected protistan phylotypes. Addressing some important questions regarding this diversity will soon become possible. How many protistan species are there? Are these species globally distributed or is there a high degree of endemism among these species? The answers to these questions are presently unresolved and actively debated (Finlay and Fenchel 1999).

How do environmental perturbations affect protistan assemblages? How might changes in these assemblages affect ecosystem function? Answers to these questions have implications that range from the design and implementation of experiments with natural protistan assemblages (e.g. What are the effects on protistan species and their activities due to the containment of samples in incubation bottles?) to more far-reaching ecological issues (e.g. How do protistan assemblages respond to environ-

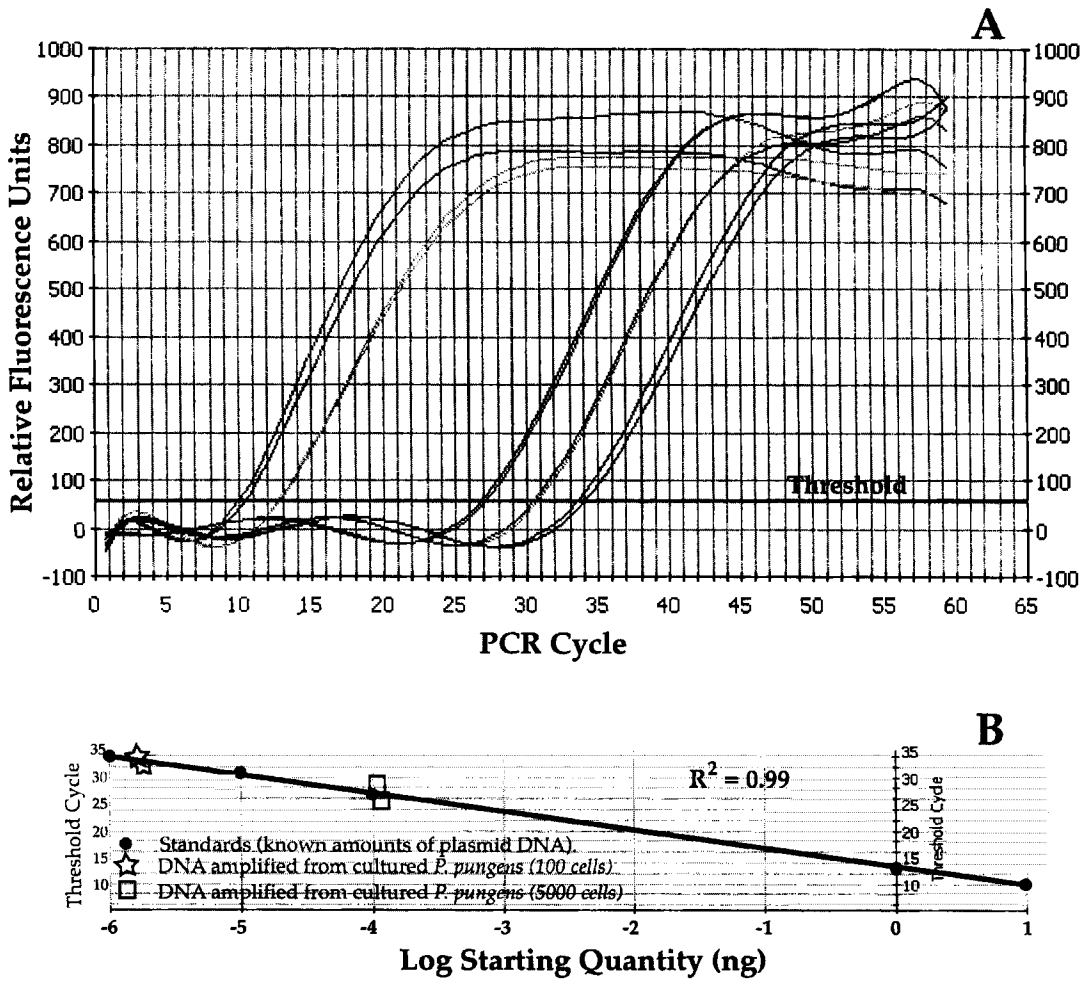

Fig. 5. Quantitative, real-time PCR for *Pseudo-nitzschia pungens*. (A) Amplification of cloned target DNA in PCR reactions conducted in duplicate at five dilutions spanning seven orders of magnitude. Duplicate reactions are shown for each dilution. (B) Standard curve relating PCR threshold cycle to log starting concentration of plasmid DNA (solid circles) and two samples of a *P. pungens* culture (open stars and squares; duplicate subsamples). Amplification of a 40-base pair fragment of *P. pungens* large subunit (LSU) rDNA was accomplished from plasmid DNA. Extraction of DNA from cultured cells was performed as mentioned previously (Fig. 1, legend). Approximately 872 base pairs of the LSU was amplified using primers DIR-F (Scholin et al. 1994) and D3B-R (Nunn et al. 1996). Amplification products were purified using Zymo DNA clean and concentrator kits (Zymo Research) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI). Plasmid inserts were sequenced using the amplification primers to verify species identity. Quantitative PCR was performed using the genus level primers pnqpcr-fwd (CTT TGG AAG AGC GCA G) and pnqpcr-rev (CTA GCA ACA GAC ATC AAC TC). Reactions were performed using an iCycler iQ Real-Time PCR Detection System and IQ SYBR Green Supermix (BIO-RAD Co., Richmond, CA). Reaction conditions were 40 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec.

