Nota Científica

New record of *Prymnesium parvum* f. *patelliferum* (Green, Hibberd & Piennar) Larsen stat. nov. (Prymnesiophyceae) from Valparaíso Bay

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ABSTRACT. An “unidentified flagellate” with a short non coiling haptonema was found by chance in a living phytoplankton sample taken from Valparaíso Bay, Chile. Monocultures were obtained and kept in Provasoli medium (PES), at salinity 34.0, $16 \pm 2^\circ\text{C}$, $40 \mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and 11:13 h light:darkness. The strain (MLB298) was obtained by the capillary method. Cells were harvested by centrifugation, and pigments were extracted and examined using HPLC. Cells were examined with both light and electron microscopy. The genus was identified as *Prymnesium* Conrad, due to its characteristic pigments (Chl c_3 plus fucoxanthin) and cellular features, mainly haptonema and scale characteristics. Further cultured studies of the cells revealed asexual and sexual reproduction. Electron microscopy observations of the scales showed the radial pattern of *P. patellifera* and the concentric fibrillar pattern of *P. parvum*, confirming that *P. patellifera* and *P. parvum* are alternating generations of the same species (conspecificity). *P. parvum* f. *patelliferum* (Green, Hibberd & Piennar) Larsen stat. nov. is cited herein for the first time for the Chilean coast.

Key words: *Prymnesium parvum* f. *patelliferum*, fine structure, HPLC, culture, Chile.

Nuevo registro de *Prymnesium parvum* f. *patelliferum* (Green, Hibberd & Piennar) Larsen stat. nov. (Prymnesiophyceae) en la bahía de Valparaíso

RESUMEN. Un “flagelado no identificado”, caracterizado por presentar un haptonema corto y rígido, fue casualmente encontrado en muestras vivas de fitoplancton de la bahía de Valparaíso, Chile. Se obtuvieron monocultivos que fueron mantenidos en medio Provasoli (PES), 34 de salinidad, $16 \pm 2^\circ\text{C}$, $40 \mu\text{mol photon m}^{-2}\cdot\text{s}^{-1}$, 11:13 h (luz:oscuridad). La cepa (MLB298) fue obtenida por el método de capilaridad. Las células fueron cosechadas por centrifugación y los pigmentos extraídos fueron examinados por HPLC. Las células fueron observadas bajo microscopio óptico y electrónico de transmisión. El género fue identificado como *Prymnesium* Conrad, de acuerdo a los pigmentos característicos, Chl c_3 más fucoxantina y a caracteres celulares, principalmente el haptonema y características de las escamas. Observaciones de las células en cultivo demostraron reproducción asexual y sexual. Observaciones de las escamas bajo microscopio electrónico de transmisión mostraron patrones radiales propios de *P. patellifera* y patrones fibrilares concéntricos propios de *P. parvum*. Estos caracteres confirmarían que *P. patellifera* y *P. parvum* son generaciones alternas de la misma especie (coespecificidad). En este trabajo *P. parvum* f. *patelliferum* (Green, Hibberd & Piennar) Larsen stat. nov. es citado por primera vez para las costas de Chile.

Palabras clave: *P. parvum* f. *patelliferum*, ultraestructura, HPLC, cultivo, Chile.

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In Chile, the light microscope is the most commonly used tool for qualitative and quantitative analyses of marine phytoplankton. Less is known about the relative importance of planktonic “naked” phytoflagellates and “other microalgae” due to the requirements of observing living material, using

an electron microscope, and being familiar with the characteristics of a number of species. The few studies available cover *Heterosigma akashiwo* from Chiloe (Parra *et al.*, 1991) and *Asteromonas gracilis* and *Chlorella neustonica* from central Chilean waters (Catalán & Collantes, 1997, 2000).

