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Phylogenetic analyses of *Prorocentrum* spp. and *Alexandrium* spp. isolated from Northern coast of Vietnam based on 18S rDNA sequence

Dang Diem Hong*, Hoang Thi Minh Hien, Ngo Hoai Thu, Hoang Lan Anh and Quoc Hai Luyen

Institute of Biotechnology, Vietnamese Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay - 1000, Ha Noi, Vietnam

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Abstract: *Some species of marine dinoflagellates belonging to genera Alexandrium and Prorocentrum have been responsible for paralytic shellfish poisoning (PSP) and diarrheic shellfish poisoning (DSP), respectively. Morphological and molecular studies of 4 species including Alexandrium sp. 5, Alexandrium sp. 16, Prorocentrum sp. 1 and Prorocentrum sp. 3 that were collected in Northern coast of Vietnam were presented for the first time. By morphologic observations, we identified Alexandrium sp. 5 and Alexandrium sp. 16 as Alexandrium minutum, Alexandrium affine, respectively; Prorocentrum sp. 1 and Prorocentrum sp. 3 as Prorocentrum mexicanum. Sequence data from the partial 18S riboxomal RNA genes have been used to generate a phylogenetic framework with database of GenBank. The obtained results of phylogenetic analyses of species of Prorocentrum spp. and Alexandrium spp. based on 18S rDNA sequences are similar to morphological observations. Thus, molecular tool would be helpful for the identification of dinoflagellate species and further taxonomic studies in Vietnam.*

Key words: *Alexandrium, Phylogenetic analysis, Prorocentrum, 18S rRNA gene*
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Introduction

Some microalgae produce toxins which can cause mass mortality of other marine flora and fauna including aquaculture. Algal toxins may also accumulate in shellfish or finfish, thereby posing serious human health risks upon consumption of contaminated seafood. If uncontrolled, these effects may have devastating impact on the seafood industry. An improved understanding of the occurrence and distribution of the causative species, the population dynamics (growth rates, nutrient requirements, life cycles, *etc.*), the ecophysiology, better methodologies for detection of toxins and testing of toxicity, will improve our ability to forecast the events and reduce their effects, and thereby develop still better management tools. This is how we can minimize the impacts of harmful algal blooms on human health and economy (Touzet *et al.*, 2007).

Although Vietnam has the great biodiversity in freshwater and marine microalgae, studies on harmful microalgae in Vietnam began only a few years ago. In general, for harmful algal composition in Vietnamese coastal waters, a list of species including the distribution and cell density of mainly dinoflagellate (genera *Alexandrium*, *Prorocentrum*), diatoms (genus *Pseudonitzschia)* and cyanobacteria (genus *Trichodesmium*) was given by Nguyen and Doan (1997). Therefore, having an overview of microalgae resources as species composition, distribution, and available stock and taxonomic system especially is a necessary requirement. Many taxonomic investigations of microalgae have been carried out by Vietnamese scientists for years, but they are still mainly based on the morphologic observation, which requires considerable taxonomic experience and is sometimes laborious and time-consuming (Ki and Han, 2005; Hong *et al.*, 2007; Yoo, 2004). However, these works have met a serious matter with species sharing similar morphological features that are easily changed by environmental factors. In some difficult groups, *e.g.* the dinoflagellate genus *Alexandrium*, identification of species can be done in the light microscope, but observation of the very fine detail that distinguish the species requires considerable expertise. It should be noted, however, that harmful effects are not necessarily associated only with toxin production by the microalgae. Some species may cause fish kills or other harmful events through secondary effects of intensive blooms, *e.g.* oxygen depletion or physical damage to fish gills. Such species include for example, the dinoflagellate *Prorocentrum micans*, several species of the diatom genus *Chaetoceros* and a number of other (Hallegraeff *et al.*, 2004).

The recent trend in taxonomy and phylogenetic studies are based on not only the morphological characters but also on the rDNA analysis. In general, rDNA consist almost conservative genes, however, there were certain regions 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 an intergenic spacer (IGS). They have proved useful tools for species identification (Zang *et al.*, 2007; Ki and Han, 2007; Godhe *et al.*, 2007; Touzet *et al.*, 2007; Hong *et al.*, 2002, 2007). In this paper, the first time in Vietnam, the morphological and molecular studies of some species belonging to dinoflagellates that collected in Northern coast of Vietnam are presented. Microscopic observations of species showed that they belong to *Prorocentrum* sp. and *Alexandrium* sp. Subsequently, we have used partial gene of 18S rDNA to determine clearly and to establish a phylogenetic framework for these species.

