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A comparative study between *Prorocentrum shikokuense* and *P. donghaiense* (Prorocentrales, Dinophyceae) based on morphology and DNA sequences

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Abstract: The taxonomic position of an armored dinoflagellate *Prorocentrum donghaiense* Lu, 2001, which is one of the causative species of large red tides occurring off Changjiang River mouth was examined and compared to a similar species *P. shikokuense*. In both species, the following morphological features were shared; a weakly concave cell with a tiny apical spine around the periflagellar area, one side of the cell being slightly extended on the anterior end, a rounded posterior end, a rounded nucleus located in the posterior region and similar size of cells. DNA sequences of small subunit rDNA and internal transcribed spacer 1 (ITS1)-5.8S rDNA-ITS2 regions of these two species were largely the same. Morphological and molecular data suggest that *P. donghaiense* and *P. shikokuense* are synonymous and *P. shikokuense* has priority over *P. donghaiense*.

Key words: dinoflagellate, HAB, *Prorocentrum dentatum*, *Prorocentrum donghaiense*, *Prorocentrum shikokuense*

Introduction

Since the 1990s, a small thecate dinoflagellate has been known to form vast red tides off the Changjiang River mouth in the East China Sea. In 1995, particularly the red tides caused by this species expanded over more than 100 km. The organism causing this huge red tide off the Chanjiang River mouth in the East China Sea was described as a new species, *Prorocentrum donghaiense* Lu, 2001 (Lu & Goebel 2001). However, a species which is morphologically similar to *P. donghaiense* was earlier described as *Prorocentrum shikokuense* Hada, 1975 based on cells collected from Iwamatsu Bay in the Bungo Channel on the western coast of Shikoku, Ehime Prefecture, Japan on 8 June 1973 by Hada (1975). *Prorocentrum donghaiense* has now been observed in China and Korea, while *P. shikokuense* is reported from Japan.

Lu et al. (2005) reported variation of cell shape with careful examination of many cells of *P. donghaiense* using light and scanning electron microscopy (LM & SEM), and also compared the morphology of *P. donghaiense* with that of the original description of *P. shikokuense*. Based on these observations, Lu et al. (2005) concluded that it is difficult to determine whether or not the two species are the same. The reason for this difficulty is due to limited information on *P. shikokuense,* because of the description and handwritten illustrations based on only LM observations. For adequate identification, information on the fine morphology of *P. shikokuense* using LM and SEM is required. In addition, molecular data on *P. shikokuense* are lacking, although ribosomal DNA (rDNA) sequences of *P. donghaiense* were determined.

In this study, in order to compare *P. shikokuense* with *P. donghaiense* based on morphological and molecular data, we collected cells of *P. shikokuense* from the Iwamatsu Bay type locality as well as Uwajima Bay close to the type locality where Hada also found them in 1973, and from some additional locations where the specimens had been identified as *Prorocentrum dentatum* Stein, 1883 we then observed them with a light microscope and a scanning electron microscope and obtained molecular data (small subunit rDNA and internal transcribed spacer 1 (ITS1)-5.8S rDNA-ITS2 sequences).

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Materials and Methods

Sampling and culture

Plankton samples containing *Prorocentrum shikokuense* cells were collected from the surface waters of Iwamatsu Bay in July 2010. Cells of *P. shikokuense* were collected with a 10 µm-mesh plankton net from Uwajima Bay in June 2005. The plankton samples from Iwamatsu Bay were fixed with formalin at a final concentration of 2%. Glutaraldehyde was added to part of the field samples collected from Uwajima Bay (final concentration 2%) and they were kept as stock samples. For enrichment cultures, samples were added to a plastic cup containing Daigo's IMK Medium for Marine Microalgae (Daigo, Tokyo, Japan) and placed in a culture cabinet at 15°C with a photon flux density of about 50 µmol photons $\cdot m^{-2} \cdot s^{-1}$ under a 16 h : 8 h light : dark regime. Clonal cultures were established by capillary-pipette isolation and maintained under the conditions described above. Other clonal cultures of the targeted species "*Prorocentrum dentatum*" (Culture No. ND117 and ND118) were established using plankton cells collected from Ago Bay, Mie Prefecture in July 2005. Several additional samples for morphological observation of the species called *Prorocentrum* sp. aff. *dentatum* by Matsuoka et al. (2006) were examined from the following locations; off Mie Fishing port in Nagasaki Prefecture, around the Danjo Islands, and from Imari Bay in Nagasaki Prefecture (Table 1; Fig. 1).

