ISSR as new markers for genetic characterization and evaluation of relationships among phytoplankton

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Abstract: Abstract In order to increase the molecular tools and markers needed for the identification of phytoplankton species, the inter simple sequence repeat (ISSR) fingerprinting was adapted to micro-algae and its use in genetic analysis was demonstrated. Twelve strains, 6 Alexandrium, 4 Pseudo-nitzschia, 1 Skeletonema and 1 Tetraselmis were analysed for the first time with ISSR amplifications. The patterns were highly polymorphic and very reproducible. The 6 primers gave 223 polymorphic markers that clearly and easily distinguished all 12 strains (mainly toxic ones) and gave 187 polymorphic markers among the Alexandrium and the Pseudo-nitzschia species. ISSR amplifications also indicated a large occurrence of simple sequence repeat (SSR) in phytoplankton genomes, especially in Pseudo-nitzschia, and show their usefulness to cluster intra and inter species. ISSR markers were found to be good markers for genetic characterization and diversity study and led to consider them as new tools for the survey of phytoplankton.

Keywords: Alexandrium - fingerprints - genetic characterization and diversity - ISSR - Pseudo-nitzschia - Skeletonema - Tetraselmis

Abbreviations

DGGE, Denatured Gradient Gel Electrophoresis; HMA, Heteroduplex Mobility Assay; ITS, Internal Transcribed Spacer, LSU, Large Sub-Unit; RAPD, Random Amplified Polymorphic DNA; RFLP-PCR, Restriction Fragment Length Polymorphism-Polymerase Chain Reaction; SSCP, Single Strand Conformation Polymorphism; SSU, Small Sub-Unit.

Introduction

Phytoplankton represents a major component of marine ecosystem, with around 3400 to 4000 species including toxic strains thus needing monitoring program to prevent economical and health consequences. Identification of phytoplankton is currently based on morphological characterization (light and electronic microscopy), which is time consuming, difficult and observer dependent. As a consequence, the development of new reliable and efficient tools based on molecular analysis has been increasing for the last 15 years because species identification is a critical step. Molecular biology techniques are powerful methods and good candidates because of their consistency independently of environmental parameters and developmental stages. During the last decade, molecular characterization and genetic relationships were mainly based on the analysis of rRNA genes: sequence comparison of SSU, LSU and ITS regions, RFLP-PCR and nucleic acid hybridization technologies (e.g. Scholin et al.1994, Walsh et al. 1998, Guillou et al. 2002). More recently, some studies have used the heterogeneity of a few characteristic genes: *rbcL* and *rbcS* or *psbA* (Chesnick et al. 1996, Takishita and Uchida 1999) or reported the use of molecular markers: RAPD, SSCP, DGGE and HMA (Adachi et al. 1997, Murayama-Kayano et al. 1998, Bolch et al. 1999, Uribe et al. 1999, Oldach et al. 2000, Coyne et al. 2001). These studies were able to characterize some species but lacked polymorphism to identify all the tested species.

   In higher plants or animals, Inter Simple Sequence Repeat or ISSR (Zietkiewicz et al. 1994) markers are more and more in demand, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz et al. 1994, Bornet and Branchard 2001, Bornet et al. 2002). ISSR uses the presence through out the genome of Simple Sequence Repeats (SSR) which are ubiquitous, abundant and highly polymorphic tandem repeat motifs composed of 1 to 7 nucleotides. A unique PCR primer composed of a few SSR repeats (with or without anchored end) was used to amplify DNA sequences between two inverted SSR composed of the same units and spaced out by less than around 4 Kbp. Neither sequence information nor prior genetic studies were required for these analyses, unlike for SSR markers. So far, ISSR has not been used to study phytoplankton although it has the potential to fill up the need of more polymorphic tools to distinguish micro-algae. The aim of the study

esported here was to evaluate the use of ISSR markers to characterize and to estimate genetic diversity between 12 cultured strains: 6, 4, 1 and 1 *Alexandrium*, *Pseudonitzschia*, *Skeletonema* and *Tetraselmis* genera, respectively.

