Morphology and phylogeny of *Prorocentrum venetum* Tolomio & Cavolo (Dinophyceae)

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Running title: Study of *Prorocentrum venetum*

Abstract

Prorocentrum venetum was one of the first species of the genus *Prorocentrum* described by scanning electron microscopy by Tolomio and Cavolo in 1985. Since the first observation of the species in the Venice Lagoon (Italy) in summer 1981, it has not been found reported again in published phytoplankton records in Mediterranean waters or elsewhere. Two strains were isolated from a French Mediterranean lagoon, which were morphologically identified as *P. venetum* by microscopy. Based on rDNA sequences (spanning from the 18S rDNA to the D3 region of the 28S rDNA), the phylogenetic analysis demonstrated that *P. venetum* belongs to the same clade as *Prorocentrum triestinum* and *Prorocentrum redfieldii*. The analysis of scanning electron micrographs provided an in-depth morphological description of the theca, particularly on the pore pattern and new structural details of the platelets in the periflagellar

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area. These morphological characteristics were compared with the closely related species within the *P. triestinum* clade, which showed synapomorphic characters in the periflagellar area (small accessory pore, platelet pattern, shape of the apical wing). Further comparison of characteristics varying between species in this clade and in the sister clade encompassing species related to *Prorocentrum micans* suggested some features of morphological evolution within this part of the genus.

Highlights

- Detailed morphological description with new structural details of the periflagellar area of *Prorocentrum venetum*.
- First rDNA sequence and phylogenetic analysis of *P. venetum*.
- *P. venetum* belongs to the same clade as *P. triestinum* and *P. redfieldii*.

Keywords

rDNA sequence, morphology, phylogeny, taxonomy

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52 **1. Introduction**

53

54 *Prorocentrum* (Ehrenberg, 1834) is a thecate dinoflagellate genus with a peculiar morphology
55 within this lineage. The theca is composed of two large lateral plates joined by the sagittal
56 suture and of the periflagellar area which is a set of small platelets around the flagellar and
57 accessory pores inserted apically between these two plates (Hoppenrath et al., 2013). The genus
58 is distributed worldwide, mostly marine. It is diverse in terms of morphology and habitats,
59 comprising planktonic and benthic species. For a century and half after the description of the
60 type species *Prorocentrum micans* Ehrenberg (Ehrenberg, 1834), all species were described by
61 light microscopy. With the advent of new technologies, first for observation by scanning
62 electron microscopy, and then for genetic analysis mainly by rDNA sequencing, the new species
63 were then gradually described with more and more fine and precise criteria. Thus, even for old,
64 well-established species with worldwide distribution, it has become obvious that a new clarified
65 description may be needed (e.g., Tillmann et al., 2019, 2022). For *P. micans* reinvestigated by
66 Tillmann et al. (2019), this came after new cryptic species were erected (Han et al. 20016,
67 Henrichs et al., 2013), to some extent challenging of the limits of accepted morphological
68 plasticity in many *Prorocentrum* species (e.g., Bursa, 1959, 1962; Hulburt, 1965; Cohen-
69 Fernandez et al., 2006). In addition, new *Prorocentrum* species are still regularly described
70 (e.g., Arteaga-Sogamoso et al., 2023; Tillmann et al., 2023a, b; Gómez et al., 2023).
71 The small planktonic species *Prorocentrum venetum* Tolomio & Cavolo was first described
72 from waters collected in Venice Lagoon (Italy), near Alberoni fort along the Canale del
73 Lemento, during summer 1981, when the organism formed a dense and almost monospecific
74 bloom (Tolomio & Cavolo, 1985). Original material had been stored by the authors in the
75 cryptogamy herbarium of the Biology Department of the University of Padua (Padova, Italy),
76 but upon request to the Biology Department, it was established that this material was obviously
77 lost (L. Trainotti, pers. com.) and was therefore no longer available for further analysis. The
78 description of *P. venetum* was one of the first made using scanning electron microscopy (SEM)
79 for a species of *Prorocentrum*. All observations were made from formalin-fixed material: light
80 microscopy (LM) and SEM observations (two and six published micrographs, respectively)
81 were reported under relatively low magnifications. Cell orientation (i.e., the designation of right
82 and left lateral thecal plates) was done following Bergh (1881) and Loeblich et al. (1979),
83 whereas most species were described with the reverse orientation following Stein (1883), which
84 has now become the conventional designation (Taylor, 1980; Fensome et al., 1993; Hoppenrath
85 et al., 2013).

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86 Since the first description of *P. venetum*, the distribution of this species has remained
87 undocumented, as it seemed not to have been reported again in published phytoplankton records
88 in Mediterranean waters or elsewhere (Gómez, 2008; Guiry, 2022). The validity of
89 phytoplankton taxa never reported again after their original description can sometimes be
90 challenged (e.g., Thessen et al., 2012). In a review examining the taxonomical status of
91 *Prorocentrum mexicanum*, Gómez et al. (2017) proposed that *P. venetum* should be considered
92 a synonym of *P. mexicanum*, given the similarity in cell shape and morphology of the two
93 species. In contrast, Tillmann et al. (2022) suggested that *P. venetum* could belong to the
94 *Prorocentrum triestinum*/*Prorocentrum redfieldii* lineage based on similar morphological
95 features including the position of the periflagellar area and the pattern of thecal pores. No
96 genetic data from specimens identified as *P. venetum* have yet been reported to date to establish
97 the phylogenetic position of the species in the genus *Prorocentrum*. Therefore, a morphological
98 reinvestigation of *P. venetum* and its genetic characterization with rDNA sequencing were
99 needed.
100 In this paper, the isolation of two *Prorocentrum* strains from a Mediterranean coastal lagoon is
101 reported. The general morphology, ultrastructure and rDNA phylogenetics have been
102 investigated and LM and especially SEM morphological data were consistent with the
103 description of *P. venetum* by Tolomio & Cavolo (1985). High magnification SEM enabled
104 clarifying ultrastructural details of the species and the closest *Prorocentrum* species were
105 identified by phylogenetic analysis of ribosomal DNA (rDNA).
106
107 **2. Material and methods**
108
109 *2.1. Sampling, strain isolation and culture methods*
110 Two *Prorocentrum venetum* strains, AYR20082019-3C4 and AYR20082019-3E9 (abbreviated
111 AYR19-3C4 and AYR19-3E9), were isolated on 20 August 2019 from the Ayrolle Lagoon
112 (43°04'44"N, 3°04'12"E). This shallow oligotrophic coastal Mediterranean lagoon has a
113 surface area of 13.2 km² and an average depth of 0.5 m. Its water shows important annual
114 variations in salinity and temperature (Bec et al., 2011; Grzebyk et al., 2017); on sampling day,
115 the water temperature was 20 °C and salinity was 43. Seawater (20 l) was filtered through a 20-
116 µm sieve, passed onto a 5-µm sieve, and the 5-20 µm phytoplankton thus collected were then
117 used for cell isolations. Clonal cultures were obtained by isolating a single cell by micro-
118 pipetting using a sharpened Pasteur pipette under a Axiovert inverted microscope (Zeiss, Jena,
119 Germany), and inoculation in a well of a 96-well culture plate containing enriched natural

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120 seawater (ENSW) culture medium (Andersen, 2005) at a salinity of 34. Cells were grown at 121 18.5 °C in a climate chamber with a 12:12 h light-dark photoperiod. Well-developed cultures 122 were selected and transferred to wells of 24-well plates containing 2 ml of culture medium, and 123 subsequently in 50-ml suspension culture flasks (Greiner Bio-One, Frickenhausen, Germany), 124 and grown as described before.

125

126 *2.2 Morphological analyses*

127 Observation of living or fixed cells (formaldehyde: 1% final concentration, or neutral Lugol-128 fixed: 1% final concentration) was carried out using an inverted microscope (Axiovert 200M; 129 Zeiss, Jena, Germany) and a compound microscope (Axiovert 2; Zeiss), both equipped with 130 epifluorescence and differential interference contrast optics. Light microscopy examination of 131 thecal plates of cells stained with Solophenyl Flavine 7GFE500 (Chomérat et al., 2017) was 132 performed using epifluorescence microscopy with epifluorescence filter set 09 (Zeiss; BP 450-133 490; FT 510; LP 515). The shape and location of the nucleus was determined after staining of 134 formalin-fixed cells with 4'-6-diamidino-2-phenylindole (DAPI, 0.1 µg ml^{-1} final 135 concentration) for 10 min. Images were taken either with a digital camera (Axiocam MRc5; 136 Zeiss), or videos were recorded using a digital camera (Gryphax; Jenoptik, Jena, Germany) at 137 full-HD resolution. Single frames were extracted using Corel Video Studio software (Version 138 X8; Coral, Ottawa, Canada). Cell length and depth of freshly fixed cells (neutral Lugol, 1 % 139 final concentration) from dense but healthy and growing strains during late exponential phase 140 were measured at microscopic magnification of 1000x using the compound microscope and the 141 Axiovision software (Zeiss).