Light microscopy

Cells were observed using an Olympus BX51 microscope equipped with Nomarski interference optics (Olympus Co. Ltd., Tokyo, Japan), and micrographs were taken with an Olympus DP50 digital camera attached to the microscope.

Scanning electron microscopy

The selected cells were allowed to adhere to a 0.1% poly-L-lysine coated glass plate. Thereafter, to remove mucilage on the cell surface, 2 N sodium hydroxide solution was used. The cells on the glass plate were rinsed a few times in distilled water, dehydrated in an ethanol series, and critical point dried using a critical point dryer HCP-2 (Hitachi Koki, Tokyo, Japan). Dried cells were coated with platinum-palladium in a JFC-1600 ion-sputter (JEOL, Tokyo, Japan). The materials were examined with a JEOL JSM-6390 scanning electron microscope.

Molecular analysis

Cells of *Prorocentrum shikokuense* collected from Iwamatsu Bay, Uwajima Bay, and "*P. dentatum*" cultures (ND117 and ND118) from Ago Bay were centrifuged, homogenized using a plastic stick, and then used as DNA templates. We used external primers SR1, LSUR2 and KOD Plus ver. 2 PCR kit (TOYOBO, Japan) in that order for the first round of PCR (Takano & Horiguchi 2006). In the second round of PCR, 0.5 µL of the PCR products was used as a DNA template with combinations of internal primers and TaKaRa Ex Taq™ (TAKARA BIO INC., Japan). Details of the PCR and sequencing protocol are provided by Takano & Horiguchi (2004, 2006).

We used two sets of alignments i.e. SSU rDNA and ITS regions, respectively. The SSU rDNA sequences were aligned manually based on the published secondary structure of the SSU rRNA molecule, available at the rRNA Web server (http://www.psb.ugent.be/rRNA/ssu/index.html) and alignments were refined manually. The ITS1-5.8S rDNA-ITS2 sequences were aligned manually with only a few gaps. Neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods were implemented by PAUP version 4.0b10 (Swofford 2002) for the phylogenetic analyses. The program Modeltest version 3.7 (Posada & Crandall 1998) was used to explore the model of sequence evolution that best fits the data set by the Akaike Information Criterion (AIC) (Akaike 1974). The model selected for the SSU alignment set was the GTR+*I*+*G*; the general time-reversible (GTR) model plus the proportion of invariable sites (*I*) and the variable sites (*G*; gamma distribution with shape parameter) with the following parameters: assumed nucleotide frequencies A=0.2565, C=0.2008, G=0.2652, and T=0.2775; substitution-rate matrix with AC=1.1158, AG=3.5526, AT=1.4707, CG=0.7050, CT=8.5060, and GT=1; *I*=0.4701; *G*=0.6959, and the number of rate categories=4, and that for the ITS regions alignment set was the K81uf (Kimura three-parameters with unequal base frequencies, Kimura 1981) with the following parameters: assumed nucleotide frequencies A=0.1977, C=0.2445, G=0.2623, and T=0.2955; substitution-rate matrix with AC=1, AG=2.9693, AT=0.4181, CG=0. 4181, CT=2.9693, and GT=1. ML was performed using the heuristic search option with a branch-swapping algorithm (tree bisection-reconnection; TBR) and the starting tree obtained by stepwise addition with the random addition of sequences (10 replicates). The distance matrix was calculated using the Kimura two-parameters distances (Kimura 1980), and the distance tree was constructed using the NJ method (Saitou & Nei 1987). MP was performed using the heuristic search option with random addition of sequences (1000 replicates) and the branch-swapping algorithm (TBR). All characters were weighted equally and gaps were treated as missing. Bootstrap analyses with 100 and 1,000 replicates for ML (using the heuristic search option with a branch swapping algorithm, nearest neighbor interchange (NNI) and a starting tree obtained by neighbor joining) of the SSU and the ITS regions, respectively, and 1,000 replicates of both sets for NJ and MP (with the random addition of sequences, 10 replicates, and TBR) were applied to examine the robustness and statistical reliability of the topologies (Felsenstein 1985).

Table 1. Occurrence records of *Prorocentrum shikokuense* ("*P. shokokuensis*" by Hada, 1975, *P. donghaiense* Lu, 2001 and *P. obtsusidens* by Adachi 1972) around the East China Sea.