Materials and methods

Micro-algal cells and cultures

Twelve species of micro-algae were used: *Alexandrium* [*minutum* (France), A. *fundyense* (Canada), A.*tamarense* (UK and Japan) and A. *catenella* (France and Spain)], *Pseudonitzschia* [*pseudodelicatisima* (France), P. *pungens* (Spain), P. *multiseries* (France) and P. *fraudulenta* (Spain)], *Skeletonema costatum* (France) and *Tetraselmis suesica* (UK). Monoclonal cultures were maintained, in ESP medium of Provasoli (1968) and f/2 medium (Guillard & Ryther,1962) for *Alexandrium*, *Skeletonema* and *Tetraselmis* or in ESP-K modified medium (J. Fresnel, Université de Caen, France, pers. comm.) for *Pseudonitzschia*. All cultures were grown under controlled conditions: 16°C, 12/12 h light/dark photoperiod and 50 +/- 4 μE m^{-2} s^{-1} light intensity.

DNA extraction

Micro-algae were grown to the mid exponential phase and cells were harvested by centrifugation at 6 000 g for 10 min. Cells were re-suspended in 1 ml of CTAB extraction buffer (100 mM Tris [pH 8.0], 1.4 M NaCl, 20 mM EDTA [pH 8.0], 0.2% β-mercaptoethanol, 1% SDS, 180 μg proteinase K, 2% CTAB) and heated at 65°C for 90 min. DNA was purified successively by one chloroform:isoamyl alcohol (24:1), one phenol:chloroform:isoamyl alcohol (12:12:1) and one chloroform:isoamyl alcohol (24:1) extractions. After precipitation with 0.6 ml of cold isopropanol, the DNA pellet was washed with ethanol (70%)-ammonium acetate (10 mM) mix and ethanol (70%). DNA was dissolved in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]) and RNA was digested using 10 ng of Rnase (Promega, USA). The DNA concentrations were calculated with a fluorometer (DyNA Quant 200, Hoefler, Amersham Biosciences, USA) using bisbenzimide (Amersham Biosciences, USA) as the fluorescent dye.

ISSR amplifications

Five primers without anchored end and one with a 5'-anchored end, were tested using specific and optimal annealing temperatures (table 1). ISSR-PCR amplifications and analyses were those described by Bornet and Branchard (2001) with the following modifications: the first denaturation step was increased to 6 min. and the number of cycles were increased to 32, the

primer and dNTP concentrations were increased to 6 µM and 400 µM, respectively. PCR was performed in triplicate using a Mastercycler apparatus (Eppendorf, Germany).

Table 1. List of the six primers used to amplify ISSR markers from phytoplankton DNA.

Primer	T_A (°C)	Range of amplified fragments	Total number of selected fragments
$(CCA)_5$	57	200-2200 bp	39
$(CAA)_5$	52	180-2500 bp	37
$(CAG)_5$	60	100-2300 bp	43
$(ATG)_5$	45	100-2550 bp	44
$(GACA)_4$	52	175-1850 bp	37
$GTC(CT)_8$	55	200-1950 bp	23

Data analysis

ISSR patterns were compared and markers were scored twice as being present or absent using ImageMaster 1D software (Amersham Biosciences). Only well separated bands with high intensity were selected as markers. Pairwise comparisons were calculated using Jaccard's coefficient (Jaccard. 1901). The similarity values found were used to generate a consensus tree using the Unweighted Pair Group Method Analysis (UPGMA), analyses were performed with NTSYSpc version 2.0 (Rohlf 1998). ISSR data were also subjected to genetic analysis using POPGENE 1.32 software (http://www.ualberta.ca/~fyeh).

Results

Six primers were used: four non-anchored tri-nucleotides, one non-anchored tetra-nucleotide and one anchored di-nucleotide (Table 1). PCR amplifications were performed in triplicate and from different DNA extractions for each strain and the results obtained for a same specie gave identical electrophoresis patterns, i.e. ISSR fingerprints. Each primer gave rich and clear patterns with selected bands from 100 to 2500 bp. Representative ISSR fingerprints obtained with primer $(ATG)_5$ are shown in Figure 1. A total of 223 bands of high intensity and well separated were selected as markers. All the ISSR markers were polymorphic among the 12 strains. The average of amplified polymorphic markers was of 37 per primer, with a minimum of 23 and a maximum of 44 (Figure 1). Primer $(CAA)_5$ gave no or one band with *Alexandrium* DNA. No significant difference of the number of amplified bands was observed with the other primers among the

