Random Amplified Polymorphic DNA (RAPD) Identification of Genetic Variation in *Chlorella* species

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The random amplified polymorphic DNA (RAPD) technique was used to characterize 18 reference strains of microalgae, mostly *Chlorella* species, collected from various localities around Korea peninsular. Eighteen strains consist of four genera of the family marine *Chlorella* from 12 samples, two genera of fresh water *Chlorella* from three samples, and three genera on *Nannochloris*. Twenty 10-mer anonymous primers were screened for amplification of genomic DNA extracted from samples using the CTAB extraction method.

Nineteen of these oligonucleotide primers were positive or band producing. Three of 20 random primers (OPA 10, OPA 12, and OPA 18) resulted in both clear band and a high degree of reproducibility and showed some potential to be used to discriminate individual samples of both genetically hetero-and homogeneous populations, in determining phylogenetic relationships between species within a genus and developing individual fingerprints for each samples.

Key words : *Chlorella*, polymerase chain reaction, polymorphic DNA, polymorphisms

Introduction

The family of *Chlorella* is one of the most important microalgae groups in marine environments. Some members of this family are important as live foods to culture rotifers and larvae in aquaculture. Members of the *Chlorella* often predominating in sea water have been shown to have the nutritive elements, mainly highly unsaturated fatty acids and amino acids that are required for fish larvae and crustaceans (Snell et al., 1983; Watanabe et al., 1983; 1984).

Recently, genetic engineering and genetic manipulation techniques have been used in applied biotechnology programs for agriculture and aquaculture. Especially, a new technique using the polymerase chain reaction (PCR) (Innis et al., 1990) has been developed to differentially amplify anonymous regions of genomic DNA fragments using oligonucleotide primers, referred as random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Hadrys et al., 1992). Amplification fragment length polymorphism (AFLP) have been proven useful in identifying both inter-and intra-specific genomic DNA variation and in determining genetic compatibility profiles (Kapraun et al., 1992; Hadrys et al., 1992; Baird et al., 1992). Taxonomic studies are important to ensure that microalgae of interest are correctly identified and recognized to enhance utilization of microalgae resources for the development of phycoculture and aquaculture industry.

The family of *Chlorella* is highly differentiated. The phenotype of this family exhibits complexity and variability on several factors: temperature, intra-and inter-individual, salinity, environment and geographical distribution, and chemical composition (Hur and Kim, 1988; Oh-Hama and Miyachi, 1988). Phenotypic classification systems of this family results taxonomic inconsistencies and arises many problems. To complement the existing classification systems, new genetic and molecular approaches are recently introduced (Dutcher and Kapraun, 1994; Ho et al., 1995). The

RAPD technique for fingerprinting or comparing polymorphisms in arbitrary nucleotide sequences could be used for the discrimination among individuals.

The present investigation was conducted to access the usefulness of the polymerase chain reaction using anonymous nucleotide primers in differentiation and characterization of *Chlorlla* species. The amplification product banding patterns would provide comparisons between genomic profiles and analysis of similarity matrix data.

Materials and Methods

Materials

Collection data for 13 samples of the *Chlorella* and *Nannochloris*. species obtained from localities around Korea Peninsular and are given in Table 1. In addition, 5 other *Chlorella* samples collected from other countries were used for comparison purpose. Preliminary identification based on morphological characteristics was carried out with the photomicroscope (Hur, 1992). Restriction enzymes were purchased from New England Biolabs Inc. and United States Biochemicals Corp. T4 DNA ligase and *Taq* DNA polymerase were purchased from United States Biochemicals Corp.

Preparation, preservation and culture of samples

Chlorella and *Nannochloris* species were cultivated in an f/2 medium (Guillard and Ryther, 1962) at 20℃ and 26℃, respectively, with aeration under continuous illumination (ca. 5,000 lux) using a cool-white fluorescent lamp. Cells were harvested using a centrifuge and stored at -25℃.