142 Observations using scanning electron microscopy (SEM) were performed as described in 143 Tillmann et al. (2023b).

144 For transmission electron microscopy (TEM), cells from strain AYR19-3C4 were concentrated 145 in a microfuge tube by slow centrifugation (8 g for 1.5 min). The pellet was prefixed with 2.5% 146 glutaraldehyde in filtered seawater at 4 °C for 60 min. Cells were washed twice in filtered 147 seawater before post-fixation with 2% OsO_4 in filtered seawater at room temperature for 60 148 min. Fixed cells were dehydrated through a graded series of ethanol (30%, 50%, 70%, 10 min 149 each; 85%, 90%, 95%, 2x 100%, 15 min each), then in 100% propylene oxide (twice, 15 min 150 each), infiltrated with propylene oxide-resin mixtures (2:1, 1:1, 1:2), and embedded in EMBed-151 812 resin (Science Services, Munich, Germany). The block was polymerized at 60 °C for 22 h 152 and sectioned with a diamond knife on a Reichert Ultracut microtome (Reichert-Jung, Vienna, 153 Austria). Thin sections were directly viewed under an EM 900N TEM (Zeiss) operated at 80

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154 kV. Digitized images were taken with a *sharp:eye* Wide-angle Dual Speed CCD-camera (TRS,
155 Dünzelbach, Germany) operated by the ImageSP software (TRS, Dünzelbach, Germany).
156 Terminology of cell orientation and designation of thecal plates and platelets follows
157 Hoppenrath et al. (2013) supplemented by Tillmann et al. (2019).
158
159 *2.3 Sequencing and phylogenetic analyses*
160 Genomic DNA (gDNA) extraction and purification was performed using the PureLink Plant
161 Total DNA Purification kit (Invitrogen, Carlsbad, California, USA). Pelleted cells (1.0 ml of
162 culture) were resuspended in 250 µl of provided lysis buffer R2, then after the addition of 15
163 µl of SDS, were incubated at 55 °C for 15 min in a in a water bath sonicator Sonorex RK100H
164 (Bandelin, Berlin, Germany) working at 35 kHz frequency. RNAs were then digested after
165 RNase addition and incubating again at 55 °C for 15 min in the water bath sonicator.
166 Manufacturer's instructions were then followed, allowing gDNA adsorption onto a purification
167 column, cleaning steps with a washing buffer, and ultimately, gDNA was eluted with 100 µl of
168 the provided elution buffer (10 mmol l^{-1} Tris at pH 8.0). The eluted gDNA solution was
169 supplemented with 0.2 µl of EDTA solution (0.5 mol l^{-1} at pH 8.0) and then stored at -24 °C.
170 An assembled rDNA sequence that included the 18S rRNA gene, the internal transcribed
171 spacer (ITS) region (ITS1, the 5.8S rDNA, and ITS2), and the D1-D3 region of the 28S rRNA
172 gene (hereafter called 18S-D3 sequence) was constructed with sequencing data obtained from
173 two overlapping PCR-amplified rDNA fragments (Grzebyk et al., 2022). The PCR primers used
174 for PCR and sequencing are described in supplementary material (Table S1). The 18S rDNA
175 fragment was amplified with the primer pair 18S-F and 18S-PROT-R. The second rDNA
176 fragment, which included the 18S rDNA end, the ITS region and the beginning of the 28S
177 rDNA, was obtained with the 18S-I3F forward primer that hybridizes to the 3' end of 18S rDNA
178 (allowing a ~600-bp overlap with the 18S rDNA amplicon sequence) and the D3b-R reverse
179 primer hydridizing at the end of D3 domain of 28S rDNA. The PCR reactions were performed
180 using and the PrimeSTAR GXL DNA Polymerase Kit (Takara Bio Inc., Japan) with a high-
181 fidelity enzyme, in a Mastercycler Ep Gradient S thermal cycler (Eppendorf, Germany). The
182 PCR programme consisted of 40 cycles: 98 °C for 15 s, 52 °C for 15 s, and 68 °C for 2 min,
183 followed by a final elongation period at 68 °C for 2 min. The amplicons were purified with the
184 QIAquick PCR Purification Kit (Qiagen), and sequenced with the appropriate sequencing
185 primers (Table S1), the Big Dye Terminator V3.1 kit and an ABI 3500XL Genetic Analyzer
186 (Applied Biosystem, Foster City, CA, USA) at the ISEM-Labex CEMEB sequencing facility

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187 (Montpellier University, France). The sequence chromatograms were checked by eye and the
188 DNA fragments were assembled using the BioEdit v7.2.6.0 program (Hall, 1999).
189 Due to the heterogeneous distribution of reference sequence data for the genus *Prorocentrum*
190 concerning the 18S rDNA, the ITS region and the D1-D3 region of 28S rDNA, their sequencing
191 data were used in separate phylogenetic analyses. The 28S rDNA phylogenetic analysis focused
192 on the D1-D2 region because the D3 domain was not determined in many reference sequences,
193 and given the powerful phylogenetic information retained in this D1-D2 rDNA barcode region
194 (Grzebyk et al., 2017). Reference sequences were selected by BLASTN similarity analyses
195 (Altschul et al., 1990) using the web interface NCBI BLAST (Johnson et al., 2008) and the
196 GenBank nucleotide database. Alignments were generated with CLUSTAL X 2.1 (Larkin et
197 al., 2007) and were refined by eye using the BioEdit program (Hall, 1999). Phylogenetic
198 analyses were performed with the online application Phylogeny.fr (Dereeper et al., 2008) run
199 by the ATGC bioinformatics facility (http://www.atgc-montpellier.fr/). The "A la Carte" mode
200 was used with the corrected alignment, in which the phylogenetic analysis pipeline
201 implemented PHYML 3.0 (Guindon et al., 2010) with applied settings including the HKY85
202 substitution model and four categories of substitution rates, and the estimation by the program
203 of the Gamma distribution parameter, the proportion of invariable sites and the
204 transition/transversion ratio. The estimation of branch support in the phylogenetic tree was
205 statistically tested with the SH-like approximate likelihood-ratio test (Anisimova & Gascuel,
206 2006).
207
208 **3. Results**
209
210 *3.1. Detailed description*
211 In culture, the cells of both strains AYR19-3C4 and AYR19-3E9 were photosynthetic and had
212 a planktonic lifestyle. Both strains shared identical morphological details. The LM and SEM
213 morphological descriptions and all morphometric analyses were based on the study of these two
214 strains, while the TEM ultrastructural study was carried out only with the AYR19-3C4 strain.
215 This strain is described here (Figs 1–6), and micrographs obtained with strain AYR19-3E9 are
216 shown in the supplementary material (Figs S1–S3).
217 Cells were 19.0-24.4 μm long, 11.4-17.4 μm deep and 8.1-15.0 μm wide; the length to depth
218 ratio (l:d) was 1.52-2.52 (Table 1). Using light microscopy, cells observed in lateral view had
219 an oval shape with a barely visible anterior projection (Figs 1A-H, Figs S1A, B). The dorsal
220 curvature was elliptic whereas the ventral curvature was more swollen and formed a ventral