Number	Location	Species name	Age of appearance	Reference	Supplment
1	Near Gouqi Islad in the Chanjeang River Estuary	as *P. donghaiense*		Lu D.-D. 2001	Original description
2	Off Chanjiang River	as *P. donghaiense*		Lu D.-D, et al. 2005	29–32°N×122–123.5°E
	China?	as *P. dentatum*		Han X.-T. et al. 2005 (unpublished)	AY551273 (GenBank)
3	Chanjeang River Estuary - West of Jeju Iskand, Korea	as *P. donghaiense*		Yoon Y.-H. et al. 2003	
4	Masan Bay of Korea	as *P. donghaiense*		Jeong et al. 2004 (unpublished)	AJ841810 (GenBank)
5	Masan Bay of Korea	as *P. dentatum*	June 1985, July 1986, July 1990	Park, S.-J. 1991	
5	Hengan Bay of Korea	as *P. dentatum*	June 1985, July 1990	Park, S.-J. 1991	
5	Bukshin Bay of Korea	as *P. dentatum*	July 1990	Park, S.-J. 1991	
5	Gohyun Bay of Korea	as *P. dentatum*	July 1990	Park, S.-J. 1991	
6	Iwamatsu Bay of the Bungo Channel	as *P. shikokuensis*	July 1973	Hada 1975	Original description
6	Iwamatsu Bay of the Bungo Channel	*P. shikokuense*	July 2010	This study	DNA analysis; Onservation
7	Bungo Channel	as *P. dentatum*	2008	Natl. Res. Inst. Fish. & Environ. Inland Sea 2008	
8	Suo Nada, Yamaguchi Pref	as *P. dentatum*	2003, 2004, 2005, 2007	Natl. Res. Inst. Fish. & Environ. Inland Sea 2007	
9	Seto Inland Sea	as *P. dentatum*	2003	Natl. Res. Inst. Fish. & Environ. Inland Sea 2003	
10	Fukuoka Bay	as *P. dentatum*	2005	Natl. Res. Inst. Fish. & Environ. Inland Sea 2005	
11	Imari Bay		July 2006	This study	
12	Omura Bay, Nagasaki Pref.	as *P. dentatum*	2005	Natl. Res. Inst. Fish. & Environ. Inland Sea 2005	
13	Off Mie Fishing Port, Nagasaki Pref.		May 2006	This study	
14	Ariake Bay, Kumamoto Pref.	as *P. dentatum*	2005	Natl. Res. Inst. Fish. & Environ. Inland Sea 2005	
15	Yatsushiro Sea, Kumamoto Pref.	as *P. dentatum*	2005	Natl. Res. Inst. Fish. & Environ. Inland Sea 2005	
16	Danjo Island West		April of 2006	This study	
17	Nomi Bay, Kochi Pref.	as *P. dentatum*	2008	Natl. Res. Inst. Fish. & Environ. Inland Sea 2008	
18	Tachibana Bay of the Kii Channel	as *P. shikokuensis*	May 1974	Hada 1975	
19	Kumano Nada, Mie Pref.	as *P. dentatum*	2006	Natl. Res. Inst. Fish. & Environ. Inland Sea 2006	
20	Owase Bay, Mie Pref.	as *P. dentatum*	2003, 2005	Natl. Res. Inst. Fish. & Environ. Inland Sea 2005	
21	Ago Bay, Mie Pref.	as *P. dentatum*	2002, August 2005	Hata 2005 (personal com.)	ND 117, ND 118; DNA analysis; Observation
21	Ago Bay, Mie Pref.	as *P. obtusidens*		Adachi 1972	
	South Pacific, South America	as *P. dentatum*			AY803742 CCMP1517
	Canary Island, the Atlantic	as *P. donghaiense*	2009	Ellegaard, M. (coll.), Hansen, G. (Iso.)	SCCAP K-1260

