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Detection of *Prorocentrum shikokuense* in the Mediterranean Sea and evidence that *P. dentatum*, *P. obtusidens* and *P. shikokuense* are three different species (Prorocentrales, Dinophyceae)

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Abstract: A dinoflagellate identified as *Prorocentrum dentatum*, *P. shikokuense* or *P. donghaiense* is responsible of massive harmful events. Blooms of a species identified as *P. shikokuense* have been recently reported in the Mediterranean Sea, and an exotic origin, tentatively introduced by ballast waters from Asia, has been hypothesized. The molecular data based on the small-, large subunit, and internal transcriber spacers ribosomal RNA gene (SSU-, LSU-, ITS rRNA) sequences confirmed *P. shikokuense* in the Mediterranean Sea. The Mediterranean ribotype is identical to a subtropical North Atlantic ribotype, and with slight divergence from the numerous sequences from the Pacific Ocean. To revisit the relationship between *P. shikokuense* (=*P. donghaiense*) and *P. obtusidens*, we provide the first micrographs of *P. dentatum* and *P. obtusidens*, the latter collected from the type locality. Our observations indicate that *P. dentatum*, *P. obtusidens*, and *P. shikokuense* are three different species. Their diagnostic morphological characters are: *Prorocentrum dentatum* is 44–60 µm long, leaf-shaped, pointed and central posterior end, conspicuous anterior shoulder; *P. obtusidens* is 33–41 µm long, irregular parallelepiped, almost parallel valve margins, eccentric and pointed posterior end, moderate anterior shoulder; and *P. shikokuense* is <25 µm long, sunflower seed-shaped, round and centric posterior end, inconspicuous anterior shoulder.

Keywords: Dinophyta, harmful algae bloom, invasive alien species, non-indigenous species, molecular phylogenetics, toxic phytoplankton

INTRODUCTION

Prorocentrum Ehrenberg is a common dinoflagellate genus distributed worldwide. The cells have two dissimilar flagella emerging from the anterior part, and a laterally compressed cell body covered by two large thecal plates. *Prorocentrum* comprises both planktonic and benthic species. Numerous benthic species are known to produce toxins, and some planktonic species are also toxic, or responsible for massive blooms in areas affected by fresh-water and/or anthropogenic inputs of abundant nutrients and dissolved organic matter (Lu and Goebel 2001; Lu et al. 2005; Takano and Matsuoka 2011; Su-Myat and Koike 2013; Shin et al. 2019; Roselli et al. 2019; Madhu et al. 2020). Correctly recog-

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nizing the taxonomic identities and relationships of the species within the genera is of pivotal importance to understanding the biogeography and bloom ecology of the species. However, questions remain regarding the taxonomic positions and biogeography of some species, particularly with respect to *P. dentatum* F.Stein, *P. obtusidens* J.Schiller, and *P. shikokuense* Hada (Takano and Matsuoka 2011; Shin et al. 2019 and references therein).

Stein (1883) described the oceanic species *Prorocentrum dentatum* from an indeterminate locality of the Atlantic Ocean as a leaf-shaped cell, with a pointed posterior margin, and a prominent anterior extension in one side, referred as the shoulder. From the southern Adriatic Sea, Schiller (1928) described *P. obtusidens* as a cell resembling an irregular parallelogram, almost parallel sides of the valves, and the posterior margin was pointed and displaced towards the opposite side of a moderate shoulder. The cell dimensions were 36 µm long and 16–20 µm wide. Dodge (1975) considered *P. obtusidens* as a junior synonym of *P. dentatum*, and he reported light and electron microscopy images of cells of a culture isolated at Plymouth, U.K. From blooms at the coasts of south Japan, Hada (1975) proposed *P. shikokuense* for sunflower seed-shaped cells of 20–27 µm long and 7–10 µm wide, and a tiny anterior spine. Hada's publication went unnoticed or his description based on line drawings was considered insufficient, and that species continued being identified as *P. dentatum* in Japan (Fukuyo et al. 1990) and other regions (Hernández-Becerril et al. 2000; Gómez et al. 2008).

The progressive eutrophication of the estuary of the Yangtze River resulted on massive blooms of a *Prorocentrum* species. It was described as *P. donghaiense* Lu based on individuals with a more robust appearance than the original description of *P. shikokuense* (Lu and Goebel 2001; Lu et al. 2005). Further morphological and molecular studies of isolates from China, Korea and Japan demonstrated that *P. donghaiense* was a junior synonym of *P. shikokuense* (Takano and Matsuoka 2015). Blooms of this species were also reported in other coastal waters of the Indo-Pacific basin (Su-Myat and Koike 2013; Madhu et al. 2020). In the Mediterranean Sea, Percopo et al. (2011) has commented that *P. shikokuense* (as *P. donghaiense*) is common in the eutrophic waters of the Bay of Naples, southern Italy, but no morphological or molecular evidence is available to verify its identification. Roselli et al. (2019) reported the proliferation of *P. shikokuense* in the port of Brindisi, southern Italy, speculating on the recent introduction via ballast waters from Asia. However, molecular data are not available to confirm the identity and to evaluate the biogeographic conductivity of the Mediterranean population of *P. shikokuense*.

Shin et al. (2019) extended the work of Takano and Matsuoka (2011) and proposed that *Prorocentrum shikokuense* and *P. donghaiense* were junior synonyms of *P. obtusidens* but distinct from *P. dentatum*. However, no new data or micrographs of *P. obtusidens* or *P. dentatum* were included to substantiate the assignments. Based on the interpretation of the literature alone, Shin et al. concluded that *P. obtusidens* is conspecific with *P. shikokuense*, although the former was described as a cell of 36 µm long and the latter has a distinct shape and is significantly smaller, ~20 µm long.

In this study, the presence of *P. shikokuense* in the Mediterranean Sea is documented with molecular data. We provide the first micrographs of *Prorocentrum dentatum* and *P. obtusidens*. Further assessment based on new and literature morphological data reveal that *Prorocentrum dentatum*, *P. obtusidens* and *P. shikokuense* are three different species.