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221 bulge near the anterior end, making the contour of thecal plates slightly asymmetrical. This
222 asymmetry was accentuated by the small spine-like apical projection located in a slightly central
223 to dorsal subapical position (Figs 1A-E). At the posterior end, the cell shape was variable,
224 showing nearly rounded shapes (Fig. 1C; Figs S1C, P), or slightly pointed with rounded tip
225 close to the vertical axis (Fig. 1V; Fig. S1O), or with a short, flattened edge with a barely visible
226 tip slightly oriented ventrally (Figs 1E, G; Figs S1E, N, P). In dorsal/ventral view, the cell shape
227 was lenticular (Figs 1I-N). The posterior end was pointed in narrow cells (Figs 1I-J; Figs S1G,
228 Q) but was truncate in enlarged cells (Figs 1K-N; Figs S1J, K, S). The pusule having the
229 appearance of a hyaline sack structure was located in the anterior part of the cell (Figs 1A-F,
230 H, I; Figs S1B-E). Golden-brown coloration in light microscopy and red fluorescence of
231 chlorophyll under the epifluorescence microscope showed two lobed chloroplasts extending
232 laterally under each thecal plate (Figs 1A-J, P-S, Z; Figs S1B-E, O-U, W), often having an
233 indentation occupied by the pusule system in the anterior end of cell (Fig. 1Aa). In lateral view,
234 a putative roundish pyrenoid was sometimes visible in central position of cells, between the
235 pusule and the nucleus (Figs 1B, D, F, G, Aa). The round nucleus occupied most of the posterior
236 half of the cell (Figs 1C, E, G-L, Ab-Ad; Figs S1C, H-N, X). The nucleus shape could be more
237 irregular probably in early stage of nuclear division (Fig. 1Ad). Thecal pores were difficult to
238 recognize and visible only without cellular content (Figs 1W, X) or after theca staining (Figs
239 1T-V). Rod-shaped structures (trichocysts) were not observed in the anterior cell area.
240 Scanning electron microscopy observations were obtained from both strains (strain AYR-3C4:
241 Figs 2-3; strain AYR-3E9: Figs S2-S3). Morphometric data showed moderate variations in the
242 morphological features of the cells within and between the two strains (Table 1). The thecal
243 surface of the two plates and the periflagellar platelets was smooth (Figs 2, 3, S2, S3). The
244 intercalary band at the suture of thecal plates was almost lacking in narrow cells (Fig. 2A, E-F;
245 Fig. S2C), and was broad and smooth with transverse striation in large cells (Figs 2C, I, L; Figs
246 S2G-I). Both lateral plates were perforated with pores except in their central area (Figs 2A-I,
247 S2A-G). There were three different types of pores (Figs 2K-N, 3F-J; Fig. S2M). The first two,
248 denoted as large and small pores, were each in similar numbers on the right and left plates
249 (Table 1). The large pores were numerous (24-39 per thecal plate) and consisted of a circular
250 depression (0.31-0.61 μm outside diameter) at the bottom of which was a small round orifice
251 (0.11-0.22 μm in diameter) at the top of a tubular structure. The internal tubular structure could
252 be oblique (Figs 3G, H). There were fewer small pores (7-17 per plate) which were flush with
253 the plate surface and were generally scattered near the large pores. The mean diameter of the
254 pore orifice of small pores (0.11-0.16 μm) was similar to that of large pores (Table 1), but the

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orifice diameter of large pores was more variable (up to 0.22 µm, Table 1). The third type of pore (denominated as mini-pore) was a tiny, single pore located at the slightly protruding tip at the posterior end of the cell (Figs 3I, J). Its diameter < 0.1 µm was significantly smaller than the orifice of the other pores. The mini-pore was difficult to identify or was not always observed on examined plates.

On the two lateral plates, large and small pores were arranged together in groups, which positions were approximately mirror-arranged on the right and the left plates (Figs 4A-B, E). Groups were numbered clockwise on the right plate starting from the ventral edge of the periflagellar area and ending at the dorsal edge (from R1 to R10), and symmetrically counter-clockwise on the left plate (L1 to L10) (Fig. 4E). In each group, the number of pores varied by a few units between cells of each strain and between the two strains (Supplementary Tables S2, S3), and the location of each pore varied more or less on the plate and in relation to neighbouring pores. Most groups contained a single small pore, but there were often two in R2/L2 and R9/L9. Only R6/L6 did not have a small pore but sometimes a mini-pore instead. In pore groups, pores were either scattered or arranged in short rows (Fig. 2A-I). On the right plate, the first row of pores R1 was located on the ventral edge of the periflagellar area along platelets 4 and 5 (Figs 2K, L, 3A, B), while pores in the homologous row L1 were aligned approximately perpendicular to the edge of plate (Fig. 2N). The anterior half of plates had three other groups with scattered pores, one on the ventral edge (R2/L2) and two on the dorsal edge (R9/L9 and R10/L10) (Figs 2A-I). On the posterior half, the smallest group of pores (R5/L5) contained only one large and one small pore (Figs 3F, I, J) and there were five rows of pores (Figs 2A-I). Four rows radiated almost perpendicularly from the plate edge: two rows from the ventral curvature (R3/L3 and R4/L4) and two from the dorsal curvature (R7/L7 and R8/L8). In the last row (R6/L6), the large pores were arranged evenly spaced along the dorsal edge of the posterior end (Figs 3F, I, J).

The periflagellar area was enclosed in a shallow V- to U-shaped recess in the right thecal plate, much of it located ventrally to the longitudinal axis of the cell. It was approximately lenticular in shape, with dimensions of 3.4-4.3 µm in depth and 1.7-2.5 µm in width (Table 1). It was composed of eight platelets: 1, 2, 3, 4, 5, 6, 7, 8 (Figs 3A-E; Fig. 4F; Fig. S3). The dorsally located platelet 1 was the largest, with a size making nearly half of the periflagellar area. It was roughly triangular in shape and partly elevated (forming a sort of hump) above to the edge of the lateral plates (Figs 2K, M, N). Platelet 1 bore the most prominent apical projection (0.80-1.43 µm high; Table 1), rising on the internal edge along platelets 2, 7, 8 and 6 (Figs 3A-D). This very broad projection was a wing with a visor shape, rising above platelet 7 and the

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289 accessory pore (Figs 2K, 3A). The ventral platelet 4 was flat (Figs 3A-E). The flagellar pore
290 was surrounded by platelets 3, 5, 6 and 8, all of which bore a low list bordering the pore as a
291 sort of collar (Figs 3A-E). The list on platelet 6 pointed in the form of a small protrusion rising
292 slightly above the pore (Figs 3B, D, E). The flagellar pore was oval in shape, measuring 0.9-
293 1.3 μm long and 0.6-0.9 μm wide (Table 1); it was closed by two lip-like structures (Fig. 3A-
294 B). The accessory pore was inserted between platelets 7 and 8; it was approximately round and
295 tiny in size with a diameter of ~0.15 μm (Fig. 3B-D).
296 Intracellular ultrastructure examined by TEM (Figs 5, 6) complemented observations made by
297 light and epifluorescence microscopy. The large dinokaryotic nucleus in the posterior half of
298 the cell contained the typical condensed chromosomes and a nucleolus (Figs 5A, B D).
299 Mitochondria with tubular cristae and oil droplets were distributed in the cytoplasm (Figs 5A,
300 B), and the Golgi apparatus was observed (Fig. 5F). The membrane system of the pusule was
301 visible below the flagellar pore region (Fig. 5C). Trichocyst cross sections of different diameter
302 were spread in the cell (Figs 5B, E). Chloroplast parts (lobes) were located in the cell periphery
303 directly below the thecal plates (Fig. 5A, B). The pyrenoid can be of an irregular interlamellar
304 (immersed, internal; Fig. 6A, B) or more often of a bulging (terminal) type without starch sheath
305 (Fig. 6 C-F). Single thylakoids or stacks of two traversed the pyrenoid matrix in no ordered
306 manner (Fig. 6).
307
308 *3.2. Molecular phylogenetics*
309 The size of the rDNA sequence of *P. venetum* strain AYR19-3C4 (accession number PP258975)
310 - comprising the 18S rDNA, the ITS region and the D1-D3 region of the 28S rDNA - was 3326
311 base pairs (bp) between the two external PCR primer-binding sites (abbreviated as 18S-D3),
312 which was the same length as for the *P. redfieldii* strain CCMP1919 (accession number
313 ON491170). The obtained sequence from strain AYR19-3E9 (from end of 18S to the end of D3
314 region of the 28S rDNA, accession number PP258976) was identical to those from strain
315 AYR19-3C4.
316 Phylogenetic analysis of the genus *Prorocentrum* based on 18S-D3 sequences (Fig. 7) showed
317 that *P. venetum* belonged to a well-supported clade formed with *P. triestinum* and *P. redfieldii*.
318 This triadic clade was sister to a clade containing the species *P. micans*, *P. koreanum*, *P.*
319 *texanum*, *P. rhathymum* and *P. steidingerae*. The alignment of nucleotide sequences from *P.*
320 *venetum*, *P. triestinum* and *P. redfieldii*, showed an identical pattern with many shared gap
321 positions and specific substitutions in this clade with respect to sequences from other
322 *Prorocentrum* species (Supplementary Fig. S4). Between the three species, some shared

