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Evaluation of Denaturing Gradient Gel Electrophoresis (DGGE) for the Determination of Marine Phytoplankton Genetic Diversity

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A study was conducted to optimize gradient gel electrophoresis (DGGE) protocols for determination of marine eukaryotic phytoplankton genetic diversity. The target genetic marker was a 330-bp fragment of the 18S subunit of the ribosomal RNA gene. PCR products incorporating a 40 base pair (bp) GC-clamp were successfully obtained from clonal cultures of eleven dinoflagellate and one diatom species. All the DGGE-PCR products denatured within a denaturant concentration range of 30% to 55%. Optimum DGGE run conditions were 10 hours at a constant 150V and a temperature of 60º C in TAE buffer. DGGE-PCR product with the lowest GC content denatured first, but not all products from different species separated out as expected. Products from *Alexandrium affine* and *A. leei*, which differed in nucleotide sequence but had identical GC content were separable but products from *Cochlodinium* sp., *Gyrodinium instriatum* and *Karenia sanguinea*, which differed in both nucleotide sequence and GC content, were not separable. The minimum number of cells in a mixed population required to obtain detectable DGGE-PCR product varied widely between species. Analysis of natural phytoplankton samples from six different locations by DGGE showed 28 bands, of which 13 were unique. In contrast, microscopic analysis of the samples showed the presence of at least 45 morphospecies belonging to 38 genera. These results suggest that DGGE will underestimate the diversity in a natural phytoplankton community.

Key words: 18S ribosomal RNA, DGGE, Genetic diversity, Marine phytoplankton.

Determination of phytoplankton species composition and diversity is one of the parameters often incorporated in aquatic ecology studies. This is often carried out by microscopy. Data obtained from such studies are extremely valuable and provide highly useful information on the structure and functioning of an aquatic community. However, the work involved in obtaining such data is very tedious, especially when many samples are involved. It also requires a certain level of taxonomic expertise, especially when size classes smaller than net phytoplankton are included in the analysis.

There are certain types of studies where detailed phytoplankton species identity may not be, but data on diversity and potential changes in community composition are required. One example is its potential application in environmental impact assessment (EIA) studies. In such studies the potential immediate impact of pollutants on the diversity of a phytoplankton community to be affected might be tested in micro- or mesocosms. Since these studies are often carried out as contract research, thus rapid data turnover is often required.

Advances in molecular biology have greatly facilitated procurement of genetic diversity information. Data are normally obtained by the polymerase chain reaction (PCR) followed by RAPD, RFLP or direct sequencing analyses (Coleman and Goff 1991). In the case of phytoplankton this would often require

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establishment of pure cultures of the species to be studied. These types of analyses are suitable for comparison of strains of the same species, different species within the same genus, or at other similar closely related level. They are less suitable or applicable at the whole community level. Establishing cultures of most phytoplankton species present in a community for subsequent genetic analysis is not an easy task. However, it was recently reported that PCR products could be obtained from a single phytoplankton cell (Bolch 2001).

One approach that is gaining popularity in studies on the diversity of bacteria and archaea natural communities is gradient gel electrophoresis (GGE), using either a chemical denaturant gradient (DGGE), or a heat gradient (TGGE). This technique, which was originally developed to detect mutations, has been widely applied in studies on prokaryote communities from diverse environments (Muyzer and Smalla 1998; Galand *etal*. 2002; Araya *etal*. 2003; Fasoli *etal*. 2003; Hein *etal*. 2003). In contrast there has been very few applications of GGE in studies on either freshwater or marine phytoplankton (Van Hannen *et al*. 1998; Diez *etal*. 2001) and reliable protocols still need to be developed. In theory, GGE could provide a rapid means of assessing the genetic diversity of microbes in a particular community without the need for detailed taxonomic analysis or establishment of cultures (Dorigo *etal*. 2002).
Here we present results from DGGE methods optimization using clonal laboratory culturesof marine phytoplankton. The DGGE protocols were then applied in a study to compare diversity data obtained for natural marine phytoplankton samples. DGGE variability information obtained was thereafter compared with observations based on traditional microscopy.

METHODS

Test species

Clonal cultures of 11 marine dinoflagellates and a marine diatom (Table 1) were established from material collected from various locations in Malaysia. Cultures were grown in ES-DK medium (Kokinos and Anderson 1995) at 26^{o} C under a 14:10 hour light dark photoperiod. Exponential phase cultures were harvested by centrifugation for DNA extraction.