Materials and Methods

Isolation and establishment of cultures: The 4 clonal cultures, which were isolated from natural seawater in Northern coast of Vietnam from 1st December 2005 to 31st April 2006 (Fig. 1 and Table 1), were

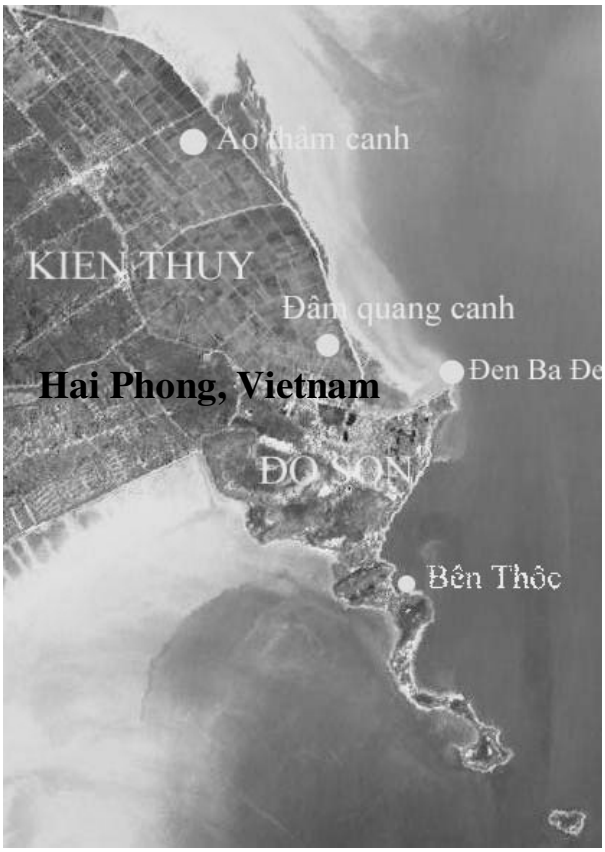

Fig. 1: A map showing sample collection locations along the Northern Vietnam coast

kindly donated by Dr. Thuoc C.V. (Hai Phong Institute of Ocenology, Vietnamese Academy of Science and Technology). Each clonal culture was obtained by the isolation of a single cell by micropipette, followed by several rinse in sterile culture medium. *Alexandrium* spp. and *Prorocentrum* spp. were cultured in IMK medium (Taigo IMK medium, Nihon chemical Co, Japan). The algae were grown in batch cultures at 20°C under a 12:12 hr L/D cycle and a photon flux density of ca. 90 $\mu molm^{-2}s^{-1}$.

Morphological identification: Samples were examined under a microscope using light microscope (LM), scanning electron microscope (SEM) with model JSM-5410L (Jeol Company, Japan) and epifluorescence (Olympus BX51, Japan). For epifluorescence, fixed samples were stained with calcofluor white (Sigma, USA) and viewed under UV with a UV filter set. Images were captured with a cooled CCD camera (SIS Colorview F12, Germany).

DNA primer design: Partial and full-sequences of the 18S rRNA gene were aligned to design PCR primers. By comparison of the sequences of 5 species of *Alexandrium*: *A. minutum* (GenBank accession no. AJ535388), *A. ostenfeldii* (AJ535383), *A. affine* (AJ535375), *A. tamarense* (X 54946), *A. cohorticula* (AF113935); 7 species of *Prorocentrum*: *P. lima* (Y16235), *P. maculosum* (Y16236), *P. concavum* (Y16237), *P. micans* (M14649), *P. minimum* (Y16238), *P. mexicanum* (Y16232), *P. emarginatum* (Y16239); the Alex18F-U18R (Alex18F 5'- GAGAGGGAGCTTGAGAAATG-3'; U18R 5'- GGCATCACAGACCTGTTATTGC-3') and UPro18F-U18R (UPro18F 5'- TACCACATCTAAGGAAGGCAGCAG-3') primer pairs were designed to be specific for *Alexandrium* and *Prorocentrum* species, respectively.

