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Morphological and genetic comparison of two strains of a *Prorocentrum* species isolated from Zhejiang coastal water of China and Masan Bay of Korea*

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Abstract In this paper, we examined the detailed morphology of two strains of *Prorocentrum* isolated from the coastal waters of Zhejiang (Wenling area), China, and Masan Bay of Korea. A taxonomic comparison was made among strains on the basis of morphological and molecular data. The cellular dimensions of the Chinese Wenling strain (LAMB090508) and Korean strain (PDKS0206) were similar and the cells of both strains were of asymmetric and elongated shape. The posterior end of most cells was rounded. Megacytic zones of aged cells were broader with dense tiny knobs. The roundish nucleus was located in the posterior part of the cell. A few irregular shaped chloroplasts were distributed within the cell. The nucleotide similarity of the two strains, determined from the 5.8S rDNA-ITS sequences, was 99.83%. The comparative results of morphology and molecular analysis suggest that both strains isolated from China and Korea were the identical species, *Prorocentrum donghaiense* Lu.

Keyword: *Prorocentrum*; morphology; 5.8S rDNA-ITS; coastal waters of China; Korea

1 INTRODUCTION

Globally, the evidence of changes in the distribution of harmful algal bloom (HAB) species is recognized. Identifying key HAB species is helpful for revealing changes of their bio-geographic distributions. Understanding the reasons for the changes in the distribution of key HAB species in Asia is one of the important challenges for GEOHAB Asia (GEOHAB, 2010). Some species in the genus of *Prorocentrum* are among the major organisms causing harmful "red tides" in Chinese and Korean coastal waters (Zhu et al., 1994; Lu and Goebel, 2001; Zhou, 2003; Qi and Wang, 2003; Lu et al., 2005; Lee et al., personal communication). In the last decade, *Prorocentrum donghaiense* Lu, a high biomass bloom-forming species, has been observed in the East China Sea (ECS). It has formed large scale blooms in spring, covering ten thousand square kilometers of coastal waters of the Zhejiang Province of China. A similar species has been recorded as *Prorocentrum dentatum* Stein in routine monitoring work in the coastal waters of Korea. It is supposed that they are the same species (Lu et al., 2005). In order to confirm whether this is the case and to better understand its bio-geographic distribution, two strains of the targeted species were isolated from the Wenling area in Zhejiang coastal water of China (the ECS) and the Masan Bay of Korea. The morphological structure and genetic information of both strains are carefully compared in the present study.

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2 MATERIAL AND METHOD

2.1 Strain isolation and cultivation

Original samples were collected at the Wenling coastal water of Zhejiang and Masan Bay of Korea (Fig.1). Both strains of *Prorocentrum* were isolated using the serial dilution method of Throndsen (1978). The strains were maintained in culture flasks in f/2 medium, at salinity of 30–32, light-dark cycle 12:12 h at photosynthetically active radiation (PAR) of 9.6–10.6 μmol/m^2·s, light intensity of 810 lx and at 20°C. Cells of different life stages were examined for demonstration of cell shape change and size measurements.

2.2 Morphological observation

For observation with light microscope (LM), living and preserved *Prorocentrum* cells were observed at 20×, 40× objective and 100× oil objective using an OLYMPUS CX31 and LEICA DM 5000B light microscope equipped with a digital camera (LEICA DFC 300FX). The cells were fixed with 3% Lugol's solution. Cell length and width were measured from at least 80 cells in exponential growth phase and were photographed using a calibrated objective. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) was used for staining the nuclei of the cells. A ZEISS LSM 510 Laser scanning microscope (LSM) was employed for observing the nucleus and chloroplasts. For examination with scanning electron microscopy (SEM), the samples were preserved with 3% neutralized formaldehyde. Specimens were filtered on nucleopore filters with 1 and 3 μm pore size, dehydrated in an alcohol series and critical-point-dried (BALZERS CP0303 critical point dryer) using CO_2 as the intermediary fluid. Samples were sputter-coated with gold-palladium (Lu et al., 2005). A ZEISS DSM940 scanning electron microscope was employed for analysis and photography. The identification of targeted species were based on cell shape and size, position of nucleus, surface micro-topography, ornamentation of thecal plates, the architecture of the periflagellar area as well as the intercalary bands under LM and SEM (Steidinger and Tangen, 1996; Lu et al., 2005).