MATERIALS AND METHODS

Sample collection

Cells of *Prorocentrum dentatum* were observed from Lugol's solution preserved samples collected in several locations of the open Pacific Ocean in 2002 and 2003 according to the method reported in Gómez and Furuya (2007). Cells of *Prorocentrum obtusidens* were observed from a sample collected on May 13th, 2021 offshore Taranto, Ionian Sea (40'433 N, 17'239 E) and on October 6th, 2021 offshore Bari, southern Adriatic Sea (41'152 N, 16'864 E). The plankton samples were collected in a vertical tow from 10 m depth to the surface using a 10 µm mess plankton net. Cells were preserved with acid Lugol's solution and examined with an inverted microscope (Eclipse Ti-S, Nikon, Tokyo, Japan) connected with a digital camera. Cells of *P. shikokuense* were obtained from a sample collected from surface waters of the harbor of Brindisi (40'654 N, 19'980 E), southern Adriatic Sea, on September 20th, 2018 following the same protocol as in Roselli et al. (2019). Cells were examined with the same protocol as described for *P. obtusidens.*

DNA extraction, PCR amplification, and sequencing

Molecular data are restricted to *Prorocentrum shikokuense* because numerous cells were available from coastal blooms. The cells of *P. dentatum* that were observed in 2002 and 2003 are not available, and the number of observed cells of *P. obtusidens* were insufficient for molecular analyses. The cells of *P. shikokuense* were micropipetted individually with a fine capillary into a clean chamber filled sterilized seawater contain small amounts sodium thiosulfate

for removing the iodine. Then, the cells were micropipetted individually into a clean chamber filled with autoclaved Milli-Q water. The same procedure was repeated twice to remove any source of contamination and salts. Finally, 50 cells were deposited in a 0.2-ml Eppendorf tube filled with absolute ethanol and was kept at room temperature and in darkness.

The ethanol preserved cells were transferred into a 2-ml Eppendorf tube, and centrifuged at 10,000g for 2 min. After the supernatant was removed carefully, approximately 100 mg of 0.5 mm-zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK, USA) were added to the pellet and bead-beaten at 6 m s^{-1} for 30 s using an MP Fast Prep-24 Tissue and Cell Homogenizer (MP Biomedicals LLC, Santa Ana, CA, USA). DNA was extracted using the CTAB (Cetyl Trimethyl Ammonium Bromide) protocol (Zhang and Lin 2005). 250 µL CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 0.2% β-mercaptoethanol, 0.1 mg ml^{-1} proteinase K) was added into the samples, and the tubes were incubated for six hours at 55 °C with gentle mixing every hour. For each sample, 250 µl of chloroform was added and mixed well, centrifuged for 10 min at 15,000g, and the supernatants transferred to a new 2-ml Eppendorf tube. The genomic DNA was then purified using DNA Clean & Concentrator Kit (Zymo Research Corp., Irvine, CA, USA) and eluted in 20 µl of 10 mM Tris-HCl (pH 8). One to two µl of extracted DNA was used as the template to amplify the gene fragment of small subunit (SSU), internal transcriber spacer (ITS) and large subunit (LSU) rDNA by using primer pairs of 18ScomF1 (GCTTGTCTCAAAGATTAAGCCATGC) and 18ScomR1 (CCACCTACGGAAACCTTGTTACGAC) (Zhang et al. 2005) and of 18ScomF-3 end: GTCGTAACAAGGTTTCCGTAGGTG with com28SR1: TCACGCATAGTTCACCATCTTTCG (Wang et al. 2014). PCR conditions were as follows: 94 °C for 2 min followed by 5 cycles of 95 °C for 20 s, 52 °C for 30 s, 72 °C for 40 s, then 35 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 40 s, and a final step of extension at 72 °C for 7 min. The PCR products with the expected size were purified using DNA Clean & Concentrator Kit and directly sequenced as reported Zhang et al. (2005). The sequences were deposited in DDBJ/EMBL/GenBank under accession numbers MZ593905–MZ593908.

Phylogenetic analyses

The small and large subunit, and internal transcriber spacers of the rRNA gene sequences were analyzed using Basic Local Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database. The SSU, LSU and ITS rRNA gene sequences of *Prorocentrum shikokuense* were aligned in large multiple sequence alignments containing distinct groups of dinoflagellates using ClustalW (Larkin et al. 2007) and the evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the General Time Reversible (GTR) model with Gamma Distributed rates in MEGA7 software (Kumar et al. 2016). The final trees were built including all the available sequences identified as *Prorocentrum dentatum*, *P. donghaiense*, *P. shikokuense* and other close sequences found from a BLAST search. Other Prorocentrales species, and representatives of other dinoflagellate groups were included. The SSU rRNA gene sequence with accession number EF492500 retrieved from GenBank as *Heterocapsa pygmea* CCMP1322 clustered in the clade of *P. shikokuense*. However, it was omitted because the sequence showed a long branch suggesting a tentative error. ML bootstrap values (BS) were obtained after 1,000 replications. The sequences of *Scrippsiella acuminata* were used for rooting the SSU, LSU and ITS rRNA gene phylogenetic trees. Bayesian posterior probabilities (PP) were calculated from a Bayesian analysis performed using MrBayes 3.2 (Ronquist et al. 2012) under the GTR + Gamma substitution model and the random-addition-sequence method with 10 replicates. Two independent analyses of four chains (one cold and three heated) were run with 20,000,000 generations, sampled every 1,000th cycle, with an appropriate burn-in (10%).

RESULTS

Morphology of *P. dentatum*, *P. obtusidens* and *P. shikokuense*

The records of *Prorocentrum dentatum* were scarce and restricted to samples from the open ocean. Lugol's solution preserved cells were observed in the equatorial Pacific, the Celebes Sea and the offshore waters in the south of Japan (Table 1). The cells were leaf-shaped with curved concave sides of the valves (Fig. 1C–I). The valves were tapering progressively into a posterior margin with centrally and pointed end. A distinctive character was the asymmetry of the anterior margin, with an extension in one side that is referred as the shoulder. Dimensions were 44–56 µm long (mean 49.6, n = 10), 25–28 µm wide (mean 26.6 µm), and the length/wide ratio varied from 1.6 to 2.0 (mean 1.8). The observations fit well with the Stein's original description (Fig. 1A–B).