Table - 1: Species designation, collection date and sites of *Prorocentrum* and *Alexandrium* used in this study

Number	Species	Collection date	Collection site
1	*Prorocentrum* sp. 1	December 2005	Do Son, Hai Phong, Vietnam
2	*Prorocentrum* sp. 3	March 2006	Do Son, Hai Phong, Vietnam
3	*Alexandrium* sp. 5	January 2006	Do Son, Hai Phong, Vietnam
4	*Alexandrium* sp. 16	April 2006	Do Son, Hai Phong, Vietnam

Table - 2: Morphological characters in the two species of *Prorocentrum* used in this study. Specific characters of each species in this work are underlined

Species	Length (µm)	Width (µm)	Valves			Apical periflagellar area	
			Shape	Ornamentation	Pore pattern	Shape in apical view	Ornamentation
Prorocentrum mexicanum Osorio Tafall	30-40	20-25	Nearly oval	Depressions	Pores in radial lines; pores scattered on valves	Ovoid	Wing spine and protruding apical plate
Prorocentrum sp. 1	30	20-22	Nearly oval	Barely visible depressions	Pores in radial lines	Ovoid	Wing spine and protruding apical plate
Prorocentrum sp. 3	33-35	23-24	oval	Depressions	Pores in radial lines	Ovoid	Wing spine and protruding apical plate

Table - 3: Strains of *Prorocentrum* and *Alexandrium* species used in the phylogenetic analysis, with origin of isolates, 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 $8^0389'$ N, $79^002'$ W	B. Berland	Y16237	Grzebyk *et al.* (1998)
P. emarginatum Fukuyo	PREU2	Re´union Island, S.W. Indian Ocean $21^010'$ S, $55^017'$ E	B. Berland	Y16239	Grzebyk *et al.* (1998)
P. lima (Ehrenberg) Dodge	#151	Tokushima, Mugi Ooshima, Japan, Pacific coast $33^037'$ N, $134^029'$ E	S. Yoshimatsu	Y16235	Grzebyk *et al.* (1998)
P. maculosum Faust	PPAN20	Contadora Island, Panama, $8^038'$ N, $79^002'$ 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 $43^024'$ N, $3^042'$ E	B. Berland	Y16238	Grzebyk *et al.* (1998)
P. mexicanum Osorio Tafall					
	PMOO04	Moorea Island, French Polynesia, Pacific Ocean $17^030'$ S, $149^050'$ W	B. Berland	Y16232	Grzebyk *et al.* (1998)
	sp. 1*	Do Son, Hai Phong (Vietnam)	D. Hong		Hong *et al.* (2005)
	sp. 3*	Do Son, Hai Phong (Vietnam)	D. Hong	DQ174089	Hong *et al.* (2005)
Alexandrium					
A. affine (H. Inoue and 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. cohorticular		Malaysia	G. Usup	AF113935	
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)
	sp.16*	Do Son, Hai Phong (Vietnam)	D. Hong	DQ171879	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	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)
Noctiluca scintillans (Macartney) Kofoid et Swezy	Noct.ilu			AF022200	John *et al.* (2003)
Peridinium sp.				F022202	Sauders *et al.* (1997)
Pyrocystis noctiluca Murray ex Haeckel	Pyrocyst			AF022156	John *et al.* (2003)
Sarcocystis muris	SARRR16S			M64244	Gajadhar *et al.* (1991)

*The isolated strain in this study

Table - 4: Location of different nucleotide positions in the sequence of the 18S rRNA gene among *Prorocentrum mexicanum* (Accession number: Y16232), *Prorocentrum* sp. 1 and *Prorocentrum* sp. 3; between *Alexandrium minitum* (AJ535388) and *Alexandrium* sp. 5 and between *Alexandrium affine* (AJ 535375) and *Alexandrium* sp. 16

Species	Nucleotide positions				
	231	380	750	797	952
Prorocentrum mexicanum					
- Y16232			A	-	A
- Strain sp. 1			A	G	A
- Strain sp. 3			G	G	G
Alexandrium minitum					
- AJ535388	C				
- Strain sp. 5	T				
Alexandrium affine					
- AJ 535375		A			
- Strain sp. 16		G			