An "unidentified flagellate" with a short, flexible, non coiling haptonema similar to *Prymnesium* was found by chance during the isolation and characterization of native microalgae from Valparaíso Bay, Chile (Collantes *et al.*, 2001). The taxonomy of the genus *Prymnesium* Conrad (1926) is of interest because its representatives are widely distributed and have been identified as the causal agents of extensive fish mortalities in brackish waters from northern Europe to the Middle East, mostly Israel (Green *et al.*, 1982), and China (Guo *et al.*, 1996). Ten species have been described: *P. saltans* (Conrad, 1926), *P. minutum* (Carter, 1937), *P. czosnowskii* (Starmach, 1968), *P. parvum*, and *P. patellifera* (Green *et al.*, 1982) = *P. patelliferum* (Green *et al.*, 1982) orthog. emend. (Jordan & Green, 1994), *P. annuliferum* and *P. zebrinum* (Billard, 1983), *P. calathiferum* (Chang & Ryan, 1985), *P. nemamethecum* (Pienaar & Birkhead, 1994), and *P. flaveolatum* (Fresnel *et al.*, 2001).

A summary of the signature pigments that are useful as markers of algal groups and oceanic processes demonstrated Chl c_3 pigment in some prymnesiophytes, one chrysophyte, and several diatoms and dinoflagellates (Stauber & Jeffrey, 1988; Jeffrey, 1997). Research on the Prymnesiophyceae revealed three combinations of Chl c pigments: Chl c_1 and c_2 (Jeffrey, 1976), Chl c_2 and c_3 (Jeffrey & Wright, 1987) and Chl c_1 , c_2 , and c_3 (Fawley, 1989). Graham & Wilcox (2000) mentioned at least five variations in additional pigment contents occurring among haptophytes: fucoxanthin; Chl c_3 plus fucoxanthin; Chl c_3 plus 19'-hexanoyloxyfucoxanthin; Chl c_3 plus 19'-butanoyloxyfucoxanthin; 19'-hexanoyloxyfucoxanthin; and fucoxanthin (Jeffrey & Wright, 1994) and other haptophytes such as *Isochrysis* characterized by Chl c that has a phytol tail (Zapata & Garrido, 1997).

The taxonomy of these species is confusing due to their similar appearance under a light microscope. Green *et al.* (1982) distinguished the *Prymnesium* species by small ultrastructural differences in their organic body scales. *P. parvum* and *P. patellifera*, both toxin producers (Moestrup & Thomsen, 1995) that can be distinguished by such scale criteria, were found to be very closely related when molecular techniques were applied. They are suspected to be alternating generations of the same species (Larsen & Medlin, 1997; Larsen, 1999).

The culture examined in this study was originated from a single cell isolated from a water sample

collected on 1 August 1998 from Valparaíso Bay, Chile (32°57'S, 71°33'W). The clonal culture MLB298-CCAM-UV (Collantes *et al.*, 2001) was maintained in Provasoli medium (PES) at ambient temperature with natural light from a south-facing window. Cultures of the strain (MLB298) were maintained in Provasoli medium (PES), at salinity 34.0, $16 \pm 2^\circ\text{C}$, with $40 \mu\text{mol photon m}^{-2}\cdot\text{s}^{-1}$, and 11:13 h light:darkness. Cell counts were performed using a Haemocytometer (0.1 mm deep). Algal growth rates were determined by least squares fit of a straight line to the logarithmically transformed data (Guillard, 1973). A culture in an exponential growth phase ($60 \cdot 10^4 \text{ cell} \cdot \text{mL}^{-1}$) was used for pigment extractions.

Methods for cell harvests, pigment extracts, and pigment analyses were performed as detailed in Fawley (1989). Pigment analyses were adapted to the RP-HPLC system (Lachrom, Merk-Hitachi, L-7100 pump), reverse phase, and linear gradient system with a C18 column (Lichrospher 100 RP-18 endcapped (5 μm), LichoCart 250-4 HPLC-Cartridge). Pigments were detected at 440 nm (Lachrom, Merk-Hitachi, L-7400 model). The identity parameters used for chromatographic identification were the retention time of cochromatography Chl a Sigma and authentic pigments that we obtained from *Phaeodactylum tricornutum* (reference culture). Furthermore, the retention time values were compared with chromatographic analyses of *P. tricornutum*, *Emiliana huxleyi*, *Pavlova gyraans*, *Pavlova lutheri*, and *P. parvum* (Fawley, 1989; van Heukelem *et al.*, 1992; Garrido *et al.*, 1995; Jeffrey & Wright, 1997; Wright & Jeffrey, 1997; Zapata *et al.*, 2000).