A total of fourteen individuals of *Prorocentrum obtusidens* were found in our plankton net sample collected in coast of Taranto, south Italy (Table 1). The cell contour was broadly oblong, attenuated posteriorly, resembling an irregular parallelogram with straight valve sides from anterior to median point (Fig. 1K–AB). The cells showed a short pointed anterior extension, the shoulder, and a posterior end pointed and slightly displaced towards the opposite side of the shoulder. Cell dimensions of the individuals from the Ionian Sea were 33–42 µm long (mean 37.6 µm, n = 14), 13–19 µm wide (mean 16.1 µm), and the length/wide ratio varied from 2.0 to 2.9 (mean 2.3). The more elongated individuals fit well with Schiller's original description (Fig. 1J), while other individuals showed a more robust appearance (Fig. 1R). In order to facilitate the comparisons, a cell of *P. obtusidens* was placed alongside cells of *P. shikokuense* (Fig. 1AB). Another cell collected from the southern Adriatic Sea, type locality, was 34 µm long and 14 µm wide (Fig. 1AC).

Table 1. Location, geographical coordinates, depth and collection date of the records of *Prorocentrum* spp.

Taxon	Ocean region	Coordinates	Depth (m)	Collection date	Figure
P. dentatum	Celebes Sea	5.100 N 120.883 E	20	Dec 2002	1C
P. dentatum	South Japan	31.500 N 138.000 E	20	May 2002	1D
P. dentatum	South Japan	34.250 N 138.000 E	175	Jul 2002	1E
P. dentatum	South Japan	32.500 N 138.000 E	50	May 2002	1F
P. dentatum	Equatorial Pacific	0.000 N 175.000 W	50	Jan 2003	1G
P. dentatum	South Japan	32.000 N 138.000 E	50	Jul 2002	1H
P. obtusidens	Ionian Sea	40.433 N 17.239 E	0–10	May 2021	1K–AB
P. obtusidens	Adriatic Sea	41.152 N 16.864 E	0–10	Sep 2021	1AC
P. shikokuense	Adriatic Sea	40.654 N 19.980 E	0–1	Sep 2018	1AB, AE–AH

Individuals of *Prorocentrum shikokuense* were obtained from a bloom in the port of Brindisi, south Italy on September 20th, 2018 with an abundance of 9.9 × 10^4 cells L^{-1}. The cells were sunflower seed-shaped with a round posterior end, and an inconspicuous shoulder (Fig.1AB, AE–AG). Dimensions were 19.5–23.3 µm (mean 21.6 µm, n = 135) and 7.1–12.9 µm (mean 9.3 µm), except for megacytic cells that reached 14 µm wide (Fig. 1AH). The length/wide ratio varied from 1.7 to 3.0 (mean 2.3). Cell pairs or occasionally four-celled chains were observed (Fig. 1AE–AG).

Molecular phylogeny

We have obtained SSU-, LSU- and ITS rRNA gene sequences of four isolates of *Prorocentrum shikokuense* from the sample collected in September 2018 in the port of Brindisi, Italy. In all the phylogenies, the sequences retrieved from GenBank as *P. dentatum*, *P. shikokuense* and *P. donghaiense* clustered as a sister group to *P. cordatum* (Ostenfeld) J.D.Dodge [=*P. minimum* (Pavillard) J.Schiller, *P. balticum* (Lohmann) A.R.Loeblich] (Figs 2–4). In the SSU rRNA gene phylogeny, nearly all the sequences of *P. shikokuense* are from isolates of the Pacific Ocean (Fig. 2). The exceptions are our new sequences from the Mediterranean Sea, one sequence identified as *P. donghaiense* reported as the strain K-1260 from the subtropical North Atlantic Ocean (accession number MK713637), and other sequence identified as *P. cordatum* from Sweden, Baltic Sea (MH976698). The sequence from the Baltic Sea was identical (100%) to a sequence from Korea (AJ841810). This sequence of *P.* '*cordatum*' was shorter (only 1482 base pairs), and other molecular markers of the same strain are not available. The SSU rRNA gene sequence of *P. shikokuense* from the Mediterranean Sea only differed from the strain K-1260 by three nucleotides that were missing in our new sequences. The topology was similar in the LSU and ITS rRNA gene phylogeny (Figs 3, 4). The sequences of *P. shikokuense* from the Mediterranean Sea were 100% identical to the LSU and ITS rRNA gene sequences of strain K-1260 from the subtropical Atlantic Ocean. The geographical coverage of the samples of *P. shikokuense* was slightly wider in the ITS rRNA gene phylogeny. Among the sequences from isolates of the Indo-Pacific Ocean, there was a sequence identified as *P. rostratum* F.Stein (EU244471) that corresponded to the strain PR1V isolated at Vigo, NW coast of Spain (Fig. 4).