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323 substitutions alternated two by two (Supplementary Fig. S4). The nucleotide sequences of *P. venetum* and *P. triestinum* showed fewer nucleotide substitutions between them as compared to *P. redfieldii* (Table 2).
326 In the phylogenetic trees obtained with nucleotide sequences of the three rDNA regions (18S, D1-D2 region of 28S, and ITS region) (Figs 8-10), the relationships were not unequivocally resolved within the triadic clade within the clade between *P. venetum*, *P. triestinum* and *P. redfieldii*. In the 18S rDNA analysis (Fig. 8), the range of genetic variations within the clade between the three species (within which the *P. redfieldii* strains exhibited genetic heterogeneity) was similar to that inside the large clade encompassing *P. micans* with five other documented species (*P. gracile*, *P. texanum*, *P. koreanum*, *P. rhathymum* and *P. steidingerae*). The analyses based on D1-D2 region of 28S rDNA (Fig. 9) and the ITS hypervariable region (Fig. 10) showed slightly greater genetic difference between *P. venetum* and *P. triestinum* and the species clade of *P. redfieldii*. In both of these rDNA regions, unlike the 18S rDNA, the range of genetic variation between species clades was much smaller in the *P. triestinum* clade than in the sister clade encompassing *P. micans*.
338
339 **4. Discussion**
340
341 From the examination of the oval cell shape, the size of cells, the distribution pattern of large thecal pores, the location and shape of the periflagellar area, and the visor shape of the apical wing (spine-like in LM), the morphological observations presented herein were consistent with the description of specimens of *P. venetum* by Tolomio and Cavolo (1985). Thus, both strains (AYR19-3C4 and AYR19-3C9) could be unambiguously identified to species level. Therefore, the first genetic fingerprint of *P. venetum* can be provided in the present study, even when the reinvestigated strains were not collected from the type locality of Venice (Italy) but from another Mediterranean lagoon environment.
349 A comparison of morphological details of physical type material was not possible (see Introduction). Overall, the size and oval shape of the cell contour when viewed laterally, asymmetrical at the anterior and posterior ends relative to the long axis of the cell, matched the description by Tolomio and Cavolo (1985). Generally, the shape of the posterior end of the cells varied slightly and was not well rounded (Figs 1A, E, G, 2G-I). Although Tolomio and Cavolo (1985) did not explicitly mention such variability and deviation from the oval contour, this can be seen by carefully examining the two light micrographs (Figs. 1 and 2) provided by these authors. The strains of *P. venetum* showed a wider range in the number of large pores per plate

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357 (24-39), overlapping the range (35-40) reported in the original description. In their original
358 description, Tolomio and Cavolo (1985) did not mention the presence of different types of pores
359 (in particular, no differentiation of small pores), but this could be explained by the lower
360 magnification and resolution applied in their SEM study: the small pores and especially the
361 mini-pore at the posterior end could simply have been overlooked.
362 The phylogenetic analysis showed that *P. venetum*, *P. triestinum* and *P. redfieldii* formed a
363 well-supported clade. *P. venetum* and *P. triestinum* were closer to each other with fewer
364 nucleotide substitutions between them, while *P. redfieldii* appeared more distant and more
365 recently derived (Table 2). The analysis also strongly supported the sistership of the *P.*
366 *triestinum* clade and a large clade encompassing species related to *P. micans*.
367 Morphologically, *P. venetum* shares similarities with *P. triestinum* with regard to the cell size
368 and the subapical position of the periflagellar area, but the thecal plates of *P. triestinum* are
369 more strongly asymmetrical, have an acute posterior end and the periflagellar area is positioned
370 dorsal to the longitudinal axis (Tillmann et al., 2022).Other features identified as being shared
371 between *P. venetum*, *P. triestinum* and *P. redfieldii* are in the thecal pore pattern and the
372 arrangement of the periflagellar area (see below). Furthermore, regarding intracellular
373 ultrastructure, *P. venetum* has the same type of pyrenoid as *P. redfieldii* (Ndhlovu et al., 2017).
374 In *P. micans* and *P. texanum*, the ultrastructure of the pyrenoid is different (Kowallik, 1969;
375 Henrichs et al., 2017): it is a compound interlamellar pyrenoid that is crossed by parallel
376 thylakoid lamellae in a regular and organized manner; this part of the chloroplast can be swollen
377 and then projects into the cell (Dodge and Crawford, 1971).
378 The three types of thecal pores seen in *P. venetum* are similar in size and shape to those present
379 in *P. triestinum* and *P. redfieldii* (Tillmann et al., 2022). The three types of pores were also
380 described in *P. micans* (Tillmann et al., 2019), and were likely observed in other species where
381 small pores were described with a large variability in their diameter, including *P. texanum*
382 (range 0.08-0.13 μm; Henrichs et al., 2013), *P. elegans* (range 0.09-0.16 μm; David et al.,
383 2014), and as observed in *Prorocentrum caribaeum* (Faust, 1993, in Fig. 22).
384 Regarding the arrangement of large pores (trichocyst pores) on the thecal plates, *P. venetum*
385 has the highest number of pores in the *P. triestinum* clade, about twice as many as in the two
386 other species and the same is observed for small pores. All three species have a similar row of
387 pores at the posterior end along the dorsal edge of the thecal plates (i.e., corresponding to pore
388 groups R6/L6 of *P. venetum*), a configuration not observed elsewhere in the genus. In contrast,
389 *P. venetum* is the only species of its clade with two pairs of radial rows of pores (with ≥3 large
390 pores) on the dorsal and ventral edges of plates, although some pores are present in similar

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391 locations in the pore patterns of *P. triestinum* and *P. redfieldii*.
392 The periflagellar areas are very similar and have an identical eight-platelet pattern between *P.*
393 *venetum*, *P. triestinum* and *P. redfieldii* (Tillmann et al., 2022). The most specific common
394 feature of this *P. triestinum* clade is that the accessory pore is very small in size and is therefore
395 hardly visible in the middle of junction between the very small platelet 7 and the platelet 8. In
396 contrast in species of the sister clade encompassing *P. micans*, the large accessory pore has a
397 lenticular shape whose long axis is parallel to the junction between platelets 7 and 8
398 (Hoppenrath et al., 2013; Tillmann et al., 2019). In addition in the *P. triestinum* clade, platelets
399 7 and 8 are significantly smaller and differently shaped compared to the *P. micans* clade, and
400 the platelet 1 is relatively larger in size while platelets 2 and 6 have a reduced size. Additionally,
401 in the *P. triestinum* clade, the apical projection rises from an almost linear base along the inner
402 edge of platelet 1, between the right and left thecal plates, which configuration is also present
403 in various other *Prorocentrum* species (Hoppenrath et al., 2013). In contrast, in the *P. micans*
404 clade, the base of the spine first rises along the right inner edge of platelet 1 then turns toward
405 the outer dorsal end of platelet 1 following the edge of the left plate, giving the spine its
406 characteristic curved shape (named "long spine with sail" in *P. micans*, Tillmann et al., 2019),
407 as it is clearly observable in *P. texanum* (Henrichs et al., 2013) and *P. koreanum* (Han et al.,
408 2016), *P. gracile* (Pei et al., 2022), *P. caribaeum* (Faust, 1993), *P. gibbosum* (Tolomio, 1988)
409 and *P. arcuatum* (Skejić et al., 2017), and with the wing of *P. rhathymum* (Loeblich et al., 1979;
410 Lim et al., 2013) and *P. steidingerae* (Figs. 5-10 in Faust, 1990, after Gómez et al., 2017). On
411 the ventral side of the periflagellar area, the platelet 4 is flat in the *P. triestinum* clade whereas
412 it is flanged with a short wing along its inner edge (next to platelets 3 and 5) in the *P. micans*
413 clade.
414 As already discussed by Tolomio and Cavolo (1985), *P. venetum* presents similarities in shape
415 and size with *P. brochii* J.Schiller which was described by Schiller (1918) in Adriatic waters,
416 but this species has a more pointed posterior end, although the cell shape of *P. venetum*
417 conforms better to another drawing of *P. brochii* published by Schiller in 1928 (pl. 3, fig. 3).
418 However, *P. brochii* is clearly different to *P. venetum* by the presence of (very) large pores
419 scattered over the thecal plate (as shown in the drawing reproduced in Schiller 1918, 1928 and
420 1933) and sometimes having thick plates. Schiller (1933, pp. 41-42) synonymized *P. brochii*
421 with *Prorocentrum maximum* (Gourret) J.Schiller, (basionym *Postprorocentrum maximum*
422 Gourret), although the latter (whose size was not precisely given) had a different cell shape
423 (Gourret, 1883; Schiller, 1933, p. 41, fig. 44c). Overall, it is possible that *P. brochii* as described
424 by Schiller (1918, 1928) is closely related to *P. venetum* and *P. triestinum* (Schiller, 1918;

