Characterization of *Prorocentrum elegans* and *Prorocentrum levis* (Dinophyceae) from the southeastern Bay of Biscay by morphology and molecular phylogeny[1]

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Abstract

Benthic *Prorocentrum* species can produce toxins that adversely affect animals and human health. They are known to co-occur with other bloom-forming potential toxic benthic dinoflagellates of the genera *Ostreopsis*, *Coolia* and *Gambierdiscus*. In this study, we report on the presence of *P. elegans* M.Faust and *P. levis* M.A.Faust, Kibler, Vandersea, P.A. Tester & Litaker from the southeastern Bay of Biscay. Although sampling was carried out in Summer-Autumn 2010 - 2012 along the Atlantic coast of the Iberian Peninsula, these two species were only found in the north-eastern part of the Peninsula. Strains were isolated from macroalgae collected from rocky-shore areas bordering accessible beaches. Morphological traits of isolated strains were analysed by LM and SEM, whereas molecular analyses were performed using the LSU and internal transcribed spacer (ITS)1-5.8S-ITS2 regions of the rDNA. A bioassay with *Artemia fransciscana* and liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analyses were used to check the toxicity of the species, whose results were negative. The strains mostly corresponded to their species original morphological characterization, which is supported by the phylogenetic analyses in the case of *P. levis*, whereas for *P. elegans* this it is the first known molecular characterization. It is also the second known report of *P. elegans*.

Key words: Bay of Biscay, ITS1-5.8S-ITS2, LSU, Morphology, Phylogeny, *Prorocentrum elegans*, *Prorocentrum levis*.

List of Abreviations: Bayesian Analysis (BA); 4', 6-diamidino-2-phenylindole (DAPI); Dinophysistoxins (DTX); Internal Transcribed Spacer (ITS); Liquid chromatography–

high-resolution mass spectrometry (LC-HRMS); Maximum Likelihood (ML); Okadaic Acid (OA).

Introduction

The cosmopolitan genus *Prorocentrum* (Dinophyceae) was first established by Ehrenberg in 1834 with *P. micans* as type species. Around 60 species have so far been described, most of them from marine waters and only two are known to inhabit freshwater (Hoppenrath et al. 2013). Species in the genus can be benthic, epibenthic or planktonic and some strains produce toxins, such as okadaic acid (OA), dinophysistoxins - 1, 2, 4, borbotoxins, other OA derivates and prorocentrolides (Hu et al. 1992, Caillaud et al. 2010, Glibert et al. 2012). These toxins can cause harmful effects on animals and human health (Heredia-Tapia et al. 2002). Out of all the *Prorocentrum* species, 29 are known to be benthic and can co-occur with other potentially toxic benthic species of the genera *Coolia*, *Ostreopsis*, and *Gambierdiscus*. During recent decades, there has been an increase in the knowledge of benthic dinoflagellates (Hoppenrath et al. 2013). Consequently, several new species have been described from tropical (e.g., Faust 1991, 1993a, Faust et al. 2008) as well as temperate areas (e.g., Murray et al. 2007, Chomérat et al. 2010, 2011, 2012).

The classification of this genus has been based mostly on cell shape and size and thecal plates' ornamentations including pore patterns, intercalary band morphology and the periflagellar area. According to the original description by Faust et al. (2008), *P. levis* has a round shape (40 - 44 µm long, 37 - 40 µm wide), smooth surface and discrete distribution of round small pores whilst in contrast, *P. elegans* (Faust 1993a) is a small species (15 – 20 µm long, 10 – 14 µm wide) with an ovate cell shape and a smooth surface characterized by a set of large thecal pores arranged in a distinct pattern and

smaller pores arranged along the intercalary band. Its periflagellar area is V-shaped and accommodates an angled protrusion inexistent in *P. levis*. Platelets identification in this study was based on the new Hoppenrath et al. (2013) system. Another characteristic of the cells is the transversely striated intercalary band in *P. elegans* and smooth in *P. levis*. Both species have the tropical Twin Cays in Belize as type locality.

The main objective of this study was to contribute to the knowledge of the diversity of benthic dinoflagellates occurring as epiphytes on macroalgae and forming part of the assemblage of benthic species that are potentially toxic. The combination of morphological (LM and SEM) and molecular methods (sequences of LSU and ITS1-5.8S-ITS2) allowed us to delineate these two species whose distribution outside tropical waters was not well known.

Methods

The strains described in this study were obtained from the localities of Arrigorri (43.323172, -2.410617) and Zierbena (43.352724, -3.077975), both located in the Southeastern Bay of Biscay. These locations are part of a larger study area which includes the Atlantic coast of the Iberian Peninsula (David et al. 2012). Strain Dn153EHU of *Prorocentrum levis* was isolated from Zierbena in August 2010 and strain Dn208EHU of *P. elegans* was isolated from Arrigorri in September 2012. Strains were isolated from macroalgae at low depths and posterior cell isolation was achieved by micropippeting under the light microscope (Nikon Eclipse T2000-UT). Isolated cells were first grown in a 24-multiwell culture plate with F/2 Guillard's marine water enrichment (Sigma) and then passed to Nuclon^{TM} culture flasks containing 20 mL of medium. They were grown at a salinity of 35 and 20 ºC under a 12:12 light:dark cycle with a white fluorescent light and photon flux rate of 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$.