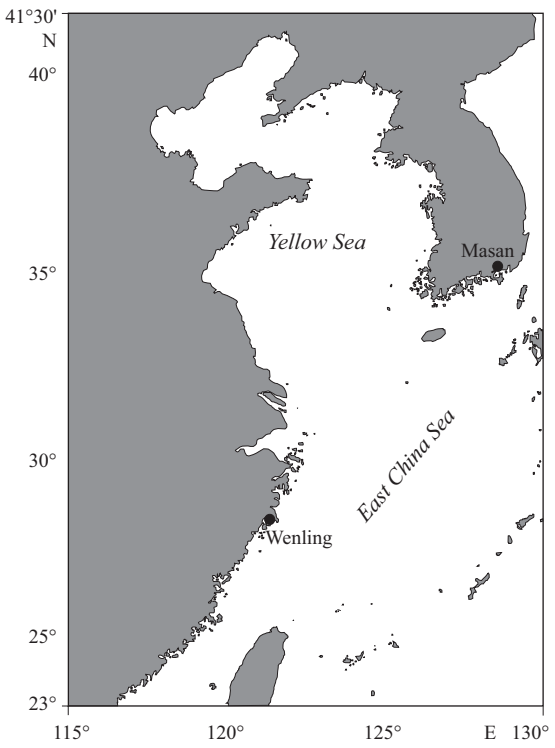

Fig.1 Sampling sites in the coastal water of Zhejiang Province, China and Korea

2.3 DNA extraction, amplification and sequencing

The total algal DNA was extracted from 100 ml of exponentially growing cultures using a UNIQ-10 plant DNA extraction kit (Sangon Company, Shanghai), on the basis of the manufacturer's protocol. The ITS sequence was specifically amplified using the PCR with the primers ITS 1 (forward, 5′-GGGATCCGTTTCCGTAGGTGAACC TGC-3′) and ITS 2 (reverse, 5′-GGGATCCATATGC TTAAGTTCAGCGGG-3′) (Coleman et al., 1994, 1997), or ITS 4 (forward, 5′-TCCTCCGCTTATTGAT ATGC-3′) and ITS 5 (reverse, 5′-GGAAGTAAA AGTCGTAACA AGG-3′) (White, 1990). The PCR mixture of 50 μL contained: 0.5 μL Taq polymerase (5 unit/μL), 5 μL 10×Buffer, 4 μL dNTP Mixture (1 mmol/L), 1 μL of each primer, 2 μL of genomic DNA template. The PCR was performed as follows: initial denaturing at 95°C for 5 min; addition of polymerase followed by 30 cycles of 94°C for 1 min; 55°C for 1 min and 72°C for 2 min; followed by a final extension step at 72°C for 5 min. The PCR product was sequenced at Sangon Company, Shanghai.

The sequence data was initially evaluated against published sequences in GenBank using the BLAST program. The ITS sequences were aligned with CLUSTAL W and a few minor corrections were made manually. Pairwise evolutionary distances were computed using Jukes and Cantor algorithm implemented in the MEGA 4.1. The phylogenetic tree was constructed by using the neighbor-joining method with Kimura's two-parameter model. The

tree was rooted using the ITS sequence of *Scrippsiella trochoidea* (EU325959) as an outgroup.