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425 Tillmann et al., 2022), but detailed morphological and molecular studies on strains from the
426 type locality of *P. brochii* are needed.
427 Compared to *P. venetum*, *P. mexicanum* (Osorio Tafall, 1942) is larger (39 µm long, 29 µm in
428 depth). The drawing of a cell in right lateral view shows that the ventral bulge is less prominent;
429 moreover, the dorsal and ventral curvatures are almost elliptical and symmetrical with respect
430 to the transverse axis of lateral plates whereas the ventral curvature is asymmetrical in *P.*
431 *venetum*. On the surface of the thecal plate, the drawing shows scattered large pores (described
432 by the Spanish term "puntuaciones") and series of striations (described as tiny spines). The
433 apical spine of *P. mexicanum* is also different from the winged projection of *P. venetum*: it
434 points straight forward and is longer with a length about one tenth of the length of the cell. The
435 author indicated that it was thin and slightly curved, and with a delicate "wing" visible in
436 dorsal/ventral view.
437 Comparing *P. venetum* to *Prorocentrum rhathymum* A.R.Loeblich III, Sherley et Schmidt
438 (1979), in relation to their oval shape, Tolomio and Cavolo (1985) highlighted several
439 differences including the dimensions (larger in *P. rhathymum*, with the length of cell in the
440 range 32-39 µm), the shape without prominent ventral bulge, the distribution of more numerous
441 trichocyst (large) pores in *P. rhathymum* (but not indicating precisely what differences), and
442 the structure of the anterior spine (as described above). Indeed, *P. rhathymum* is notably
443 distinguished by having four pairs of radial rows of pores and the absence of the dorsal row of
444 pores on the two lateral plates.
445 Comparison of morphological characteristics and rDNA phylogenetical analyses has been used
446 to discuss evolution within the genus *Prorocentrum* (e.g., Grzebyk et al., 1998; Murray et al.,
447 2007, 2009; Boopathi et al. 2015; Zhang et al., 2015; Chomérat et al., 2019). Based on nuclear
448 rDNA or mitochondrial genes, analyses revealed the existence of various clades within the
449 genus *Prorocentrum*, establishing very early the singularity of a clade of benthic species
450 (including *P. concavum*, *P. hoffmannianum* or *P. lima*) with the symmetrical contour of the
451 lateral plates (Grzebyk et al., 1998; Murray et al., 2009; Hoppenrath et al., 2013; Boopathi et
452 al., 2015; Chomérat et al., 2019; this study, Fig 7). The other species, which generally exhibit
453 an asymmetric component in their morphology, are distributed across various clades and
454 subclades within the genus, the number of which increased as the number of species analyzed
455 increased (Hoppenrath et al., 2013; Chomérat et al., 2019).
456 The clade formed of *P. venetum* with *P. triestinum* and *P. redfieldii* is strongly supported despite
457 the conspicuously difference in cell shape, with an approximately oval shape in *P. venetum* as
458 opposed to a lanceolate shape with a more distinctly pointed posterior end in the other two

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459 species. The proximity of oval shape species with species with a pointed posterior end is also
460 observed in the sister clade encompassing *P. micans*, with *P. rhathymum* and *P. steidingerae*
461 on one hand, and *P. micans*, *P. gracile* and *P. koreanum* on the other hand (Fig. 7). In the
462 *Prorocentrum* phylogenetic tree (Fig. 7), basal clades and taxa mostly show a thecal plate shape
463 with a rounded posterior end whereas the *P. triestinum* and *P. mican*s clades appear to be among
464 the most recently diverged in the genus, suggesting that the pointed posterior end was a late-
465 emerging feature in the genus. The variation in the shape of posterior end between the two
466 varieties described in *P. texanum* within the *P. micans* clade (Henrichs et al., 2013), one with a
467 rounded posterior end (var. *texanum*) and the other with a pointed posterior end (var.
468 *cuspidatum*), appears to be some kind of vestigial mark of the evolutionary emergence of the
469 morphological transition of the posterior end in this branch of the genus *Prorocentrum*.
470 Changes in the number and distribution of large pores also appear to reflect an evolutionary
471 pattern in the two sister clades. The higher number of large pores of *P. venetum* in its clade is
472 partly due to the two radial rows of pores. Similarly in the clade encompassing *P. micans*, radial
473 rows of large pores (between 2 and 6 pairs depending on taxa) contribute to a generally higher
474 number of pores. Thus, the reduction in the number of large pores (especially those arranged in
475 rows) appears to be an evolutionary derived feature in this branch of the genus *Prorocentrum*.
476 Altogether, thecal plates of *P. venetum* seems to have retained more ancestral features as
477 compared to *P. triestinum* and *P. redfieldii*.
478 The phylogenetic analyses of the three considered rDNA regions provided different metric
479 scales that appeared differently useful for the genetic delimitation of related *Prorocentrum*
480 species. The few units of nucleotide substitutions on these rDNA regions may be sufficient to
481 separate sister clades or species, however, relationships between species clades were not always
482 well resolved. Besides for some species clades, genetic variability carried by a similar number
483 of substitutions, may suggest internal subclades or peripheral clades possibly representing
484 cryptic species. In the analysis with the three combined rDNA regions (18S-D3 analysis, Fig.
485 7), the delimitation of species and the resolution of relationships between species appeared to
486 have been improved within the genus *Prorocentrum*, despite the small number of such data
487 available to date (especially those obtained in the form of fully assembled sequences).
488
489 **5. Conclusions**
490
491 The morphological description of *P. venetum* is complemented with fine details on thecal plate
492 morphology and periflagellar platelets. The phylogenetic analysis demonstrated that *P. venetum*

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493 belongs to the same lineage as *P. triestinum* and *P. redfieldii*. Morphologically, this lineage can
494 be defined by several synapomorphic characters including the significant size-reduction of the
495 accessory pore and the adjacent platelet 7, and the presence of a row of large pores along the
496 dorsal edge of the posterior end of both thecal plates. Both morphologically and
497 phylogenetically, *P. venetum* is not a synonym of *P. mexicanum*, contrary to what was
498 suggested in Gómez et al. (2017), and this is consistent with the description of this organism as
499 a distinct species by Tolomio and Cavolo (1985).
500
501 **CRediT authorship contribution statement**
502 **D. Grzebyk:** isolated the *Prorocentrum venetum* strains, performed the genetic study and the
503 phylogenetic analyses, contributed to the morphological description, discussion, drafting and
504 editing manuscript. **U. Tillmann:** morphological analysis (light microscopy, scanning electron
505 microscopy), discussion, drafting and editing manuscript. **M. Hoppenrath:** transmission
506 electron microscopy, discussion, drafting and editing manuscript.
507
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513
514 **Disclosure statement**
515 No potential conflict of interest was reported by the authors.
516
517
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519 **Supplementary material**

520 Table S1. PCR and sequencing primers used in this study.

521

522 Table S2. Variability and frequency in the number of large pores in the pore groups on the right
523 and left thecal plates between cells in the two *P. venetum* strains.

524

525 Table S3. Variability and frequency in the number of small pores in the pore groups on the right
526 and left thecal plates between cells in the two *P. venetum* strains.

527

528

529 Fig. S1. *Prorocentrum venetum* (strain AYR19-3E9), LM. **A–G**. Living cells. **H–X**.
530 Formaldehyde fixed cells. **O–U**. Cells stained with solophenylflavine in epifluorescence and
531 blue-light excitation. **V–X**. The same cell stained with DAPI in brightfield (**V**), with blue-light
532 excitation (**W**) or with UV excitation (**X**), showing shape and position of the chloroplast and
533 the posterior position of nucleus (in blue color). Scale bars: 5 µm.