Morphological features were examined in detail using SEM for which specimens were fixed using 4% formaldehyde (final concentration) and filtered on an Isopore polycarbonate membrane filter (Millipore TMTP, 5.0 µm of pore size). Filters were then rinsed twice with distilled water and dehydrated through an ethanol series (10%, 30%, 50%, 70%, 80%, 95% and 3 times with absolute) for 10 min each. The filter was dried with Hexamethyldisilazane (HMDS 98º) for 5 min and then mounted on a stub (Agar Scientific Lt.), coated with chromium and observed in a Hitachi S-4800 SEM. For the periflagellar platelets identification, it was decided to use the new system purposed by Hoppenrath et al. (2013), which is partly based on Murray et al. (2007). Cells were also examined with LM using 4', 6-diamidino-2-phenylindole (DAPI) staining method which binds to the rich AT regions of the DNA allowing us to locate the nucleus within the cell.

For DNA extraction and amplification, 1-2 mL of clonal cultures were centrifuged and genomic DNA was extracted from the cell pellet using the DNeasy® Plant Mini DNA extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) amplification for 50 µL reactions was performed using a BioMix™ (Bioline, London, UK) following the manufacturers' instructions and using the primers ITS1F - ITS1R (Leaw et al. 2001) for the ITS region and D1R – D2C (Scholin et al. 1994) for the D1-D2 region of the LSU. The thermocycler (model TC-24/H, Bioer Technology CO., LTD, China) program consisted of one pre-cycle of denaturation at 95ºC for 2 min, annealing at 50ºC by 30 s and elongation at 72ºC by 45 s. This was followed by 35 cycles of denaturation steps at 94ºC for 30 s, annealing at 50 ºC for 90 s and the elongation step by 30 s. These cycles were followed by a final elongation step of 72ºC for 10 min. Amplification products were purified using the kit MultiScreen HTS PCR 96-well filtration system (Millipore)

and quantified with the spectrophotometer Nanodrop. Sequencing was carried out with ABI PRISM™ BIGDYE v3.1® Terminator Sequencing Reaction® (Applied Biosystems) and an automatic sequencer ABI PRISM 3130xl Genetic Analyzer. The sequences were then edited using BioEdit v7.0.9 software (Hall 1999).

All sequences were aligned using the E-INS-I strategy implemented in MAFFT 6.833 (Katoh and Toh 2008) to optimize the alignment within the conserved regions. The ambiguous positions were then discarded using G-blocks (Castreana 2000), with the following parameters: minimum number of sequences for a conserved position (22 for LSU and 16 for ITS); minimum number of sequences for a flank position (22 for LSU and 16 for ITS); maximum number of contiguous non-conserved positions (10 for both); minimum length of a block (5 for both); and allowed gap positions (half for both). Based on this alignment, two phylogenetic approaches were used: a maximum likelihood (ML) analysis carried out with RAxML (Stamatakis 2006), with GTR+G+I model and 1000 bootstrap samples; and a Bayesian analysis (BA) carried out with MrBayes 3.2 (Ronquist and Huelsenbeck 2003), using 6 rate categories and gamma distribution, 10^6 generations and discarding the first 25% of the trees. The LSU phylogenetic analysis comprised 43 sequences from which two were from our study and 41 were retrieved from GenBank. The ITS dataset had 31 sequences, where three were from this study and 28 from GenBank. Sequences of *Ostreopsis* cf. *siamensis* were used as the outgroup. Molecular sequences from the two regions of the gene and corresponding to the clonal cultures of *P. levis* (Dn153EHU) and *P. elegans* (Dn208EHU) generated in this study were deposited in GenBank (Table 1).

Artemia franciscana was used to test for nauplii survivorship with cell-free medium and grazing experiments as described in Ajuzie (2007) with minor changes concerning the number of cells (5, 15, 30, 50, 400) and nauplii (10) per well.

Subsequently, LC-HRMS analyses (carried out in positive mode with a Thermo Scientific Dionex High-Speed LC coupled to an Exactive mass spectrometer equipped with an Orbitrap mass analyzer and a HESI-II probe for electrospray ionization) were performed to test for the presence of OA, Dinophysistoxin 1, Dinophysistoxin 2, and Okadaic esters. To do so, 200 mL of culture were harvested with cell densities of 9472 cells $\cdot$ mL^{-1} for *P. levis* and 20707 cells $\cdot$ mL^{-1} for *P. elegans* using glass fiber filters (Whatman GF/C). Samples were extracted with MeOH, sonicated and centrifuged at 5065*g* for 10 min. Toxins were separated using a X-Bridge C18 column (2.1 x 100 mm, 2.5 µm particle size) maintained at 35ºC with a flow rate of 200 $\mu L \cdot min^{-1}$. The mobile phase consisted of 2 mM amonium acetate with a 5.8 pH (A) and 100% MeOH (B). An elution from 60% B to 70% B was run during 5 min; 80% B was reached in minute 10 and held for 5 min; 100% B was reached in minute 20 and held for 5 min; then B decreased to 60% during 0.1 min and this was held until min 30. Standard solutions of OA, (Dinophysistoxins) DTX1, DTX2 and PTX2 (containing 0.7124, 0.4048, 0.2016 and 0.4295 ng $\cdot$ μL^{-1} respectively) were used for toxins identification. To identify okadaic esters, a solution obtained from cultures of *P. lima* and *P. belizeanum* was used. This contained Norokadanone; 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate; diol-ester, 7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate; and 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate, kindly provided by Professor Javier Fernández from IUBO (La Laguna University, Tenerife, Spain). Two separate samples of *P. lima* and *Dinophysis* were also analyzed in parallel as positive controls. In order to reduce matrix effects in the analyses, samples were cleaned by solid phase extraction (SPE) with 60 mg Oasis HLB cartridges (Waters, Eschbom, Germany) following the procedure developed by These et al. (2009). Both crude extracts and eluates from SPE, were analyzed.