3 RESULT

3.1 Morphology of the strains, LAMB090508 and PDKS0206

The cellular dimensions of the Wenling strain (LAMB090508) and Korean strain (PDKS0206) are presented in Table 1. The mean cell length of 80 measured cells of the strain LAMB090508 was 19.29 μm with a standard deviation of ±1.39 μm, the mean width of cells was 10.64 μm with a standard deviation of ±1.39 μm. The cellular dimensions of 122 measured cells of the PDKS0206 strain were negligibly different with 19.72 μm mean length (standard deviation of ±1.57 μm) and 11.44 μm mean width (standard deviation of ±1.66 μm).

Targeted strains appeared same morphological characteristic with *Prorocentrum donhaiense*. The cell shape was asymmetric and elongated, one side at the anterior end with a slight extension. The posterior end of cells was rounded, but few cells were more or less pointed (Fig.2). The periflagellar area on the right valve was v-shaped with flagellar pores surrounded by an apical collar that varied in shape and size depending on the individual cell and the cell age (Fig.3a, b). The spines on the thecal plates were knob-like. The trichocyst pores were distributed mainly along the valve margins. Megacytic zones of aged cells became wider with dense tiny knobs (Fig.3c, d). The roundish nucleus was located in the posterior part of the cell. Irregular-shaped chloroplasts were distributed within the cell (Fig.4).

3.2 Molecular identification by 5.8S rDNA-ITS sequences

591 base pairs (bp) of 5.8S rDNA-ITS sequences were determined for strains LAMB090508 and PDKS0206. The GC content was 49%. Only one base of 591 bp was different between the two strains (Fig.5), making the nucleotide similarity 99.83%.

The ITS sequences of *P. donghaiense* (AY465116), *P. minimum* (AF208244), *P. balticum* (EU927548), *P. micans* (DQ485145), *P. rhathymum* (EU244466), *P. cassubicum* (EU244475), *P. lima* (FJ823582) and *Scrippsiella trochoidea* (EU325959) were downloaded from GenBank for comparison. The phylogenetic tree based on ITS sequence was constructed using the Neighbor-joining method with bootstrap values (Fig.6). Based on the phylogenetic tree, the *Prorocentrum* strains LAMB090508, PDKS0206 and AY465116 were clustered together. The cluster was supported by a high bootstrap value (100%).

The ITS sequences of ten species (strains) were aligned and their Jukes-Cantor corrected distances were calculated, as shown in Table 2. The nucleotide divergence between strain LAMB090508 and PDKS0206 was only 0.002; that between strain LAMB090508 and AY465116 was 0.005; that between strain PDKS0206 and AY465116 was 0.004. According to the ITS data, the Korean strain and the ECS strains (LAMB090508 and AY465116) represent an identical species, *Prorocentrum donghaiense* Lu. The results from the Jukes-Cantor distance matrix showed that there are distinct differences among the sequences of *P. donghaiense*, *P. minimum*, *P. balticum* and other *Prorocentrum* species.

3.3 Bio-geographical distribution

The different strains of species identified as *P. donghaiense* occurs in the coastal waters of the East China Sea (Lu and Goebel, 2001; Qi and Wang, 2003; Lu et al., 2005; this study). It is report as *P. dentatum* in the Hong Kong coastal waters and in the South China Sea (Hodgkiss and Yang, 2001), Korean, and Japanese coastal waters (Horiguchi, 1990; Lee et al., personal communication). It is also recorded in the Northern Arabian Sea (Munir, 2010). However, the patterns of its global distribution still remains uncertainty and needs to be studied.

3.4 Population dynamics

Prorocentrum donghaiense, first described by Lu and Goebel (2001), is a dinoflagellate which has

Table 1 Cellular dimensions of *P.donghaiense* isolated from ECS and Korea

Cell strain	Mean of *L*±standard deviation	Mean of *W*±standard deviation	Number of cells
Wenling of Zhejiang (LAMB090508)	19.29±1.39	10.64±1.39	80
Masan Bay of Korea (PDKS0206)	19.72±1.57	11.44±1.66	122