Genomic DNA extraction

For DNA extraction cells were lysed by osmotic shock lysis (Scholin *etal*. 1993) in NET buffer (15 mM NaCl, 10 mM EDTA pH 8.0, 10 mM Tris HCl pH 7.5) containing 1% SDS. This was followed by cleaning with CTAB. DNA was initially extracted in chloroform : isoamyl alcohol (C:I, 24:1). After the first extraction proteinase K (Amresco) was added at 100 mg mL^{-1} and the mixture was incubated at 37^{o} C for 1 h. DNA was precipitated with cold ethanol (EtOH). The DNA pellet was redissolved in 500 mL TE buffer containing 100 mg mL^{-1} Rnase A (Promega) and incubated at 37^{o} C for 1 h. DNA was reextracted in P:C:I (25:24:1) and precipitated in cold EtOH. The DNA was dissolved in 50 mL TE buffer and stored at -20^{o} C. The amount of extracted genomic DNA was quantified from a standard curve prepared using the dsDNA PicoGreen DNA quantitation kit (Molecular Probes Inc.) for the Turner Design TD700 fluorometer according to the manufacturer's instructions.

Primers

DGGE-PCR primers were designed targeting a short fragment of the phytoplankton 18S subunit of the ribosomal RNA gene. For this purpose, published sequences from 44 marine dinoflagellate species and 12 marine diatom species were aligned using Clustal-X (Thompson *et al*. 1997) to find a common highly conserved region but with enough variability so that fragments can be resolved by DGGE. NetPrimer software (http://www.premierbiosoft.com/netprimer/netprimer.html) was used to determine the optimum primer pair. The eventual primers used were EUK1-LSJ with a 40 bases-long GC clamp (5'-[cgc ccg ccg cgc ccc gcg ccc ggc ccg ccg ccc ccg ccc g] gta tgg tcg caa ggc tga aa -3'), EUK2-LSJ (5'-ggg cat cac aga cct gtt att gc -3') and EUK3-LSJ, which was the same as EUK1-LSJ but without the GC clamp. Primers EUK1-LSJ and EUK3-LSJ corresponded to base positions 1109 – 1129 of the *Gonyaulaxspinifera* 18S rDNA sequence while primer EUK2-LSJ corresponded to positions 1416 – 1439 of the same sequence (Saunders *et al*. 1997).

PCR

DGGE-PCR was carried out on a PTC-100 thermal cycler (MJ Research). Each 50 mL reaction mix contained genomic DNA template, 1× reaction buffer, 1.5 mM $MgCl_2$, 200 mM

each dNTP (Promega), 0.5 mM each primer, and 1.5U *Taq* polymerase (Promega). Genomic DNA concentrations tested were 15ng, 30ng, 45ng, 60ng, 90ng, 120ng, 150ng, and 180ng. Genomic DNA of the gram negative bacterium *Aeromonas hydrophila* and gram positive *Staphylococcusaureus* were used as prokaryotic controls. When the primer pair EUK1-LSJ and EUK2-LSJ was used the PCR cycle consisted of preheating at 95° C for 5 min, followed by 35 cycles at 94° C for 45 s, 65° C for 45 s, and 72° C for 45 s, ending with a final extension at 72° C for 7 min. When primer pair EUK3-LSJ and EUK2-LSJ was used, the annealing temperature was changed to 60° C. Specificity and size of the PCR product was examined by electrophoresis in 1% agarose gel in TBE buffer. The gel was stained with ethidium bromide (EtBr) and viewed under UV light. Images were captured on Polaroid film. PCR products were stored at -20° C.

Optimization of DGGE

The melting behavior of each DGGE-PCR product was determined by perpendicular DGGE. The gel contained 8% (w/v) polyacrylamide (37.5:1, acrylamide : bisacrylamide, Promega) and a linear gradient of 0% - 70% denaturant was established from left to right. A 100% denaturant concentration was defined as 7 M urea (Promega) and 40% (v/v) deionised formamide (Promega). The gel was polymerised with 0.09% (v/v) ammonium persulfate (Sigma) and 0.07% (v/v) TEMED (Sigma). A 75 mL mix of each DGGE-PCR product with and without GC clamp was loaded into the single well of the gel. The gel was electrophoresed in a Universal Dcode DGGE apparatus (Biorad) at a constant voltage of 150 V and temperature of 60° C for 6 h. The gel was then stained with EtBr and viewed under UV light.

The optimum resolving time for the DGGE was determined using a parallel DGGE gel. An 8% polyacrylamide gel was prepared containing a linear gradient of 25% - 55% denaturantA 10 mL mix of each DGGE-PCR product from *Gonyaulax spinifera* and *Leptocylindrus minimus* was loaded into the first well of the gel. Then every subsequent hour for the next 12 h, successive wells of the gel were loaded with the same mix. Electrophoresis was carried out at 150 V and 60° C. The gel was then stained with EtBr and viewed under UV light.