177

178 Results

179 The two species of *Prorocentrum* were found in only one out of the 18 visited
180 sites located throughout the Atlantic side of the Iberian Peninsula. *Prorocentrum levis*
181 was found in Zierbena and *Prorocentrum elegans* in Arrigorri, both located in the
182 southeastern part of the Bay of Biscay. These appeared together with other potentially
183 toxic epibenthic dinoflagellates such as *Prorocentrum lima*, *Prorocentrum emarginatum*
184 - complex, *Prorocentrum rhathymum*, *Coolia monotis*, *Coolia canariensis* and
185 *Ostreopsis* cf. *siamensis*, all of them of broader distribution, except *C. canariensis*,
186 which only appeared in Zierbena.

187 Analyses using LM and DAPI staining methods allowed us to observe that both
188 species might divide by growing a membrane envelope, presented golden-brown
189 chloroplasts and had a nucleus in the posterior end of the cell (Fig. 1).

190 Cells of *P. levis* (Figs. 1, d, e, f; 2) showed two biconcave valves with smooth
191 surface and a discrete distribution of round pores. Under LM the pyrenoid was observed
192 in the center of the valve (Fig. 1e). Cells were oval (37.41 – 50.76 µm long, 45.13 ±
193 3.89 µm, n = 20; 30.20 – 42.20 µm wide, 35.97 ± 3.44 µm, n = 20), slightly excavated
194 in the center of each valve, showing a shallow cusp in the anterior end (Fig. 2, a and b).
195 Cells usually grow by asexual reproduction, attached by a hyaline envelope forming
196 long chains of cells (Fig. 2c). In cultures, dark pigmented clusters growing attached to
197 the flask walls were visible without magnification. The valves were smooth, presenting
198 foveate ornamentations (Fig. 2, d and e) with a range from 211 to 222 (217 ± 4, n = 7)
199 pores per valve. They also presented a belt of about 105 irregularly distributed marginal
200 pores (Fig. 2d) located in the periphery of both valves. The pores were unevenly
201 distributed through the valves; they were more condensed in the periphery and rare in

the center of the cell. Two sizes of pores were observed; the larger size pores varied from 0.12 – 0.19 µm (0.15 ± 0.02 µm, n = 53) whilst minute pores were varied from 0.05 - 0.10 µm (0.08 ± 0.01 µm, n =47). The diameter of foveate ornamentations varied from 0.27 - 0.59 µm (0.45 ± 0.05 µm, n = 54; Fig. 2e). The periflagellar area, situated in the anterior end of the right valve was on average 6 µm wide and 3 µm long (Fig. 2, e, f, g), moderately excavated and V-shaped. After processing for SEM, most of the cells showed a periflagellar area, which detached perfectly from the valve and maintained the platelets order. It showed 8 platelets with platelet 8 sometimes divided into two smaller platelets (Fig. 2e). The flagellar pore was large and oblong whereas the accessory pore was smaller. The thecal wall was very thick (1.27 ± 0.17 µm, n = 17) and presented a peculiar ornamentation with vertical striations. The intercalary band, which was usually smooth and thin, could be seen in detail in what could be interpreted as a cell in division (Fig. 2, h and i). It presented transverse striations of 0.92 ± 0.05 µm long (n = 14) and 0.40 ± 0.06 µm wide (n = 18) in each valve, just below the intercalary ring of 0.59 ± 0.01 µm (n = 7). It seems that the smooth intercalary ring corresponded to the intercalary band when the cells are not in division.

Cells of *P. elegans* (Figs. 1, a, b, c; 3) were small and ovate (14.78 – 21.51 µm long, 18.02 ± 1.79 µm, n = 20; and 12.49 – 18.02 µm wide, 14.81 ± 1.51 µm, n = 20). Both valves were smooth revealing two types of thecal pores, large (0.21 - 0.31 µm, 0.26 ± 0.02 µm, n = 37) and small (0.09 - 0.16 µm, 0.12 ± 0.02 µm, n = 55; Fig. 3, a-d). The large pores were arranged in a characteristic pattern that allowed us to identify the species. The smaller ones were unevenly distributed and situated in the periphery of the valves along the intercalary band. The valves presented a range of 18 to 22 (21 ± 2, n = 6) large pores and 80 to 91 (85 ± 5, n = 6) small pores and the center of the valves lacked pores. It was also possible to see a band of small pores bordering the periphery

of the valve (Fig. 3i). The periflagellar area (Fig. 3, g and h) was large relative to cell size (av. 6 µm wide) and in comparison to other *Prorocentrum* species. It is situated on the right valve in a shallow triangular depression where five apical platelets (1, 3, 4, 5, 6) could be easily distinguished (Fig. 3g). The platelets appeared smooth and included a protrusion seen in platelet 1 (1.76 x 0.80 µm) located adjacent to the accessory pore (Fig. 3h), this pore seemed smaller than the flagellar pore. The surface of the intercalary band (Fig. 3, e and f) was smooth and transversely striated with broad (from one valve to the other; 2.87 ± 0.14 µm, n = 7) evenly spaced bands (0.52 ± 0.07 µm, n = 11). Apart from the easily visible transversely striated band, some longitudinal bands could also be distinguished.