L. Length; *W*. Width; *n*. number of measured cells; the unit of *L* and *W* as well as of the standard deviation is μm

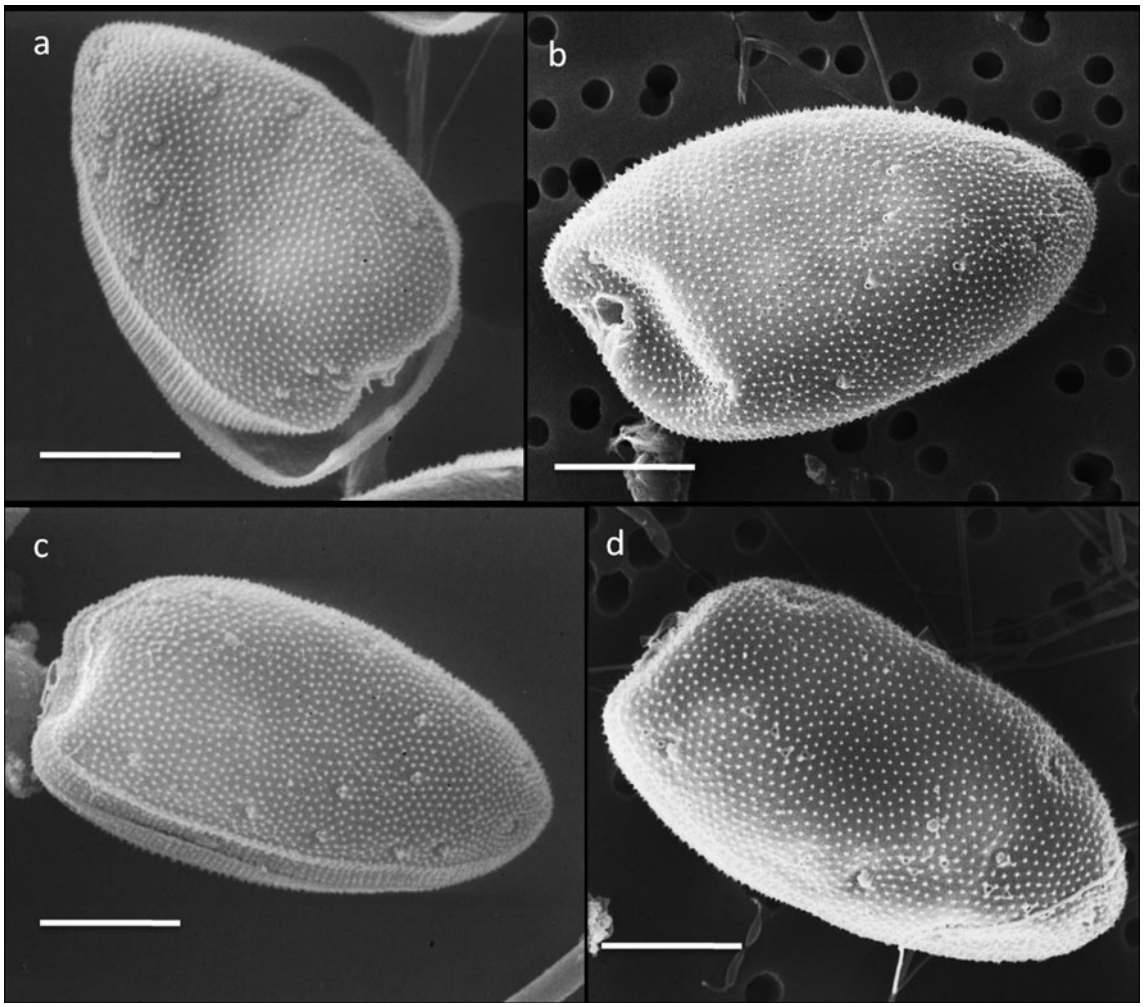

Fig.2 a, b: Right valve view of *P. donghaiense* cell under SEM; c, d: Left valve view of *P. donghaiense* cell under SEM, showing an extension at one side of apical area