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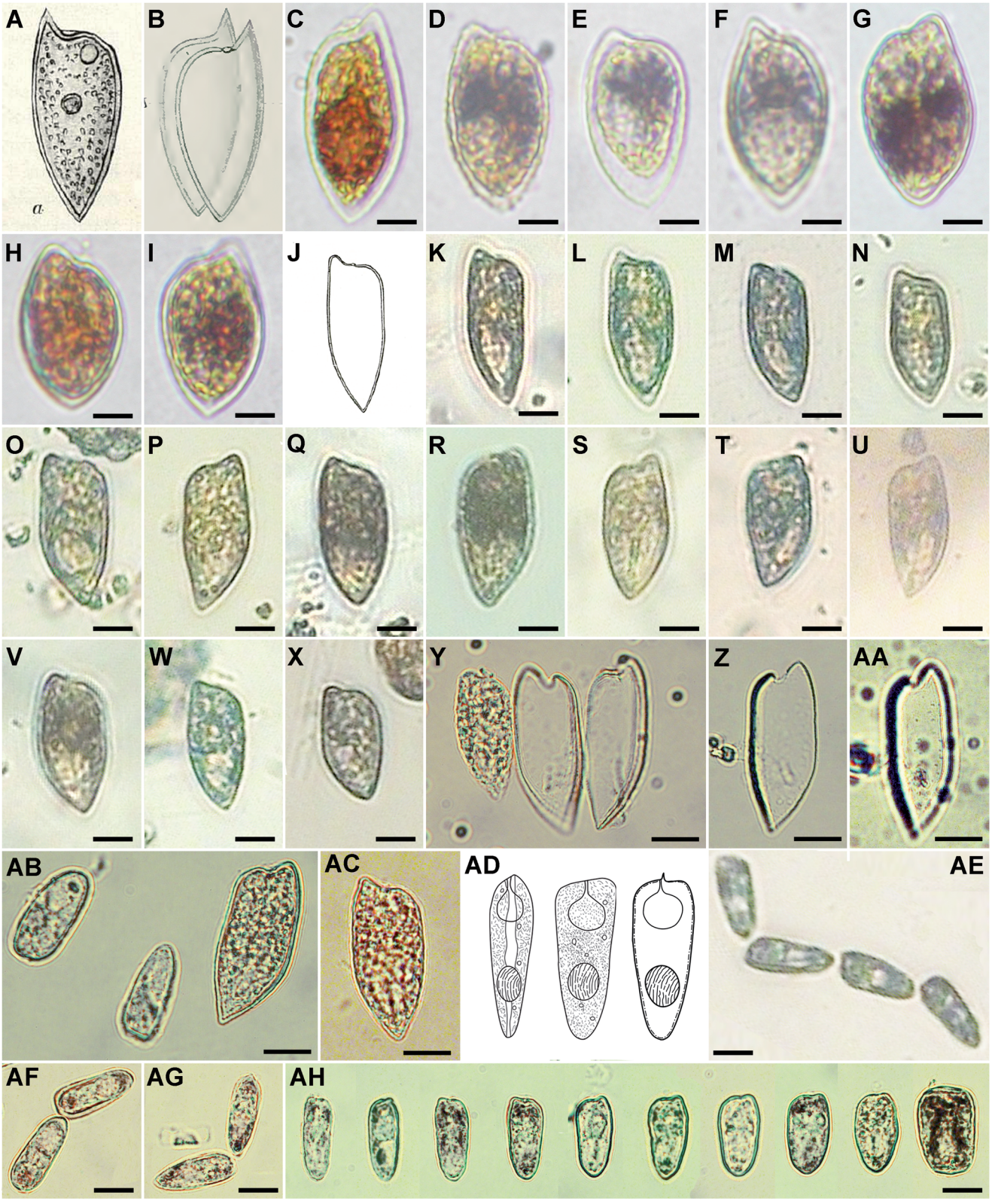
Fig. 1. Light micrographs of Lugol's solution preserved individuals and line drawings of *Prorocentrum dentatum* (A–I), *P. obtusidens* (J–AC) and *P. shikokuense* (AB, AD–AH). (A–B) *Prorocentrum dentatum* by Stein (1883). (C–I) Individuals from the Pacific Ocean. See Table 1 for location. (J) *Prorocentrum obtusidens* re-drawn from Schiller (1928). (K–AB) Individuals from a single sample collected offshore Taranto, Ionian Sea. (Y–AB) Valves of the same individual. (AB) One cell of *P. obtusidens* and two cells of *P. shikokuense*. (AC) *P. obtusidens* collected offshore Bari, Adriatic Sea. (AD) *Prorocentrum shikokuense* redrawn from Hada (1975). (AB, AE–AH) Individuals from a single sample of the harbor of Brindisi, Italy. (AE–AG) Chain-forming individuals. (AH) Note the intraspecific morphological variability. The last individual corresponds to a megacytic cell. Scale bar = 10 µm.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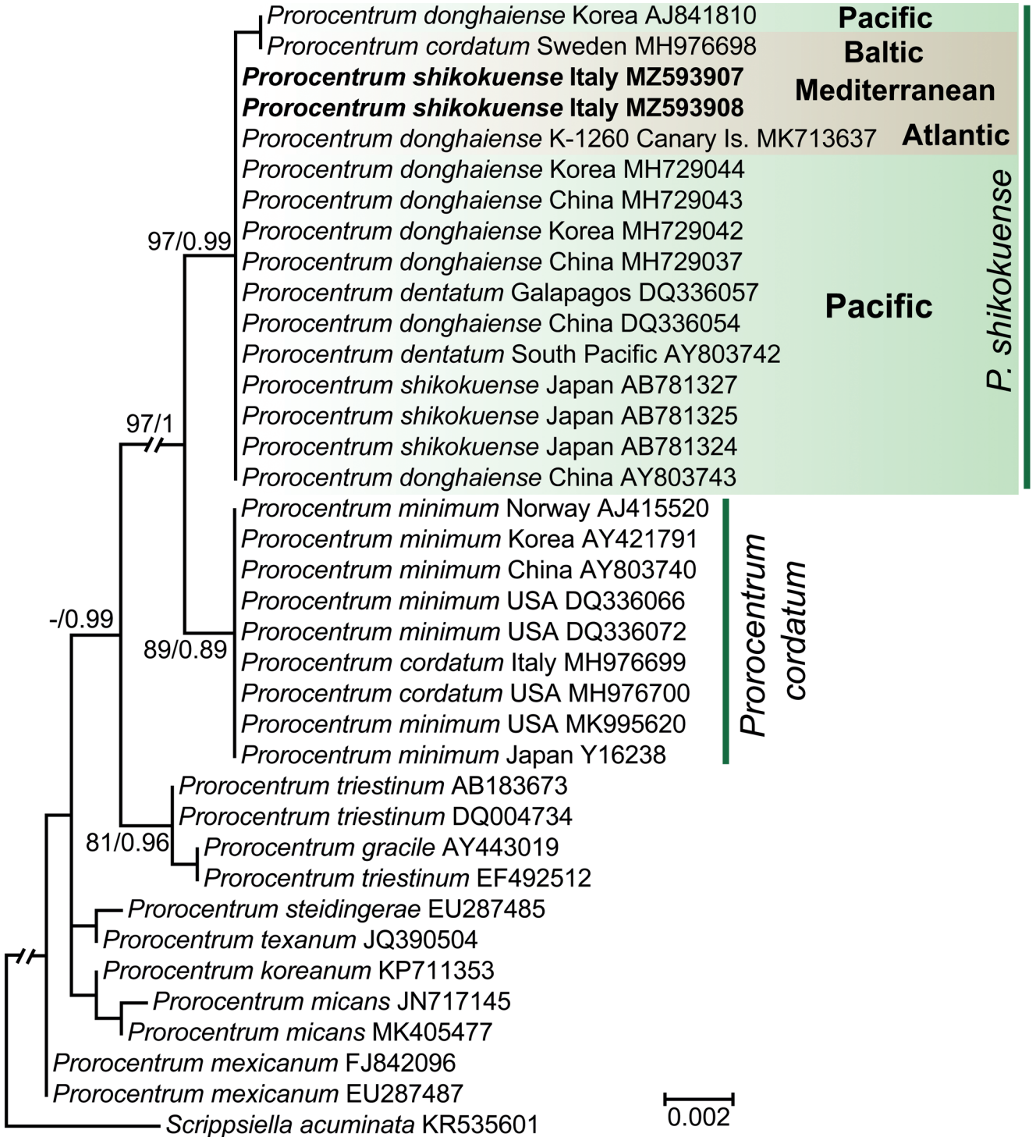

Fig. 2. Phylogenetic tree based on SSU rRNA gene sequences, showing the position of the sequence of *Prorocentrum shikokuense* by Maximum Likelihood (ML). Numbers near branches denote ML bootstrap (BS) and Bayesian posterior probability (PP) values. BS and PP values <70 and 0.8, respectively, are not shown.