Both ML and BA phylogenetic analyses revealed identical tree topologies and only the ML trees are shown. The final dataset of LSU had 870 positions (587 from variable sites, 443 parsimony informative sites and 144 singletons) and the ITS tree had 599 positions where 477 were from variable sites, 364 parsimony informative sites and 113 singletons. From the LSU tree (Fig. 4), a diversified group of benthic *Prorocentrum* species forming three main branches could be seen. One was composed of *Prorocentrum clipeus* sequences forming a well supported clade. The other contained two sequences of *P. tsawwassenense*, a clade of *P. emarginatum/fukuyoi*, our sequence of *P. elegans* Dn208EHU, a group with sequences of *P. dentatum* and *P. minimum*, a well defined clade of *P.rhathymum*, another with*P. micans* and *P. gracile*, and a sequence of *P. triestinum*. The last one was divided into two other branches. One branch included sequences of *P. playfairi*, *P. foveolatum* and *P. borbonicum*, and the other was divided into two other branches with three clusters in each. One cluster included sequences of *P. consutum* and *P. bimaculatum*, other of *P. lima*,and a third one with sequences of *P. belizeanum* and *P. hoffmannianum*.The last branch was represented by

three well-defined clusters containing sequences of *P. concavum/faustiae*, *P. foraminosum*, and *P. levis*. The ITS phylogenetic tree (Fig. 5) showed more variability than the LSU tree. It presented a group of *P. shikokuense/dentatum* which was a basal clade to other taxa forming two branches: one, only containing *P. minimum* sequences, and another more diversified. This last one was divided into eight groups and we could observe from those a well defined clade of *P. triestinum*, other of *P. rhathymum* with a sequence of *P. cassubicum* and other of *P. micans* with *P. texanum*. Our sequence of *P. elegans* appeared as a sister taxa of these last two clades. Furthermore, two well-defined clades were also observed, where one was composed of *P. levis* sequences and the other was divided into two subclades. One of these contained sequences of *P. belizeanum* and *P. hoffmannianum* and the other, sequences of *P. arenarium* and *P.lima*. Our sequence corresponding to the strain Dn209EHU of *P. emarginatum* – complex, appeared as a sister taxa of these last two clades.

The tests with *Artemia franciscana* showed that the cell-free medium was not toxic for both species. The grazing experiment showed no direct effects on the nauplii which, after 48 h individuals were still avid swimmers and presenting cells in their guts. However, at the highest cells concentration, some of the nauplii in the *P. levis* wells would get trapped in the fibers of the hyaline envelope. LC-HRMS analysis of crude extracts and eluates of both species showed that toxins as OA, DTX1, DTX2, or Okadaic esters listed in (Paz et al. 2007) were not detected.

Discussion

This paper reports on the presence of *Prorocentrum elegans* and *Prorocentrum levis* in the Southeastern Bay of Biscay. Previously, other *Prorocentrum* species were identified in the area including *P. lima*, *P. rhathymum* and 3 different lineages of the *P.*

277 *emarginatum* complex (Laza-Martínez et al. 2011), which makes seven out of the 29
278 known benthic *Prorocentrum* species. *Prorocentrum levis* was originally described
279 from Belize in the Caribbean Sea (Faust et al. 2008) and then reported in the
280 Mediterranean Sea. Additionally, sequences from strains isolated from the Catalan
281 Coast (western Mediterranean Sea) were deposited in GenBank in 2008 (unpublished,
282 i.e., FJ489619), in Greek coastal waters (Aligizaki et al. 2009) and later in the Adriatic
283 Sea (Pistocchi et al. 2012). In this study, *P. levis* was found in Zierbena (northern
284 Iberian Peninsula), a semi-enclosed bay with relatively shallow areas located adjacent to
285 one of the main harbors of Bilbao. *P. elegans* was found in Arrigorri, which is also
286 located near a harbor in the north of the Iberian Peninsula. This is the first report of *P.*
287 *elegans* after its description by Faust (1993a) and its nucleotide sequence is provided for
288 the first time. Despite the fact that Faust (1993a) reported *P. elegans* to be a bloom-
289 forming species in its type locality, no other sightings of this species have been
290 reported.

291 Species of *P. levis* were distinguished from other *Prorocentrum* based on size,
292 shape, periflagellar area, intercalary band, and the number, shape and location of several
293 valve pores (Faust et al. 2008). Furthermore, cells usually grow in a hyaline envelope
294 forming chains of cells and did not present valve ornamentation. Our cell sizes
295 presented a larger range of values than in the original description (Faust et al. 2008) and
296 similar values to Aligizaki et al. (2009), although we present a larger range of width
297 values. The number of pores per valve and the number of marginal pores seems to
298 match the description of Faust et al. (2008). These authors only found one size of pores,
299 which corresponded to the range of our larger pores, although we also found minute
300 pores. It seems that the number of valve pores cannot be used as characteristic of the
301 species since it is a highly variable trait within the species (Aligizaki et al. 2009). The

