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THE PHYLOGENETIC TREE OF *ALEXANDRIUM*, *PROROCENTRUM* AND *PSEUDO-NITZSCHIA* OF HARMFUL AND TOXIC ALGAE IN VIETNAM COASTAL WATERS BASED ON SEQUENCES OF 18S rDNA, ITS1-5.8S - ITS2 GENE FRAGMENTS AND SINGLE CELL – PCR METHOD

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ABSTRACT

Molecular biological techniques support the identification of microalgae of Vietnam. *Prorocentrum*, *Alexandrium* and *Pseudo-nitzschia* are main harmful and toxic microalgal genera found in Vietnam coastal waters. The results of morphology and nucleotide sequence analysis of 18S rDNA and ITS1-5.8S-ITS2 gene obtained from genomic DNA have shown that the *Prorocentrum* sp. 3 (isolated from Cat Ba, Hai Phong on October, 2004), *Alexandrium* sp. 5 (collected on October, 2004) and *Pseudo-nitzschia* sp. G3 (colleted in Do Son, Hai Phong on December, 2005) belonging to *Prorocentrum mexicanum* (the homological percent of 99.9% with sequencing of *P. mexicanum* in Genbank of Y16232, AY886763), *A. minutum* (99.8% - AJ535388, DQ168664) and *Pseudo-nitzschia pungens* (98.8% - AY544769, DQ166533), respectively. The obtained results indicated that the exceptional fresh samples, for *Prorocentrum* genus which may be preserved at 25% ethanol, 4% formaldehyde, 1% glutaraldehyde, while *Alexandrium* genus – at 25% ethanol, and *Pseudo-nitzschia* genus - 4% formaldehyde and 1% glutaraldehyde for two weeks have not effected on their analysis of sequences generated by Single Cell PCR method. In these studied samples, the nucleotide sequences obtained from genomic DNA and Single-cell PCR methods were the same with the homological percent more than 99%. Application of this method to samples collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006 showed that *Prorocentrum mican* and *P. sigmoisdes* were found.

Keywords: *Prorocentrum*, *Alexandrium*, *Pseudo-nitzschia*, phylogenetic analysis, SC-PCR method

INTRODUCTION

Although Vietnam has a great biodiversity in freshwater and marine microalgae, studies on harmful microalgae began only a few years ago. In general, for harmful algae, a list of harmful and toxic algal species including the distribution and cell density of mainly dinoflagellate (genera *Alexandrium*, *Prorocentrum*), diatoms (genus *Pseudo – nitzschia)* and cyanobacteria (genus *Trichodesmium*) was just given by Nguyen & Doan, (1997) and Nguyen, (2004). Therefore, having an overview of microalgae resources as species composition, distribution, and available stock and taxonomic system especially is a necessary requirement. Identification at species rank in these genera are difficult, because identification criteria used in conventional taxonomic system, i.e. morphological difference among species, are not easily observed under regular laboratory condition. It needs observation under an electron or a light microscope with specific sophisticated attachments such as an epifluorescence or a differential interference contrast. Without them critical identification is

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almost impossible (Ki and Han, 2005). However, these works have met a serious problem with species having similar morphological features that is easily changed by environmental factors (Hallegraeff *et al.*, 2004). The recent trend in the world, taxonomy and phylogenetic studies based on not only the morphological characters but also on the Ribosomal DNA (rDNA) analysis. The accumulation of species specific gene sequence data in these genera provides useful and powerful molecular biological method for their identification.

rDNA consist conservative genes; however, there were certain of the rDNA, which were highly variable. These variable regions generally occur in the spacer sequences – an external transcribed spacer (ETS), two internal transcribed spacers (ITS1/ITS2) and intergenic spacer (IGS). They have proved useful tools for species identification. In this paper, the morphological and molecular studies of some species belonging to Dinoflagellates and diatom collected in Northern coast of Vietnam are presented. Observations with microscope showed that our studied species belong to *Prorocentrum* spp., *Alexandrium* spp., and *Pseudo-nitzschia* spp. As a sequence, we have used partial gene of 18S rDNA, the ITS region (ITS1 and ITS2) which are intervened by 5.8S rDNA - a coding region to determine clearly and generate a phylogenetic framework for these species. The two non-coding regions exhibit greater genetic variation than the coding regions and have used for phylogenetic analysis at the intraspecific, interspecific, and intergeneric level (Coleman and Mai, 1997). Recently, Single cell PCR (SC-PCR) method was applied, because it can determine the presence of the harmful or toxic algal species without culturing them and is probably the most rapid and convenient method. In this paper, species belonging to *Prorocentrum*, *Alexandrium* and *Pseudo-nitzschia* isolated from Haiphong coast, Northern part of Vietnam, in 2004 and 2005 had been used. Success in getting the sequence was also compared among plankton samples fresh or preserved at different conditions such as 4% formaldehyde, 25% ethanol, 1% glutaraldehyde for two weeks. SC-PCR method have also applied to identify samples collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006, *Prorocentrum micans* and *P. sigmoides* were found.

MATERIALS AND METHODS

Strains and Culturing Conditions

The cells of *Alexandrium* sp. 5 and *Prorocentrum* sp. 3 used in this study were isolated from shrimp ponds in Do Son, Hai Phong, Vietnam in October 2004 and *Pseudonitzschia* sp. G3 from Cat Ba, Hai Phong, Vietnam in December 2005. *Prorocentrum micans*, *P. sigmoides* were collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006. Unialgal culture of *Prorocentrum* sp. 3, *Alexandrium* sp. 5 and *Pseudonitzschia* sp. G3 were obtained by isolation of single cell by micropipette, followed by several rinsed in sterile culture medium. Cells were cultured in f/2-enriched seawater (Fritz Chemical Co., Dallas, Tex.). The alga was grown in batch cultures at 20^{0}C under a 12:12 light: dark cycle and density a photon flux of ca. 100μmol m^{-2}s^{-1}. Cell densities were estimated by averaging the numbers cells counted by light microscopy in three to five separate 5 ml in Lugol's iodine solution.

Morphological Identification

The valve morphology of *Pseudo-nitzschia* sp. G3 was examined as described previously (Lundholm *et. al.*, 2003; 2006; Dang *et al.*, 2007). Species was identified following Skov *et al.*, (1999), after determining the width of cells and the density of poroid rows, poroids, fibulae, and interstriae of at least 30 randomly selected cells.

Samples of *Prorocentrum* sp. 3 and *Alexandrium* sp. 5 were examined under a microscope using Light Microscope (LM), scanning electron microscope (SEM) with model JSM-5410LV (Jeol Company, Japan) and epifluorescence (Olympus BX51). For epifluorescence, fixed samples were stained with calcofluor white (Sigma) and viewed under UV with a UV filter set. Images were captured with a cooled CCD camera (SIS Color view F12, Germany).

DNA Extraction, Amplification, Purification, Cloning and Sequencing of PCR Products

For DNA extraction, cultured cells of *Prorocentrum* sp. 3, *Alexandrium* sp. 5 and *Pseudonitzschia* sp. G3 in exponential growth phase were harvested and the supernatant was removed according to reports of Dang *et al.*,

(2005, 2007); Ngo *et al.*, (2005) and Hoang *et al.*, (2006). Maintenance of genomic DNA preparation was described in Dang *et al.*, 2002. The length of PCR product of *Prorocentrum* sp. 3 and *Alexandrium* sp. 5 were approximately 1.1kp (partial 18S rDNA gene fragments) and of *Pseudonitzschia* sp. G3 – 825bp (of ITS1-5.8S-ITS2 gene). The PCR mixture (20 ìL) contained 2 ìL 10X PCR buffer, 0.75 mM of deoxyribonucleoside triphosphate (dNTP) mixture (Takara, Japan), 0.5 mM of each primer, 50-100 ng of genomic DNA, 0.075 units of *Taq* DNA polymerase and distilled water. The amplification parameters were 94^{0}C for 3 min, followed by 35 cycles of 94^{0}C for 30 second, 55^{0}C for 1 min, 72^{0}C for 1 min and, finally, 72°C for 5 min, and stored at 4^{0}C. To confirm the presence of amplified DNA fragments of partial 18S rDNA and ITS, PCR products were separated electrophoretically on 1.0% agarose gel in 1X TAE buffer stained with ethidium bromide (0.5ìg/mL). Primers selected to generate PCR products of *Prorocentrum* sp. 3, *Alexandrium* sp. 5 and *Pseudo-nitzschia* sp. G3 according to that described by Dang *et al.*, (2005, 2007); Ngo *et al.*, (2005) and Hoang *et al.*, 2006.