a, c: strain LAMB090508; b, d: strain PDKS0206. Scale bar=5 μm

formed extensive high biomass blooms in the East China Sea (ECS) since 1998. Although it is neither a toxin producer nor a species associated with fish kills, it alters the ecosystem by forming dense blooms. For example, the zooplankton abundance in the vicinity of these blooms is substantially reduced. Current evidence shows that zooplankton preferentially graze other phytoplankton, suggesting poor nutritional quality for this species (GEOHAB, 2010). It occurs as a blooming species in late spring and the relatively high cell densities last during the summer months in the ECS. Blooms are initiated in the beginning of April and usually last until the middle of June. They cover large areas, in the order of 10 000 km^2, from the Changjiang to the Nanji archipelago (28°–35°N) and spread between 20 to 50 m water depth. *P. donghaiense* is highly dominant in the plankton during these bloom events, comprising more than 90% of numerical abundance (Lu et al., 2005). It also appears in autumn and winter seasons, but with low cell density.

4 DISCUSSION

The genus *Prorocentrum* was classified into five sections (Dodge, 1975). Two strains of *Prorocentrum* isolated from Wenling, China and Masan Bay, Korea are the closest to the Section E, defined by "spiny thecal plates present, trichocyst pores and anterior spine may also occur." On the basis of its taxonomic characteristics, two strains are proved to be *P. donghaiense* Lu that is close to *P. minimum*.

The cell shape of two strains in this study differs from that of *P. dentatum* described by Stein (1883). The posterior end of *P. dentatum* is remarkbly pointed and the anterior part has a triangular sharply