302 periflagellar area in the right valve had 8 platelets characteristic of this species. Platelet
303 8 sometimes seemed to be divided into 2 small platelets, a feature not observed so far in
304 the genus (Hoppenrath et al. 2013). Faust et al. (2008) described *P. levis* as having a
305 smooth intercalary band, which is in line with our observations, although a more
306 ornamented intercalary band can be seen in dividing cells. Cells of *P. levis* were
307 reported to produce OA and DTX2 (Faust et al. 2008) but we did not observe toxicity
308 with the *A. franciscana* assays and toxins were not detected by LC-HRMS analysis.
309 Aligizaki et al. (2009) also did not detect toxins when tested with a phosphatase
310 inhibition assay. However, amounts of microalgae toxicity can be a matter of
311 environmental conditions and can also depend on the physiological status of the species
312 or even on the geographic area (Guerrini et al. 2009). So in this case, if toxins were
313 present, they might not occur in sufficient concentrations to provoke visible damage to
314 the nauplii. The only observed effect was that some grazers got trapped in the mucus
315 secreted by *P. levis*.
316 Cells of *P. elegans* could be distinguished by its smaller size, fewer valve pores
317 and a transversely striated intercalary band. Cell size corresponded to the original
318 description (Faust 1993a); smooth valves with two sizes of pores. However, our large
319 valve pores (0.21 - 0.31 µm) were much larger than those of the original description
320 (0.12 µm on average) and the smallest ones (0.09 - 0.16 µm) were also different from
321 those reported by Faust (1993a). The large pores were uniformly round with smooth
322 margins and arranged in a pattern characteristic of this species. Cells showed a large
323 periflagellar area, that was situated on the right valve, in comparison to cell size, and
324 when compared to other *Prorocentrum* species (Faust 1993a). According to Faust
325 (1993a), cells present eight platelets in the diagnosis and seven platelets in the
326 protologue, but this could not be properly confirmed in our study. It presented a smooth

angled protrusion also known as apical spine, which does not exist in *P. levis* (Faust et al. 2008). Apparently, in both species the periflagellar area can be detached from the valves as a single unit, a feature only observed after the SEM procedure. This was observed mostly in *P. levis* cells and more rarely in *P. elegans* although Faust (1993a) only saw this in *P. elegans*. This could be considered an artifact of SEM as it was never observed in cultures. The intercalary band was smooth and transversely striated with broad, even spaced bands in *P. elegans* whilst being smooth in *P.levis* but still showed a characteristic striated band when dividing. The small cell size was common with other benthic species of *Prorocentrum* such as *P. sipadanense* (Mohammad-Noor et al. 2004), *P. borbonicum* (Ten-Hage et al. 2000), *P. norrisianum* (Faust 1997) and *P. formosum* (Faust 1993b) but none of these species presented the smooth protrusion or the characteristic pore pattern. *Prorocentrum elegans* was described as having the nucleus placed in the cell anterior, which was in contrast to the usual position in the cell posterior in other *Prorocentrum* species (Hoppenrath et al. 2013). Our observations with DAPI stained cells, showed that the strain Dn208EHU had the nucleus in the cell posterior. We interpret the observed discrepancy as an inaccuracy of the original description rather than as a sign pointing to a different species due to its cell size, the characteristic thecal pore pattern and large periflagellar area. There were no previous reports on the toxicity of *P. elegans*, which matched with our negative results obtained by the *A. franciscana* assays and LC-HRMS analyses.

The phylogenetic analyses confirmed the identification of *P. levis*, which appeared well separated in both ITS and LSU trees. In the case of *P. elegans*, we could not state the confirmation of the species, as this was the first molecular sequence of the species. However, it did appear differentiated from other species. The LSU analyses were congruent with Chomérat et al. (2010, 2012) which showed the existence of two

major clades separating *Prorocentrum* species by their symmetry. This is also seen in previous studies performed with other molecular markers (Grzebyk et al. 1998, Murray et al. 2007, Faust et al. 2008, Chomérat et al. 2010, 2012). *Prorocentrum elegans*, in both phylogenetic analyses, was included in the groups containing mostly asymmetric species and presented as a sister taxa of the clades containing *P. rhathymum* and *P. micans*. *Prorocentrum elegans* share morphological traits with either *P. rhathymum* or *P. emarginatum*, although its diminishing size allowed us to differentiate them. As indicated by Faust et al. (2008) and Chomérat et al. (2010) much care must be taken in the interpretation of the phylogenetic analyses of the genus *Prorocentrum* since some molecular sequences could be misidentified as can be seen with the *P.cassubicum* sequence EU244475 in the ITS tree, that is clearly a *P. rhathymum*. It is of paramount importance to provide detailed morphological descriptions in addition to molecular analyses in order to avoid this problem. In both trees, *P. levis* was represented in the clade containing the symmetric species of *Prorocentrum*. Although our strains were easily identifiable after SEM analysis and confirmed with phylogenetic analysis in the case of *P. levis*, the couples *P. emarginatum/P. fukuyoi* and *P. belizeanum/P. hoffmannianum* were subjected to more cryptic morphologies leading to some confusion that can be observed in the LSU tree (Chomérat et al. 2010). Different markers can be used to delineate species and even though the ITS marker presents much more variability than the LSU or SSU and, consequently, can be more discriminant at the species and within species levels, it has been used less than other markers with strains of *Prorocentrum*. Nevertheless, the few sequences available allowed us to confirm that our strain Dn153EHU belonged to *Prorocentrum levis*, turning this into the first report of this species in the area. We were unable to find any nucleotide sequence of *P. elegans* in GenBank, so the strain Dn208EHU was mainly identified by morphology.

This is the first report on this species after that of Faust (1993a) and the first report to deposit its sequence in a nucleotide bank.