The PCR products were purified from agarose gel with a Centriguge Kit (Promega, USA). Purified DNA was cloned into the Gene JET™ PCR cloning Kit (Fermentas, USA) and the plasmid was introduced by heat shock treatment into competent *Escherchia coli* DH5á. Transfomants were screened by transferring single white colonies according to previously reported (Dang *et al.,* 2007). Plasmid DNA was isolated using GFX ™ micro plasmid prep Kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Sequencing of the cloned PCR inserts was carried out with a Big Dye (R) Terminator v3.1 Cycle Sequencing Ready Reaction Kit and Autosequencer - ABI PRISM 3100 Avant genetic Analyzer (USA) with pJET1 forward and pJET1 reverse. Both strands of at least two different recombinants were sequenced to check for PCR errors generated in the amplification procedure. The nucleotide sequences were analyzed using the Clustal X 1.81 and BLAST programs.

Light microscopy, photographic record, SC-PCR amplification, cloning, and sequencing

Individual cells of *Prorocentrum* sp. 3, *Alexandrium* sp. 5, *Pseudonitzschia* sp. G3 were isolated from cultural samples and washed in sterilized distilled water using a micropipette under a microscope. For observation and photographic record of single cells, we used a glass slide with a frame of vinyl tape (Horiguchi *et al.*, 2000). Cells were transferred to a drop of distilled water placed in the center of a frame on a slide glass and covered with a cover slip. The cells were observed and photographed. After taking photographs, the cover slip was carefully removed. Under a microscope the cells were picked up and broken with a fine glass needle. The whole broken cell was transferred to 500 μl PCR tubes containing PCR premix. This technique allowed us to keep records of the cells used for SC-PCR.

The PCR protocols, amplification primer and sequencing protocols used in this study were a modification of those described by Nakayama *et al.*, (1996); Takano and Horiguchi, (2004) for SSU rDNA. In the first round of PCR, the single cells were used directly as template to amplify approximately 1762bp of the SSU rDNA using the terminal primers SR1F and SR12R (Table 1). In

Table 1. Oligonucleotide primers used for amplification and sequencing.

Code	Synthesis direction	Sequende (5'→ 3')	Anneals to *
SR1F	Forward	TACCTGGTTGATCCTGCCAG	1-20
SR2kawF	Forward	AAGTTTCTGACCTATCAGCT	321-302
SR3R	Reverse	AGGCTCCCTGTCCGGAATC	394-376
SR4F	Forward	AGGGCAAGTCTGGTGCCAG	548-566
SR5kawR	Reverse	ACTACGAGCTTTTTAACCGC	630-611
SR6F	Forward	GTCAGAGGTGAAATTCTTGG	891-910
SR7R	Reverse	TCCTTGGCAAATGCTTTCGC	951-932
SR8KAWF	Forward	GGATTGACAGATTGATAGCT	1224-1243
SR9R	Reverse	AACTAAGAACGGCCATGCAC	1286-1267
SR10F	Forward	AGGTCTGTGATGCCCTTAGA	1420-1439
SR11R	Reverse	CGCTTACTAGGAATTCCTCG	1582-1563
SR12R	Reverse	CCTTCCGCAGGTTCACCTAC	1781-1762

* Annealing site in the 18S rDNA of *Volvox carteri* (Rausch *et al.*, 1989)

the second round of PCR, 1μl of PCR product was used as template, and we used three or six pair of primers such as: SR1F-SR5KawR (630bp was amplified) or SR4F-SR9R (800bp was amplified) or SR8KaWF-SR12R (550bp was amplified) (Table 1).

The PCR conditions for first rounds were one initial cycle of denaturation at 95^{0}C for 10 min, followed by 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 55^{0}C for 1 min and extension at 72^{0}C for 2 min. The temperature profile was completed by a final extension cycle at 72^{0}C for 10 min. The PCR conditions for the second rounds were one initial cycle of denaturation at 95^{0}C for 10 min, followed by 45 cycles of denaturation at 94^{0}C for 30 s, annealing at 55^{0}C for 30s and extension at 72^{0}C for 1 min. The temperature profile was completed by a final extension cycle at 72^{0}C for 10 min. These PCR products were cloned according to describe in the part above mentioned.

Sequence Analysis

For sequence analysis, the data obtained, with additional 21 and 23 accessed sequences of *Alexandrium*, *Procentrum* and *Pseudo-nitzschia*, respectively, from GenBank for comparison (Table 2), were aligned with the Clustal X program (Thompson *et al.* 1997). Two-parameter distances were estimated and the phylogenic tree was inferred with the neighbor-joining (NJ) method using the MEGA program, version 2.1 (Kumar *et al.*, 2004; Saito and Nei, 1987). The bootstrapping method was performed with 1000 repetitions for NJ analysis. In this case, *Noctiluca scintillans* (GenBank accession no. AF022200), *Peridinium* sp. (AF022202), *Pyrocystis notiluca* (AF022156) and *Sarcocystis muris* (M64244) was used as outgroups (Gajadhar *et. al.*, 1991).

RESULTS

Morphological study of *Prorocentrum* sp. 3, *Alexandrium* sp. 5 and *Pseudo-nitzschia* sp. G3

***Prorocentrum* spp.** The characteristics of the species and specific characters of the strains collected from Do Son, Hai Phong and Phu Quoc Island, Kien Giang province were identified by LM and SEM (Fig. 1). According to the observations of Faust (1999) and Cohen-Fernandez *et al.*, (2006), *Prorocentrum* sp. 3 collected from Do Son, Hai Phong belongs to species *P. mexicanum* (Fig. 1A), species collected from Phu Quoc Island, Kien Giang province in 27-29, June, 2006 belong to the species *P. micans* and *P. sigmoides* (Fig. 1B and C).

***Alexandrium* sp. 5** Most species of *Alexandrium* have been described from natural material, and several of these are delimited by very minor deviations in plate morphology. In addition to the general characters such as size, shape, and chain formation, the morphology of particular the 1', 6", apical pore complex, and the posterior sulcal plates are important for species identification. By morphological observation, *Alexandrium* sp.5 isolated from Hai Phong seems to be considered under a species name, *A. minutum* (Fig. 1D, 1E and 1F). The present species is assigned to *A. minutum* because of the cell size and the shape of the 1', 6', and posterior sulcal plates. In the Vietnamese material, some cells appears to be slightly wider than long. *A. minutum* was found in few samples in the Thua Thien – Hue, Qui Nhon Bay in Binh Dinh Province, and Cam Ranh Bay, Nha Trang. It has previously been reported from Ha Long Bay (Yoshida *et al.*, 2000). It is widely distributed.

***Pseudo – nitzschia* sp. G3** The observation under light microscopy of *Pseudo – nitzschia* sp. G3 isolated from Do Son, Hai Phong, Vietnam seem to be *P. pungens* (Grunow *ex* Cleve) Hasle. The frustules are symmetrical, linear to lanceolate in both valve and girdle view, the last 1/4 of the cell sides are tapering towards the apices. The apices are rounded. The cell length is 80-116 µm, the width is 2-3.8 µm. The valves are strongly silicified. A central interspace is absent. Cell overlap 1/4 - 1/3 (data not show here).

Under TEM, the number of interstriae and fibulae in 10µm is (8)-9-12-(13) and 9-13-(15), respectively. There are consistently two rows of round periods and 2-3-(4) in 1 µm (Fig. 1G).

The observations of *P. pungens* from Vietnamese waters are in good accordance with findings from other parts of the world (Skov *et al.*, 1999). The cell shape and morphometric measures of cultured material did not change compared to natural material. *P. pungens* belongs in the *seriata*-group, but narrow cells may be less than 3 µm wide. In this case, it may be confused with and *P. cuspidate* or *P. pseudodelicatissima*, but both have a central interspace. *P. pungens* is

Table 2. Strains of *Prorocentrum, Alexandrium and Pseudo-nitzschia* species used in the phylogenetic analysis, with origin of isolate, GenBank accession number, and citation.