DNA extraction, amplification, cloning and sequencing: Cultured cells in exponential growth phase were harvested by centrifugation and the supernatant was removed. The genomic DNA was extracted by following the procedure described in Hong *et al.* (2002, 2007). After harvest, the samples were immediately suspended in extraction buffer (0.8M LiCl – Lithium chloride; 0.6% Sarkosyl; 10mM EDTA – Ethylene Diamine Tetraacetic acid (pH 8.0); 0.2% PVP – poly vinyl pyrrolidone and 5% β mercaptoethanol) and incubated for 15 minutes at 55°C. Then, it was shaken gently at 4°C for 1 hr. The supernatant was collected after centrifuging (2000 x g, 5 min, 4°C). Total DNA in the supernatant was the precipitated directly by addition of 0.1V of 3M Sodium acetate (pH 5.2) and 2V of 100% Ethanol. The precipitate was washed twice by centrifugation (1800 x g, 5 min, 4°C) with 70% ethanol. The pellet was resuspended in TE (10 mM Tris- Cl, pH 8.0 and 1mM EDTA, pH 8.0). The polymerase chain reaction (PCR) mixture (20 µl) contained 2 µl of 10X PCR buffer, 0.75 mM of deoxyribonucleoside triphosphate (dNTP) mixture (Tokyo, Osaka, Japan), 0.5 mM of each primer, 50-100ng of genomic DNA, 0.075 unit of Taq DNA polymerase and distilled water. For DNA amplification, the GeneAmp® PCR system 9700 Thermal Cycler (Applied Biosystems) was programmed for 94°C for 3 min, followed by 35 cycles of 94°C for 30 second, 55°C for 1 min, 72°C for 1 min and, finally, 72°C for 5 min, and stored at 4°C. The lengths of PCR products of *Prorocentrum* spp., *Alexandrium* spp. were approximately 1.1 kb. These PCR products were cloned according to TOPO kit (Invitrogen). Recombinant plasmids were transformed into *E. coli* DH5a T1'strain. The results of cloning were confirmed by PCR checking method and restriction analysis by. *E. co*RI enzyme according to previous study (Hong *et al.*, 2002, 2007). Then the DNA plasmids were sequenced using an autosequencer - ABI PRISM 3100 Avant genetic Analyzer (USA) with a Big Dye(R) Terminator v3.1 Cycle Sequencing Ready Reaction Kit. Both strands of at least two different recombinants were sequenced to check for PCR errors generated in the amplification procedure.

Sequence analysis: Sequences were edited and manipulated using the MEGA3 (Kumar *et al.*, 2004). For sequence comparison, multiple sequence alignment was performed with each sequence revealed from the present study and 18S rDNA sequences of 19 dinoflagellate species retrieved from DDBJ/EMBL/GenBank, using the ClustalX (Thompson *et al.*, 1997). *Noctiluca scintillans* (GenBank accession no. AF022200), *Peridinium* sp. (AF 022202), *Pyrocystis notiluca* (AF 022156) and *Sarcocystis muris* (M64244) were used as the outgroups. Genetic distance values were calculated by using the aligned DNA sequences according to the Kimura 2-parameter model (Kimura *et al.*, 1980). Phylogenetic tree was inferred by use of the neighbor-joining (NJ) algorithm (Saito and Nei, 1987) in MEGA3 and applied bootstrap analysis from 1,000 bootstrap replications.

Results and Discussion

Morphological study of *Prorocentrum* and *Alexandrium*: Among the microalgae, dinoflagellates comprise the largest number of toxic species. Taxonomic accounts of more than 70 species of *Prorocentrum* and *Exuviaella* have been described (Faust *et al.*, 1999). The characters used to differentiate the species of *Prorocentrum* relate to the following features: cell shape and size, apical spine, pyrenoids, nucleus, and in particular surface structure and ornamentation of the valves which in some cases can be observed only by scanning electron microscopy. According to the morphological features described by Faust (1999), Cohen-Fernandez *et al.* (2006) and Grzebyk *et al.* (1998) and microscopic observations of *Prorocentrum* sp. 1 and *Prorocentrum* sp. 3 shown in Table 2, they seem to belong to species of *P. mexicanum* (Fig. 2A, B, C and D).

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 (s.p.) plates are important for species identification. Based on morphological features, Balech (1995) recognized 29 species of *Alexandrium*, and recently MacKenzie and Todd (2002) and Larsen and Nguyen (2004) described 2 new species of *Alexandrium* from New Zealand and Vietnam waters, respectively. These 31 species are assigned to two subgenera grouping as *Alexandrium* nom. nud. and *Gessnerium*. *Alexandrium affine* has been recorded commonly in the coastal waters of the Thua Thien – Hue and Hai Phong is from December to February and from the Van Phong and Cam Ranh Bays (Nha Trang) during dry season (Larsen and Nguyen, 2004; Nguyen, 2004). The *Alexandrium* sp. 16 differs from the original features of *A. affine* described by Fukuyo *et al.* (1985) in only a minor detail. By LM and Epi-fluorescent micrographs observations, the cell of *Alexandrium* sp. 16 collecting in Do Son, Hai Phong, Vietnam was about 36 µm in diameter, rounded-pentagonal in shape, sometimes a little longer than wide, chain-forming. The epi- and hypotheca are about equal in size, cingulum descending, displaced about one girdle width. Both the cingulum and the sulcus are deeply incised and bordered by pronounced list (Fig. 2E). Scattered trichocysts pores