534

535 Fig. S2. *Prorocentrum venetum* (strain AYR19-3E9), SEM. **A**, **D–G**, **J**. Right lateral view. **B**.
536 Left lateral view. **C**. Left dorsal view of a young cell with narrow intercalary band. **H–I**. Ventral
537 view of an enlarged cell. **K**. Internal face of left plate. **L–M**. The three types of pores at the
538 posterior end of cells: large pores, small pores (black arrows) and the mini-pore (white
539 arrowhead). Scale bars: 5 µm (**A–K**) or 1 µm (**L–M**).

540

541 Fig. S3. *Prorocentrum venetum* (strain AYR19-3E9), SEM. **A–E**. Detail views of the apical
542 periflagellar area, with platelet numbering, in right apical view (**A–B**, **D–E**) and in apical view
543 (**C**). ap = accessory pore, fp = flagellar pore. Scale bars: 1 µm.

544

545 Fig. S4. Nucleotide alignment of 18SD3 rDNA sequences from species belonging to sister
546 clades encompassing *P. venetum* and *P. micans*. Continuous full length sequences for strains
547 with a single accession numbers; combination of discontinuous partial sequences (two or three
548 joint accession numbers for the other strains. Phylogenetic analyses were conducted with
549 fragments between nucleotide positions 1-1749 for 18S rDNA, positions 1770-2369 for ITS
550 region, and positions 2390-3073 for 28S D1D2 region.

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552 **References**

553

554 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment
555 search tool. J. Molec. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

556

557 Andersen, R.A., 2005. Algal culturing techniques. 578 pp. Academic Press.

558

559 Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast,
560 accurate, and powerful alternative. System. Biol., 55: 539–52.
561 https://doi.org/10.1080/10635150600755453

562

563 Arteaga-Sogamoso, E., Rodríguez, F., Amato, A., Ben-Gigirey, B., Fraga, S., Mafra, L.L.,
564 Fernandes, L.F., de Azevedo Tibiriçá, C.E.J., Chomérat, N., Nishimura, T., Homma, C.,
565 Adachi, M., Mancera-Pineda, J.E., 2023. Morphology and phylogeny of *Prorocentrum*
566 *porosum* sp. nov. (Dinophyceae): A new benthic toxic dinoflagellate from the Atlantic and
567 Pacific Oceans. Harmful Algae 121, 102356. https://doi.org/10.1016/j.hal.2022.102356

568

569 Bec, B., Collos, Y., Souchu, P., Vaquer, A., Lautier, J., Fiandrino, A., Benau, L., Orsoni, V.,
570 Laugier, T., 2011. Distribution of picophytoplankton and nanophytoplankton along an
571 anthropogenic eutrophication gradient in French Mediterranean coastal lagoons. *Aquat.*
572 *Microb. Ecol.* 63:29–45. https://doi.org/10.3354/ame01480

573

574 Bergh, R.S., 1881. Der Organismus der Cilioflagellaten. Eine Phylogenetische Studie.
575 Morphologisches Jahrbuch 7: 177-288, pl. 12-16.

576

577 Boopathi, T., Faria, D.G., Cheon, J.-Y., Youn, S.H., Ki, J.-S., 2015. Implications of high
578 molecular divergence of nuclear rRNA and phylogenetic structure for the dinoflagellate
579 *Prorocentrum* (Dinophyceae, Prorocentrales). J. Eukaryot. Microbiol. 62, 519-531
580 https://doi.org/10.1111/jeu.12206

581

582 Bursa, A., 1959. The genus *Prorocentrum* Ehrenberg. Morphodynamics, protoplasmatic
583 structures, and taxonomy. Can. J. Botany 37, 1-31. https://doi.org/10.1139/b59-001

584

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

585 Bursa, A.S., 1962. Some morphogenetic factors in taxonomy of dinoflagellates. Grana Palynol.
586 3, 54-66. https://doi.org/10.1080/00173136209429104
587
588 Chomérat, N.; Gatti, C.M.I.; Nézan, E.; Chinain, M. 2017. Studies on the benthic genus
589 *Sinophysis* (Dinophysales, Dinophyceae) II. *S. canaliculata* from Rapa Island (French
590 Polynesia). Phycologia 56, 193-203. https://doi.org/10.2216/16-24.1
591
592 Chomérat, N., Bilien, G., Zentz, F., 2019. A taxonomical study of benthic *Prorocentrum* species
593 (Prorocentrales, Dinophyceae) from Anse Dufour (Martinique Island, eastern Caribbean
594 Sea). Mar. Biodiversity 49, 1299-1319. https://doi.org/10.1007/s12526-018-0913-6
595
596 Cohen-Fernandez, E.J., Meave Del Castillo, E., Salgado Ugarte, I.H., Pedroche, F.F., 2006.
597 Contribution of external morphology in solving a species complex: The case of
598 *Prorocentrum micans*, *Prorocentrum gracile* and *Prorocentrum sigmoides* (Dinoflagellata)
599 from the Mexican Pacific Coast. Phycol. Res. 54, 330-340. http://dx.doi.org/10.1111/j.1440-
600 1835.2006.00440.x
601
602 David, H., Laza-Martínez, A., García-Etxebarria, K., Riobó, P., Orive, E., 2014.
603 Characterization of *Prorocentrum elegans* and *Prorocentrum levis* (Dinophyceae) from the
604 southeastern Bay of Biscay by morphology and molecular phylogeny. J. Phycol. 50, 718-
605 726. http://dx.doi.org/10.1111/jpy.12200
606
607 Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F.,
608 Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny.fr: robust
609 phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36: W465–W469.
610 https://doi.org/10.1093/nar/gkn180
611
612 Dodge, J.D., Crawford, R.M., 1971. A fine-structural survey of dinoflagellate pyrenoids and
613 food-reserves. Bot. J. Linn. Soc. 64, 105-115. https://doi.org/10.1111/j.1095-
614 8339.1971.tb02138.x
615
616 Ehrenberg, C.G., 1834. Dritter Beitrag zur Erkenntnis großer Organisation in der Richtung des
617 kleinsten Raumes. Abhandlungen der Physikalisch-Mathematischen Klasse der Königlichen
618 Akademie der Wissenschaften zu Berlin 1833. Pp. 145-336.

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

619
620 Faust, M.A., 1990. Morphologic details of six benthic species of *Prorocentrum* (Pyrrophyta)
621 from a mangrove island, Twin Cays, Belize, including two new species. J. Phycol. 26, 548-
622 558. https://doi.org/10.1111/j.0022-3646.1990.00548.x
623
624 Faust, M.A., 1993. *Prorocentum belizeanum*, *Prorocentrum elegans*, and *Prorocentrum*
625 *caribbaeum*, three new benthic species (Dinophyceae) from a mangrove island, Twin Cays,
626 Belize. J. Phycol. 29, 100-107. https://doi.org/10.1111/j.1529-8817.1993.tb00287.x
627
628 Gómez, F., 2008. Phytoplankton invasions: comments on the validity of categorizing the non-
629 indigenous dinoflagellates and diatoms in European Seas. Mar. Poll. Bull. 56, 620-628.
630 https://doi.org/10.1016/j.marpolbul.2007.12.014
631
632 Gómez, F., Qiu, D., Lin, S., 2017. The synonymy of the toxic dinoflagellates *Prorocentrum*
633 *mexicanum* and *P. rhathymum* and the description of *P. steidingerae* sp. nov.
634 (Prorocentrales, Dinophyceae). J. Eukaryot. Microbiol. 64, 668-677.
635 https://doi.org/10.1111/jeu.12403
636
637 Gómez, F., Gourvil, P., Li, T., Huang, Y., Zhang, H., Courcot, L., Artigas, L.F., Soler Onís, E.,
638 Gutiérrez-Rodríguez, A., Lin, S., 2023. Molecular phylogeny of the spiny-surfaced species
639 of the dinoflagellate *Prorocentrum* with the description of *P. thermophilum* sp. nov. and *P.*
640 *criophilum* sp. nov. (Prorocentrales, Dinophyceae). J. Phycol. 59, 70-86.
641 https://doi.org/10.1111/jpy.13298
642
643 Gourret, P., 1883. Les Péridiniens du Golfe de Marseille. Annales Mus. Hist. Nat. Marseille
644 (Zool.) 1, 1-114.
645
646 Grzebyk, D., Sako, Y., Berland, B., 1998. Phylogenetic analysis of nine species of
647 *Prorocentrum* (Dinophyceae) inferred from 18S ribosomal DNA sequences, morphological
648 comparisons, and description of *Prorocentrum panamensis*, sp. nov. J. Phycol. 34, 1055–68.
649 https://doi.org/10.1046/j.1529-8817.1998.341055.x
650
651 Grzebyk, D., Audic, S., Lasserre, B., Abadie, E., de Vargas, C., Bec, B., 2017. Insights into the
652 harmful algal flora in northwestern Mediterranean coastal lagoons revealed by