DNA Isolation

Genomic DNA was isolated using the CTAB extraction method (Ho et al., 1995; Rogers et al., 1988; Murray and Thompson, 1980). Approximately, 5 g (wet weight) of samples was ground in liquid nitrogen before addition of 30 ml of 1 x CTAB (cetyltrimethyl ammonium bromide) extraction buffer [1% (w/v) CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.7 M NaCl, 1% polyvinylpyrolidine (PVP)] and incubated for 15 min at 65℃. An equal volume of chloroform-isoamylalcohol (24 : 1) was added, mixed and centrifuged at 11,000 g for 3 min. The top aqueous phase was collected and one tenth volume of CTAB solution [10% (w/v) CTAB, 0.7 M NaCl] was added to the top aqueous phase. A second chloroform-isoamylalcohol extraction was performed and centrifuged again. One volume of the top phase was added to two volumes of CTAB precipitation buffer [1% CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0)], and placed on ice for 30 min and then centrifuged at 11,000 g for 3 min. The pellet was resuspended in 100 μl TE buffer [0.1 M Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] containing 1 M NaCl and heated to 65℃ for 5 min. DNA was precipitated, washed, dried and dissolved in TE buffer.

Random amplified polymorphic DNA PCR (RAPD-PCR)

Polymerase chain reaction (PCR) amplification was performed in 25 μl volume containing 2.5 μl 5 x *Taq* DNA polymerase buffer (USB), 100 μM each of dATP, dGTP, dCTP, dTTP, 5 pmol primer (Operon Kit A, Operon Technologies Inc., California, USA), 200 ng of genomic DNA and 2.5 mM $MgCl_2$ and 1.0 unit *Tag* DNA polymerase.

Amplification was perofrmed in a Ericom DNA Delta Cycler I programmed for 45 cycles of 1 min denaturation at 95℃, 1 min annealing at 30℃(when screening for primers) or 37℃(all other experiments) and 2 min extension at 72℃(Kim and Richardson, 1993; 1994). Twenty microlitres of the reaction products were separated by electrophoresis through 1.5% agarose gels and stained with etidium bromide.

Agarose gels were photographed with a Kodak Polaroid camera over a UV transilluminator (312 nm) using 667 film.

Table 1. List of species, habitat and collection sites

Collection number	Species	Collection sites	Chemical composition[1]		
			Protein	Lipid	Ash
KMCC C-19	*C. stigmatophora*	UTEX 993, USA	20.10	1.80	47.09
KMCC C-20	*C. ellipsoidea*	Japan	26.46	8.66	38.67
KMCC C-21	*C. ellipsoidea*[2]	UTEX 247, USA	29.48	0.12	32.67
KMCC C-22	*C. vulgaris*	Nacdong	33.11	1.03	30.05
KMCC C-23	*C.* sp.	Kamcheon	41.16	6.85	30.05
KMCC C-24	*C.* sp.	Suncheon	23.66	10.85	41.18
KMCC C-25	*C.* sp.	Suncheon	30.87	3.69	34.68
KMCC C-26	*C.* sp.	Suncheon	31.70	6.77	30.80
KMCC C-27	*C.* sp.	Yeocheon	24.96	2.21	37.66
KMCC C-28	*C.* sp.	Nacdong	26.65	4.93	34.46
KMCC C-29	*C.* sp.	Nacdong	21.64	7.44	35.62
KMCC C-30	*C.* sp.	Nacdong	21.60	6.99	31.71
KMCC C-31	*Nannochloris oculata*	UTEX LB 1988, USA	33.55	1.87	40.76
KMCC C-32	*Nannochloris salina*	Seri-2, USA	25.02	8.38	37.99
KMCC C-33	*Nannochloropsis* sp.	Israel	35.19	3.65	29.49
KMCC FC-4	*C. pyrenoidosa*	UTEX 26, USA		N.D.[3]	
KMCC FC-5	*C. ellipsoidea*	UTEX 27, USA		N.D.[3]	
KMCC FC-6	*C. elliposidea*	UTEX 20, USA		N.D.[3]	

[1] Hur and Lee. (1996) Chemical composition of microalgae. J. Aquaculture, 9 (4) *in press.*
[2] Fresh water Chlorella acclimatized in sea water.
[3] Not determined.