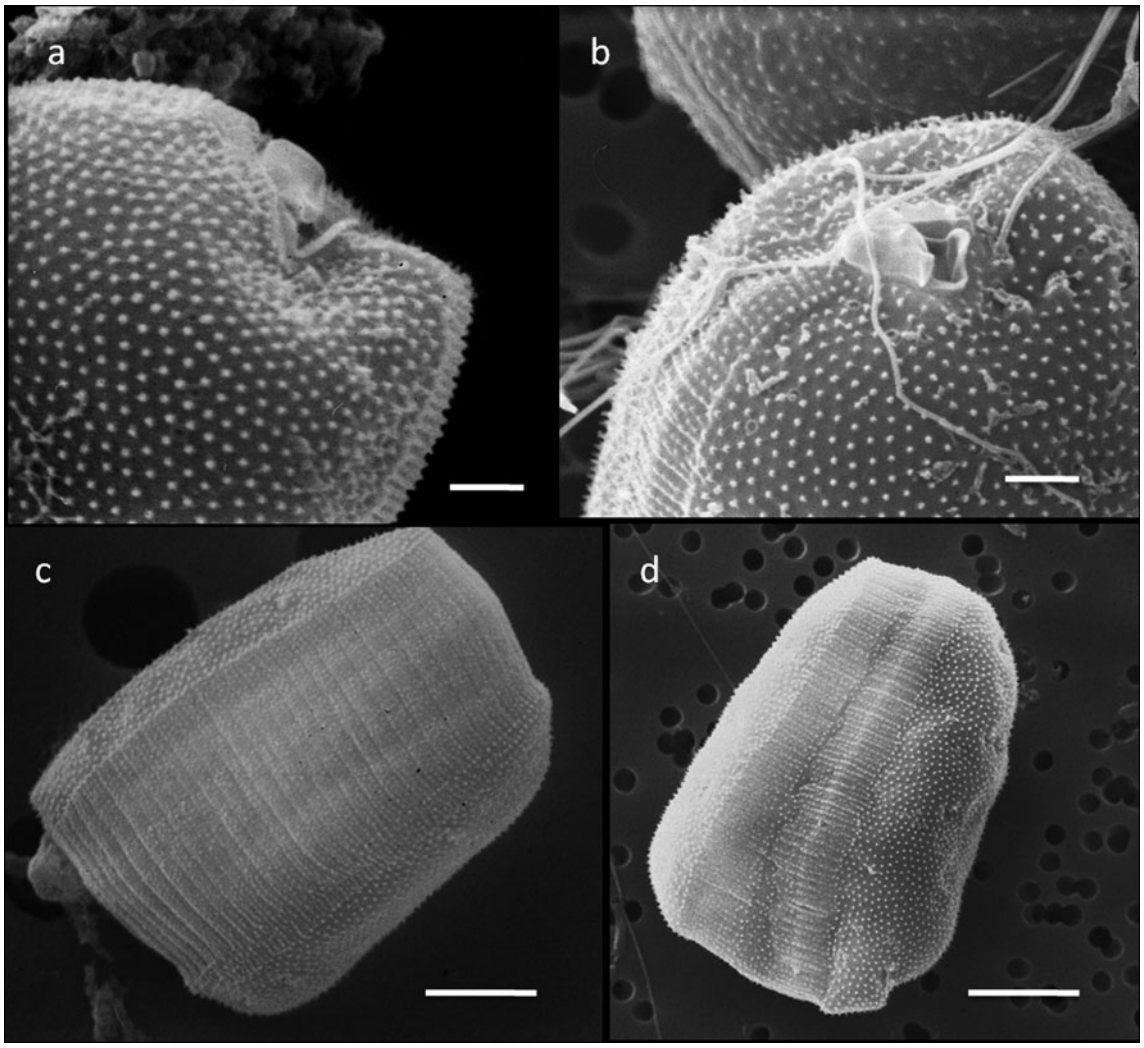

Fig.3 a, b: Ear-shaped extensions of *P. donghaiense* are clearly visible at the periflagellar area (a. strain LAMB090508; b. strain PDKS0206). Scale bar = 2 μm; c, d: Old *P. donghaiense* cell (dividing stage) with broad megacytic zones (a, c. strain LAMB090508; b, d. strain PDKS0206). Scale bar=5 μm

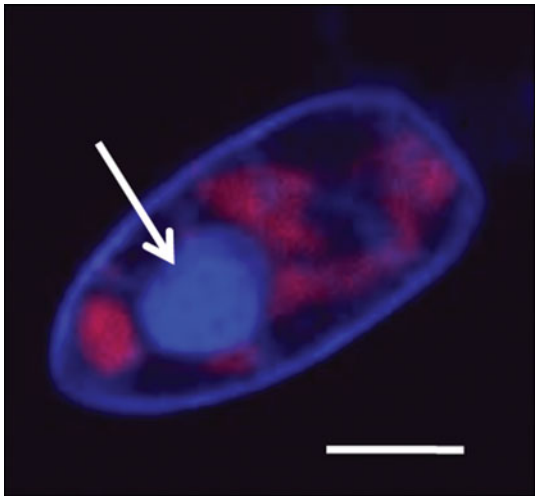

Fig.4 Laser scanning microscope (LSM) photo showing nucleus (arrow) and chloroplasts (red color) of *P. donghaiense* (Scale bar=5 μm)

pointed extension at one side. Dodge (1975) considered *Prorocentrum obtusidens* Schiller, *P. veloi* Tafall and *P. monacense* Kufferath as *P. dentatum* Stein and gave 36–60 μm as the cell length, which is much larger than the cell length in *P. donghaiense*. The outline of the cell illustrated by Dodge (1975) was characterized by parallel sides towards the anterior. Species presented by Horiguchi (1990) was much more elongated. *P. dentatum* described by Stein (1883) is a relatively large *Prorocentrum s*pecies of 50–60 μm length (Schiller, 1918, 1928, 1933). The species in this study is much smaller (<20 μm) which is close to the cell size (15–30 μm) described by Yoo and Lee (1986) and Horiguchi (1990). The shape of the cells in East Asia waters does not coincide with that of *P. dentatum* as described by Stein (1883) and Schiller (1928, 1933). *P. donghaiense* differs from *P. minimum*