mental change? What are the effects of these changes?). These latter questions might apply to natural changes (season, depth) or to anthropogenic effects such as eutrophication, pollution or even purposeful perturbations such as iron addition to oceanic waters to promote phytoplankton blooms (Landry et al. 2000). Community resilience, stability, and the degree of ecological redundancy within protistan assemblages are largely unknown but of vital importance to understanding and predicting ecosystem response to environmental change (McGrady-Steed, Harris, and Morin 1997; Naeem and Shibin 1997).

Studies of protistan species interactions will be greatly benefited by current and future detection strategies discussed herein. Simplified methods for species identification and enumeration will facilitate studies of predation, competition, mutualism, and parasitism involving protists. For example, these protocols will aid monitoring programs designed to understand the conditions that promote the growth and dominance of particular species within natural assemblages (e.g. bloom-forming species of algae), and enable experimental studies to examine if these factors can be manipulated to prevent such outcomes.

In addition to advances in our knowledge that are taking place now, a number of long-term goals in protistan ecology are also becoming achievable. For example, information on the presence and abundance of species in near-real-time is becoming a reality using both immunological and genetic approaches (Fig. 5) to identify and count these microorganisms (Caron et al. 2003; Coyne et al. 2001; Scholin et al. 1999). The next major step in this arena will be the development and application of true, real-time methodology that will allow monitoring of protistan species in-situ, and will complement near-real-time optical methods for assessing these assemblages (Olson, Zettler, and DuRand 1993; Rose et al., in press; Sieracki, Sieracki, and Yentsch 1998).

Assaying the growth rates of microorganisms in situ is also

a long term goal of microbial ecologists. Establishing growth rates for species of particular interest can provide important ecological information on the environmental conditions that promote the growth of these species. Novel molecular approaches based in the analysis of genes associated with cell division hold promise for such a method (Lin, Chang, and Carpenter 1995, 1997). Combined with a rapid assay for population abundance, such a tool could provide a powerful means of predicting population dynamics of individual species in nature, and responding to important emerging events.

Stumbling blocks, traditional and novel approaches for identification, and the species concept. Modern genetic and immunological approaches offer an enormous potential for enhancing traditional methods for studying protistan ecology. Indeed, future improvements in detection (specificity, sensitivity, dynamic range), miniaturization, and automation are beginning to revolutionize ecological studies of individual species and natural protistan assemblages. Nevertheless, there are significant impediments slowing the adoption of these approaches. These obstacles include technical and intellectual considerations. Molecular technology advances at a rapid pace. Acquiring proficiency and maintaining abreast of technological improvements in the field are not trivial tasks. In addition, technical proficiency must be complemented with a sound theoretical training in order to be able to properly evaluate results, and recognize the benefits and inadequacies of specific methodological approaches.

The technical and intellectual issues mentioned above complicate the application of molecular approaches to ecological studies of protists, but they do not create insurmountable problems. A more fundamental problem is how we define species based on DNA sequence information or antigenic characters. Traditional, morphology-based taxonomies of protists provide a rich history and sound foundation for species identifications. For ecologists, the goal should not be to create a separate taxonomy that is disconnected from traditional, morphology-based taxonomic schemes, but rather to correlate molecular 'signatures' with morphologically-defined species. In addition, the ecological species concept must be taken into account. Ultimately, the use of molecular signatures as tools to study the distributions, abundances, and activities of protists in nature makes sense only if the ecological criteria by which we define these molecular markers are well-defined.

Thus, correlations with morphology and physiological ability are pivotal for the application of molecular markers as proxies for species identifications in ecological studies. This is unlikely to be a trivial task because the degree of intraspecific variability inherent in all of these aspects (sequence information, antigenic character, morphology, physiology) may be considerable (Petrushevskaya and Swanberg 1990; Weisse 2002). Nevertheless, there is a growing realization that the relationship between these features must be defined for ecological studies to move forward (Modeo et al. 2003). That correlative work remains to be done for the vast majority of protistan species. Schemes presently under consideration or construction to provide a register for all biological species should take this goal into account (Patterson 2003).

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