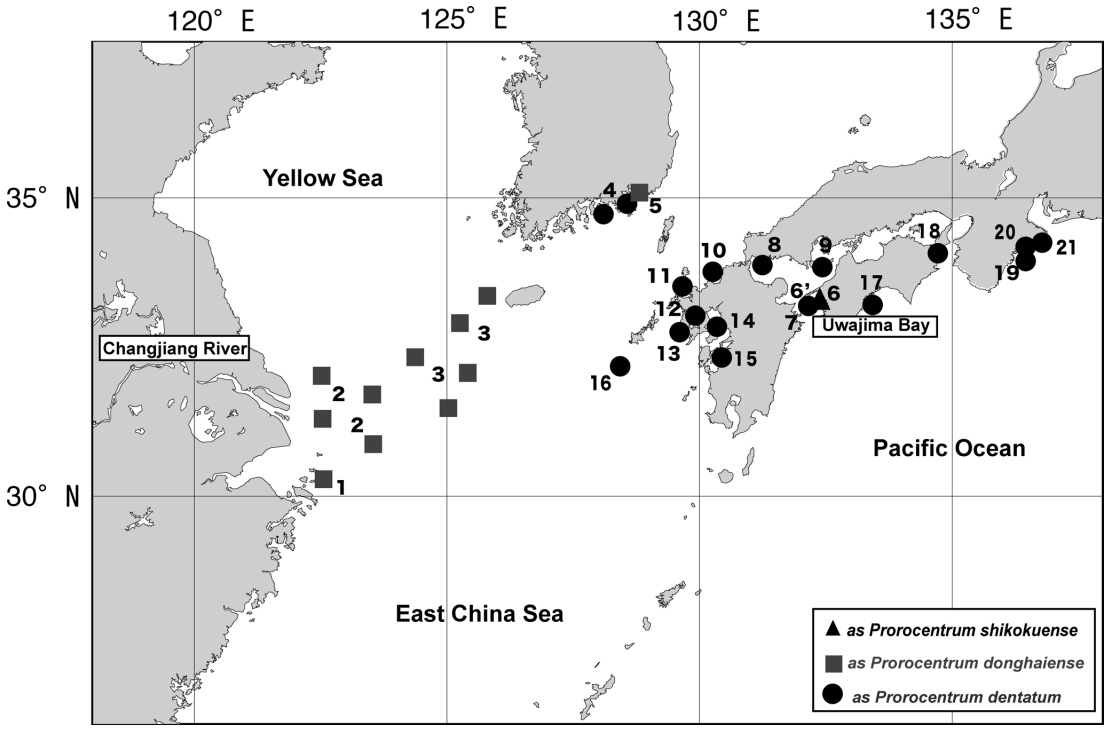

Fig. 1. Geographical distribution of *Prorocentrum shikokuense* including those formally referred to *P. donghaiense*, *P. dentatum* and *P.* sp. aff. *dentatum* in and around the East China Sea. Locations with numbers are shown in Table 1.

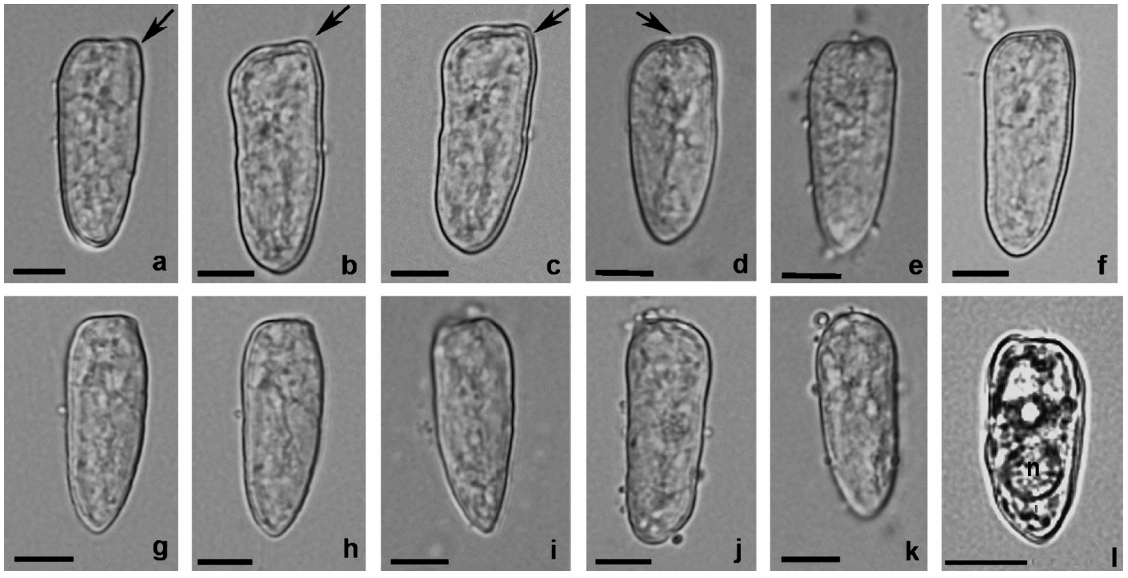

Fig. 2. Light micrographs of fixed cells of *Prorocentrum shikokuense* collected from Iwamatsu Bay (a–k) and Uwajima Bay (l) showing individual variations in cell shape and size. a–c. A black arrow indicates shoulder slightly developed on the anterior end. d. A black arrow indicates shallow indentation around the flagellar pore. l. "n" indicates a spherical nucleus. Scale bars = 10 µm.