Alexandrium and *Pseudonitzschia* genomes (Table 2). Among the 223 markers, 187 were amplified only in *Alexandrium* or *Pseudonitzschia* and 42 were common to these two genera. Forty four ISSR markers were amplified in *Alexandrium* strains (one or more strains) only and 101 in *Pseudonitzschia* strains (one or more strains) only (Table 2). ISSR markers clearly and easily distinguish all the tested species and also discriminate between toxic/non toxic or geographical origins of strains from the same species.

The similarity values of the phenogram (Figure 2) obtained from 223 ISSR markers ranged from 0.0546 to 0.2143 and the cophenetic coefficient was 0.97. Two main distinct clusters were observed and diverged at 0.0546 phenon level. The first one, including the seven strains of *Alexandrium* (dinoflagellate), can be subdivided in two groups: the *A. minutum*, *A. fundyense*, *A. tamarense* (Japan and U.K.) group and the *A. catenella* (France and Spain) group, these groups being separated at a similarity level of 0.1000. The second cluster, included the last six strains: four *Pseudonitzschia*, one *Tetraselmis* and one *Skeletonema*, which corresponded to five diatoms and 1 flagellate. *Pseudonitzschia* species have been clustered into two groups composed of *P. pungens*/*P. fraudulenta* and *P. pseudodelicatisima*/*P. multiseries* respectively.

Table 2. Mean genetic characteristics of *Alexandrium* and *Pseudonitzschia* populations from ISSR data obtained with the software Popgene 1.32, numerical data in italics correspond to standard deviation.

Analyse population	Nt	Ns	Ne	h	I	Ht	Hs	Gst	Nm
Alexandrium	86	44	1.299 *0.203*	0.215 *0.097*	0.365 *0.117*	-	-	-	-
Pseudonitzschia	142	101	1.438 *0.223*	0.290 *0.093*	0.4604 *0.0106*	-	-	-	-
Alexandrium and *Pseudonitzschia*	187	42	1.215 *0.153*	0.166 *0.088*	0.298 *0.117*	0.179 *0.008*	0.159 *0.005*	0.1084	4.1139