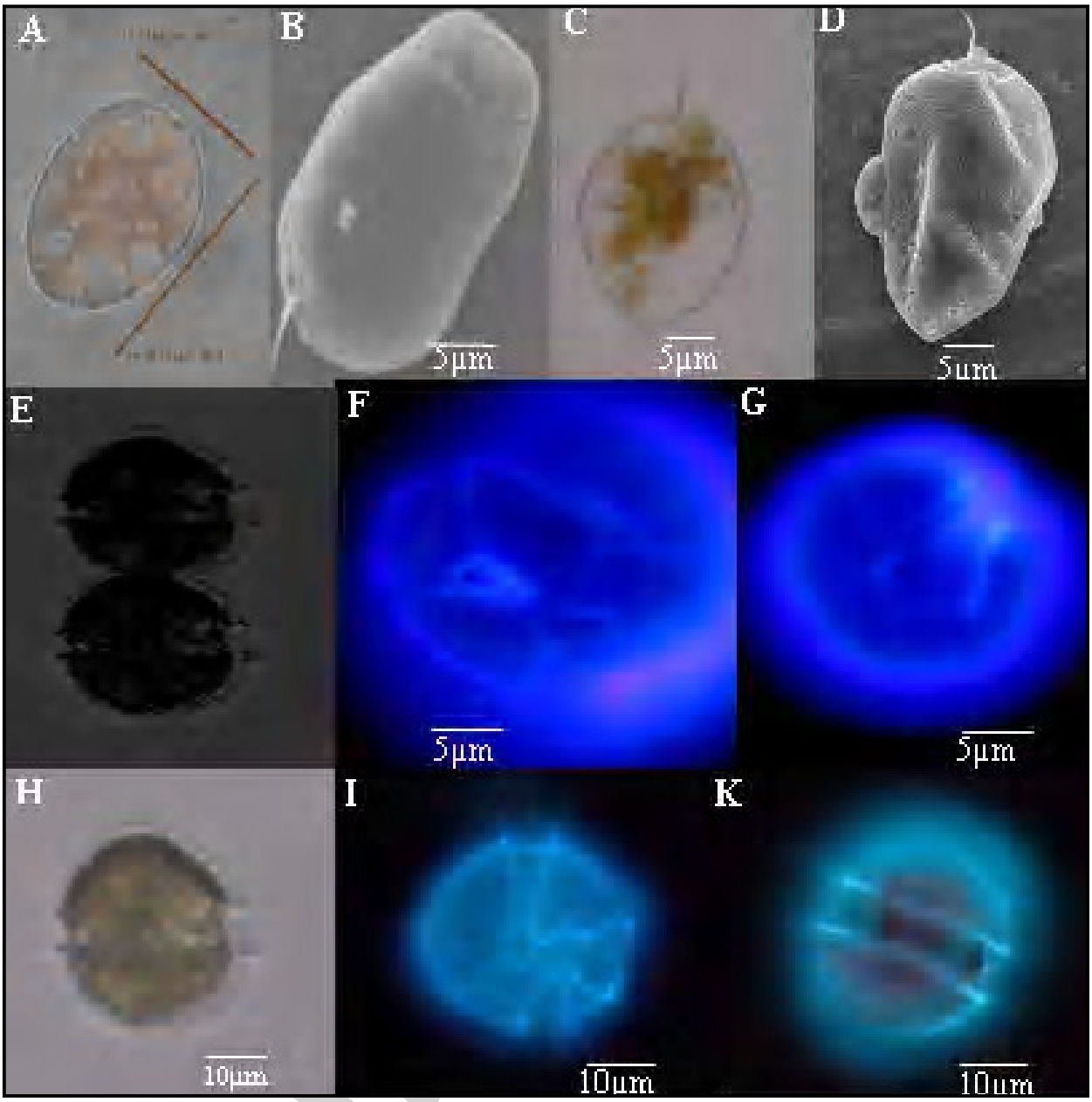

Fig. 2: Morphological image of *Prorocentrum mexicanum* strain sp. 1 and sp. 3, *Alexandrium affine* strain sp. 16 and *Alexandrium minitum* strain sp. 5. (A, B) LM and SEM image of *Prorocentrum mixecanum* strain sp. 3; (C, D) LM and SEM image of *Prorocentrum mixecanum* strain sp. 1; (E) LM image of *Alexandrium affine* strain sp. 16; (F and G) Epi-fluorescent micrographs of *Alexandrium affine* strain sp. 16; (H) LM image of *Alexandrium minitum* strain sp. 5; (I, K) Epi-fluorescent micrographs of *Alexandrium minitum* strain sp. 5

occur on the thecal plates. The 1' plate connects directly to the apical pore complex (APC), with a small ventral pore at the margin of the 1'-4' suture, and usually situated on the posterior half of the suture. The APC has a large connecting pore situated dorsally relative to the comma-shaped apical pore, marginal pores not observed. The anterior sulcal (s.a.) plate has no pre-cingular part; the (s.p.) plate has an attachment pore, which connects to the right margin of the plate by a tiny channel (Fig. 2F and G). By morphological observations, *Alexandrium* sp. 16 isolated from Hai Phong seems to be considered under a species name, *A. affine*.

With *Alexandrium* sp. 5 isolated from Hai Phong, Vietnam, its cells were oval in shape, small with transdiameter between 20 and 28 µm (Fig. 2H). Gamete was approximately half the size of vegetative cell. The first apical plate (1') was rhomboidal with ventral pore (v.p.) located on the anterior right margin of the plate. Some cells showed

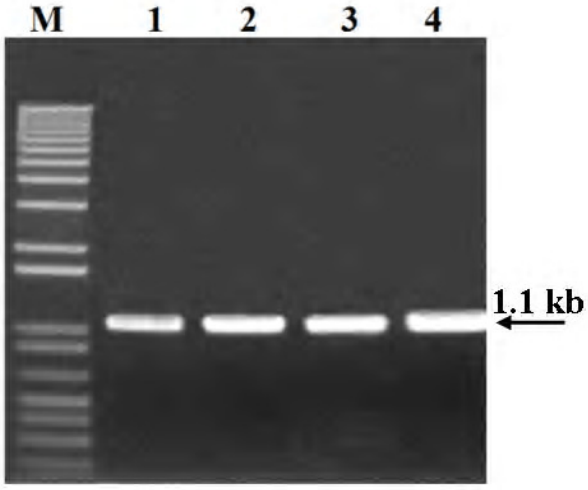