Results

Morphology of *Prorocentrum shikokuense*

Light microscopy. Cells are variable in shape, elongated and asymmetric, narrowing toward the posterior ends, with the shape being somewhat similar to that of a sunflower seed (Fig. 2a–l). In blooming seasons, chains consisting of more than four cells are frequently observed. One side of the anterior end is often extends more than the other (Fig. 2a–c; a black arrow). The periflagellar area of the cell is slightly concave with a tiny apical spine that is rarely visible with a light microscope (Fig. 2d; a black arrow). The posterior end of most cells is generally rounded, but that of some cells is attenuated (Fig. 2g–l, k). Cells from Iwamatsu

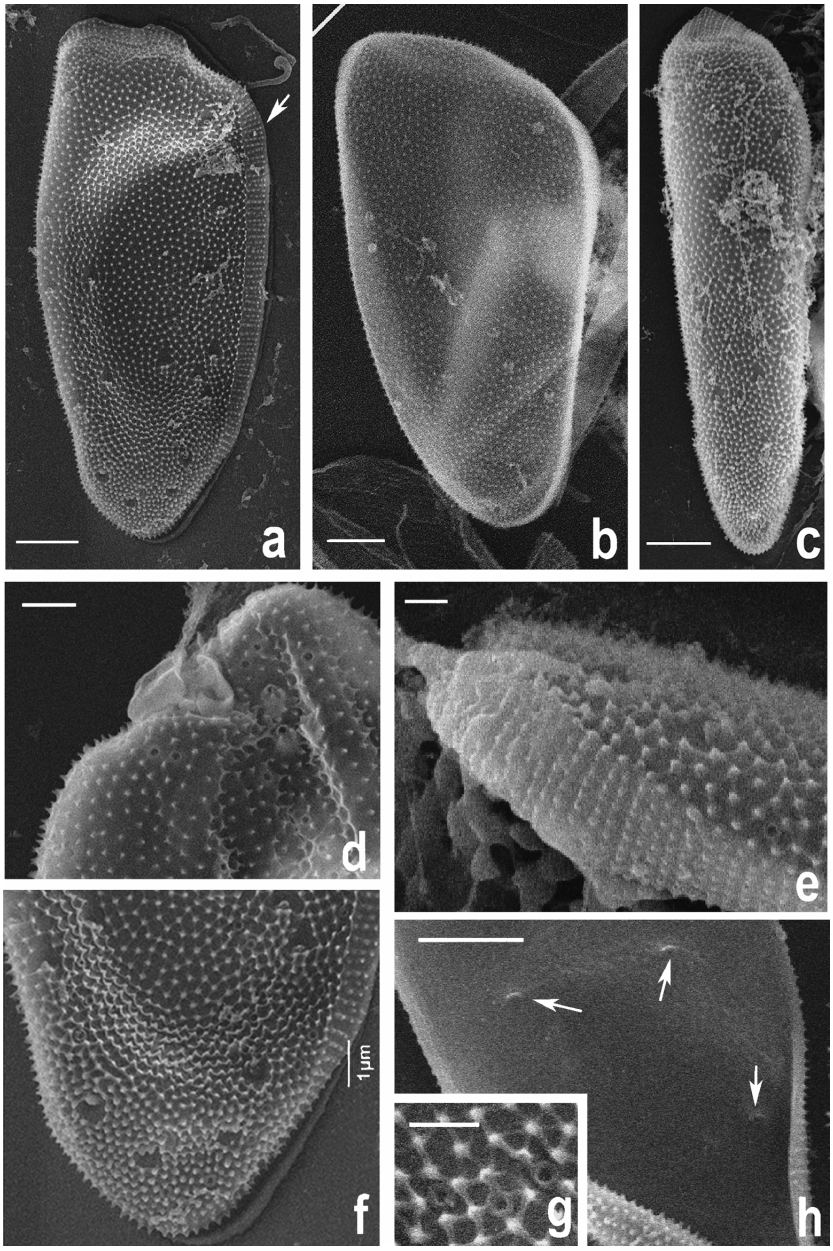

Fig. 3. Scanning electron micrographs of fixed cells of *Prorocentrum shikokuense* collected from Iwamatsu Bay. a; Right valve view showing megacytic zones (arrow) (Scale bar=5 µm). B; Left valve view showing gently swelling shoulder (Scale bar=5 µm). c; Typically slender cell (Scale bar=5 µm). d; 'ear-shaped' apical collar structure around the periflagellar area and trichocyst pores (Scale bar=1 µm. e; High magnification view of the cell showing tiny knobs distributed perpendicularly to the antero-posterior axis (Scale bar=1 µm). f; High magnification view of the posterior region showing trichocyst pores mainly distributed around the cell margin (Scale bar=0.5 µm). g; Small valve pores and knob-like spines densely distributed over the surface (Scale bar=1 µm). h; Inner surface of the valve showing small hollows probably corresponding to trichocyst pores (Scale bar=1 µm).