P = percentage of polymorphism
Nt = Number total of makers (100% polymorphic in this study)
Ns = Number of specific makers amplified only in the genus (100% polymorphic in this study)
Ne = effective number of alleles (Kimura and Craw, 1964)
h = Nei's (1973) gene diversity
I = Shannon's information index (Lewontin, 1972)
Figure 1. ISSR patterns of the 12 strains of phytoplankton (Dinoflagellates, Flagellate, Diatoms) amplified with primer $(ATG)_5$.
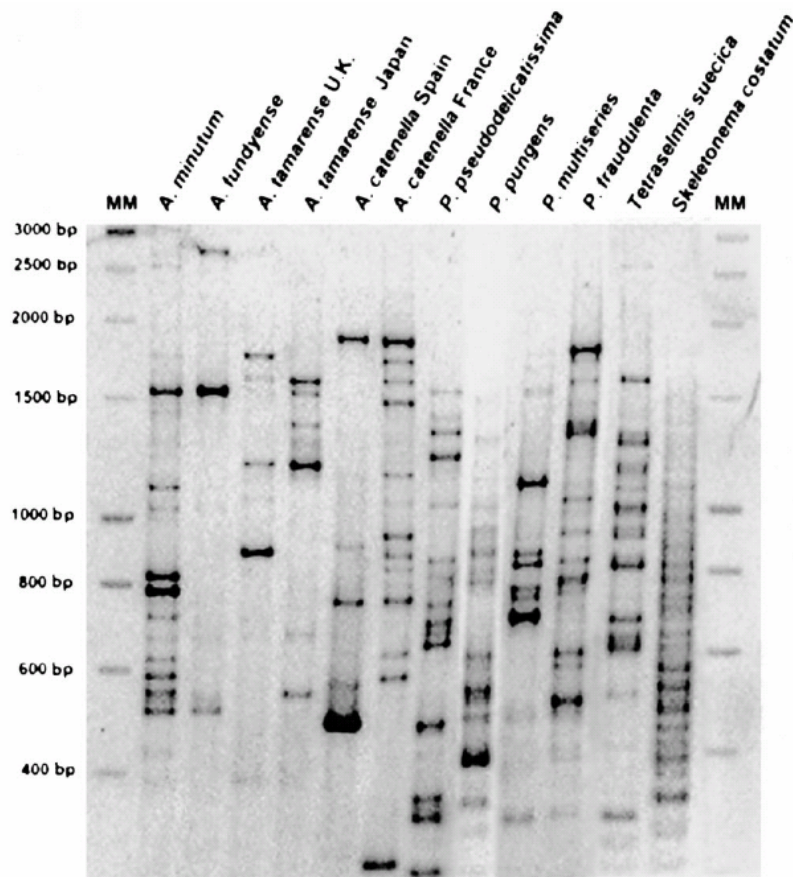
Figure 1. ISSR patterns of the 12 strains of phytoplankton (dinoflagellates, flagellate, diatoms) amplified with primer (ATG)5.
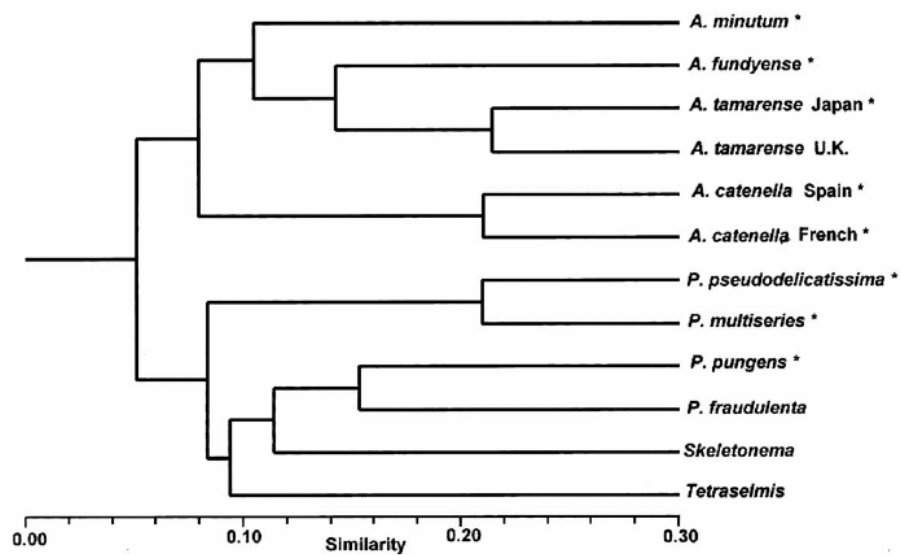

Figure 2. Consensus cluster analysis of the 12 cultivated strains of toxic (*) and non-toxic phytoplankton using ISSR fingerprints data (223 polymorphic markers) from a Jaccard similarity matrix and the UPGMA method.

Discussion

ISSR fingerprints were previously amplified, with more or less success, in animal and higher plants using different approaches. In this study, the use of ISSR fingerprints, described by Bornet and Branchard (2001), needed some slight modifications to give optimal patterns in phytoplankton because of the genetic and chemical characteristics of micro-algae. Six primers were selected and have shown the ability of ISSR markers to provide robust, complete and polymorphic fingerprints among micro-algal species (Table 1 and Figure 1). Primer $(ATG)_5$ proved to be the best one due to the sharpness of its patterns and the high number of polymorphic markers provided. ISSR amplification denoted a very low abundance of CAA repeats in the *Alexandrium* genomes and an unequal abundance of CCA, CAG, ATG, GACA and CT repeats in *Alexandrium* and *Pseudonitzschia* genomes. SSR were less abundant in *Alexandrium* than in *Pseudonitzschia*.