DISCUSSION

Prorocentrum shikokuense in the Mediterranean Sea

Marampouti et al. (2021) listed *Prorocentrum shikokuense* among the twenty alien harmful microalgae in the Mediterranean Sea. However, the analyses of the available molecular data reveal that the Mediterranean or Atlanto-Mediterranean ribotypes of most of these 'alien' microalgae are genetically distinct from the supposed original exotic populations. This suggests that most of the Mediterranean populations of these microalgae are genetically independent populations rather than recent introductions from exotic ocean regions (Gómez and Galil 2021). The port of Brindisi receives a considerable ship traffic from Asia, apparently supporting the hypothesis of the introduction of exotic phytoplankton via ballast waters. Molecular data are needed, however, to resolve the biogeographical affinity of the Mediterranean population of *P. shikokuense*. The problem is that available rRNA gene sequences show a strong bias towards isolates from the Pacific Ocean. Another problem is the limited genetic divergence between the isolates because their rRNA gene sequences were almost identical even using variable molecular markers such as the ITS rRNA gene sequence. Nearly all the available sequences of *P. shikokuense* are from the Pacific Ocean (Figs 2–4). An exception in the SSU- and LSU rRNA gene phylogeny is the strain K-1260 of the Norwegian Culture Collection of Algae (accession numbers MK713637-9) that was isolated in a port of the La Gomera Island in the Canary Archipelago (subtropical North Atlantic). This small port does not receive big ships, and the Canary Archipelago is not known as a route of ship traffic from Asia. The LSU and ITS rRNA gene sequences of the strain K-1260 were 100% identical to the Mediterranean ribotype of *P. shikokuense*. This suggests a distinct population of *P. shikokuense* exists in the tropical Atlantic and Mediterranean Sea, at odds with the hypothesis of a recent introduction from Asian waters. However, because most of the available sequences are from isolates from the Pacific Ocean, and the resolution of the molecular marker is insufficient, an unequivocal conclusion on the biogeographical affinities of this species remain to emerge.

Percopo et al. (2011) reported a scanning electron microscopy (SEM) image of *Prorocentrum shikokuense* (identified as *P. donghaiense*) from the offshore waters of the Gulf of Lions, NW Mediterranean Sea. Cell dimensions were 16.5 µm long and 9.5 µm wide. The individual was shorter, and relatively wider (lower length/wide ratio) than the cells of *P. shikokuense* during a bloom in the port of Brindisi (mean 21.6 µm long, 9.3 µm wide). It should be noted that the drying in the SEM treatment reduces the cell size (Pertola et al. 2003). The robust appearance of the individuals reported in Percopo et al. (2011) matches well with smaller individuals of *P. donghaiense* in the species original description (16–22 µm long, 9–14 µm wide; Lu and Goebel 2001). This suggests that the morphotype observed in offshore waters differed from the blooming cells in neritic eutrophic waters (Fig. 1AH). Percopo et al. (2011) hypothesized that *Prorocentrum maximum* (Gourret) J.Schiller, described from the Gulf of Lions, could be a senior synonym of *P. shikokuense*. However, the shape of the cell illustrated by Gourret (1883) as *Postprorocentrum maximum* may correspond to an individual of *P. micans* without apical spine. Later, Schiller (1931) transferred it into *Prorocentrum* as *P. maximum*. Schiller (1931) reported a line drawing of *P. maximum* that is considered an earlier illustration of *P. mexicanum* B.F.Osorio (Gómez et al. 2017).

Prorocentrum shikokuense in the North Atlantic Ocean

The molecular data suggest the presence of *P. shikokuense* in the European Atlantic (Figs 2, 4), but studies combining molecular and morphological data remain missing. In addition to the sequence from the subtropical Canary Islands, there is other SSU rRNA gene sequence from a strain isolated in the Baltic Sea that clustered in the clade of *P. shikokuense* (accession number MH976698) (Fig. 2). Monti-Birkenmeier et al. (2019, their fig. 2c) provided a SEM image of this Baltic strain submitted to GenBank as *Prorocentrum cordatum*. The cell was oval with a length of 10 µm that fit well with *P. cordatum* (Monti-Birkenmeier et al. 2019), while cells of *P. shikokuense* are more elongated and ~20 µm long. There is no coherence between the morphology of the illustrated cell and the topology of the sequence MH976698 in the molecular phylogeny (Fig. 2). According Xu et al. (2010), *P. shikokuense* showed an optimal growth at 27 ºC in the East China Sea. In the Mediterranean Sea, blooms of *P. shikokuense* were observed at temperatures of 28–32 ºC (Roselli et al. 2019). The Baltic Sea is characterized by low temperatures, and huge blooms are not expected. Further studies are needed to confirm the occurrence of *P. shikokuense* in the Baltic Sea.