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

Preprint not peer reviewed

653 pyrosequencing metabarcodes of the 28S rRNA gene. Harmful Algae, 68: 1–16.
654 http://dx.doi.org/10.1016/j.hal.2017.06.003
655
656 Grzebyk, D., Pasqualini, V., Garrido, M., Quilichini, Y., Pereto, C., Cecchi, P., 2022. Insight
657 into the morphology and genetic diversity of the *Chaetoceros tenuissimus* (Bacillariophyta)
658 species complex. Eur. J. Phycol. 57: 507-525.
659 https://doi.org/10.1080/09670262.2022.2029949
660
661 Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New
662 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the
663 performance of PhyML 3.0. Syst. Biol. 59: 307–321. https://doi.org/10.1093/sysbio/syq010
664
665 Guiry, M.D., 2022. *Prorocentrum venetum* Tolomio & Cavolo 1985. In Guiry, M.D., Guiry,
666 G.M. (Eds), *AlgaeBase*, World-wide electronic publication, National University of Ireland,
667 Galway. http://www.algaebase.org (accessed 24 November 2022).
668
669 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
670 program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41: 95–98.
671
672 Han, M.-S., Wang, P., Kim, J.H., Cho, S.-Y., Park, B.S., Kim, J.-H., Katano, T., Kim, B.-H.,
673 2016. Morphological and molecular phylogenetic position of *Prorocentrum micans* sensu
674 stricto and description of *Prorocentrum koreanum* sp. nov. from Southern coastal waters in
675 Korea and Japan. Protist 167, 32-50. http://dx.doi.org/10.1016/j.protis.2015.12.001
676
677 Henrichs, D.W., Scott, P.S., Steidinger, K.A., Errera, R.M., Abraham, A., Campbell, L., 2013.
678 Morphology and phylogeny of *Prorocentrum texanum* sp. nov. (Dinophyceae): a new toxic
679 dinoflagellate from the Gulf of Mexico coastal waters exhibiting two distinct morphologies.
680 J. Phycol. 49, 143-155. http://dx.doi.org/10.1111/jpy.12030
681
682 Hoppenrath, M., Chomérat, N., Horiguchi, T., Schweikert, M., Nagahama, Y., Murray, S.,
683 2013. Taxonomy and phylogeny of the benthic *Prorocentrum* species (Dinophyceae)—A
684 proposal and review. Harmful Algae 27, 1-28. http://dx.doi.org/10.1016/j.hal.2013.03.006
685

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

686 Hulburt, E.M., 1965. Three closely allied dinoflagellates. J. Phycol. 1, 95-96.
687 https://doi.org/10.1111/j.1529-8817.1965.tb04564.x
688
689 Kowallic, K., 1969. The crystal lattice of the pyrenoid matrix of *Prorocentrum micans*. J. Cell.
690 Sci. 5, 251-269. https://doi.org/10.1242/jcs.5.1.251
691
692 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.,
693 Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins,
694 D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.
695 https://doi.org/10.1093/bioinformatics/btm404
696
697 Lim, A.S., Jeong, H.J., Jang, T.Y., Kang, N.S., Lee, S.Y., Yoo, Y.D., Kim, H.S., 2013.
698 Morphology and molecular characterization of the epiphytic dinoflagellate *Prorocentrum* cf.
699 *rhathymum* in temperate waters off Jeju Island, Korea. Ocean Sci. J. 48, 1-17.
700 https://doi.org/10.1007/s12601-013-0001-6
701
702 Loeblich, A.R., III, Sherley, J.L., Schmidt, R.J., 1979. The correct position of flagellar insertion
703 in *Prorocentrum* and description of *Prorocentrum rhathymum* sp. nov. (Pyrrhophyta). J.
704 Plankton Res. 1, 113-120. https://doi.org/10.1093/plankt/1.2.113
705
706 Murray, S., Nagahama, Y., Fukuyo, Y., 2007. Phylogenetic study of benthic, spine-bearing
707 prorocentroids, including *Prorocentrum fukuyoi* sp. nov. Phycol. Res. 55, 91-102.
708 https://doi.org/10.1111/j.1440-1835.2007.00452.x
709
710 Murray, S., Ip, C.L.C., Moore, R., Nagahama, Y., Fukuyo, Y., 2009. Are prorocentroid
711 dinoflagellates monophyletic? A study of 25 species based on nuclear and mitochondrial
712 genes. Protist 160, 245-264 https://doi.org/10.1016/j.protis.2008.12.004
713
714 Ndhlovu, A., Dhar, N., Garg, N., Xuma, T., Pitcher, G.C., Sym, S.D., Durand, P.M., 2017. A
715 red tide forming dinoflagellate *Prorocentrum triestinum*: identification, phylogeny and
716 impacts on St Helena Bay, South Africa. Phycologia 56, 649-665.
717 https://doi.org/10.2216/16-114.1
718

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

Osorio Tafall, B.F., 1942. Notas sobre dinoflagelados planctónicos marinos de México, con descripción de nuevas especies. Anal. Esc. Nal. Cien. Biol. Méx. **2**: 435-447 (Lám. 34-36).

Pei, L., Hu, W., Wang, P., Kang, J., Mohamed, H.F., Wang, C., Liu, L., Luo, Z., 2022. Morphologic and phylogenic characterization of two bloom-forming planktonic *Prorocentrum* (Dinophyceae) species and their potential distribution in the China Sea. Algal Res. 66, 102788. https://doi.org/10.1016/j.algal.2022.102788

Schiller, J., 1918. Über neue *Prorocentrum*– und *Exuviaella*–Arten aus der Adria. Archiv für Protistenkunde 38, 250-262.

Schiller, J., 1928. Die plaktische vegetationen des Adriatischen Meeres. C. Dinoflagellata I Teil. Adiniferidae, Dinophysidaceae. Archiv für Protistenkunde 61, 46-91.

Schiller, J., 1933. Dinoflagellatae (Peridineae) in monographischer Behandlung. In Dr. L. Rabenhorst's Kryptogamen–Flora von Deutschland, Österreich und der Schweiz. Zehnter band Flagellatae, Kolkwitz, R. editor. 615 pp. Akademische Verlagsgesellschaft, Leipzig.

Skejić, S., Car, A., Marasović, I., Jozić, S., Bužančić, M., Arapov, J., Ninčević Gladan, Ž., Bakrač, A., Kušpilić, G., Vidjac, O., Larsen, J., 2017. Morphology and ecology of the poorly known dinoflagellate *Prorocentrum arcuatum* (Dinophyceae) from the Medulin Bay (eastern Adriatic Sea). Acta Adriat. 58, 41-52. https://doi.org/10.32582/aa.58.1.3

Tillmann, U., Hoppenrath, M., Gottschling, M., 2019. Reliable determination of *Prorocentrum micans* Ehrenb. (Prorocentrales, Dinophyceae) based on newly collected material from the type locality. Eur. J. Phycol. 54: 417-431. https://doi.org/10.1080/09670262.2019.1579925

Tillmann, U., Beran, A., Gottschling, M., Wietkamp, S., Hoppenrath, M., 2022. Clarifying confusion – *Prorocentrum triestinum* J.Schiller and *Prorocentrum redfieldii* Bursa (Prorocentrales, Dinophyceae) are two different species. Eur. J. Phycol. 57: 207-226. https://doi.org/10.1080/09670262.2021.1948614

Tillmann, U., Gottschling, M., Wietkamp, S., Hoppenrath, M., 2023a. Morphological and phylogenetic characterisation of *Prorocentrum spinulentum*, sp. nov. (Prorocentrales,

Preprint not peer reviewed

This preprint research paper has not been peer reviewed. Electronic copy available at: https://ssrn.com/abstract=4961632

753 Dinophyceae), a small spiny species from the North Atlantic. Microorganisms 11, 271. https://doi.org/10.3390/microorganisms11020271
754

755

756 Tillmann, U., Wietkamp, S., Gottschling, M., Hoppenrath, M., 2023b. *Prorocentrum*
757 *pervagatum* sp. nov. (Prorocentrales, Dinophyceae): A new, small, planktonic species with
758 a global distribution. Phycol. Res. 71, 56-71. https://doi.org/10.1111/pre.12502