Fig. 3: PCR products of 18S rRNA gene fragment of genera *Prorocentrum* and *Alexandrium*. Lane M: Marker 1Kb Plus DNA Ladder; Lane 1-2: PCR products of *Prorocentrum* sp. 1, *Prorocentrum* sp. 3; Lane 3-4: PCR products of *Alexandrium* sp. 5, *Alexandrium* sp. 16, respectively

long and narrow 1', with almost parallel right and left margins. APC was comma in shape without anterior attachment pore. Sixth precingular plate (6") was longer than wide (length width ratio = 1.5-1.8). S.p. plate was wider than long (Fig. 2I and K). The present species is assigned to *Alexandrium minitum* because of the cell size and the shape of the 1', 6" and s.p. plates. In the Vietnamese samples, 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 also has previously been reported in species isolated from Ha Long Bay (Yoshida *et al.*, 2000; Lim *et al.*, 2007).

Phylogenetic analysis based on 18S rDNA sequences: Ribosomal RNA gene data are also useful tools for taxonomy and phylogeny at the levels of genus and species. The identification of dinoflagellate species has been established, mainly using morphological features; however, these sometimes vary in response to changing environmental conditions (Grzebyk, 1998). Molecular studies using rDNA sequence data indicate that several *Alexandrium* and *Prorocentrum* species are more closely related by geographical origin than by clear morphological criteria (Ki and Han, 2007; Lin *et al.*, 2006; Faust *et al.*, 2008; Rogers *et al.*, 2006). In addition, the rDNA sequences allowed separating two types of *Prorocentrum* as benthic and planktonic behavior (Grzebyk, 1998).