Bay, type locality, and Uwajima Bay near the type locality are from 20.2 to 31.7 µm long (mean=25.9 µm, n=40) and from 8.0 to 14.3 µm wide (mean=10.5, n=40). The range of long/wide ratio is from 1.79 to 3.88. The rounded nucleus is located in the posterior region of the cell (Fig. 2l).

Scanning electron microscopy. The thecal plates have many knob-like spines spread densely over the surface (Fig. 3a–f) and trichocyst pores (approx. 240 nm in diameter; Fig. 3g), that are mainly distributed around the peripheral margin of the cells (Fig. 3a, b), particularly around the antapex (Fig. 3f). The periflagellar area of the right thecal plate is V-shaped and concave with an ear-shaped collar structure that varies in shape and size (Fig. 3d), and one of the left thecal plats is slightly concave (Fig. 3a, b). Inside the cells, there are small hollows, probably corresponding to the trichocyst pores outside. The megacytic zones are well developed in natural cells (Fig. 3a, e)

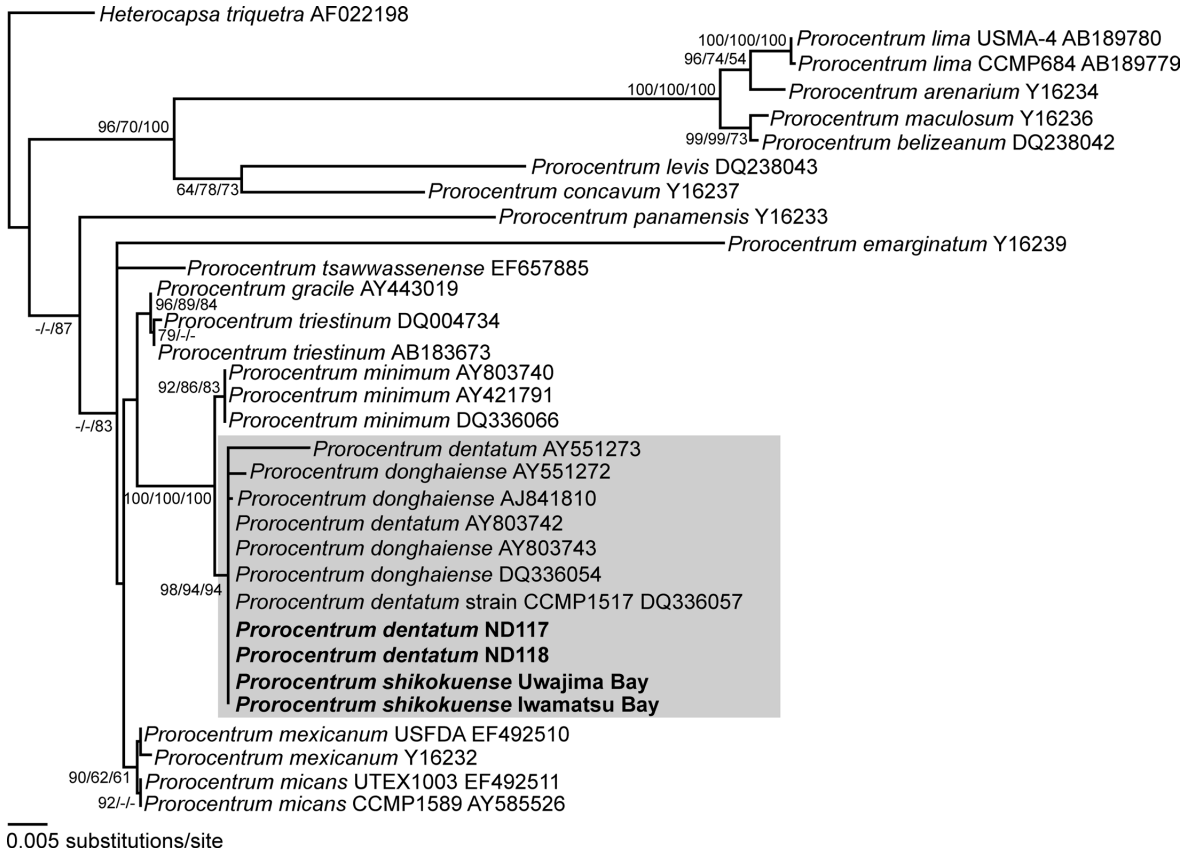

Fig. 4. Maximum likelihood tree constructed from SSU rDNA sequences, showing phylogenetic affinities of *Prorocentrum shikokuense* collected from Iwamatsu Bay and Uwajima Bay and "*P. dentatum"* (ND117 and ND118) collected from Ago Bay indicated with bold face, to sequences deposited as *P. donghaiense* and *"P. dentatum"*. For details of the evolutionary model and parameters used, see Materials and Methods. Bootstrap percentages (>50%) for NJ/MP/ML methods are presented at each node.