DATA Analysis

Band sharing analysis was carried out for RAPD data using pairwise comparison of the samples according to the formula of Nei and Li (1979). The formula of coefficient similarity is $F=2n_{xy}/(n_x+n_y)$, wher n_x is the total number of DNA fragments from sample X, n_y is the total number of DNA fragments from sample Y and n_{xy} is the number of DNA fragments that were identical in the two samples. In a comparison of two samples, *F* values close to 1.0 indicate high degree of genetic similarity. An *F* values of 1.0 indicate that the two samples are identical.

Results

In order to assess the usefulness of the polymerase chain reaction using random amplifed polymorphic DNA (RAPD) primers in the differentiation and characterization of *Chlorella* and *Nannochloris* species collected from various localities around the Korea peninsular, DNA from samples given in Table 1 were isolated by the CTAB extraction method as described in "Materials and Methods." Chemical compositions of the collected *Chlorella* and *Nannochloris* species are also listed in Table 1. As shown in Fig. 1, genomic DNA was successfully isolated and purity of DNA was an $OD_{260/280}$ ratio ranging from 0.85 to 1.3

Twenty 10-mer anonymous primers were screened for amplification of genomic DNA extracted from samples using the template DNA from *Chlorella ellipsoidea* (KMCC C-20). Of the 20 primers screened, Nineteen of these oligonucleotide primers were able to generate amplification products or band producing, ex-

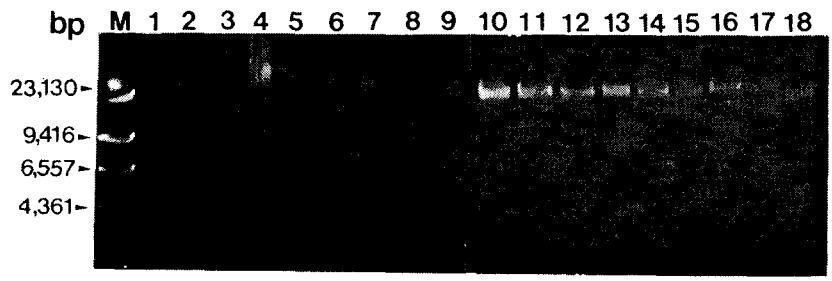

Fig. 1. Genomic DNA isolated from *Chlorella* and *Nannochloris* species.
Lane M indicates a molecular weight marker, lane 1, KMCC C-19 (*C. stigmatophora*); lane 2, KMCC C-20 (*C. ellipsoidea*); lane 3, KMCC C-21 (*C. ellipsoidea*); lane 4, KMCC C-22 (*C. vulgaris*); lane 5, KMCC C-23 (*C.* sp.); lane 6, KMCC C-24 (*C.* sp.); lane 7, KMCC C-25 (*C.* sp.); lane 8, KMCC C-26 (*C.* sp.); lane 9, KMCC C-27 (*C.* sp.); lane 10, KMCC C-28 (*C.* sp.); lane 11, KMCC C-29 (*C.* sp.); lane 12, KMCC C-30 (*C.* sp.); lane 13, KMCC C-31 (*Nannochloris oculata*); lane 14, KMCC C-32 (*Nannochloris salina*); lane 15, KMCC C-33 (*Nannochloropsis* sp.); lane 16, KMCC FC-4 (*C. pyrenoidosa*); lane 17, KMCC FC-5 (*C. ellipsoidea*); lane 18, KMCC FC-6 (*C. ellipsoidea*).