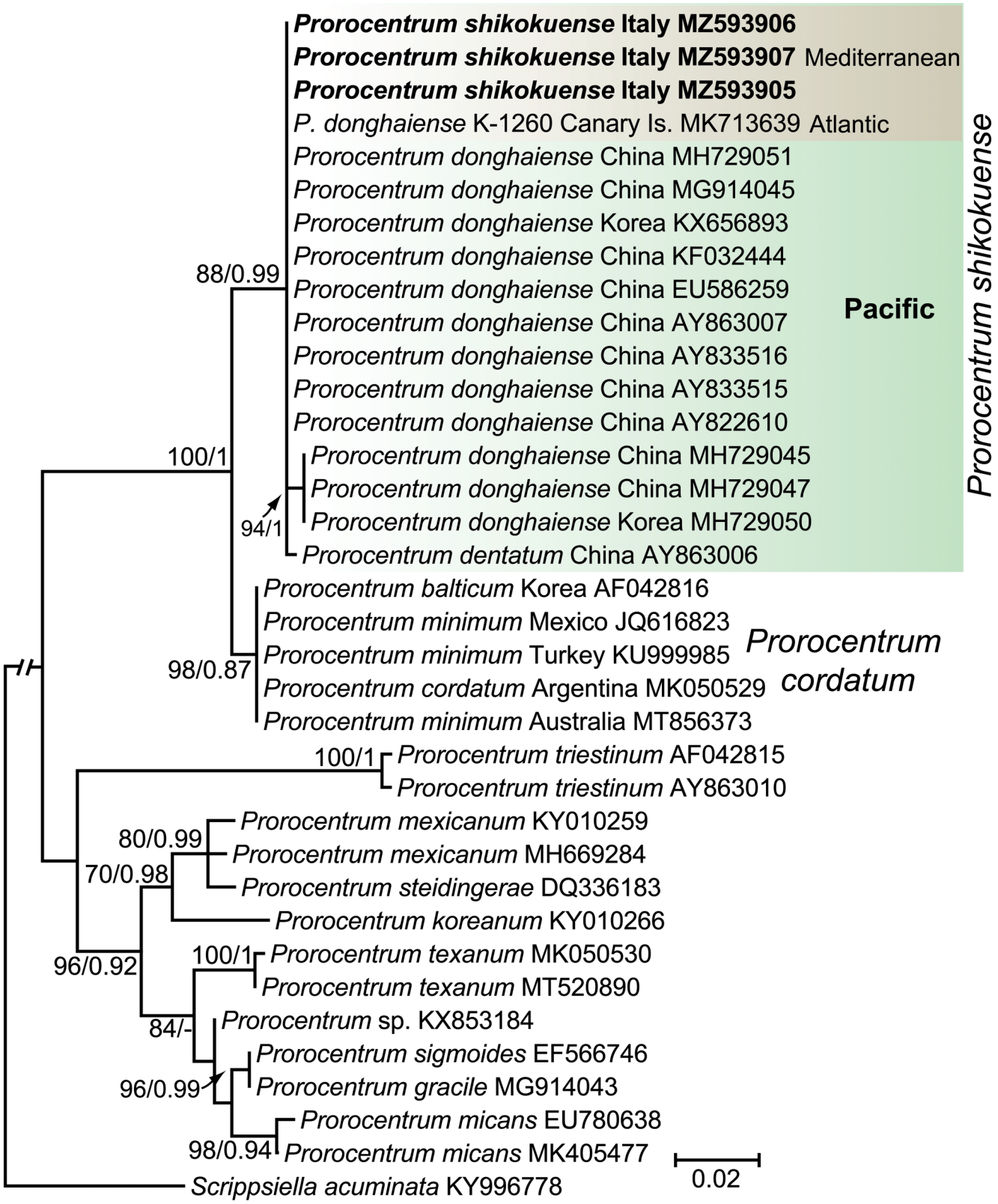

Fig. 3. Phylogenetic tree based on D1/D2 LSU rRNA gene sequences, showing the position of the sequence of *Prorocentrum shikokuense* by Maximum Likelihood (ML). Numbers near branches denote ML bootstrap (BS) and Bayesian posterior probability (PP) values. BS and PP values <70 and 0.8, respectively, are not shown.