Prorocentrum donghaiense (LAMB090508)

TTCCGTAGGTGAACCTGCGGAAGGATCATTCGCACGCATCCATTTGATTCATTGTGAATAACAGTTGGTGAGGATCTGGGTGGGGATGGAGATAGCATCGATGCCCCCAT
GCGGATACTCGAGGGCAGCAAGCCAGGCTCAGACCGTCTTCTGGGCTTGTCCTTGCTGCCAGTGTCTGTTTGATATTCTGTATGTTCCAATTTGTTCTGAGTGGTCTTCC
ACTCTTTATCTTCTTACAACTTTCAGCGACGGATGTCTCGGCTCGAACAACGATGAAGGGCGCAGCGAAGTGTGATAAGCATTGTGAATTGCAGAATTCCGTGAACCAAT
AGGGACTTGAACGTATACTGCGCTTTCGGGATATCCCTGAAAGCATGCCTGCTTCAGTGTCTATTCTTATTATTCCAGCATCTGACTTGTTCGGAATGCTTGTGTGTGTT
TGTGTGCCAGGGCGCCTCTACGGCCTCTGGCGCATTCAGTGCACAGGGTCTTCCCGCGCAAACAACTAGAAGAGTATCTCTGATGCTATCTGTTGTCTTGTTGTCGGGCT
GGGCCTTGTTGTCTAGTGCATATCGCACTCATACCAAGACA

Prorocentrum donghaiense (PDKS0206)

TTCCGTAGGTGAACCTGCGGAAGGATCATTCGCACGCATCCATTTGATTCATTGTGAATAACAGTTGGTGAGGATCTGGGTGGGGATGGAGATAGCATCGATGCCCCCAT
GCGGATACTCGAGGGCAGCAAGCCAGGCTCAGACCGTCTTCTGGGCTTGTCCTTGCTGCCAGTGTCTGTTTGATATTCTGTATGTTCCAATTTGTTCTGAGTGGTCTTCC
ACTCTTTATCTTCTTACAACTTTCAGCGACGGATGTCTCGGCTCGAACAACGATGAAGGGCGCAGCGAAGTGTGATAAGCATTGTGAATTGCAGAATTCCGTGAACCAAT
AGGGACTTGAACGTATACTGCGCTTTCGGGATATCCCTGAAAGCATGCCTGCTTCAGTGTCTATTCTTATTATTCCAGCATCTGACTTGTTCGGAATGCTTGTGTGTGTT
TGTGTGCCAGGGCGCCTCTACGGCCTCTGGCGCATTCAGTGCACAGGGTCTTCCCGCGCAAACAACTAGAAGAGTATCTCTGATGCTATCTGTTGTCTTGTTGTCGGGCT
GGGCCTTGTTGTCTAGTGCATATCGCACTTATACCAAGACA