Species	Sample designation	Origin	Source	Accession number	Reference
Prorocentrum					
P. concavum Fukuyo	PPAN04	Contadora Island, Gulf of Panama, Pacific coast 8°389' N, 79°02' W	B. Berland.	Y16237	Grzebyk *et al.*, 1998
P. emarginatum Fukuyo	PREU2	Re´union Island, S.W. Indian Ocean 21°10' S, 55°17' E	B. Berland.	Y16239	Grzebyk *et al.*, 1998
P. lima (Ehrenberg) Dodge	#151	Tokushima, Mugi Ooshima, Japan, Pacific coast 33°37' N, 134°29' E	S. Yoshimatsu	Y16235	Grzebyk *et al.*, 1998
P. maculosum Faust	PPAN20	Contadora Island, Panama, 8°38' N, 79°02' W	B. Berland.	Y16236	Grzebyk *et al.*, 1998
P. micans	clone pr10	Proc. Natl. Acad. Sci. U.S.A. 83, 8644-8648 (1986)	M. Herzog	M14649	Herzog *et al.*, 1986
P. minimum (Pavillard) Schiller	PmS1	Se`te, French Mediterranean coast 43°24' N, 3°42' E	B. Berland	Y16238	Grzebyk *et al.*, 1998
P. mexicanum Osorio Tafall					
	PMOO04	Moorea Island, French Polynesia, Pacific Ocean 17°30' S, 149°50' W	B. Berland	Y16232	Grzebyk *et al.*, 1998
	sp. 3*	Do Son, Hai Phong (Vietnam)	D. Hong	DQ174089	Dang *et al.*, 2005
Alexandrium					
A. affine (H. Inoue & Y. Fukuyo) E. Balech	CCMP112			AJ535375	John *et al.*, 2003
A. catenella (Whedon and Kofoid) Balech	BAHME217	Tarragona (Spain)	M. Delgado	AJ535392	John *et al.*, 2003
A. leei E. Balech	JHW0006-2			AY641565	Kim *et al.*, 2005
A. margalefii Balech	ALexmarg			U27498	John *et al.*, 2003
A. minutum Halim					
	AL3T	Gulf of Trieste (Italy)	A. Beran	AJ535388	John *et al.*, 2003
	Sp. 5*	Do Son, Hai Phong (Vietnam)	D. Hong	DQ168664	Ngo *et al.*, 2005
A. monilatum (Howell) F.J.R. Taylor	JR07			AY883005	Direct Submission
A. ostenfeldii (Paulsen) Balech and Tangen	BAHME136	Timaru (New Zealand)	N. Berkett	AJ535383	John *et al.*, 2003
A. tamarense (Lebour) Balech	Alextama			X54946	John *et al.*, 2003
A. tamiyavanichii Balech					
	Atamiy			AF113935	John *et al.*, 2003
	TAMI2207, type H			AB088325	Kim *et al.*, 2004
A. sp. tamutum	SZN28			AJ535379	John *et al.*, 2003
A. taylorii Balech	AY1T	Lagoon of Marano (Italy)	A. Beran	AJ535390	John *et al.*, 2003
Pseudonitzschia					
P. pseudodelicatissima (Hasle) Hasle	P-11	Gafahna, Portugal	N. Lundholm	AY257854	Lundholm *et al.*, 2003

Table 2 (Continued)

Species	Sample designation	Origin	Source	Accession number	Reference
P. calliantha Lundholm, Moestrup et Hasle 2003					
	DS2	Do Son, North Vietnam	J. Skov	AY257856	Lundholm *et al.*, 2003
	HA-D4	Ha Long Bay, North Vietnam	J. Skov	AY257857	Lundholm *et al.*, 2003
	TA-1	Thuan An, Central Vietnam	J. Skov	AY257855	Lundholm *et al.*, 2003
P. cuspidata (Hasle) Hasle 1993	Tenerife8	Tenerife, Canary Islands	N. Lundholm	AY257853	Lundholm *et al.*, 2003
Pseudo-nitzschia sp. Hobart5	Hobart5	Hobart, Tasmania, Australia	L. Holtegaard	AY257851	Lundholm *et al.*, 2003
P. caciantha Lundholm, Moestrup and Hasle 2003	Mex20	Near Tuxpam, Mexico	N. Lundholm/ Ø. Moestrup	AY257861	Lundholm *et al.*, 2003
P. delicatissima (Cleve) Heiden	LæsØ 5	Kattegat, Denmark	N. Lundholm	AY257849	Lundholm *et al.*, 2003
P. fraudulenta (Cleve) G.R. Hasle	Limens1	Limens, Spain	K. GrotØl/N. Lundholm	AY257840	Lundholm *et al.*, 2003
P. galaxiae Lundholm et Moestrup	Mex23	Near Tuxpam, Mexico	N. Lundholm/ Ø. Moestrup	AY257850	Lundholm *et al.*, 2003
P. micropora Priisholm, Moestrup and Lundholm	VPB-B3	Van Phong Bay, Vietnam	J. Skov	AY257847	Lundholm *et al.*, 2003
P. multiseries (Hasle) Hasle	Mu3	Monterey Bay, CA, USA	P. miller, C. Scholin	AY257844	Lundholm *et al.*, 2003
P. multistriata	Korea A	Chinhae Bay, Korea	E. Cho	AY257843	Lundholm *et al.*, 2003
P. seriata (Hasle) Hasle	Nissum3	Nissum Bredning, Denmark	N. Lundholm	AY257841	Lundholm *et al.*, 2003
P. cf. *subpacifica*	RdA8	Ria de Arousa, Spain	N. Lundholm	AY257860	Lundholm *et al.*, 2003
P. turgiduloides G.R. Hasle	3-19	Ross Sea, 75°59'S, 145°019'W	N. Lundholm / N. Daugbjerg	AY257839	Lundholm *et al.*, 2003
P. pungens (Grunow ex Cleve) Hasle					
	G3*	Do Son, Vietnam	D. Hong	DQ166533	Dang *et al.*, 2007
	P-24	Costa Nova, Portugal	N. Lundholm	AY257845	Lundholm *et al.*, 2003
	Mex18	Near Tuxpam, Mexico	Ø. Moestrup	AY257846	Lundholm *et al.*, 2003
P. australis Frenguelli	PLYSt54B		J. Fehling	AY452528	Fehling *et al.*, 2004
P. inflatula (G.R. Hasle) G.R. Hasle	No7	Phuket, Thailand	K. Priisholm	DQ329204	Lundholm *et al.*, 2006
P. dolorosa Frenguelli	BP2	Boca Piccola, Italy	N. Lundholm	DQ336155	Lundholm *et al.*, 2006
P. cf. *australis*	6/24/03 B	Monterey Bay, CA, USA	H. A. Bowers	AY559850	Direct Submission
Pyrocystis noctiluca Murray ex Haeckel	Pyrocyst			AF022156	John *et al.*, 2003
Noctiluca scintillans (Macartney) Kofoid et Swezy	Noct.ilu			AF022200	John *et al.*, 2003
Peridinium sp.				F022202	Saunders *et al.*, 1997
Sarcocystis muris	SARRR16S			M64244	Gajadhar *et al.*, 1991