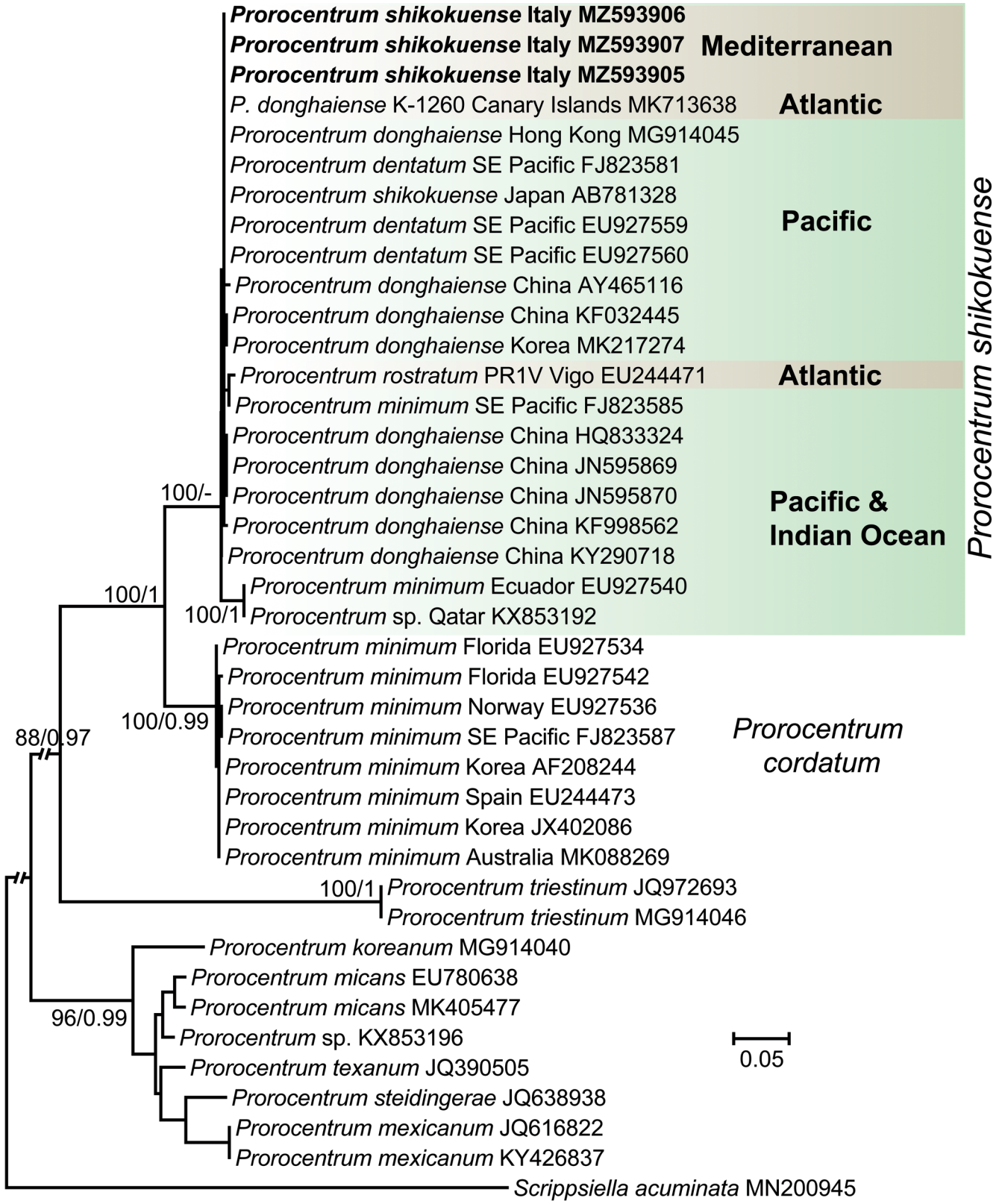

Fig. 4. Phylogenetic tree based on ITS rRNA gene sequences, showing the position of the sequence of *Prorocentrum shikokuense* by Maximum Likelihood (ML). Numbers near branches denote ML bootstrap (BS) and Bayesian posterior probability (PP) values. BS and PP values <70 and 0.8, respectively, are not shown.

In the ITS rRNA gene phylogeny, a sequence identified as *Prorocentrum rostratum* with accession number EU244471 clustered in the clade of *P. shikokuense* among sequences from the Asian Pacific Ocean (Fig. 4). *Prorocentrum rostratum* is a distinctive species known from tropical waters (Stein 1883). The cells are elongated, 5–6 times as long as broad (~60 µm long), valves ending at anterior end in long pointed process, notched in the side view. *Prorocentrum rostratum* is easily distinguishable from *P. shikokuense* that is smaller, ~20 µm long, with an inconspicuous shoulder, and a round posterior margin (Fig. 1AB, AE–AH). The strain *P. rostratum* PR1V (EU244471) was isolated at Vigo, NW Spain. It is not expected to find the tropical species *P. rostratum* in the cold waters of the Galician Rias. No morphological information of the strain PR1V is available currently. Beyond the problem in the identification associated with the sequences of *P. shikokuense* in Europe, the presence of *P. shikokuense* in the European Atlantic Ocean is not unexpected, but a definite conclusion awaits combined morphological and molecular analyses. While the molecular data reveal that *P. cordatum* is present in the American and European coasts of the North Atlantic Ocean (Figs 2–4), there is no evidence for the presence of *P. shikokuense* in the American Atlantic coasts.

The distinction of *Prorocentrum dentatum*, *P. obtusidens* and *P. shikokuense*

Numerous sequences of *Prorocentrum shikokuense* are retrieved as *P. dentatum* in GenBank (Figs 2–4). In a pioneer study on the oceanic dinoflagellates, Stein (1883) described *P. dentatum* from an unknown location of the Atlantic Ocean without size data. He illustrated a cell with leaf-shaped outline, tapered posterior margin with a pointed end. The anterior margin was asymmetric with the shoulder as a sharply pointed anterior prolongation in one side (Fig. 1A–B). There are no observations of cells identical to Stein's line drawings because this author's drawing style tends to exaggerate the acuteness of the cell appendices. Examples can be found in Stein's illustrations of *Tripos eugrammus* (Ehrenberg) F.Gómez or *Dinophysis tripos* Gourret (Fig. 5). Lohmann (1920) reported as *P. dentatum* as cells with prominent but blunt shoulder, which is closer to the real morphology of this species (Fig. 1C–I). Our observations reveal that *P. dentatum* is a rare species. The few records were located between depths of 20–175 m in the open ocean (Table 1). This renders the studies when compared to the other easily accessible bloom-forming coastal species of *Prorocentrum*.

Schiller (1918) cited *P. dentatum* in the list of species observed from research cruises in the Adriatic Sea, but he did not report any illustration or morphological data to verify the identity. Schiller (1928) described *P. obtusidens* from the southern Adriatic Sea as elongated cell of 36 µm long and 16–20 µm wide, with parallel valve sides tapering posteriorly in an acute eccentric end, located in the opposite side of the shoulder (Fig. 1J). In his monograph, Schiller (1931) illustrated *P. dentatum* and *P. obtusidens* as two distinct species. Schiller reproduced the illustrations of *P. dentatum* by Stein (1883) and Lohmann (1920), and he reported dimensions of 50–60 µm long for this oceanic species with low abundances. In the Italian coasts, Rampi (1969, his plate 1, his figure 1) showed a micrograph of *P. dentatum* that fit with Stein's original description. Rampi (1969) reported cell dimensions of 48–55 µm long that is in the range of 50–60 µm long reported by Schiller (1931) or in the present study (44–56 µm long) (Table 2).

Compared with Schiller (1928) description of *P. obtusidens* from the southern Adriatic Sea, our observations from southern Italy, including the type locality, fit well in shape and size with Schiller's original description (Fig. 1J–AC). Our individuals of *P. obtusidens*, 33–42 µm long and 13–19 µm wide, are within the range of 36 µm and 16–20 µm of Schiller's original description. The size is significantly smaller than *P. dentatum* (44–56 µm, Fig. 1C–I). *Prorocentrum obtusidens* shows some morphological variability (Fig. 1K–AC), but the diagnostic characters that distinguish *P. dentatum* and *P. obtusidens* are stable such as the parallel valve contour, the eccentric end of the posterior margin, and the less prominent shoulder in the latter species. Böhm (1936) and Wood (1963) reported illustrations of *P. obtusidens.* Authors such as Margalef (1973) have considered *P. dentatum* and *P. obtusidens* as distinct species.

Dodge (1975) reviewed the genus *Prorocentrum*, but at that time he was not aware of the description of *P. shikokuense* by Hada (1975). Dodge proposed *P. obtusidens* as a junior synonym of *P. dentatum*, based on data from the literature and his own observations from a culture isolated in the 1960's by Dr. Parke at Plymouth, U.K. Dodge (1975) reported that *P. dentatum* ranged from 36–60 µm long, the upper limit matching the value by Schiller (1931) for *P. dentatum*, and the lower limit matching the length of *P. obtusidens* reported by Schiller (1928, 1931).