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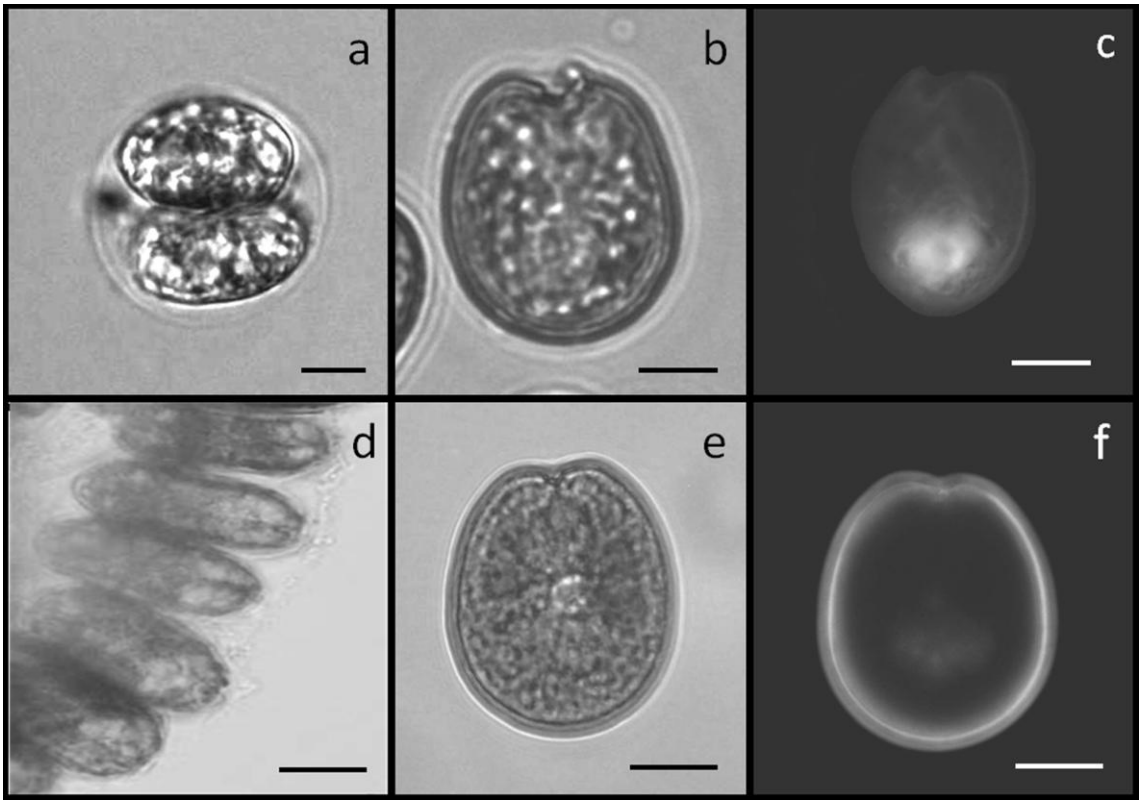


FIG. 1. Light microscopy (LM) micrographs. (a–c) *Prorocentrum elegans* strain Dn208EHU; (d–f) *Prorocentrum levis* strain Dn153EHU. (a and d) dividing cells surrounded by a membranous envelope; (b and e) valve view; (c and f) nucleus view stained with DAPI. Scale bars (a–c) = 5 µm;(d) = 20 µm; (e and f) = 15 µm.