* The isolated strain in this study

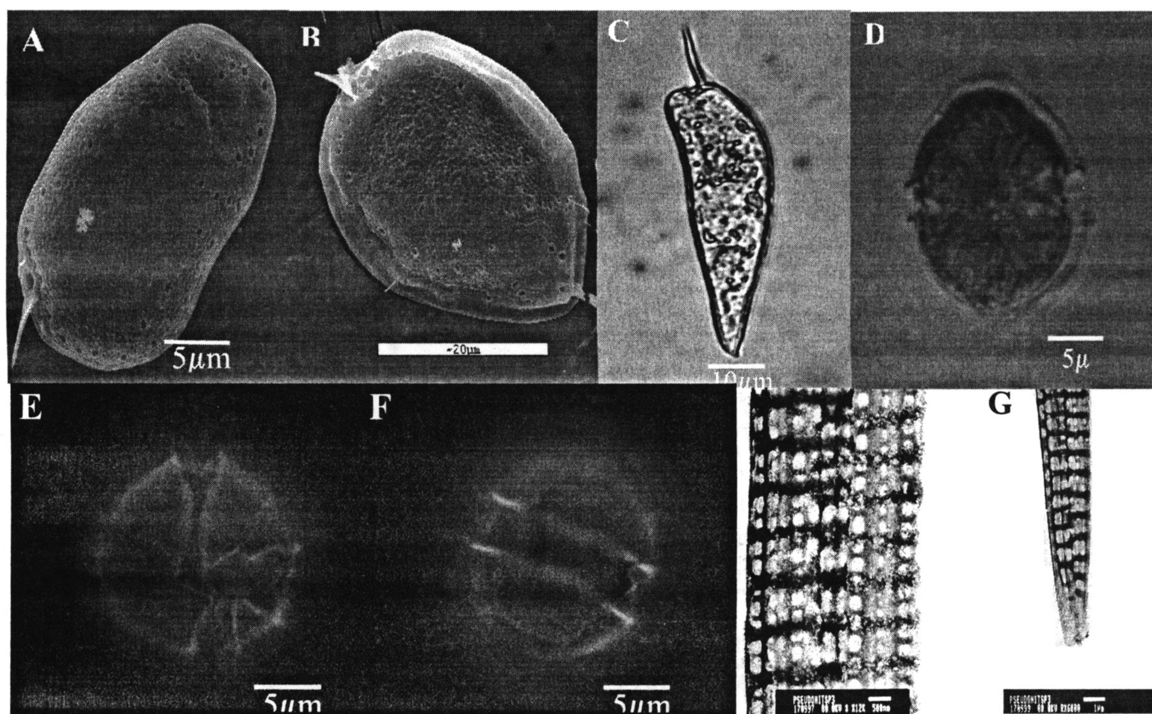

Figure 1. Morphological image of *Prorocentrum mexicanum*, *P. mican*, *P. sigmoides*, *Alexandrium minitum*, *Pseudo-nitzschia pungen*. (**A**) SEM image of *Prorocentrum mixecanum* strain, isolated from Do Son, Hai Phong city (Vietnam). (**B**) SEM image of *Prorocentrum mican* isolated from Phu Quoc Island, Kien Giang province (Vietnam). (**C**) LM image of *Prorocentrum sigmoides* isolated from Phu Quoc Island, Kien Giang province (Vietnam). (**D**) LM image of *Alexandrium minimum* shows the cell shape and cingular displacement. (**E, F**) Epi-fluorescent micrographs of *Alexandrium minimum*. (**G**) TEM image of *Pseudo-nitzschia pungen*.

very common species in Vietnam coastal waters. It occurred more or less all year round at all the sites from the north to the south of Vietnam. It was found at temperatures from 20 – 30^{0}C, and all salinities from 14.5 – 34.5 ‰. Fryxell and Haslle, (2004) indicate *P. pungens* as a neritic, cosmopolitan species.

Phylogenetic Analysis *of Alexandrium* sp. *5*, *Prorocentrum* sp. 3 Based on Sequences of 18S rDNA Gene Fragment

For reconstructing the phylogenetic tree were used the different sequences from DNA databases from FASTA searches such as 18S rDNA sequences of 21 dinoflagellate species of genera *Alexandrium* and *Prorocentrum*. In the reconstructions of dinoflagellate phylogeny (Fig. 2) using the apicomplexan *Sarcocystis muris, Peridinium* sp. and *Pyrocystis notiluca* as the outgroup, *Noctiluca scintillans* is found to be a primitive dinoflagellate, as previously reported from other molecular data (Sauder *et al.* 1997).

Genetic homogenous coefficient matrix and phylogenetic tree have showed *Prorocentrum* sp.3 have the highest homogeneous coefficient with *P. mexicanum* (99.8%), then with *P. minimum* (99.5%), *P. micans* (99.2%), *P. concavum* (96.6%), *P. emarginatum* (95.9%), *P. maculosum* (94.3%), *P. eridinium* sp. (93.9%), *P. lima* (93.8%). On the phylogenetic tree, *Prorocentrum* sp. 3 possesses length of equal evolutionary branches in *P. mexicanum* and lying beside *P. mexicanum*. Thus based on genetic homogeneous coefficient of 18S rDNA sequence and distribution on phylogenetic tree, the obtained result allowed us to make the conclusion that *Prorocentrum*. sp. 3 was *P. mexicanum*.

Homogeneous level between species belonging to *Alexandrium* genus was from 92.5% (between *A. monilatum* and *A. leei*) to 99.9% (between *Alexandrium* sp.5 and *A. minutum* and *A. affine*; *A. minutum* and *A. ostenfeldii*). NJ phylogenetic tree was constructed using method of Kimura of 2 parameters (Kimura, 1980). All species of *Alexandrium* genus were devised into two groups.

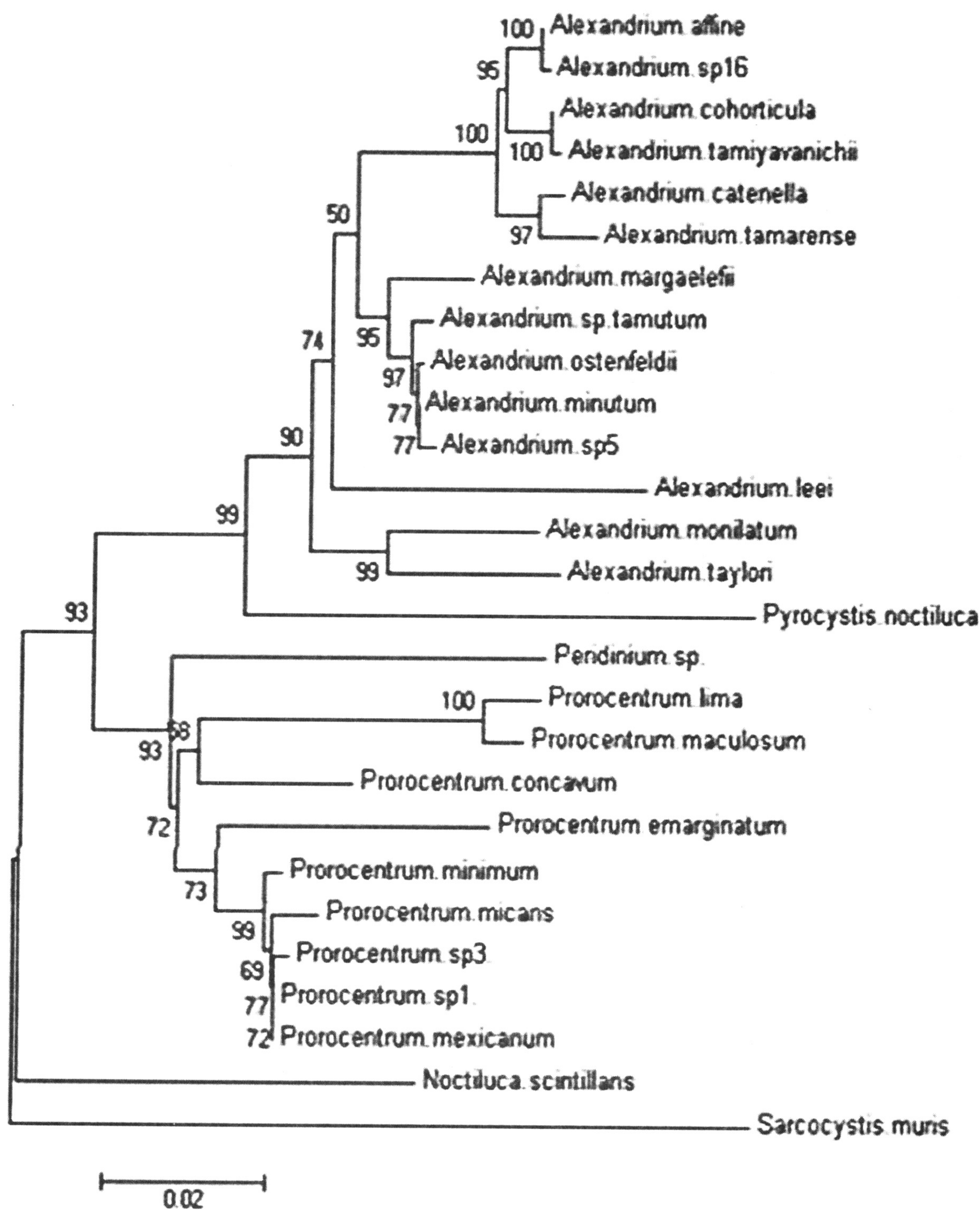

Figure 2. Phylogenetic relationship of *Prorocentrum* and *Alexandrium* species based on the completely aligned 18S rDNA sequences (21 strands). DNA sequences determined in this study are in underline. The tree was constructed using the Kimura 2-parameter method (Kimura *et al.* 1980) and 1,000 bootstrapped replicates in MEGA3. *Pyrocystis noctiluca*, *Peridinium* sp., *Noctiluca scintillans*, *Sarcocystis muris* was included as outgroup. Bootstrap values of less than 50% are not shown. Branch lengths represent genetic distance among the taxa. The bar denotes substitution per nucleotide position.