Living cells were observed with Leica DMRBE and Olympus Vanox photomicroscopes equipped with differential interference contrast optics (DIC). Samples of actively growing cells were prepared for transmission electron microscopy (TEM) using three fixation protocols: a) pre-fixation with 3% glutaraldehyde (GTA) in filtered and sterile sea water; b) fixation (2.0 h) with 2.5% GTA (0.1 M sodium cacodylate buffer pH 7.4 and 4% sucrose pH 8), after which samples were washed in the buffer; and c) post-fixation (2.0 h) with 2% OsO_4 and 3% $\text{K}_3\text{Fe}(\text{CN})_6$ (v/v). Samples were then stained with 2% uranyl acetate and dehydrated in a graded series of ethyl alcohol and two times in 100% cetona. The samples were infiltrated with Eponate and embedded in shallow silicone rubber embedding moulds at 60°C for 72 h. Sections were cut using Reichert OM-U2 ultramicrotome, double-stained with uranyl acetate

and lead citrate, then examined using a Zeiss EM 900 electron microscope (modified from Cáceres, 1995).

Under the culture conditions used, the k value of MLB298 was $1.05 \text{ cell division day}^{-1}$. The separation of pigments from *P. tricornutum* (reference culture) and *Prymnesium* extracts by reversed-phase, high-performance liquid chromatography showed the following clearly resolved pigments: 1) chlorophyll c_3 , 2) chlorophyll c_1 , 3) chlorophyll c_2 , 4) fucoxanthin, 5) cis-fucoxanthin, 6) diadinoxanthin, 7) diatoxanthin, and 8) chlorophyll a (Fig. 1a,b). The β -carotene fraction was not detected with this last methodology.

Under a light microscope, the vegetative cells of *Prymnesium* from Valparaíso Bay were 6-10 μm long and 4-6 broad. Most are sub-spherical to elongated, with more or less parallel sides and a rounded posterior end, although some were bent or pyriform or had a tapered posterior end; the anterior end is obliquely truncate. The two chloroplasts were lateral and parietal, yellow-green to olive, lobed or dissected, each with an immersed pyrenoid. The two flagella were equal or sub-equal, distally tapered, heterodynamic, and 10.0-14.5 μm in length. The haptonema was very short (3.0-4.0 μm), flexible and non coiling, arising sub-apically from a groove or depression in the truncate face (Fig. 2); during

Figure 1. Separation of pigments from extracts of a) *P. tricornutum* and b) *P. parvum* f. *patelliferum* from Valparaíso Bay by reversed phase high performance liquid chromatography: 1: chlorophyll c_3 , 2: chlorophyll c_1 , 3: chlorophyll c_2 , 4: fucoxanthin, 5: cis-fucoxanthin, 6: diadinoxanthin, 7: diatoxanthin, and 8: chlorophyll a .

Figura 1. Separación de pigmentos a partir de extractos de a) *P. tricornutum* y b) *P. parvum* f. *patelliferum* de la bahía de Valparaíso, mediante cromatografía líquida de alta resolución. 1: clorofila c_3 , 2: clorofila c_1 , 3: clorofila c_2 , 4: fucoxantina, 5: cis-fucoxantina, 6: diadinoxantina, 7: diatoxantina, y 8: clorofila a .

mitosis, the flagella were cast off. The cells were heart-shaped, dividing them longitudinally. Sexual reproduction between cultured flagellate cells was observed for the first time. Cellular unions occurred with the posterior ends sticking together and the flagella were cast off. Zygotes divided into four progeny cells.

Figure 2. *P. parvum* f. *patelliferum*. Light microscopy. Vegetative cell showing the haptonema (h).

Figura 2. *P. parvum* f. *patelliferum*. Microscopía óptica. Célula vegetativa mostrando el haptonema (h).

The swimming motion was smooth. The cell rotates around its longitudinal axis and the anterior (flagellar) pole describes a helix around the path travelled; the flagella were directed in the posterior direction. The onset of cyst formation begins when the flagella were cast off and then a large vesicle appeared in the posterior region of the cell just inside the cell membrane. During cyst development, the cell wall gradually thickens. Cysts were ovoid (9.3-10.8 μm x 6.0-6.4 μm) with a simple sub-anterior pore (2.75-3.00 μm in diameter).