Fig.5 5.8S rDNA-ITS of strain LAMB090508 and PDKS0206

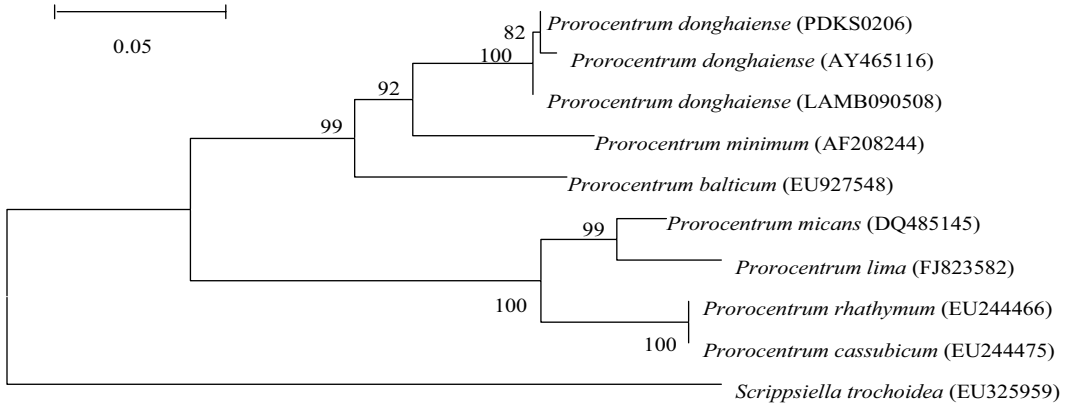

Fig.6 Phylogenetic tree based on 5.8S rDNA-ITS sequence data

Table 2 Jukes-Cantor corrected distance based on 5.8S rDNA-ITS

	1	2	3	4	5	6	7	8	9
P. donghaiense (LAMB090508)									
P. donghaiense (PDKS0206)	0.002								
P. donghaiense (AY465116)	0.005	0.004							
P. minimum (AF208244)	0.089	0.091	0.095						
P. balticum (EU927548)	0.113	0.111	0.120	0.137					
P. micans (DQ485145)	0.234	0.237	0.262	0.248	0.262				
P. rhathymum (EU244466)	0.235	0.238	0.263	0.257	0.260	0.084			
P. cassubicum (EU244475)	0.235	0.238	0.263	0.257	0.260	0.084	0.000		
P. lima (FJ823582)	0.253	0.255	0.268	0.264	0.274	0.046	0.094	0.094	
Scrippsiella trochoidea (EU325959)	0.364	0.361	0.368	0.372	0.364	0.385	0.406	0.406	0.411

in several features. They can be distinguished by their shape, valve micro-morphology and intercalary bands. (Lu et al., 2005).

On the basis of field samples collected from western coastal waters of Shikoko, Ehime Ken and Hiroshima Bay, Japan, Hada (1975) described two species, *Prorocentrum shikokuensis* Hada (Figs.1, 2) and *Prorocentrum setouti* Hada (Figs.3, 4). The cells of *P. shikokuensis* are 20–27 μm in length, 7–10 μm in width and 5 μm in thickness. The cell length of *P. setouti* is 23–30 μm and the width is between 12–17 μm. The two species are similar, but differ in cell thickness (Hada, 1975). However, megacytic zones of senescent cells of *Prorocentrum* can became broader within the same strain (Lu et al., 2005). The presence of apical spines in the periflagellar area is considered to be a conservative characteristic for identifying *Prorocentrum* species (Steidinger and Tangen, 1996). There are several species in the genus of *Prorocentrum* with apical spines. The morphology of *Prorocentrum micans* and *Prorocentrum minimum* are clearly distinct from Hada's species although they also contain apical spines. *Prorocentrum triestinum* has a pointed posterior end although the cell size is close to that of *Prorocentrum shikokuensis* and *Prorocentrum setouti*. All of them have an apical spine which can be observed under LM. On this basis, the two species of *Prorocentrum* described by Hada were considered as *P. triestinum* by Toriumi (1980). *P. donghaiense* Lu has no apical spine. Instead, an apical collar in the periflagellar area can be observed under SEM, but not under LM. Thus, *P. shikokuensis* Hada, *P. setouti* Hada and *P. donghaiense* Lu should not be considered as the same species.

The ITS sequences have proved to be good molecular tools for species identification (Zhang et al., 2004). In this research, the nucleotide similarity of ITS sequence from two studied strains is very high. The phylogenetic tree shows that three strains of *P. donghaiense* from a clade with 100% bootstrap support and *P. donghaiense* has a close relationship with *P. minimum*.

5 CONCLUSION

On the basis of the detailed morphology and molecular data analysis, both strains of *Prorocentrum* isolated from Wenling coastal water of Zhejiang Province, China and Masan Bay of Korea are confirmed as *Prorocentrum donghaiense* Lu. This species should be kept separate from *P. shikokuensis* Hada and *Prorocentrum setouti* Hada.

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