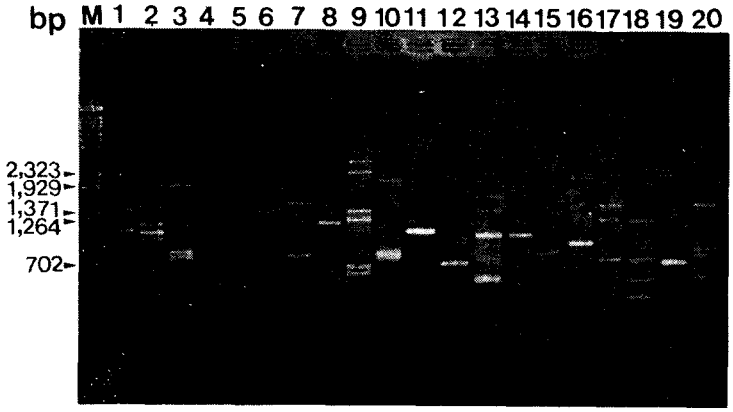

Fig. 2. PCR amplification of genomic DNA from KMCC C-20 (*Chlorella ellipsoidea*) with primers OPA-01-OPA-20.
Lane M indicates a molecular weight marker; lane 1 was RAPD bands using the primer OPA 01; lane 2, OPA 2; lane 3, OPA 3; lane 4, OPA 4; lane 5, OPA 5; lane 6, OPA 6; lane 7, OPA 7; lane 8; OPA 8; lane 9, OPA 9; lane 10, OPA 10; lane 11, OPA 11; lane 12, OPA 12; lane 13, OPA 13; lane 14, OPA 14; lane 15, OPA 15; lane 16, OPA 16; lane 17, OPA 17; lane 18, OPA 18; lane 19, OPA 19; lane 20, OPA 20.

hibiting varying degrees of polymorphism among isolates (Fig. 2).

Polymorphic DNA loci from *Chlorella* and *Nannochloris* samples were detected by three of the primers (OPA 10, OPA 12, and OPA 18) and resulted in both clear band resolution and a high degree of reproducibility (Fig. 3, 4, 5). These primers are GC rich and their seguences are listed in Table 2. The sizes of DNA fragments amplified with the three primers ranged from 0.2 to 3.0 kb. The number of PCR products generated varied from 0 to 15, depending on the combination of sample and primer used.

Table 2. Useful RAPD primers: sequences and GC contents

Primer	Sequence	GC content(%)
OPA-9	GGGTAACGCC	70
OPA-10	GTGATCGCAG	60
OPA-11	TCGGCGATAG	60
OPA-12	AGGTGACCGT	60

Especially, Fig. 5 shows a reproducible RAPD profile of different sizes of amplification products gene-

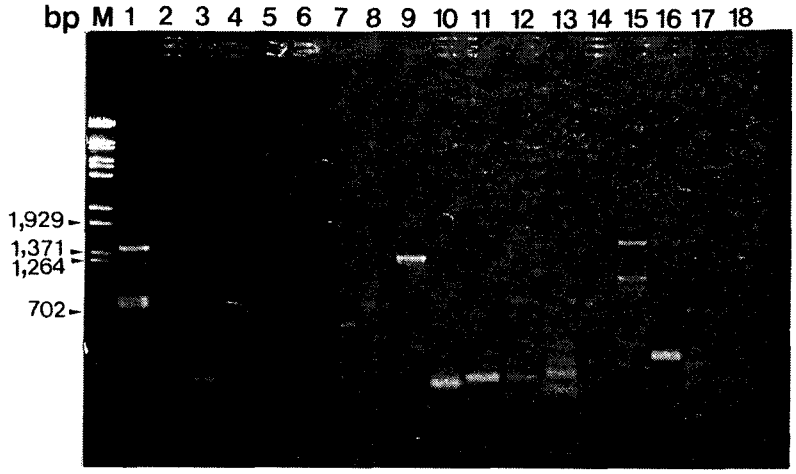

Fig. 3. PCR amplification of genomic DNA from *Chlorella* and *Nannochloris* species with the primer OPA-10.
Lane M indicates a molecular weight marker. The number above the lanes corresponds to lane explanation in Fig. 1.