In this report, the morphological studies of used species in this report belonging to genera *Alexandrium* and *Prorocentrum* were supported by phylogenetic analyses of the partial DNA sequences of the nuclear – encode small – subunit 18S rRNA. The partial 18S rRNA gene fragments of *Alexandrium* sp. 5, *Alexandrium* sp. 16, *Prorocentrum* sp. 1 and *Prorocentrum* sp. 3 were amplified by using PCR technique with specific primer pairs which shown in material and methods. PCR products of studied samples were showed in Fig. 3.

For reconstruct on the phylogenetic tree different sequences of DNA databases from FASTA searches such as 18S rDNA sequences of 19 dinoflagellate species of genera *Alexandrium* and *Prorocentrum* were used (Table 3). In the reconstructions of dinoflagellate phylogeny (Fig. 4) using the apicomplexan *Sarcocystis muris* as the outgroup, *Noctiluca scintillans* is found to be a primitive dinoflagellate, as previously reported from other molecular studies (Lenaers *et al.*, 1991; Sauder *et al.*, 1997). For phylogenetic tree of genera *Prorocentrum* and *Alexandrium*, *Peridinium* sp. and *Pyrocystis notiluca* were used as the outgroup species, respectively.

Identification of *Prorocentrum* sp. 1 and *Prorocentrum* sp. 3: Based on phylogenetic tree and genetic homogeneous coefficient matrix, the identification of *Prorocentrum* sp. 1 and *Prorocentrum* sp. 3 was carried out. *Prorocentrum* sp. 1 possess highest homogeneous coefficient with *P. mexicanum* (100%), subsequent with *Prorocentrum* sp. 3 (99.8%), *P. minimum* (99.7%), *P. micans* (99.4%), *P. concavum* (96.8%), *P. emarginatum* (95.9%), *P. maculosum* (94.4%), *Peridinium* sp. (94.1%), *P. lima* (93.9%). Within the 18S rDNA region (1100 bp), 1 base difference was found between *Prorocentrum* sp. 1 and *P. mexicanum* (Y16232) (such as positions 797 bp) (Table 4). On the phylogenetic tree, *Prorocentrum* sp. 1 possesses length of equal evolutionary branch in *P. mexicanum* lying beside *P. mexicanum*. The obtained results above mentioned allowed us to make the conclusion that *Prorocentum* sp. 1 was identified as *P. mexicanum*.

Prorocentrum sp. 3 have the highest homogeneous coefficient with *Prorocentrum* sp. 1 and *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%), *Peridinium* sp. (93.9%), *P. lima* (93.8%). Table 4 showed that pair-wise sequence divergence between *Prorocentrum mexicanum* (Y16232) and *Prorocentrum* sp. 3 was 0.29% (3 base differences). In addition, *Prorocentrum* sp. 3 possesses length of equal evolutionary branch in *P. mexicanum* lying beside *Prorocentrum* sp. 1 (which was confirmed by *P. mexicanum* as mentioned above) on the phylogenetic tree. Thus, based on genetic homogeneous coefficient of 18S rDNA sequence and distribution on phylogenetic tree, we realized that *Prorocentrum* sp. 3 is rightly *P. mexicanum*.

Identification of *Alexandrium* sp. 5 and *Alexandrium* sp. 16: 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*; *Alexandrium* sp. 16 and *A. affine*; *A. minutum* and *A. ostenfeldii*). NJ phylogenetic tree was constructed using method of Kimura of 2 parameters. All species of *Alexandrium* genus were divided into two groups. The first group has only one species of *Alexandrium 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.* sp. *tamutum* and *A. margalefii*. Among 4 sequences of *Alexandrium* sp. 5, *A. minutum*, *A. ostenfeldii* and *A.* sp. *tamutum* they have very high homogeneous level (> 99.5%); the second subgroup consist of *Alexandrium* sp.