DGGE of clonal laboratory cultures

Separation of DGGE-PCR products from the 12 test species was tested by parallel DGGE in 8% polyacrylamide gel containing 25% - 55% denaturant. Individual wells were loaded with 10 mL DGGE-PCR product of each species, a mixture of 5 mL product from each species, and 35 mL of DGGE-PCR product obtained using mixed genomic DNA template from each species. DGGE was carried out at 150 V at 60° C for 10 h. Subsequently, the gel was stained with EtBr and viewed under UV light.

The effect of variable cell density on the ability to detect a species in a mixed population was investigated by mixing together equal culture volumes of *Coolia*. *tropicalis*, *Ostreopsis ovata*, *Alexandriumleei*, *Cochlodinium*sp., and *Gambierdiscus belizeanus*. Mixtures were prepared containing 50 mL, 25 mL, 10 mL, 5 mL, 2 mL, 1 mL and 100 µL of each culture. Cell density in the culture was 11383 cells mL^{-1} for *A. leei*, 4689 cells mL^{-1} for *Cochlodinium* sp., 9255 cells mL^{-1} for *C. tropicalis*, 608 cells mL^{-1} for *G. belizeanus* and 4704 cells mL^{-1} for *O. ovata*. Genomic DNA extracted from each mixture using methods already described was used as templates for DGGE-PCR. The DGGE-PCR products were then separated by parallel DGGE using protocols already described. Products from individual clonal cultures of all species were also electrophoresed for comparison.

Direct sequencing

For sequencing genomic DNA of each test species was amplified by PCR using primers EUK3-LSJ and EUK2-LSJ. The PCR products were purified using QIAquick purification columns (Qiagen Inc.) according to the manufacturer's recommendations. Sequencing PCR was carried out using the ABI Prism BigDye terminator cycle sequencing kit (PE-ABI). A 20 mL reaction mix contained 3.2 pmol primer, 4 mL BigDye, 1x reaction buffer and 50 ng purified PCR product. PCR cycle used was: heating at 96° C for 45 s, followed by 35 cycles at 96° C for 30 s, 50° C for 15 s and 60° C for 1 min. The product was purified by the normal EtOH NaOAc method. Sequencing was carried out for both strands on an ABI 377 automated DNA sequencer (PE-ABI).

DGGE of field samples

Water samples were collected with a 5L water bottle at six locations in Sebatu in the Straits of Malacca. A one litre subsample was taken and passed through a 120 mm mesh sieve to exclude

most zooplankton. Material that passed through the sieve was further passed through a 20 mm mesh sieve. Material that was retained by the sieve was collected and the volume made up to 100 mL. The sample was preserved in 4% neutral formaldehyde. A second 1 litre subsample was processed in the same manner but without preservation. Duplicate 5L samples were collected from each location.

In the laboratory, the preserved sample was settled in a 50 mL settling chamber and cells were identified and counted using an inverted microscope. The unpreserved sample was centrifuged at 5,000 x *g* for 10 minutes to obtain a pellet. Genomic DNA was extracted from the cell pellet using methods already described. The DNA was used as template for DGGE-PCR and the product was resolved by parallel DGGE using the protocols already described. Unique bands in the DGGE gel were excised and the DNA was extracted. This was used as a template for sequencing PCR. The resultant product was sequenced using the ABI 377 autosequencer. The nucelotide sequences were analysed by BLAST and compared with previously published sequences. A genetic distance matrix based on the nucleotide sequences was calculated using Kimura's 2-parameter model (Kimura 1980) in the DNADIST module of PHYLIP (Felsenstein 1993). The distance matrix was used to construct a phylogenetic tree using a neigbor-joining algorithm (Saitou and Nei 1987).

RESULTS

A specific, single-sized product was obtained from each of the 12 clonal marine phytoplankton species tested using each primer pair. For primers EUK1-LSJ and EUK2-LSJ, the product size was 370 bp, while for primers EUK3-LSJ and EUK2-LSJ the product size was 330 bp. All these products were of the sizes expected. No product was obtained when bacterial DNA was used as the template. DGGE-PCR products from all the species denatured within a denaturant range of 25% to 55%, as exemplified by products from *G. spinifera* and *L. minimus* (Fig. 1).

Result for DGGE run time optimization is shown in Fig. 2 using products from the dinoflagellate, *G. spinifera* and the diatom, *L. minimus*. The product from *L. minimus* denatured at a higher denaturant concentration than that of *G. spinifera*. Migration for both products was completewithin 10 hours.