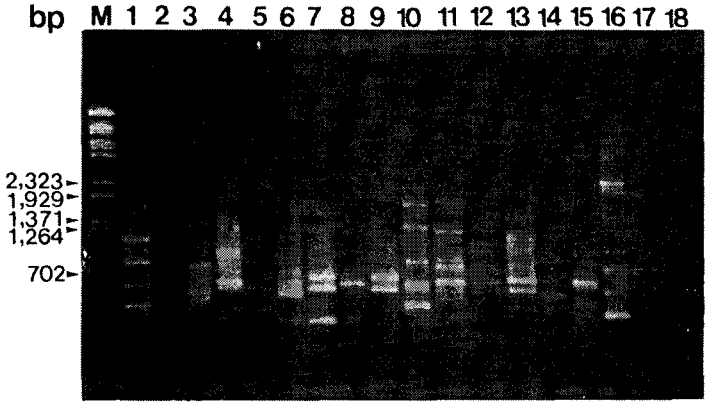

Fig. 4. Random amplified DNA polymorphisms of 18 samples of genomic DNA from *Chlorella* and *Nannochloris* species with primers OPA-12.
Lane M indicates a molecular weight marker. The number above the lanes corresponds to lane explanation in Fig. 1.

rated using primer OPA-12 and tested samples. The DNA banding patterns resulted from the primer provide evidence for both genetically hetero-and homogeneous populations in different species and isolates.

Although use of the single primer revealed polymorphic loci, no species-specific bands were apparent (Fig. 4). However, there was at least one characteristic band or PCR product unique to the individual populations of each species. There are bands that are shared by samples within the species as well as bands unique to individual samples. There are also bands that are restricted to or unique to only one or a few samples.

OPA-12 primer failed to generate any amplification product only with the template DNA from KMCC FC-6 (Fig. 4; lane 18).

The degree of similarity between various RAPD-PCR patterns for different samples was assessed by

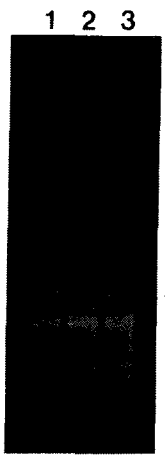

Fig. 5. Reproducibility of RAPD-PCR.
Individual PCR amplifications using DNA from KMCC C-20 (*Chlorella ellipsoidea*) using the primer OPA-18.

calculation F (coefficient of similarity) values according to the method of Nei and Li (1979). These calculations were based on the degree of sharing of bands among the samples (Table 3). The calculated value of F among the samples within *Chlorella* and *Nannochloris* species ranged from 0 to 1.0. An F value of 1.0 indicates that the two samples are identical. Some of the samples in the group are totally different (F= 0) and the highest F value was obtained with 3 samples KMCC C-23 and C-25 and C-27. Interestingly, these 3 samples of *Chlorella* species were collected from 3 different localities at the southern coast of the Korea peninsular (Table 1). Especially, C-25 and C-27 are genetically identical even though the chemical composition are different each other (Table 1). Interestingly, *Chlorella* and *Nannochloris* species collected from different localities also shared some genetic similarity (Table 3).

To assess the reproducibility of RAPD-PCR, PCR amplifications were performed on multiple identical samples. The DNA bands patterns produced by RARD-PCR showed good reproducibility although some minor variability was noted (Fig. 5).

Discussion

RAPD-PCR is a relatively simple and reliable technique for genomic mapping, identification of isolates, screening of molecular markers, and applications in population biology. In this experiment, RAPD polymorphism generated with arbitrary primers gave reliable phylogenetic grouping of *Chlorella* and *Nannochloris* species collected from different localities around the Korea peninsular. Genetic diversity within a collection of eighteen isolates was analyzed based on their polymorphisms with RAPD-PCR markers. The band pattern analysis by the RAPD method revealed polymorphism within species of *Chlorella* and Nannochloris and established DNA fingerprints useful for phylogenetic characterization.