759

760 Thessen, A.E., Patterson, D.J., Murray, S.A., 2012. The taxonomic significance of species that
761 have only been observed once: the genus *Gymnodinium* (Dinoflagellata) as an example.
762 PLoS ONE 7, e44015. https://doi.org/10.1371/journal.pone.0044015

763

764 Tolomio, C., Cavolo, F., 1985. Description de *Prorocentrum venetum* sp. nov. (Dinophyceae)
765 trouvé dans la Lagune de Venise. Botanica Marina, 28: 345-350.
766 https://doi.org/10.1515/botm.1985.28.8.345

767

768 Tolomio, C., 1988. Observations taxinomiques et ultrastructurales sur des exemplaires de
769 *Prorocentrum* (Dinophyceae) récoltés dans les eaux côtières de la Corse du Sud-Est.
770 Botanica Marina 31, 223-230. https://doi.org/10.1515/botm.1988.31.3.223

771

772 Zhang, H., Li, Y., Cen, J., Wang, H., Cui, L., Dong, Y., Lu, S., 2015. Morphotypes of
773 *Prorocentrum lima* (Dinophyceae) from Hainan Island, South China Sea: morphological and
774 molecular characterization. Phycologia 54: 503-516. https://doi.org/10.2216/15-8.1

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783 **Figure captions**

784

785 Fig. 1. *Prorocentrum venetum* (strain AYR19-3C4), LM. **A–J**. Living cells. **O–Ad**.
786 Formaldehyde fixed cells. **A–N**. Differential interference contrast LM, general size and shape
787 of cells in lateral view of the right thecal plate (**A, B**), the left thecal plate (**C–F**) and in
788 ventral/dorsal view (**I–N**). Note the round to broadly oval pusule (p) labelled in **E**, and the
789 central round pyrenoid (arrow) labelled in **B**. Also note the pointed antapical end of a
790 presumably newly divided cell (**J**) and the truncated antapical end in broader cells (**K–N**). **O–**
791 **V**. Cells stained with solophenylflavine in epifluorescence and under blue-light excitation, to
792 illustrate plates and plate sutures (visualized by green fluorescence), and the shape and location
793 of chloroplasts (red fluorescence), in ventral/dorsal view (**O–U**) or right lateral view (**V**). Note
794 the lateral position of the two lobed chloroplasts (**R**, **S**). **V–X**. Visibility of thecal pores (arrows)
795 when plates are stained (**V**), or when the contracted cell content allow a partial view of the
796 plates (**W**) or on an empty theca (**X**). **Y–Ab**. The same cell stained with DAPI in brightfield
797 (**Y**), with blue-light excitation (**Z, Aa**, two different focal planes) or with UV excitation (**Ab**),
798 to illustrate shape and position of the chloroplasts and the nucleus (blue fluorescence). Note the
799 central pyrenoid (arrow in **Aa**), the apical oval area void of chloroplast in **Aa**, and the antapical
800 position of the round nucleus (arrow in **Ab**). **Ac**, **Ad**. DAPI stained cells with epifluorescence
801 and UV excitation to illustrate shape and posterior position of the nucleus; the enlarged nucleus
802 in **Ad** is presumably undergoing nuclear division. Scale bars: 5 µm.

803

804 Fig. 2. *Prorocentrum venetum* (strain AYR19-3C4), SEM. **A–I**. Entire cells in right thecal view
805 (**A, F, G, I**), in left thecal view (**B, D, E**), or in dorsal apical view (**C**, an enlarged cell with a
806 broad, transversely striated, intercalary band). **J**. Flattened right thecal plate showing the apical
807 V-shaped insertion of the periflagellar area (arrow). **K–N**. Detail views of the apical area in
808 right apical view (**K**), right dorsal view (**L**), in right lateral view (**M**) and in left lateral view
809 (**N**). Scale bars: 5 µm (**A–J**) or 1 µm (**K–N**).

810

811 Fig. 3. *Prorocentrum venetum* (strain AYR19-3C4), detailed SEM of the periflagellar area,
812 surface structure and pores. **A–E**. The periflagellar area in apical view (**A–C**), right lateral view
813 (**D**) or apical ventral view (**E**). **F–J**. General appearance of thecal pores (large pores: white
814 arrows; small pores: black arrows) in external view (**F, I, J**) or in internal view showing the
815 internal tubular shape of large pores (**G, H**). At the posterior end of cells (**I–J**), note the presence
816 of a mini-pore (white arrowhead) located at the slightly protruding tip of the thecal plate, the

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817 diameter of which is distinctly smaller than that of the small pores (black arrows). Platelet
818 numbering according to Hoppenrath et al. (2013); ap = accessory pore, fp = flagellar pore. Scale
819 bars: 1 µm.
820

821 Fig. 4. Schematic drawings of *P. venetum*. The numbers of pores (large and small) are
822 representative of the mean/median numbers for strain AYR19-3C4 (Table 1, Tables S2 and S3).
823 **A.** Right lateral view. **B.** Left lateral view. **C** Ventral view. **D.** Dorsal view. **E.** Labeling of pore
824 groups on the right (R1 to R10 groups) and left (L1 to L10 groups) lateral plates. **F.** Periflagellar
825 area in apical view (the gray surfaces correspond to the base of the erect structures above the
826 platelets). Platelet numbering according to Hoppenrath et al. (2013); ap = accessory pore, fp =
827 flagellar pore.
828

829 Fig. 5. *Prorocentrum venetum* (strain AYR19-3C4), transmission electron microscopy of the
830 general cell ultrastructure. **A.** Longitudinal section showing part of the anterior periflagellar
831 area with flagellar pore (white arrow) and the posterior nucleus (n), peripherally arranged
832 chloroplast parts (c) and dense black oil droplets. **B.** Oblique section showing the nucleus (n),
833 chloroplast lobes (c), mitochondria (m) and trichocysts (t). **C.** Anterior cell region below the
834 periflagellar area (black arrow pointing into the flagellar pore region) with membranes of the
835 pusule system (pu). **D.** Nucleus with condensed chromosomes and nucleolus (nu). **E.** Cross
836 sections of trichocysts (t) of different diameter and an oblique longitudinal section. **F.** Part of
837 the Golgi apparatus. Scale bars: 2.5 µm (A), 5 µm (B), 1 µm (C-F).
838

839 Fig. 6. *Prorocentrum venetum* (strain AYR19-3C4), transmission electron microscopy of
840 chloroplast (c) details. A, **B**. Irregular interlamellar pyrenoid (py). **C-F**. Different morphologies
841 of the bulge-type pyrenoid (py) without starch sheath. Note the thylakoids, single or bundled in
842 pairs, which cross the pyrenoid matrix in an unordered manner. Scale bars: 1 µm.
843

844 Fig. 7. Phylogenetic tree of *Prorocentrum* genus based on 18S-D3 rDNA sequences showing
845 the position of *P. venetum*; two taxa belonging to the order Peridiniales were used as outgroups.
846 In the box, an unrooted tree with the same topology focusing on the two sister clades (containing
847 taxa related to *P. micans* and *P. venetum*, respectively) is provided to better show the genetic
848 distances between and within species clades. The *P. venetum* strain sequenced in this study is
849 in bold. The branch length is proportional to the number of substitutions per site (the scale bar

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850 represents the number of nucleotide substitutions per site). The most significant branch support
851 values are provided at the main nodes.
852
853 Fig. 8. Phylogenetic tree based on 18S rDNA sequences, of two sister clades encompassing taxa
854 related to *P. micans* and *P. venetum*. The branch length is proportional to the number of
855 substitutions per site (the scale bar represents the number of nucleotide substitutions per site).
856 The most significant branch support values are provided at the main nodes.
857
858 Fig. 9. Phylogenetic tree based on D1-D2 region of 28S rDNA sequences, of two sister clades
859 encompassing taxa related to *P. micans* and *P. venetum*. The branch length is proportional to
860 the number of substitutions per site (the scale bar represents the number of nucleotide
861 substitutions per site). The most significant branch support values are provided at the main
862 nodes.
863
864 Fig. 10. Phylogenetic tree based on the ITS region sequences (including ITS1, 5.8S rDNA and
865 ITS2), of two sister clades encompassing taxa related to *P. micans* and *P. venetum*. The branch
866 length is proportional to the number of substitutions per site (the scale bar represents the number
867 of nucleotide substitutions per site). The most significant branch support values are provided at
868 the main nodes.

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