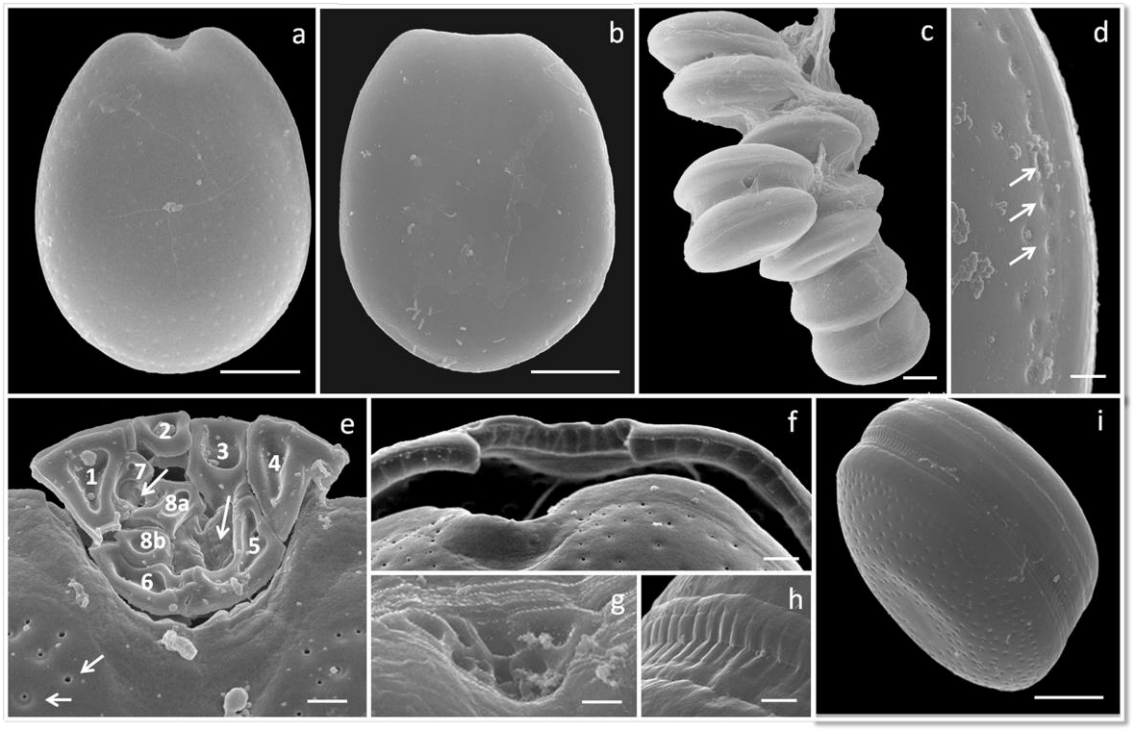


FIG. 2. Scanning electron microscope (SEM) micrographs of *Prorocentrum levis* strain Dn153EHU. (a) right valve view; (b) left valve view; (c) growing cells in a hyaline envelope; (d) periphery valve pores; (e) platelets of the periflagellar area and two types of pores; (f) thecal thickness and ornamentation; (g) periflagellar area; (h) intercalary band; (i) cell showing the intercalary band. Scale bars (a–c, i) = 10 µm; (d–h) = 1 µm.

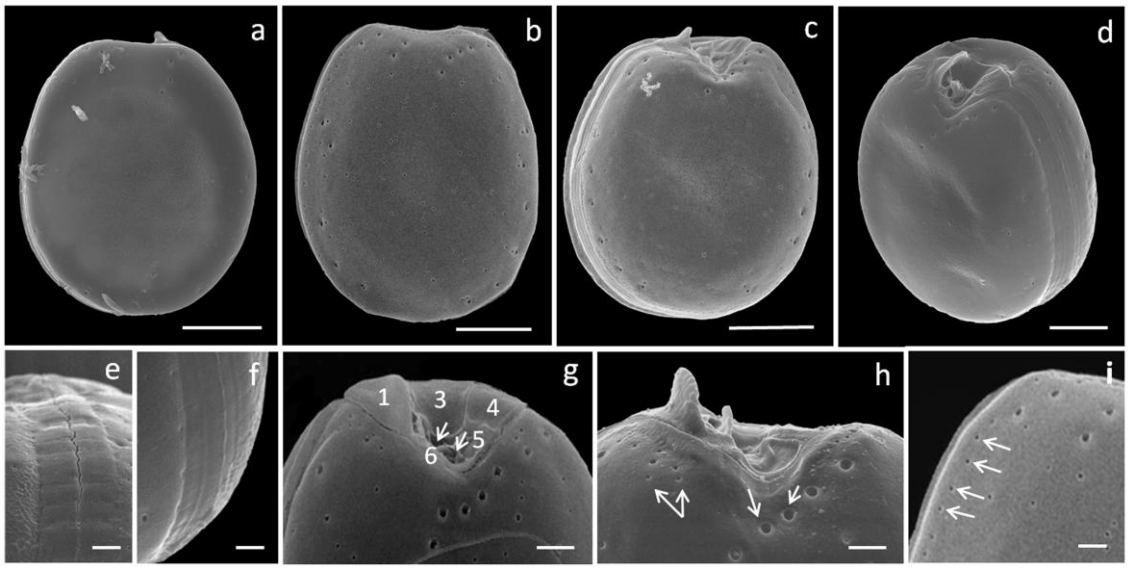
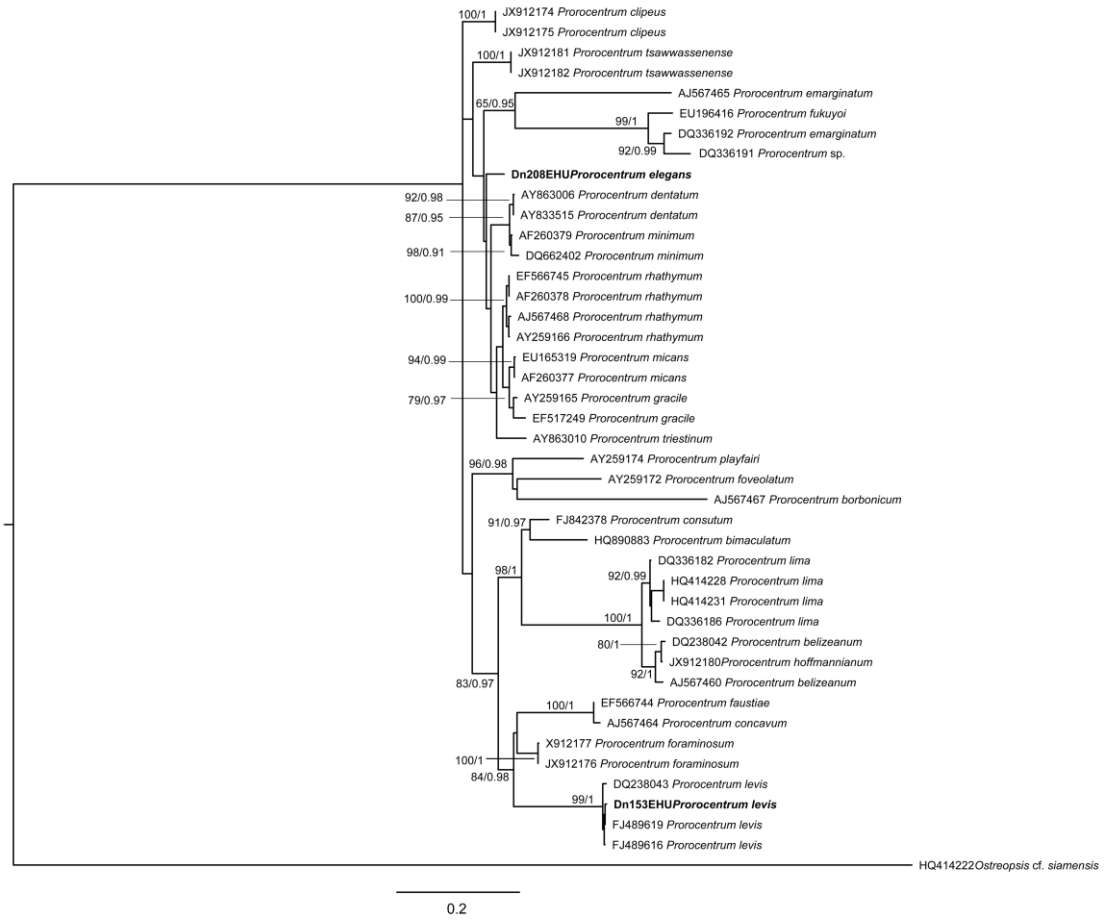