and dense rows of tiny knobs are arranged on the surface of the cell margin (Fig. 3e). Small hollows were observed on the inner surface of the valve (Fig. 3h).

Molecular data on *Prorocentrum shikokuense*

We determined the sequences of SSU rDNA (1731 bp) and the ITS regions (534 bp) of *P. shikokuense* collected from Iwamatsu Bay and Uwajima Bay, and SSU rDNA of so-called "*P. dentatum*" (ND117 and ND118). The sequences of SSU rDNA of *P. shikokuense* are identical to those of so-called "*P. dentatum*" (ND117 and ND118), *P. dentatum* CCMP1517 (AY803742 and DQ336057) and *P. donghaiense* (AY803743 and DQ336054) (Fig. 4). The sequences of the ITS regions of *P. shikokuense* from Iwamatsu Bay and Uwajima Bay are completely identical. *Prorocentrum shikokuense* shares the same sequences with *P. donghaiense* presented in Figure 11 by Lu et al. (2005) and *P. dentatum* CCMP1517. *Prorocentrum shikokuense* has 1 bp degenerate site; Y (C and T). At the same site *P. donghaiense* of Lu et al. (2005) has C and *P. dentatum* CCMP1517 has T. Therefore, *P. shikokuense* has two base pair substitutions from *P. donghaiense* (AY465116) (Fig. 5).

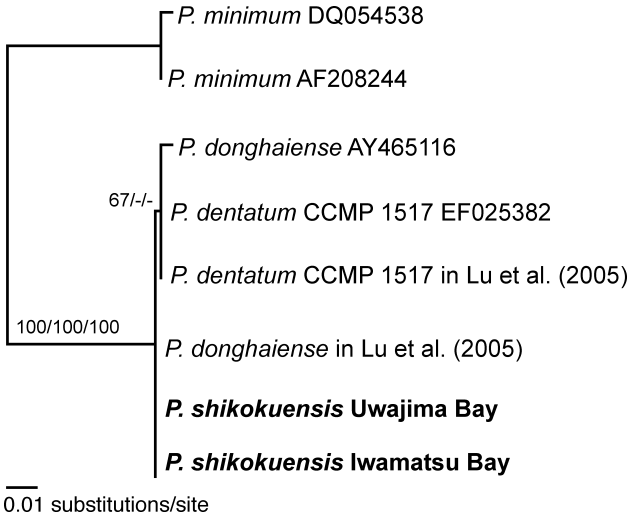

Fig. 5. Maximum likelihood tree constructed from ITS regions sequences, showing phylogenetic affinities of *Prorocentrum shikokuense* collected from Iwamatsu Bay and Uwajima Bay, indicated with bold face, to sequences deposited as *P. donghaiense* and *"P. dentatum"*. For details of the evolutionary model and parameters used, see Materials and Methods. Bootstrap percentages (>50%) for NJ/MP/ML methods are presented at each node.

Table 2. Cell size and ratios of length/width of *Prorocentrum shikokuense* collected from several different sites, including its type location.

No	Location	Length (mm)	Width (mm)	N	L/W ratio	Remarks
6	Iwamatsu Bay	20–27	7–10		2.7–3.9	*P. shikokuense* (Hada1972)
6	Iwamatsu Bay	22.7–31.7	8.5–11.6	10	2.05–3.88	Natural and culture (DAN analysis; onservation)
6′	Uwajima Bay	20.2–27.2 (25.0)	9.5–14.3 (11.7)	30	1.79–2.46	Natural and culture (DAN analysis; onservation)
21	Ago Bay	22–26.5 (23.6)	9–13 (10.5)	4	2.03–2.44	Natural and culture (DAN analysis; onservation)
13	Off Mie Fishing Port	18–22 (20.4)	8–10 (8.7)	8	2.12–2.48	Natural (Observation)
11	Imari Bay	19–23 (20.9)	8–11 (9.5)	10	2.13–2.38	Natural (Observation)
16	Danjo Islands	20–21 (20.7)	10–14 (12.0)	7	1.52–2.00	Natural (Observation)
1	East China Sea	18.6–21.6	9.6–13.0		1.7–1.96	*P. donghaiense* (Lu et al. 2005)

N: Number of cells observed

Morphometric measurement of cells reported as *Prorocentrum dentatum*

Cells collected from other areas are from 18.0 to 27.2 µm long and from 7 to 14.3 µm wide. The range of long/wide ratio is from 1.52 to 3.9 (Table 2).