DGGE profiles of the 12 phytoplankton species are shown in Fig.3a. Judging solely from the profile for mixed products (lane 13), it would seem that the sample contained eight or nine genotypes, which meant that some bands overlap. Examination of the individual lanes showed that products from *Cochlodinium* sp., *G. instriatum*, and *K. sanguinea*, as well as *O. lenticularis* and *O. ovata* could not be separated. In addition there

Table 1. Clonal cultures of dinoflagellates and a diatom used in the optimization of the DGGE protocols

Species	Clone	Origin
Thecate dinoflagellates		
Alexandrium affine (Inoue and Fukuyo) Balech	AAMS02	Straits of Malacca
Alexandrium leei Balech	ALMS02	Straits of Malacca
Gambierdiscus cf. *belizeanus* Faust	GTSA02	Sabah
Pyrodinium bahamense Plate var. *compressum* Böhm	PBSP05	Sabah
Gonyaulax spinifera (Claparède and Lachmann) Diesing	GSTL01	South China Sea
Coolia cf. *tropicalis* Faust	CMPL01	LangkawiIsland
Ostreopsis lenticularis Fukuyo	OLPR01	RedangIsland
Ostreopsis ovata Fukuyo	OVSA06	Sabah
Athecate dinoflagellates		
Cochlodinium sp.	COMS01	Straits of Malacca
Karenia sanguinea	GYMS01	Straits of Malacca
Gyrodinium instriatum Freudenthal et Lee	GIMS02	Straits of Malacca
Diatom		
Leptocylindrus minimus Gran	LMMS01	Straits of Malacca

Table 2. A+T and G+C content of a 330-bp fragment of the 18S rDNA subunit obtained by DGGE-PCR of selected species

Species	A+T%	G+C%
Alexandrium affine AAMS02	55.45	44.55
Alexandrium leei ALMS02	55.45	44.55
Gambierdiscus cf. *belizeanus* GTSA02	51.52	48.48
Pyrodinium bahamense var. *compressum* PBSP05	54.24	45.76
Cochlodinium sp. COMS01	53.33	46.67
Gyrodinium instriatum GIMS02	53.64	46.36
Karenia sanguinea GYMS01	53.03	46.97
Ostreopsis lenticularis OLPR01	55.76	44.24
Ostreopsis ovata OVSA06	56.36	43.64

was also very little separation between *A. affine* and *A. leei*. When the DGGE was repeated using a denaturant range of 30% to 45%, there was clearer separation of the *A. affine* and *A. leei* bands (Fig. 3b) but the other bands were still inseparable.

The A+T and G+C content of the test species are shown in Table 2. *Cochlodinium* sp., *G. instriatum* and *K. sanguinea* differed not only in nucleotide sequence (data not shown) but also in G+C content. Similarly *O. lenticularis* and *O. ovata* differed in both neuclotide sequence and G+C content. *A. affine* and *A. leei* had different nucleotide sequences but identical G+C content of 44.55%.

The effects of variable cell density on the efficiency of DGGE-PCR is shown in Fig. 4. For all the species tested PCR products were obtainable from even the lowest density tested, except for *Cochlodinium* sp. which did not produce detectable products from less than 10,000 cells (lane 5). In contrast, the product from *G. belizeanus* was still clearly evident even when there was only ca. 60 cells in the sample.

A total of 28 DGGE-PCR products were detected from the field samples, of which only 13 were unique (Fig. 5). In contrast, microscopic analysis showed the presence of at least 45 morphospecies belonging to 38 genera (Table 3). The dominant species were *Protoperidinium, Chaetoceros, Cylindrotheca, Ditylum, Navicula, Skeletonema, Thalassionema* and *Thalassiosira*. Phylogenetic analysis based on the nucleotide sequences showed that the unique DGGE-PCR products were most likely from the following taxa: *Chytridium* sp., *Ditylum* sp., *Skeletonema* sp., *Navicula* sp. and *Thalassiosira* spp. (Fig. 6). Two products could only be assigned as Bacillariophyceae and one as Dinophyceae.