The DNA banding patterns of *Chlorella* and *Nannochloris* species from the investigated sites provide evidence for both genetic homogeneity and diversity within indivedual populations, as well as differences between geographically separated populations. Menbers of the *Chlorella* collected in sea water show to have good balanced nutritive elements (Table 1), mainly highly unsaturated fatty acids and aminoacids that are required for fish larbvae and crustaceans. To maintain and screen for good strains of *Chlorella* for aquacultural applications, it is worthy identifying and characterizing the genetic variation in *Chlorella* species.

The presence of tough cell walls causes several problems associated with DNA extraction from *Chlorella* species. However, the CTAB method used in this study gave good results as it may be the most efficient method in removing cell walls. the obtained DNA was relatively intact and was readily amplifed by PCR. In order to differentiateacute genetic variation in tested samples, it is important to have the reproducibility of RAPD patterns. When extreme care were taken in conducting the RAPD procedure, the major banding pattern for a particular combination of primer and DNA was reproducible for replicates in experiments.

Table 3. Calculated F values among *Chlorella* species based on band sharing Analysis for primer OPA 12

	C-19	C-20	C-21	C-22	C-23	C-24	C-25	C-26	C-27	C-28	C-29	C-30	C-31	C-32	C-33	FC-4	FC-5	FC-6
C-19	–																	
C-20	0.266	–																
C-21	0.333	0.364	–															
C-22	0.421	0.000	0.125	–														
C-23	0.166	0.000	0.000	0.181	–													
C-24	0.400	0.200	0.307	0.666	0.250	–												
C-25	0.077	0.000	0.200	0.333	0.800	0.444	–											
C-26	0.181	0.000	0.000	0.200	0.000	0.286	0.000	–										
C-27	0.166	0.000	0.000	0.181	1.000	0.250	0.800	0.000	–									
C-28	0.444	0.153	0.250	0.555	0.000	0.266	0.000	0.200	0.000	–								
C-29	0.380	0.266	0.444	0.500	0.154	0.235	0.143	0.166	0.154	0.100	–							
C-30	0.352	0.444	0.461	0.266	0.250	0.166	0.222	0.000	0.250	0.133	0.588	–						
C-31	0.470	0.307	0.375	0.444	0.363	0.266	0.444	0.000	0.363	0.222	0.400	0.266	–					
C-32	0.285	0.222	0.500	0.428	0.000	0.363	0.000	0.000	0.000	0.285	0.375	0.000	0.000	–				
C-33	0.066	0.200	0.307	0.400	0.250	0.333	0.250	0.286	0.250	0.533	0.235	0.333	0.133	0.000	–			
FC-4	0.588	0.000	0.266	0.117	0.000	0.285	0.181	0.000	0.000	0.235	0.000	0.000	0.000	0.000	0.000	–		
FC-5	0.076	0.000	0.200	0.166	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.181	–	
FC-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	–

This is the first report on the use of a PCR-based polymorphism assay to differentiate and characterize the *Chlorella* species. Since genetic variation of the species starts from a minor nucleotide change and its accumulation, we applied the RAPD technique which provides highly sensitive detection in distinguishing nucleotide difference. We have detected large genetic variation among the *Chlorella* species. The degree of genetic similarities between various RAPD-PCR patterns for various samples were calculated by F (coefficient of similarity) values. Distance matrix analysis indicates considerable genetic variation among the *Chlorella* specise (Table 3). The calculated F value among the samples within *Chlorella* and *Nannochloris* species ranged from 0 to 1.0, indicating that some of the samples in the group are totally different genotypes and the two samples were identical, although the samples were collected from different localities and differred in chemical composition. Also, *Chlorella* and *Nannochloris* species collected from different localities showed some genetic similarity, suggesting that the isolates are genetically reated to the inter-species. Thus, some specific RAPD bands found in Fig. 4 simply tell us that dynamic nucleotide mutations have been occurred from the isolates.

The present study suggests that RAPD-PCR is useful in discriminating individual samples within the specific genotypes. It may also be useful in determining phylogenetic relationships between species within a genus and in developing individual fingerprints for each samples. However, additional studies using various moecular techniques should be conducted in order to draw a clear conclusion about taxonomic status of the *Chlorella* and *Nannochloris* species.

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