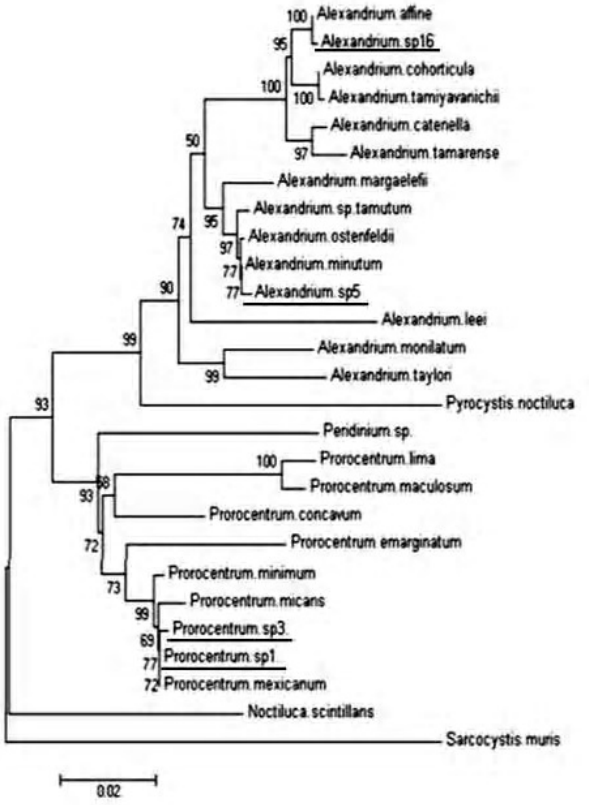

Fig. 4: Phylogenetic relationship of *Prorocentrum* and *Alexandrium* species based on the completely aligned 18S rDNA sequences (23 strains). DNA sequences determined in this study are underlined. The tree was constructed using the Kimura 2-parameter method (Kimura *et al*., 1980) and 1,000 bootstrapped replicates in MEGA3. *Noctiluca scintillans* (GenBank accession No AF 022200), *Peridinium* sp. (AF 022202), *Pyrocystis notiluca* (AF 022156) and *Sarcocystis muris* (M64 244) were used as the outgroup. Bootstrap values of less than 50% are not shown. Branch lengths represent genetic distance among the taxa. The bar denotes substitution per nucleotitde position

16, *A. affine*, *A. catenella*, *A. tamarense*, *A. cohorticula*, *A. tamiyavanichii*, *A. monilatum* and *A. taylori*.

Based on homogeneous coefficient matrix we found that *Alexandrium* sp. 5 possess the most highest homogeneous coefficient with *A. minutum* (99.9%), then *A. ostenfeldii* (99.8%), *Alexandrium* sp. *tamutum* (99.5%), *A. margalefii* (98.5%), *Alexandrium* sp. 16 (97%), *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%) and *Pyrocystis noticula* (91.6%). The result of nucleotide comparison using MEGA3 software showed that 1 base difference was found between *Alexandrium* sp. 5 and *A. minutum* (AJ535388) (such as position 231 bp) (Table 4). This represents the sequence dissimilarity (number of base differences divided by sequence length of 0.009). On the phylogenetic tree (Fig. 4), species of *Alexandrium* sp. 5 have length of evolutionary branch equal in species of *A. minutum* and lying beside *A. minutum*. Thus, based on homogeneous coefficient of 18S rDNA and the distribution to phylogenetic tree, *Alexandrium* sp. 5 was considered as under a species name, *A. minutum*.

Alexandrium sp. 16 has highest homogeneous coefficient with *A. affine* (99.9%), then *A. cohorticala* (98.9%), *A. tamiyavanichii* (98.8%), *A. catenella* (98.6%), *A. tamarense* (98.3%), *Alexandrium* sp. 5 (97%), *A. minutum* (96.9%), *A.* sp. *tamutum* (96.9%), *A. ostenfeldii* (96.8%), *A. margalefii* (96.2%), *A. monilatum* (95%), *A. taylori* (94.2%), *A. leei* (93.7%), *Pyrocystis noticula* (90.5%). Table 4 showed that pair-wise sequence divergence between *A. affine* (AJ535375) and *Alexandrium* sp. 16 was 0.09% (1 base difference). On the phylogenetic tree (Fig. 4), *Alexandrium* sp. 16 has length of evolutionary branch which is same of *A. affine* and beside itself. It allowed us to make the conclusion that *Alexandrium* sp. 16 is considered under species name, *A. affine*.

Sequences of 18S rRNA gene fragment of 3 studied species which were collected from HaiPhong - Northern coast of Vietnam in this report were submitted to GenBank with accession number as following: *Prorocentrum* sp. 3 (was identified by *P. mexicanum* – DQ174089), *Alexandrium* sp. 5 (*A. minutum* – DQ168664), *Alexandrium* sp. 16 (*A. affine* – DQ171879). These obtained results in this present study strongly suggest that morphology still is reliable tool to differentiate *Prorocentrum* and *Alexandrium* species. The species in these genera must be defined considering both the morphology and the phylogenetic relationships revealed here.

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