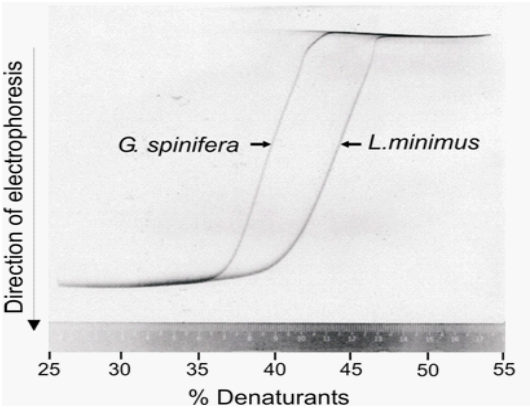

Fig. 1. Melting profiles of DGGE-PCR products from *Gonyaulaxspinifera* and *Leptocylindrusminimus* in perpendicular DGGE. Negative image of ethidium bromide-stained gel

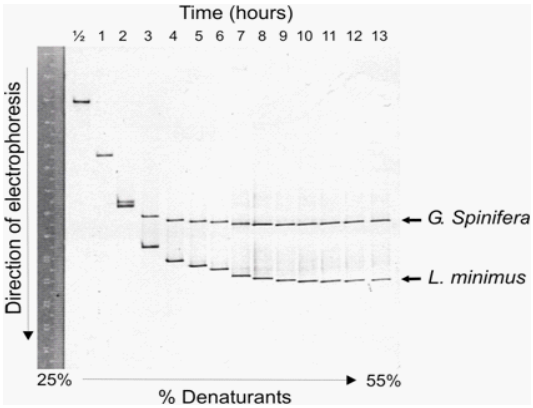

Fig. 2. Effect of running time on separation of DGGE-PCR products from *Gonyaulaxspinifera* and *Leptocylindrusminimus* in parallel DGGE. Negative image of ethidium bromide-stained gel

Table 3. Phytoplankton morphospecies abundance (x 10^3 cells L^{-1}) at six coastal sites that were also analysed by DGGE. The densities of all species belonging to the same genus were combined

Species	Sampling sites					
	I	II	III	IV	V	VI
Dinophyceae						15
Alexandrium leei	4	7	6	9	11	
A. tamiyavanichii						5
Ceratium trichoceros	9	12	9	5	8	
C. tripos						
C. furca						
C. fusus						
C. sp.						*ND*
Dinophysis caudata	1	2	1	1	1	8
Gonyaulax sp.	26	25	18	29	22	1
Gymnodinium sp.	6	1	2	ND	2	6
Prorocentrum sp.	22	21	11	15	19	47
Protoperidinium leonis	40	60	62	50	56	
P. pellucidum						
P. quinquecorne						20
Scrippsiella trochoidea	18	24	37	35	20	
Bacillariophyceae						5
Amphiprora gigantea	ND	ND	ND	3	2	6
Amphora lineolata	ND	ND	ND	7	9	8
Asterionellopsis sp.	37	14	12	12	6	1
Asterolampa sp.	ND	ND	ND	ND	ND	13
Bacteriastrum furcatum	8	27	32	29	20	
B. delicatulum						
B. sp.						ND
Bellerochea horologicales	ND	1	ND	ND	1	ND
Cerataulina sp.	ND	2	1	ND	ND	45
Chaetoceros lorenzianus	35	64	45	39	59	
C. peruvianus						ND
Corethron sp.	ND	ND	ND	1	ND	2
Coscinodiscus sp.	ND	18	3	3	6	78
Cylindrotheca closterium	80	175	250	152	169	5
Ditylum brightwellii	76	61	14	8	6	14
Eucampia zodiacus	5	10	4	13	7	3
Guinardia striata	1	4	7	6	7	3
Hemiaulus sinensis	ND	12	8	8	4	18
Lauderia annulata	ND	ND	20	19	25	6
Leptocylindrus minimum	7	26	19	4	7	ND
Lithodesmium sp.	ND	1	ND	ND	ND	ND
Melosira sp.	ND	ND	2	ND	1	2
Meuniera membranacea	ND	ND	ND	ND	3	42
Navicula sp.	68	59	56	53	52	16
Odontella sinensis	19	28	18	12	15	ND
Planktoniella sp.	ND	ND	ND	1	ND	12
Pleurosigma sp.	29	16	11	17	18	1
Pseudoguinardia recta	9	7	14	5	3	14
Pseudo-nitzschia sp.	27	34	24	23	22	17
Rhizosolenia imbricata	25	23	28	17	8	
R. setigera						28
Skeletonema costatum	4149	1071	399	31	50	42
Thalassionema frauenfeldii	35	58	69	66	54	
T. nitzschioides						231
Thalassiosira gracilis	81	133	160	161	260	
T. kushirensis						