Discussion

The morphology of our specimens collected from Iwamatsu Bay, the type locality for *Prorocentrum shikokuense* and Uwajima Bay near the type locality is identical with respect to cell shape and size to the original description of *P. shikokuense* (Hada 1975). Using a scanning electron microscope, we confirmed the characteristic distribution of trichocyst pores, development of a tiny anterior horn, and the presence of well developed megacytic zones. We were also able to newly observe several important morphological features of *P. shikokuense*, especially an ear-shaped collar structure at the apical end and valve pores. When the ear-shaped collar structure is large, it appears as a spine under LM as Hada (1975) described and illustrated, but that of most cells was not visible under LM. This structure is identical to that of *P. donghaiense* shown in Figure 6 by Lu et al. (2005). In comparison, SEM images of *P. donghaiense* given by Lu et al. (2005) have rounder posterior ends, though this feature is rather variable as shown in Figs 2 and 3. Also the long/wide ratio of the original *P. shikokuense* was slightly larger than that of *P. donghaiense*, making the former more elongate. Although the over all cell shapes of *P. shikokuense* and *P. donghaiense* are slightly different, they are not clearly distinguishable because of overlapping characters. Therefore, *P. shikokuense* and *P. donghaiense* can be considered to be the same species.

Previously, cells were reported in the East China Sea and Japanese–Korean coastal waters as "*P. dentatum"* by Yoo & Lee (1986), Park (1991), Horiguchi (1990) and the National Institute of Fisheries & Environment of the Inland Sea (2002–2008) and as *Prorocentrum* sp. aff. *dentatum* by Matsuoka et al. (2006). They also can be considered to be the same species as *P. shikokuense* based on the cell shape and size. The cell shape and the size of *P. shikokuense* are variable to some degree. Additionally, with regard to a comparison between *P. shikokuense* (as *P. donghaiense*) and the true *P. dentatum*, we agree with Lu et al. (2005) that *P. shikokuense* is a different species from *P. dentatum* (Stein 1883) in terms of cell shape and size.

In addition to morphological aspects, our molecular data, including specimens collected from the type locality, show that cells that have been identified as *P. donghaiense* and "*P. dentatum*" in the East China Sea and Japanese–Korean coastal waters are the same species as *P. shikokuense*, because their sequences, including those of *P. donghaiense* determined by Lu et al. (2005), are identical to those of *P. shikokuense*. The sequences of the ITS regions of *P. donghaiense* deposited under AY465116 have two base pairs substituted from others, but we have limited information on other data on *P. dentatum* accumulated in Genbank.

Prorocentrum shikokuense was originally described by Hada (1975, as *Shikokuensis*) under ICZN in 1975, with an English description and illustrations but without designation of a type. Lu et al. (2005) pointed out that *P. shikokuense* was an invalid name under both the International Code of Botanical Nomenclature (ICBN) and the International Code of Zoological Nomenclature (ICZN), due to lacking a Latin diagnosis and designation of a type, respectively. However, the ICZN accepts dinoflagellate species regardless of trophic nature (whether autotroph, heterotroph or mixotroph) and designation of a type was not a mandatory requirement but a recommendation before the fourth edition of the ICZN (Ride et al. 1999). The ICBN in Article 45.4 recognizes that a taxon validly described even under other nomenclatures has priority for its name and authorship. Therefore, *P. shikokuense* has validity under the ICZN and has priority over *P. donghaiense* discribed by Lu & Goebel (2001).

All of the previous occurrences of *P. shikokuense* are listed in Table 1 and arranged in Figure 1, which shows the wide distribution in and around the East China Sea and where it has formed massive blooms in the Changjiang River mouth.

Taxonomic appendix

Prorocentrum shikokuense Hada, 1975

Synonym: *Prorocentrum donghaiense*. Lu, 2001. Lu & Goebel (2001) p. 339, fig. 2.

Lectotype designated herein. figs. 1, 2 of Hada (1975) as *Prorocentrum shikokuensis*.

Remarks:

Because of an orthographic reason, the spelling of the species name, *Prorocentrum shikokuensis*, should be changed from *sihokuensis* to *shikokuense*.

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