The first group has only one species of *A. leei* which is very different from another species (homogeneous coefficient from 89.8% to 91.8%). The second group contains 2 subgroups: the first subgroup contains *Alexandrium* sp. 5, *A. minutum*, *A. ostenfeldii*, *A. tamutum* and *A. margalefii*. Sequences of *A.* sp. 5, *A. minutum*, *A. ostenfeldii* and *A.* sp. *tamutum* have very high homogeneous level (> 99.5%); the second subgroup consists of *A. affine*, *A. catenella*, *A. tamarense*, *A. cohorticula*, *A. tamiyavanichii*, *A. monilatum* and *A. taylori*.

The most highest homogeneous coefficient is between *Alexandrium* sp.5 and *A. minutum* (99.9%), then *A. ostenfeldii* (99.8%), *A.* sp. *tamutum* (99.5%), *A. margalefii* (98.5%), *A. affine* (97%), *A. cohorticula* (96.7%), *A. catenella* (96.6%), *A. tamiyavanichii* (96.6%), *A. monilatum* (96.3%), *A. tamarense* (95.9%), *A. taylori* (95.6%), *A. leei* (95%), *Pyrocystis noticula* (91.6%). In the phylogenetic tree (Fig. 2), species of *Alexandrium* sp.5 have length of evolutionary branch equal in species of *A. minutum* and lying beside *A. minutum*. The results of 18S rDNA sequence analysis clearly showed that *Alexandrium* sp.5 was *A. minutum*.

Sequences of 18S rDNA gene fragment of 2 studied species *Prorocentrum* sp.3 and *Alexandrium* sp. 5 collected from HaiPhong - Northern coast of Vietnam in this report were submitted to GenBank with accession number DQ174089 and DQ168664, respectively.

Phylogenetic Analysis of *Pseudo-nitzschia* sp. G3 based on ITS Region Sequences

The phylogenetic tree was reconstructed from the different ITS region sequences of 23 sequences of 19 *Pseudo-nitzschia* species shown on the Fig. 3.

Based on phylogenetic tree and genetic homogenous coefficient matrix, the identification of *Pseudo-nitzschia* was carried out. Homogeneous coefficient of *Pseudo-nitzschia* sp. G3 (DQ166533) with *P. pungens* (Grunow *ex* Cleve) Hasle (accession number AY257845 and AY257846) reached to the highest (98.75%), then with *P. multiseries* (Hasle) Hasle (83.2%), *P. cf autralis* Frenguelli (79.4%). Within the ITS1-5.8S-ITS2 region (693 bp), 9 base differences were found between *Pseudo-nitzschia* sp. G3 and *P. pungens* (AY257846) (such as positions 54, 86 and 135 in ITS1 region of 259 bp; 432 in 5.8S coding region of 171 bp; 455, 570, 605, 620 and 625 in ITS2 region of 263 bp). This represents a sequence dissimilarity (number of base differences divided by sequence length) of 0.0125. In addition, Fig. 3 showed that species of *Pseudo-nitzschia* sp. G3 (DQ166533) is beside *P. pungens*. The obtained results of ITS sequence analysis demonstrated clearly that *Pseudonitzschia* sp. G3 isolated from northern coast of Vietnam was *P. pungens* (Grunow *ex* Cleve) Hasle (bootstrap values 100%). The nucleotide sequence of partial 18S rDNA gene fragment and the ITS1–5.8S–ITS2 from the studied *P. pungens* species has been submitted to GenBank and given the following accession number DQ166533.

The Identification of *Alexandrium* sp. 5, *Prorocentrum* sp. 3 and *Pseudo-nitzschia* sp. G3 based on Sequences Obtained from SC-PCR Method

SC-PCR technique allows species identification rapidly and accurately from field samples. In this report, the application of SC-PCR method to *Alexandrium* sp. 5, *Prorocentrum* sp. 3 and *Pseudo-nitzschia* sp. G3 isolated from Hai Phong coast, northern part of Vietnam in October of 2004 and December of 2005 and *Prorocentrum* spp. collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006 were evaluated.

The primary aim of SC-PCR experiments was to identify preservative and fixation conditions for preserving cells without effect on the ability to amplify PCR products. Apart of fresh samples, cells of *Alexandrium* sp. 5, *Prorocentrum* sp. 3 and *Pseudo-nitzschia* sp. G3 have preserved at different conditions such as 4% formaldehyde, 25% ethanol, 1% glutaraldehyde for two weeks were tested to see if they were suitable as DNA templates. Except fresh cell for successfully SC-PCR method, positive PCR products could always be achieved from cells preserved in ethanol (25%) for *Alexandrium* sp. 5; formaldehyde (4%), ethanol (25%) and glutaraldehyde (1%) for *Prorocetrum* sp. 3 and formaldehyde (4%) and glutaraldehyde (1%) for *Pseudo-nitzschia* sp. G3 (Fig. 4 and 5). Success in getting the sequence was also compared among plankton samples fresh or preserved at different conditions for each genus

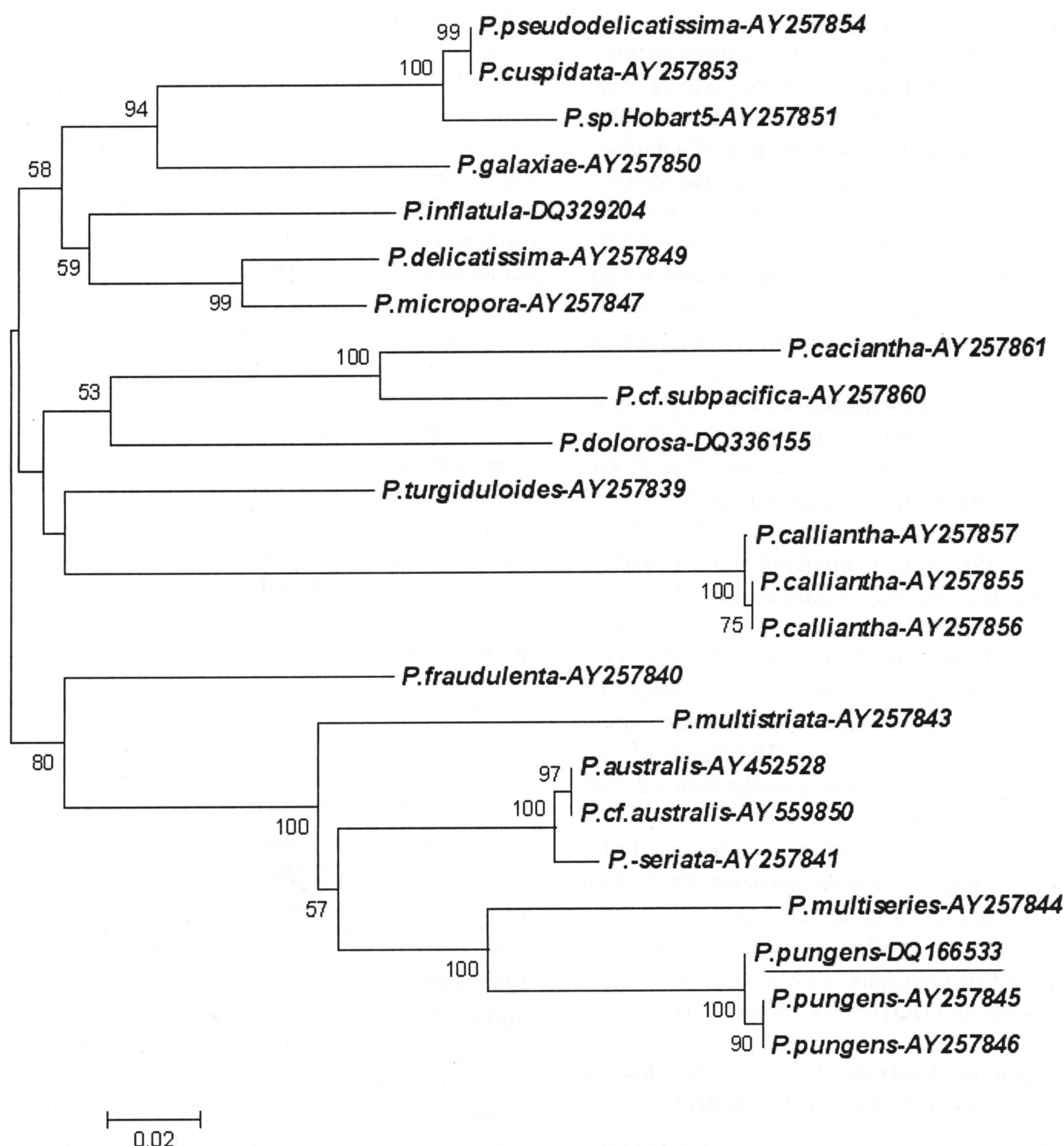

Figure 3. Phylogenetic tree of *Pseudo-nitzschia* species, based on the ITS region sequences (23 strands) with the neighbor-joining model and the Kimura 2-parameter. DNA sequences determined in this study are in underline, 1,000 bootstrapped replicates in MEGA3. Bootstrap values of less than 50% are not shown. Branch lengths represent genetic distance among the taxa. The bar denotes substitution per nucleotide position.

above mentioned such as 4% formaldehyde, 25% ethanol, 1% glutaraldehyde for two weeks.