The flagella were normally arranged as two central microtubules and nine peripheral doublets, with no fold of the endoplasmic reticulum. Cross-sections of the haptonema reveal seven singlet microtubules arranged in a crescent, surrounded by a ring of endoplasmic reticulum (Fig. 3). The surface of the cell is covered with tiny scales of organic material, two types in two layers (Figs. 3a,b). The inner layer scales (0.20 x 0.18 μm) had narrow inflexed rims and a central thickening on the distal face; most inner scales had a radial fibrillar pattern on both faces, although some had concentrically arranged fibrils on the proximal face and sometimes only in the central area of the distal face (Fig. 3b,c). The outer layer scales (0.26 x 0.21 μm) were similar but with relatively tall, upright rims. These scales are formed in the Golgi apparatus and then secreted

from the cell (Fig. 4a). The cisternae of the Golgi body were inflated centrally and often bunch together just below the flagellar basal bodies, producing a fan-shaped array in the longitudinal section (Fig. 4b). The cytoplasm of each cell was surrounded by a narrow, peripheral cisterna of endoplasmic reticulum (Fig. 4c). Each chloroplast was enclosed within a fold of endoplasmic reticulum. There was a periplastidial reticulum in the narrow space between the chloroplast ER and the chloroplast envelope. Within the chloroplasts, the thylakoids were stacked in threes to form lamellae, but there is no girdle lamella. Each chloroplast often contains a pyrenoid, which was usually penetrated by lamellae containing a few thylakoids (Fig. 4d). Where the chloroplast lies up against the nucleus, its own endoplasmic reticulum was continuous with the endoplasmic reticulum around the nucleus (Fig. 4d). The nucleus location was central between the chloroplasts and it contains a nucleolus (Fig. 4c). Reserve metabolites accumulate in many vacuoles, often concentrated in the posterior part of the cell. Several lipoid globules were present in the cytoplasm (Fig. 4c). Peripheral mucilage bodies and a pulsated vacuole were lying beneath the plasmalemma and were often discharged by adverse conditions (Fig. 4d).

The pigment contents of *Prymnesium* and *P. tricorutum* (reference culture), both from Valparaíso Bay, differ only in the presence of Chl c_3 in *Prymnesium* (Fig. 1b). The *Prymnesium* from Chilean waters are in the group producing Chl c_1 , c_2 , and c_3 (Fawley, 1989) plus fucoxanthin, all major pigments (>10%) (Jeffrey & Veski, 1997). In this study, the β -carotene fractions were not detected in either species examined. However, Fawley (1989) used the same methodology to demonstrate that *P. parvum* and *P. tricorutum* have β carotene, and Jeffrey & Veski (1997) showed that the Prymnesiophyceae possess β carotene. This contradiction suggests that the lack of β carotene is caused by the culturing conditions or the genome carotenogenic capacity of the strains, neither of which is covered in this work. Jeffrey & Veski (1997) indicated that β carotene is a trace pigment in Prymnesiophyceae and Bacillariophyta, constituting < 1% of the total chlorophylls or carotenoids.

This study found *Prymnesium* from Chilean waters to be taxonomically similar to *P. patelliferum*, given the radial fibrillar pattern on both surfaces of most scales and, in addition, the very obvious vertical rims on the outer layer scales, as well as pronounced thickening in the centre of the distal face. Nonetheless, some scales were similar to *P.*

parvum, with concentrically arranged fibrils on the distal face (Fig. 3c). In Larsen & Medlin (1997), these features were suspected to correspond to alternating generations of the same species. In view of the apparent conspecificity of the two organisms under discussion, Larsen (1999) proposed that *P. patelliferum* should be relegated to the status of a form of *P. parvum*: *Prymnesium parvum* f. *patelliferum* (Green, Hibberd & Piennar) A. Larsen *stat. nov.* Growth characteristics, toxicity, ITS1 nucleotide sequences, and CaM-1 banding patterns suggest that a separation of the two taxa at the species level is not warranted (Larsen, 1999). In this work, the observations of asexual and sexual

reproduction of the strain (MLB298) and two scale patterns confirm the organisms to be alternating generations of the same species: *Prymnesium parvum* f. *patelliferum* (Fig. 3c).