ISSR fingerprints clearly distinguished all the tested species. They allowed identification from genus level to geographical species level and allowed to separate toxic from non toxic strains of the same species. Considering *Alexandrium* and *Pseudonitzschia* strains as forming two populations, ISSR markers were more abundant and homogenous within *Pseudonitzschia*. However, their efficiency for genetic population purpose were quite identical within *Pseudonitzschia* and *Alexandrium* (especially primer $(CAA)_5$ and $(GACA)_4$), even if less strains were used for *Pseudonitzschia* (Table 2). When compared to RAPD, another multi-loci and PCR-based method, ISSR amplifications gave more markers and showed a higher level of polymorphism between phytoplankton species (Adachi et al. 1997, Murayama-Kayano et al. 1998, Bolch et al. 1999). These observations are in agreement with many studies showing the higher reproducibility and efficiency of ISSR markers (e.g. Nagaoka and Ogihara 1997, Devarumath et al. 2002, Galvan et al. 2003). This confirms the superiority of ISSR over RAPD to study phytoplankton species. ISSR technique is also more economical than other molecular marker fingerprinting methods (RAPD, RFLP, AFLP, SSCP or SSR).

ISSR fingerprints appeared to be a useful, quick and inexpensive molecular tool to solve the problems of morphological identification and strain characterization of toxic phytoplankton species especially encountered for *Pseudonitzschia* or *Alexandrium* species. For example, the identification of different strains from the same species of *Alexandrium* was impossible or very difficult based on optical observations. If *A. minutum* can be easily distinguished from *A. tamarense* and *A. catenella* due to their significant cell sizes variation but *A. tamarense* and *A. catenella* species belonging to the "tamarense complex" (*A. tamarense*, *A. catenella* and *A. fundyense*) need thecal plate dissection and microscope examination to be distinguished. Morphological observations also show that *A. catenella* and *A. fundyense* are more closely

related to each other than to *A. tamarense* (Walsh et al. 1998). Moreover molecular studies species based on rDNA (either by RFLP analysis or by sequencing) have led to more or less specific genetic profiles and sequence signatures (Scholin et al. 1994, Walsh et al. 1998, Guillou et al. 2002), no clear identification of the species from the "tamarense complex" has been achieved. ISSR fingerprints, amplified with each of the 6 primers, were able to distinguish all the strains and to discriminate between the two strains *A. catenella* and *A. tamarense*, including the toxic one from Japan and the non-toxic one from U.K. ISSR markers described in this study also proved to be powerful tools for the identification of cultured strains of *Pseudonitzschia*. For example, Scholin et al. (1999) easily detected *P. australis* and *P. pseudodelicatissima* by RNA probing analysis but they had some difficulties to identify *P. multiseries* and failed to identify *P. pungens*. All species of *Pseudonitzschia* were quickly and easily characterized with each primer.

   The ISSR markers were used to evaluate their efficiency to study genetic relationships among phytoplankton. Two main groups were observed and few differences in the clustering have been observed between ISSR data and rDNA data: for example, Scholin et al. (1994) have observed more similarities between *A. tamarense* and *A. fundyense* than between *A. tamarense* and *A. catenella* with the exception of *A. tamarense* from Western Europe. They also have shown a high divergence between *A. minutum* and *A. tamarense/A. fundyense/A. catenella.* Walsh et al. (1998) have not observed differences between *A. tamarense* and *A. fundyense* and found more similarity with *A. catenella* than with *A. minutum*. Considering *Pseudonitzschia* species, Lundholm et al. (2002) have grouped *P. pseudodelicatisima* with *P. fraudulenta* and *P. multiseries* with *P. pungens*. This can easily be explained by the different targeted regions and the high polymorphism level of scanning regions by the ISSR analysis.

   ISSR were used for the first time to characterize and to evaluate the genetic diversity within some marine phytoplankton including species causing toxic harmful blooms. ISSR-PCR gave complete, very reliable, reproducible and highly polymorphic fingerprints between phytoplankton species. This study showed the usefulness of ISSR fingerprints over rDNA data for phytoplankton analyses. ISSR amplifications also open new and interesting possibilities in the phytoplankton characterization field. In the future, the use of ISSR should be enlarged, for example, 1) for Harmful Algal Bloom monitoring by species identification of isolated cells, 2) to study genetic relationships among more species and genera to compare results with all the previous data, 3) to the management of species collections especially for strains identification or genetic stability (detection of somaclonal variations), 4) to creen quickly the most abundant SSR motifs in order to develop microsatellite markers.

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