In order to check and determine if any base alteration had occurred in both of the PCR based on the SC-PCR method or on genomic DNA of the same species, positive second round PCR products from *Alexandrium* sp. 5, *Prorocentrum* sp. 3 and *Pseudo-nitzschia* sp. G3 were cloned and sequenced. Alignment of the partial 18S rDNA gene between *Prorocentrum mexicanum* (Accession number: Y16232), *Prorocentrum* sp.3 generated by genomic DNA (with accession number DQ174089, designated by *Pro.* sp. 3_DNA_) and *Prorocentrum* sp. 3 generated by SC-PCR method (designated by *Pro.* sp. 3_Single_) showed *Prorocentrum* sp. 3 collected

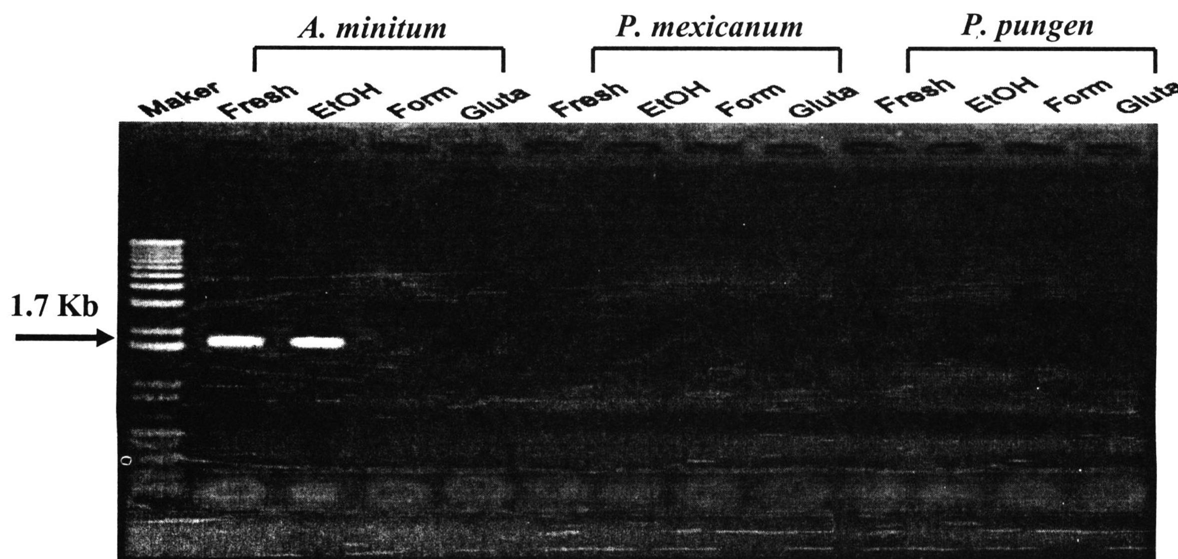

Figure 4. The effects of different preservative conditions on the first round of single cell PCR. Amplification products obtained from single cell of *Prorocentrum mexicanum*, *Alexandrium minitum*, *Pseudo-nitzschia pungen* with SR1F and SR12R primers pair. Marker contains the DNA size marker 1Kb Plus DNA ladder. Fresh designated by fresh cell, EtOH-cell preserved at 25% Ethanol; Form-cell preserved at 4% formaldehyde, Gluta-cell preserved at 1% glutaraldehyde.

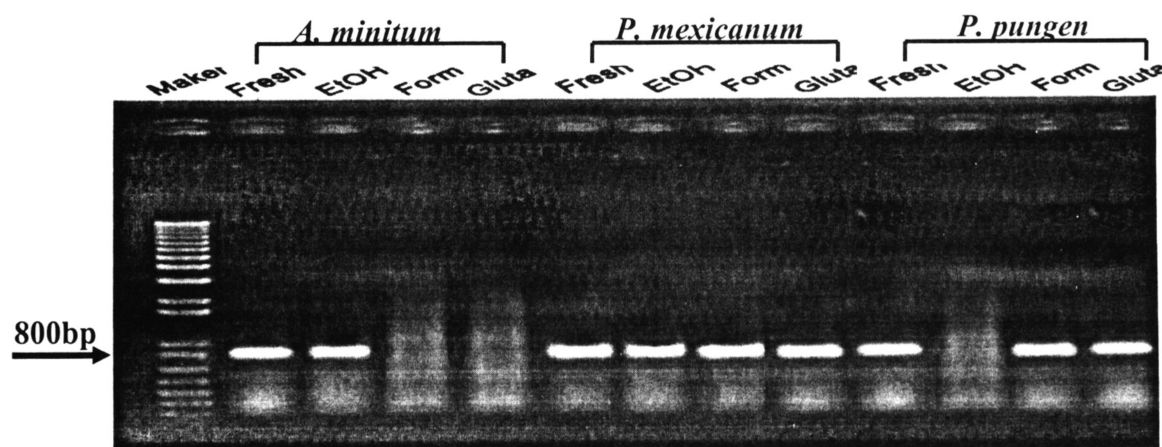

Figure 5. The effects of different preservative conditions on the second round of SC - PCR. Amplification products obtained from single cell of *Prorocentrum* sp. 3, *Alexandrium* sp. 5, *Pseudo-nitzschia sp.* G3 with SR4F and SR9R primer pair. Marker contains the DNA size marker 1Kb Plus DNA ladder. Fresh designated by fresh cell, EtOH-cell preserved at 25% Ethanol; Form-cell preserved at 4% formaldehyde, Gluta- cell preserved at 1% glutaraldehyde.

from Hai Phong has close phylogenetic relationship with the homological percent of 99.7% with *P. mexicanum* which have accession number Y16232 in GenBank and with the homological percent of 99.7% with *P.* sp. 3 generated by genomic DNA which have accession number DQ174089. Within the partial 18S rDNA gene fragment (800 bp), 2 base differences were found between *Prorocentrum* sp. 3 generated by genomic DNA and by SC-PCR method: 2 transitions (positions 65 and 722) (Table 3). Similar to the *Prorocentrum mexicanum*, sequences of *Alexandrium minitum* (with accession number: AJ535388), *A.* sp.5 generated by genomic DNA (with accession number DQ168664, designated *A.* sp.5 –DNA) and *A.* sp.5 generated by SC- PCR method

(designated *A.* sp.5-Single) were aligned. The results obtained in Table 3 showed that *Alexandrium* sp. 5 collected from Haiphong has close phylogenetic relationship with 99.6% with *Alexandrium minutum* which have accession number AJ535388 in Gen Bank and with 99,7% with *Alexandrium* sp. 5 generated by genomic DNA which have accession number DQ168664. Within studied the 18S rDNA gene fragment, 2 base differences were recognized between *Alexandrium* sp. 5 -DNA and *A.* sp. 5-Single such as in position 231 and 597.

The sequences of *Pseudo-nitzschia* sp. G3 collected from Hai Phong city which generated by SC-PCR method has close phylogenetic relationship with 98.8% with *Pseudo-nitzschia pungen* in GenBank (accession number AY257846) having 7 base difference at the position of 54, 135, 431, 454, 604, 619, 624; and 99.9% with *Pseudo-nitzschia* sp. G3 generated by genomic DNA in GenBank (accession number DQ166533) having 1 base difference at the position of 431.

In these studied samples, the nucleotide sequences that have obtained from genomic DNA and SC-PCR method were the same with the homological percent more than 99%.

SC-PCR method has also applied to identify successful samples collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006 and *Prorocentrum micans* and *P. sigmoides* were found (Fig. 1B and 1C). The nucleotide sequences of the partial 18S rDNA gene of *Prorocentrum micans* collected from Phu Quoc Island generated by SC-PCR method and by genomic DNA with *Prorocentrum micans* with accession number M14649 in GeneBank (Herzog *et al.*, 1986) were compared with the homological percent of 99.9%. Thus based on phylogenetic tree and homogeneous coefficient of 18S rDNA and the distribution to phylogenetic tree, *Prorocentrium* sp. isolated from Phu Quoc Island was considered as under a species name, *Prorocentrum micans*.