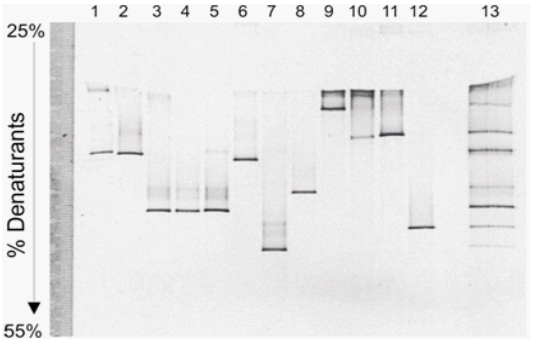

Fig. 3a. Separation of DGGE-PCR products from 12 marine dinoflagellate and a diatom species in parallel DGGE. Negative image of ethidium bromide stained-gel. Lane (1) *Alexandriumaffine*, (2) *A. leei*, (3) *Cochlodinium* sp., (4) *Gyrodiniuminstriatum*, (5) *Karenia sanguinea*, (6) *Gonyaulaxspinifera*, (7) *Gambierdiscusbelizeanus*, (8) *Pyrodiniumbahamense* var. *compressum*, (9) *Coolia* sp. (10) *Ostreopsislenticularis*, (11) *O. ovata*, (12) *Leptocylindrusminimus*, and (13) mixture of PCR products from all 12 species. For lane 13 two wells were combined into one.

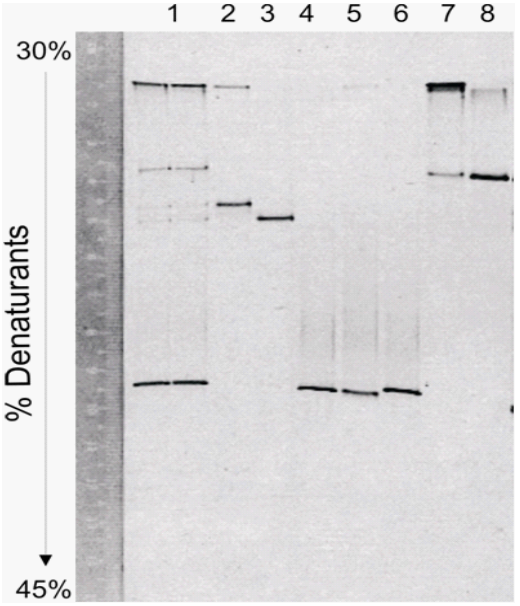

Fig. 3(b). Separation of DGGE-PCR products from seven marine dinoflagellate species in parallel DGGE over a denaturant range of 30% - 45%. Negative image of ethidium bromide-stained gel. Lane (1) mixture of PCR products from the seven species, (2) *Alexandriumaffine*, (3) *A. leei*, (4) *Cochlodinium*sp., (5) *Gyrodiniuminstriatum*, (6) *Kareniasanguinea*, (7) *Ostreopsislenticularis*, (8) *O. ovata*

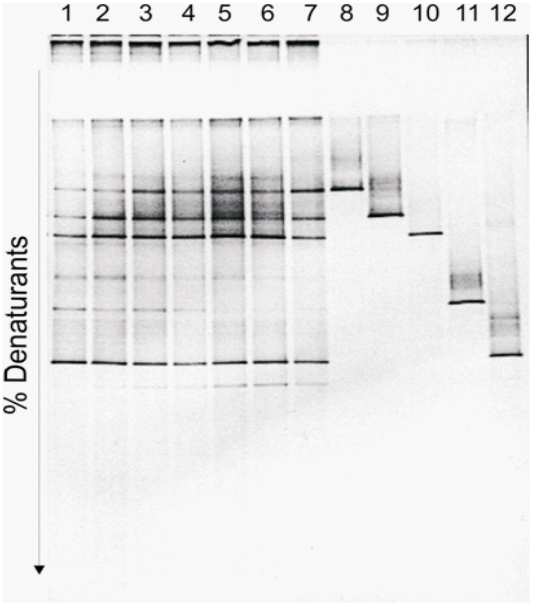

Fig. 4. Effect of cell density on DGGE-PCR product yield. Lanes 1 to 7 contained products obtained from mixes of equal culture volumes of each species, and lanes 8 to 12 contained products of individual species. Lane (1) 50 mL of each culture, (2) 25 mL, (3) 10 mL, (4) 5 mL, (5) 2 mL, (6) 1 mL, (7) 100 µL, (8) *Cooliatropicalis*, (9) *Ostreopsisovata*, (10) *Alexandriumleei*, (11) *Cochlodinium* sp., and (12) *Gambierdiscusbelizeanus*.