In this study, *P. parvum* f. *patelliferum* is recorded for the first time for the temperate Pacific coast of South America (neritic waters); *P. patelliferum* was previously known for the U.S.A. (west coast), England, Norway, Bulgaria, and Australia (Green *et al.*, 1982; Larsen & Moestrup, 1989; Larsen *et al.*, 1993). *P. parvum* is very widely distributed and found in temperate and subtropical regions of both hemispheres (Green *et al.*, 1982). Although it is characteristic of brackish waters (Tomas, 1993),

Figure 3. *P. parvum* f. *patelliferum*. Electron microscopy. a) Transversal section showing the f1,f2: flagella, h: haptonema, c: chloroplast, sc: surface of the cell covered with scales. b) Longitudinal section showing: c: chloroplasts, cer: chloroplasts endoplasmatic reticulum, vf: vestibular fossa, per: periplastidial endoplasmatic reticulum, n: nucleus, nu: nucleolus, ner: nuclear endoplasmatic reticulum, m: mitochondria, p: pyrenoids, g: Golgi body, mb: muciferous body, pv: pulsatil vacuole, sc: scales. c) r: radial and c: concentric scales.

Figura 3. *P. parvum* f. *patelliferum*. Microscopía electrónica. a) Corte transversal mostrando f1,f2: flagelos, h: haptonema, c: cloroplasto, sc: superficie de la célula cubierta con escamas. b) Corte longitudinal mostrando c: cloroplastos, cer: retículo endoplasmático cloroplastidial, vf: fosa vestibular, per: retículo endoplasmático periplastidial, n: núcleo, un: nucleolo, ner: retículo endoplasmático nuclear, m: mitocondria, p: pirenoide, g: cuerpo de Golgi, mb: cuerpos mucíferos, pv: vacuola pulsátil, sc: escamas. c) r: escamas radiales y c: concéntricas.

Figure 4. *P. parvum* f. *patelliferum*. Electron microscopy. a) sc: scale forming in the g: Golgi body, er: endoplasmic reticulum. b) g: Golgi body just below the fr: flagellar root, m: mitochondrion, n: nucleous, mb: muciferous body, cytoplasm surrounded by a narrow peripheral cisterna of per: endoplasmic reticulum. c) Transversal section showing peripheral cisterna of per: endoplasmic reticulum, n: nucleus, nu: nucleolus, m: mitochondrion, l: lipidic globules, mb: muciferous body, and sc: scales. d) Transversal section showing cer: chloroplast endoplasmic reticulum is continuous with the nucleus endoplasmic reticulum. c: chloroplast, g: Golgi body, f: flagella, p: pyrenoids, n: nucleus, pv: pulsatil vacuole, mb: muciferous body, and sc: scales.

Figura 4. *P. parvum* f. *patelliferum*. Microscopía electrónica. a) Escama formándose en el g: cuerpo de Golgi, er: retículo endoplasmático. b) g: cuerpo de Golgi ubicado bajo la fr: raíz flagelar, m: mitocondria, n: núcleo, mb: cuerpos lucíferos, citoplasma rodeado por el per: retículo endoplasmático periférico. c) Corte transversal mostrando el per: retículo endoplasmático periférico, n: núcleo, un: nucleolo, m: mitocondria, l: glóbulos lipídicos, mb: cuerpos mucíferos y sc: escamas. d) Corte transversal mostrando que el cer: retículo endoplasmático cloroplastidial es continuo con el retículo endoplasmático nuclear. c: cloroplasto, g: cuerpo de Golgi, f: flagelo, p: pirenoides, n: núcleo, pv: vacuola pulsátil, mb: cuerpo mucífero y sc: escamas.

P. parvum f. *patelliferum* from Valparaíso Bay grew under laboratory culture conditions with 34.0 salinity, similar to that of the neritic waters where the initial phytoplankton sample was obtained. Palma & Rosales (1995) indicated year-round salinity ranges of 34.4-34.7 in Valparaíso Bay.

P. patelliferum and *P. parvum* are known as a toxin-producers (Larsen *et al.*, 1993; Larsen & Bryant, 1998). In Chile, a notorious increase in the number of phytoplankton blooms has been recorded since 1970 (Muñoz & Avaria, 1997). Phenomena such as blooms of harmful microalgae have become important on national and global scales, and the proper identification of these species will improve detection systems for preventing proliferations of toxin-producers.

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Recibido: 2 mayo 2006; Aceptado: 16 enero 2007