DISCUSSION

Prorocentrum, *Alexandirum* and *Pseudo-nitzschia* are main harmful and toxic microalgal genera found in Vietnam coastal waters. Identification at species rank in these genera is difficult, because identification criteria used in conventional taxonomic system, i.e. morphological difference among species, are not easily observed under regular laboratory condition. It needs observation under an electron microscope or a light microscope with specific sophisticated attachments such as an epifluorescence or a differential interference contrast. Without them critical identification is almost impossible. But accumulation of species specific gene sequence

Table 3. Location of different nucleotide positions in the 18 S rRNA gene sequences between *Prorocentrum mexicanum* (Accession number: Y16232), *Prorocentrum* sp.3 generate by genomic DNA (with accession number DQ174089, designated by Genomic DNA) and *Prorocentrum* sp.3 generate by SC-PCR method (designated by Single Cell) and between *Alexandrium minitum* (with accession number: AJ535388), *A.*sp.5 generate by genomic DNA (with accession number DQ168664, designated Genomic DNA) and *A.* sp.5 generate by SC- PCR method (designated by Single Cell).

Species	Nucleotide positions										
	54	65	135	231	431	454	597	604	619	624	722
Prorocentrum mexicanum											
- Y16232		G									T
- Genomic DNA		G									T
- Single Cell		A									A
Alexandrium minitum											
- AJ535388				C			T				
- Genomic DNA				T			T				
- Single Cell				T			C				
Pseudo-nitzschia pungen											
- AY257845	C		T		C	T		A	G	C	
- Genomic DNA	T		C		T	C		C	C	T	
- Single Cell	T		C		A	C		C	C	T	

data in these genera provides useful and powerful molecular biological method for their identification. This study aims to identify potential HAB species belonging to *Prorocentrum*, *Alexandrium* and *Pseudo-nitzschia* endemic in Vietnamese waters by analyzing nucleotide sequences of the 18S rDNA and ITS1-5.8S-ITS2 gene fragment obtained by SC- PCR method and comparing them with known some more 40 sequences (Dang *et al.*, 2005; Dang *et al.*, 2007; Ngo *et al.*, 2005; Hoang *et al.*, 2006) which were deposited to GenBank.

Nucleotide sequences of 18S rDNA and ITS1-5.8S-ITS2 gene fragment have been applied to identify species. SC- PCR method was applied, because it can determine the presence of harmful or toxic algal species without culturing them. Species belonging to *Prorocentrum, Alexandrium* and *Pseudo-nitzschia* isolated from plankton samples collected from Hai Phong coast, northern part of Vietnam, in 2004 and 2005 had been used in this report. Success in getting the sequence was also compared among plankton samples fresh or preserved at different conditions such as 4% formaldehyde, 25% ethanol, 1% glutaraldehyde for two weeks.

Based on phylogenetic tree and homologeneous coefficient of the partial 18S rDNA gene fragment in this report, the sample of *Alexandrium* sp. 5 collected from Hai Phong, Northern part of Vietnam was identified under a species name, *A. minutum*. This result was similarity in the identification of this species based on sequence of approximately 700bp of domains 1 and 2 (D1-D2) of large subunit ribosomal (LSU) rRNA gene published by Lim *et al.*, (2007). Nucleotide sequences of domains 1 and 2 of the LSU rRNA gene showed high sequence similarity to *A. minutum* isolated from Malaysia. In this species, toxin content varied among the strains and growth stages, ranged from 3.0 to 12.5 fmol cell^{-1}. Based on the results obtained, the authors confirmed that a new gonyautoxin derivative was identified as deoxy-GTX4-12ol, and this represents the first report of this toxin analogue.

Based on the nucleotide sequences of 18S rDNA and ITS1-5.8S-ITS2 gene fragment, samples *Prorocentrum* sp. 3, *Alexandrium* sp. 5 and *Pseudo-nitzschia* sp. G3 collected from Hai Phong city of the northern part of Vietnam in 2004 and 2005 were identified under a species name, *Prorocentrum mexicanum*, *Alexandrium minutum* and *Pseudo-nitzschia pungens.* Their nucleotide sequences (*Alexandrium* sp. 5, *Prorocentrim* sp. 3 *and Pseudo-nitzschia* sp. G3) were deposited to GenBank with accession numbers as DQ168664, DQ174089 and DQ166533, respectively (Dang *et al.*, 2005; 2007, Ngo *et al.,* 2005 and Hoang *et al.*, 2006).

SC- PCR method has applied, because it can determine the presence of harmful or toxic algal species without culturing them. For successful confirmation of this method in Vietnam, species belonging to *Prorocentrum*, *Alexandrium* and *Pseudo-nitzschia* isolated from plankton samples collected from Haiphong coast, northern part of Vietnam, in 2004 and 2005 had been used. Success in getting the sequence was also compared among plankton samples fresh or preserved at different conditions such as 4% formaldehyde, 25% ethanol, 1% glutaraldehyde for two weeks.

The obtained results in this report indicated that the sequence of *Prorocentrium* sp. 3 collected from Hai Phong which generated by SC-PCR method has close phylogenetic relationship with the homological percent of 99.7% with *P. mexicanum* which have accession number Y16232 in Gen Bank (Grzebyk *et al.*, 1998) and with the homological percent of 99.7% with *Prorocentrum* sp. 3 generated by genomic DNA which have accession number DQ174089. Similarity in *Prorocentrum* sp. 3, the sequences of *Alexandrium* sp. 5 collected from Hai Phong city which generated by SC-PCR method has close phylogenetic relationship with 99.6% with *Alexandrium minutum* which have accession number AJ535388 in GenBank and with 99.7% with *Alexandrium* sp. 5 generated by genomic DNA which have accession number DQ168664.

The obtained sequences of *Pseudo-nitzschia* sp. G3 collected from Hai Phong city which generated by SC-PCR method has close phylogenetic relationship with *Pseudo-nitzschia pungen* in GenBank (accession number AY257846) with 98.8%, and 99.9% with *Pseudo-nitzschia* sp. G3 generated by genomic DNA in GenBank (accession number DQ166533).

In these studied samples, the nucleotide sequences that have obtained from genomic DNA and SC-PCR method were the same with the homological percent more than 99%.

On the other hand, each genus in different preservative condition was applied for SC- PCR method. The obtained results indicated that

available preservative conditions for *Prorocentrum* are ethanol (25%), formaldehyde (4%), glutaraldehyde (1%) and fresh sample; for *Alexandrium* are ethanol (25%) and fresh one; for *Pseudo-nitszchia* are formaldehyde (4%), glutaraldehyde (1%) and fresh one, respectively.

In this report, SC- PCR method has also applied to identify samples collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006 and *Prorocentrum micans* and *P. sigmoides* were found successfully.

CONCLUSION

These obtained results in this present study strongly suggest that morphology still is reliable tool to differentiate *Prorocentrum*, *Alexandrium* and *Pseudo-nitzschia* species. The species in these genera must be defined considering both the morphology and the phylogenetic relationships revealed here. We successfully applied SC-PCR method for correctly species identification. Except fresh cell for successful SC-PCR method, cells preserved in 25% ethanol with *Alexandrium* sp. 5; 4% formaldehyde, 25% ethanol and 1% glutaraldehyde with *Prorocetrum* sp. 3 and 4% formaldehyde and 1% glutaraldehyde with *Pseudo-nitzschia* sp. G3 were suitable as DNA templates for SC-PCR. In these studied samples, the nucleotide sequences that have obtained from genomic DNA and SC-PCR method were the same with the homological percent more than 99%. The sequences of the 18S rDNA and ITS1-5.8S-ITS2 gene fragments of 3 studied species such as *Pseudo-nitzschia* sp. G3 (collected from Cat Ba, Hai Phong, Vietnam in December, 2005 identified under scientific name of *P. pungen* (Grunow ex Cleve) Hasle), *Prorocentrum* sp. 3 and *Alexandrium* sp. 5 (isolated from shrimp ponds in Do Son, Hai Phong, Vietnam in October, 2004 were considered as under a species names, *Prorocentrium mexicanum* Osorio Tafall and *Alexandrium minutum* Halim, respectively) have been submitted to Genebank and given the following accession number: DQ166533, DQ174089 and DQ168664, respectively.