Stein (1883) described *P. dentatum* as a leaf-shaped cell with pointed centric end and a prominent shoulder

Table 2. Morphological comparison of *Prorocentrum dentatum*, *P. obtusidens* and *P. shikokuense* (=*P. donghaiense*).

Taxon	Shape	Posterior margin	Shoulder	Cell size (long, wide in µm), reference
P. dentatum	leaf, convex sides	pointed, centric	conspicuous	50–60 (Schiller 1931) 44–56, 25–28 (this study)
P. obtusidens	parallelepiped, parallel sides	pointed, eccentric	moderate	36, 16–20 (Schiller 1931) 33–42, 13–19 (this study)
P. shikokuense	sunflower seed	round centric	inconspicuous	20–27, 7–10 (Hada 1975) 19–23, 7–12 (this study)
P. donghaiense	ovate	round centric	inconspicuous	16–22, 9–14 (Lu and Goebel 2001) 19–23, 7–12 (this study)

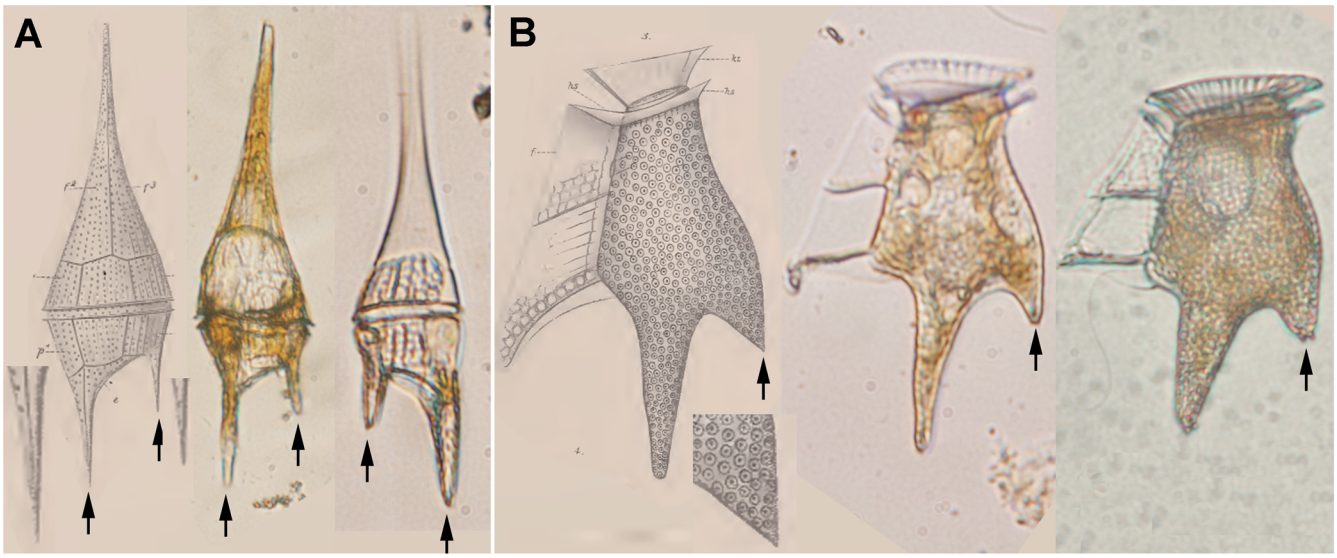

Fig. 5. A comparison of the line drawings in Stein (1883) and micrographs of *Tripos eugrammus* (A) and *Dinophysis tripos* (B). The arrows point the Stein's drawing style with acuter appendices than in the real morphology of the cells.

(Fig. 1A–B). As *P. dentatum*, Dodge (1975, his figure 4a,b) reported micrographs of cells of a culture that largely differed from Stein's original description. We do not know the age of the culture and the influence of culture conditions or culture media of the 1960's on the morphology of the cells. For example, the Plymouth culture nº18 was described as *Exuviaella mariae-lebouriae* Parke & Ballantine (=*P. cordatum*) for cells with a round to oval shapes (Parke and Ballantine 1957). Later, Bursa (1969) examined the same culture, and he described the new species *Exuviaella cordiformis* Bursa for cells that were heart-shaped. Dodge (1975) in his figure 4a reported a light micrograph of two cells with almost straight valve sides and round and eccentric posterior margin. The cells showed a distinct length-width ratio. Dodge (1975) in his figure 4b illustrated a transmission electron micrograph of a cell that showed more curved valve sides than the other individuals, and the posterior margin was centrally located. This cell shape strongly resembled *P. shikokuense*. Dodge's figure 4b showed the valve with trichocyst pores, mainly in the margin of the valve. The cell showed four pores in the posterior margin that was similar to that of *P. shikokuense* from Japan (Takano and Matsuoka 2011, their fig. 3a,f) or from the Mediterranean Sea (Roselli et al. 2019, their fig. 3b).

Dodge (1975) did not report scale bars in his figures 4a–b. Lu and Goebel (2001) discussed on the dimensions of Dodge's cells, and that according to Throndsen the cells were 23–24 µm long. Lu and Goebel (2001) commented that the cells in Dodge (1975) showed seven spines per 2 µm. Based on this, the cell of Dodge's

figure 4b is 22 µm long, while Dodge reported in the text that *P. dentatum* ranged from 36–60 µm. Based on the cell shape and size, and the arrangement of the pores, Dodge (1975) might have examined a culture of *P. shikokuense* isolated in the European coasts in the 1960's. The synonymy proposed by Dodge (1975) has influenced further work, and observations of *P. shikokuense* were identified as *P. dentatum* even in Japan (Fukuyo et al. 1990), and other regions (Hernández-Becerril et al. 2000; Gómez et al. 2008).

The report by Shin et al. (2019) did not provide original data such as micrographs of *Prorocentrum obtusidens*, *P. dentatum* or any information from the Mediterranean Sea, obscuring the certainty of the identity of the two species. Our comparisons, as illustrated in Figure 1AB, show unequivocally that *P. obtusidens* and *P. shikokuense* (=*P. donghaiense*) are different species (Table 2). Further studies are needed to obtain molecular data of the rare oceanic species *P. dentatum* and *P. obtusidens*, to determinate the geographical distribution of the neritic bloom-forming *P. shikokuense*, and to confirm the observations in the European Atlantic Ocean.

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