FIG. 3. SEM micrographs of *Prorocentrum elegans* strain Dn208EHU. (a–d) different valve views; (e and f) detail of the intercalary band; (g and h) periflagellar area in detail; (i) periphery valve pores. Scale bars (a–d) = 5 µm; (e–i) = 1 µm.



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547 FIG. 4. Phylogenetic tree of *Prorocentrum* strains by maximum likelihood (ML) method based on LSU
548 rRNA gene sequences. Numbers on the nodes represent ML (before slash) and Bayesian Analysis (BA;
549 after slash) bootstrap values. The tree is rooted using *Ostreopsis* cf. *siamensis* sequence as an outgroup.

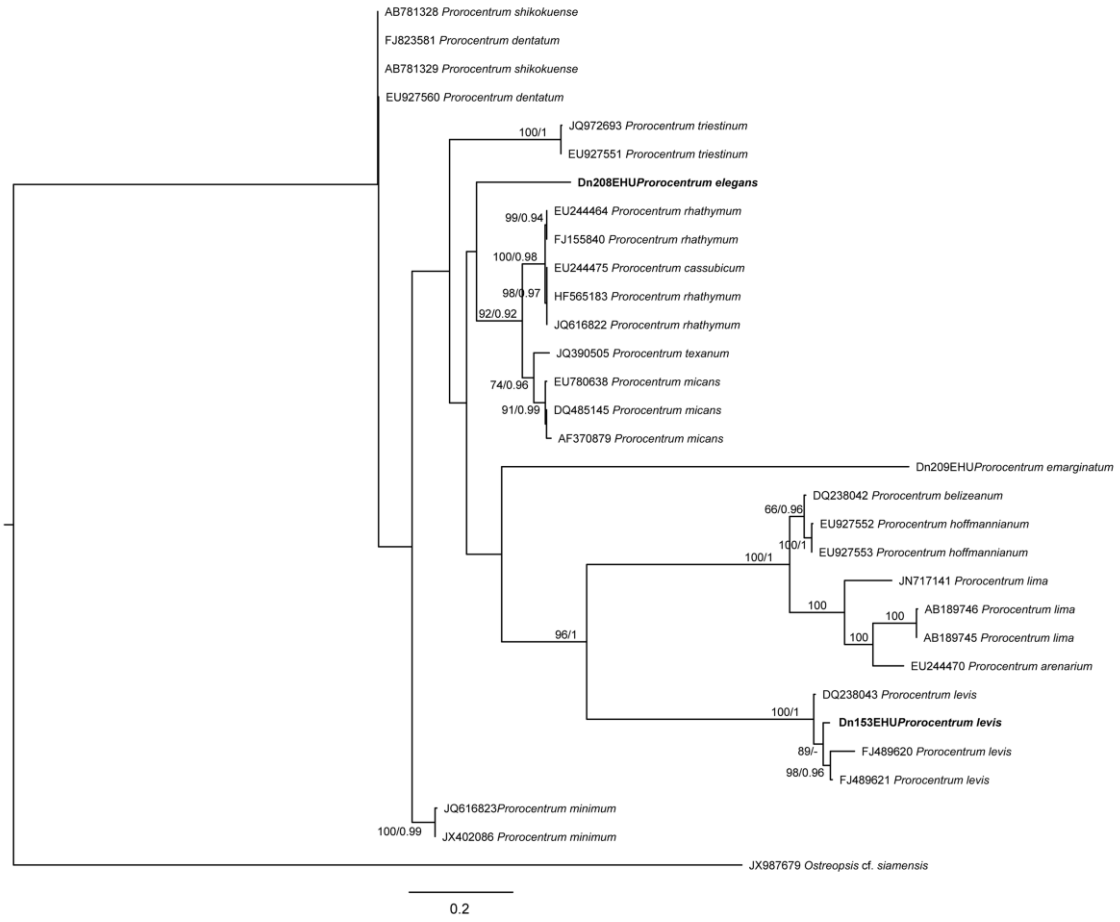

FIG. 5. Phylogenetic tree of *Prorocentrum* strains by ML method based on the ITS1-5.8S-ITS2 rRNA gene sequences. Numbers on the nodes represent ML (before slash) and BA (after slash) bootstrap values. The tree is rooted using *Ostreopsis* cf. *siamensis* sequence as an outgroup.

565 Table 1. Table with the strains found in this study and their GenBank accession codes.

566

Strain	Species	Isolation place	Isolation date	GenBank ID	
				LSU	ITS
Dn153EHU	*Prorocentrum levis*	Zierbena, Spain	August 2010	KF835599	KF835601
Dn208EHU	*Prorocentrum elegans*	Arrigorri, Spain	September 2012	KF835600	KF835602

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