Application of SC-PCR method to samples collected from Phu Quoc Island, southern part of Vietnam, in 27-29, June, 2006 showed that *Prorocentrum mica*n and *P. sigmoides* were found successfully.

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REFERENCES

Cohen-Fernandez, E.J., Castillo, E.M.D., Ugarte, H.I.S., Pedroche, F.F. 2006. Contribution of external morphology in solving a species complex: The case of *Prorocentrum micans*, *Prorocentrum gracile* and *Prorocentrum sigmoides* (Dinoflagellata) from the Mexican Pacific Coast. *Phycological Research* 54 (4): 330–340.

Coleman, A.W. & Mai, J.C. 1997. Ribosomal DNA ITS-1 and ITS-2 sequence comparisons as a tool for predicting genetic relatedness. *J. Mol. Evol.* 45: 168–177.

Dang, D.H, Hoang, M.H., Hoang, L.A., Chu, V.T. 2005. Taxonomy of *Prorocentrum* sp. Sampled from HaiPhong city by basing on the nucleotide sequences of the 18r DNA and ITS1-5,8S-ITS2 gene fragments. *J. of Biol ogy.*Vol.28, No: 1, 81–91.

Dang, D.H., Hien H.T.M., Son, P.N., Bach, N.D., Dong, N.V., (2002) Phylogenestic characterization of some Vietnamese algae based on their ITS-1 sequences. *J. of Science and Technology* 40:161–167.

Dang, D.H, Luyen, Q.H, Hoang, M.H., Ngo, H.T., Hoang, L.A. 2007. Morphological and Molecular identification of *Pseudo-nitzschia* sp. strain G3 isolated from northern coast of Vietnam based on ITS region sequences. *Korean Journal of Marine Bioscience and Biotechnology* 2(1): 60–67.

Faust, M.A., Lasersen, J., Moestrup, O. 1999. Potentially toxic phytoplankton 2. Genus *Prorocentrum* (Dinophyceae). ICES *Identification Leaflets for Plankton* 184: 1–24.

Fehling, J., Green, D.H., Davidson, K., Bolch, C.J. and Bates, S.S. 2004. Domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) in Scottish waters. *J. Phycol.* 40 (4): 622–630.

Fryxell, G.A. and Halse, G.R. 2004. Taxonomy of harmful diatoms. *In*: Hallegraeff, G. M., Anderson, D. M., Cembella, A. D. (eds), Manual on harmful marine microalgae, Monographs on oceanographic methodology 11, Unesco, Paris, 465–509.

John,U., Fensome, R.A. and Medlin, L.K. 2003. The application of a molecular clock based on molecular sequences and the fossil record to explain biogeographic distributions within the *Alexandrium tamarense* 'species complex' (Dinophyceae). *Mol. Biol. Evol.* 20 (7): 1015–1027.

Grzebyk, D., Sako, Y., and Berland, B. 1998. Phylogenetic analysis of nine species of *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological comparisons and description of *Prorocentrum panamensis* sp. nov. *J. Phycol.* 34: 1055–1068.

Gajadhar, A.A., Marquardt, W.C., Hall, R., Gunderson, J., Ariztia-Carmona, E.V. and Sogin, M.L. 1991. Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodinium cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates. *Mol. Biochem. Parasitol.* 45 (1): 147–154.

Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. 2004. Manual on harmful marine microalgae. – *Monographs on oceanographic methodology 11*, Unesco, Paris.

Herzog, M. and Maroteaux, L. 1986. Dinoflagellate 17S rRNA sequence inferred from the gene sequence: Evolutionary implications. *Proc. Natl. Acad. Sci.* U.S.A. 83: 8644–8648.

Hoang Lan Anh, Chu Van Thuoc, Dang Diem Hong. 2006. Constructing the phylogenetic tree of *Pseudonitzschia* sp.(G3) isolated from HaiPhong city based on sequence analysis of ITS1- 5,8S - ITS2 fragment. *J. of Biology* T. XXVIII. No.4. 68–73 (2006).

Horiguchi, T., Yoshizawa-Ebata, J., and nakayama, T. 2000. *Halostylodinium arenarium*, gen. et sp. Nov. (Dinophyceae), a coccoid sand- dwelling dinoflagellate from subtropical Japan. *J. Phycol.* 36: 960–971.

Ki, J.S. and Han, M.S. 2005. Molecular analysis of complete SSU to LSU rDNA sequence in the Harmful Dinoflagellate *Alexandrium tamarense* (Korean isolate, HY970328M). *Ocean Science Journal* 40(3): 155–166.

Kim, K.Y., Yoshida, M. and Kim, C.H. 2005. Molecular phylogeny of three hitherto unreported *Alexandrium* species: *A. hiranoi*, *A. leei* and *A. satoanum* (Gonyaulacales, Dinophyceae) inferred from the 18S and 26S rDNA sequence data. *Phycologia* 44: 361–368.

Kimura M. 1980 A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. of Molecular Evolution*, 16:111–120.

Kumar, S., Tamura K., and Nei M. 2004. MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics*. 5: 150–163.

Lim, P.T., Sato, S., Thuoc, C.V., Tu, P.T., Huyen, N.T.M., Takata, Y., Yoshida, M., Kobiyama, A., Koike, K., Ogata, T. 2007. Toxic *Alexandrium minutum* (Dinophyceae) from Vietnam with new gonyautoxin analogue. *Harmful Algae* 6: 321–331.

Lundholm, N., Moestrup, O., Hasle, G.R. and Hoef-Emden, K. 2003. A study of the *Pseudo-nitzschia pseudodelicatissima/cuspidate* complex (Bacillariophyceae): What is P. pseudodelicatissima?. *J. Phycol.* 39: 797–813.

Lundholm, N., Moestrup, O., Kotaki, Y., Hoef-Emden, K., Scholin, C. and Miller,P. 2006. Inter- and intraspecific variation of the *pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. *J. Phycol.* 42 (2): 464–481.

Nakayama, T., Watanabe, S., Misui, K., Uchida, H., and Inouye, I. 1996. The phylogenetic relationship between the Chlamydononadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycol. Res.* 44: 47–55.

Ngo, T.H.T., Luyen, Q.H., Dang, D.H., Chu, V.T. 2005. Identification of *Alexandrium* sp.(C) isolated from Do Son, Hai Phong based on sequence analyses of 18S rRNA. Issues of basic research in Life Science. *Proceedings, the 2005th National conference on life sciences Hanoi Medical University*, Science and Technics publishing House November 03, 2005: 1400–1403.

Nguyen, N.L. and Doan, N.H. 1997. The distribution of species composition and cell density of phytoplankton in the central Vietnam. – *In*: Dang NT, Nguyen TA, Nguyen HP, Le PT and Vo ST (eds). *Proceeding of the National Conference on Marine Biology*, Nha Trang, Vietnam, Science and Technology Publishing House, Hanoi: 195 – 208.

Nguyen, N.L. 2004. An autecological study of the potentially toxic dinoflagellate *Alexandrium affine* isolated from Vietnamese waters, *Harmful Algae* 3: 117–129.

Saito N., Nei M. 1987. The neighbor – joining method: A new method for reconstructing phylogenetic tress. *Molecular Biology and Evolution* 4: 406 – 425.

Sauders, G.W., Hill, D.R.A., Sexton, J.P., Andersen, R.A. 1997. Small – subunit RNA sequence from selected

dinoflagellates: testing classical evolutionary hypotheses in the agae of molecular systematics. *Plant. Syst. Evol.* 11: 237–59.

Skov, J., Lundholm, N., Moestrup, Ø. and Larsen, J. 1999. Potentially toxic phytoplankton 4. The diatom genus *Pseudo-nitzschia* (Diatomophyceae/ Bacillariophyceae). *In*: Lindley, J. A. [Ed.] ICES Identification Leaflets for Plankton. No.185. ICES, Palægade 2–4, DK-1261 Copenhagen. p.23.

Takano, Y., and Horiguchi, T. 2004. Surface ultrastructure and molecular phylogenetics of four unarmored heterotrophic dinoflagellates, including the type species of the genus Gyrodinium (Dinophyceae). *Phycological Research* 52: 107–116.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876–4882.

Yoshida, M., Ogata, T., Thuoc, C.V., Masuoka, K., Fukuyo, Y., Hoi, N.C. and Kodama M. 2000. The first finding of the toxic dinoflagellate *Alexandrium minutum* in Vietnam. – *Fisheries Science* 66: 177 – 179.