DISCUSSIONS

In recent years there has been increased application of DGGE and TGGE in ecological studies of aquatic microbes. While these methods are of limited use in taxonomic or biogeographic studies, they are very promising means to rapidly assess and compare genetic diversity of groups of organisms from different communities. Also, they could be potentially used to assess changes in genetic diversity in a community. Indeed these are the most common applications of the technique in bacterial studies. It has also been demonstrated for bacteria that DGGE can provide quantitative data on the abundance of genotypes in natural samples with the incorporation of an internal standard during the PCR step (Bruggermann *et al*. 2000). In contrast to studies on prokaryotic communities, GGE has so far been rarely used for phytoplankton.

Results from this study showed that it

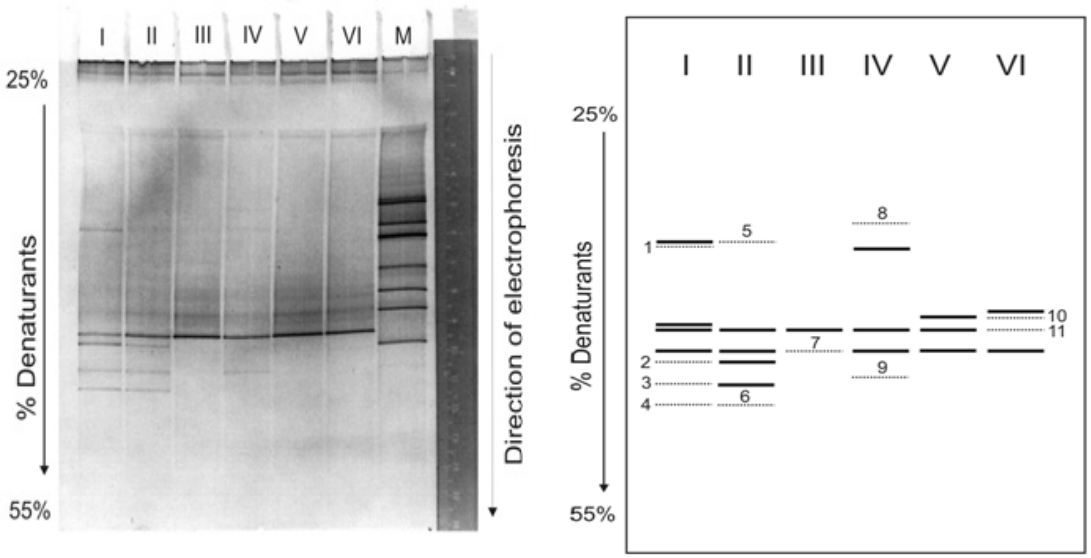

Fig. 5. DGGE profiles of natural plankton samples from six different sites. Negative image of ethidium bromide-stained gel is shown in the upper panel. The lower panel is a representation of the gel. Bands denoted by dotted lines and numbered were sequenced

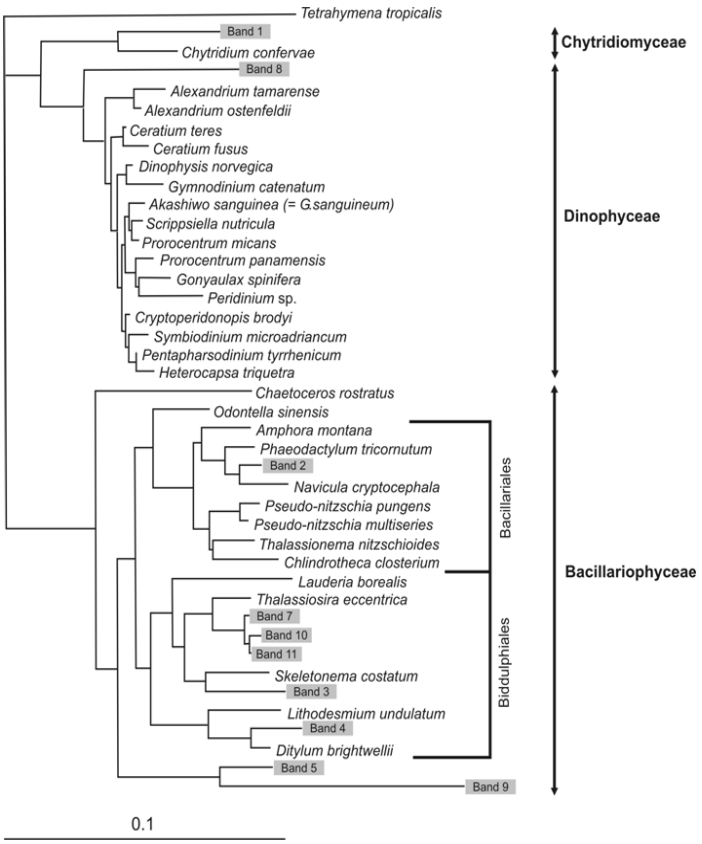

Fig. 6. Reconstruction of a phylogenetic tree incorporating nine DGGE-PCR products from natural plankton samples for which nucelotide sequences were obtained. *Tetrahymena tropicalis* was used as an outgroup

was relatively easy to obtain PCR products from phytoplankton for GGE analysis. Ideally the optimisation of methods should have included more species, especially diatoms, but lack of cultures was a problem. Based on the species studied the optimum DGGE conditions were an 8% polyacrylamide gel with 25% - 55% denaturant concentration, run at a constant 150V at a temperature of 60° C for 10 hours in TAE buffer. Running times for DGGE may take several hours but this can be significantly shortened if TGGE is used.

Not all the DGGE-PCR products resolved as expected in the DGGE gel. In theory, DGGE should be independent of taxonomy or phylogenetic affiliation. The ease of denaturing should be inversely proportional to the amount of G+C content, and this was observed in this study. For example, the product from *O. ovata* (G+C = 43.64%) denatured early while product from *G. belizeanus* (G+C = 48.48%) denatured last. However, products from *Cochlodinium* sp., *G. instriatum* and *K. sanguinea* could not be separated even though they differed in nucelotide sequence and G+C content. Meanwhile, products from *A. affine* and *A. leei* which had identical G+C content were separable.

Denaturing behavior may depend not only on total G+C content but also on the distribution of these nucleotides within the double strand. For eaxmple, at base positions 247-252 *A. affine* had TTCGA while *A. leei* had TCCGG. It has been reported that adjacent C and G could interact, for example through van der Waals forces (Hoppe *etal*. 1983), resulting in the need for more energy required to denature the double strand. This may be the reason why the product from *A. leei* denatured later than that of *A. affine* even though both had identical G+C content. However this argument could not explain the inability to separate the products from the three naked dinoflagellate species. These resolution problems might be overcome by using larger DGGE-PCR product size that incorporates more sequence variability and the use of narrower denaturant concentration ranges. DGGE studies on bacteria generally use a target gene fragment of 550 bp in size (Brinkhoff *et al*. 1999; Sahm *et al*. 1999).

Another potential problem, especially with regard to natural populations, is that for certain species high cell numbers may be required in order to obtain detectable DGGE-PCR product from mixed samples. In the case of *Cochlodinium* sp. for example no PCR product was detectable even from ca. 9000 cells whereas product from *G. belizeanus* was obtainable from less than 100 cells. Previous studies have shown that algae species differ in genome size, genome number and copy number for the small subunit of rDNA (reviewed in Coleman and Goff 1991). Most probably DGGE-PCR would be biased towards the more abundant genomic template while the less abundant might be swamped (Ward *etal*. 1992; von Wintzingerode *etal*. 1997).

It is quite simple to evaluate DGGE efficiency using pure laboratory cultures, but this is not the case with natural samples. Results from this study showed that DGGE seriously underestimated the diversity in all the samples. While the dominant diatom taxa (*Thalassiosira, Skeletonema, Dytilum, Navicula*) were detected, many of the less abundant species were not detected. Surprisingly a product from a chytrid was detected even though the taxon was very rare in the samples. Since not all the DGGE-PCR products were sequenced it was not known whether some of the overlapping bands were of different species.

The limited number of DGGE-PCR products obtained from the field samples could be due to at least three factors. First, only a very small fraction of the extracted genomic DNA was used in the PCR. Thus chances of capturing DNA from non-abundant species would be very small. Ideally all the extracted DNA should be used as template in the DGGE-PCR but this would be impractical and costly. The second factor relates to efficiency of DNA extraction from a mixed natural population sample. In contrast to bacteria, phytoplankton has more varied morphology, size, and cell wall material. At present the efficiency of DNA extraction from such varied material is not known. In addition phytoplankton cells contain various chemical components, some of which could be inhibitory to the PCR process. Thus, more rigorous cleanup procedures may be required, which would result in loss of DNA. The third factor is that some of the overlapping DGGE-PCR products may actually represent different species. The only way to ascertain this is to sequence every single DGGE-PCR product. While this is

technically simple task, it is much more expensive to achieve. These problems also probably exist in studies on bacterial communities, in which DGGE has been widely used.

In conclusion, DGGE is a promising method to rapidly assess genetic diversity of natural marine phytoplankton populations. However, more optimization work still needs to be carried out before the technique can be routinely and reliably adopted for that purpose. As it stands, the DGGE results still have to be validated by morphological examinations of the samples. The evidence suggests that DGGE will most probably underestimate the diversity present in a natural sample and results have to be interpreted with caution

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