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FIRST RECORD OF *BIECHELERIA BREVISULCATA* (SUESSIACEAE, DINOPHYCEAE) FROM ARGENTINA

Inés Sunesen[1,*], Francisco Rodríguez[2], Jonás A. Tardivo Kubis[1], Delfina Aguiar Juárez[1] & Eugenia A. Sar[1,**]

[1] *División Ficología "Dr. Sebastián A. Guarrera", FCNyM, UNLP, Paseo del Bosque s/n, 1900 La Plata, Argentina; CONICET, Argentina; * isunesen@fcnym.unlp.edu.ar ** easar@fcnym.unlp.edu.ar* (authors for correspondence).
[2] *Instituto Español de Oceanografía (IEO-CSIC), Subida a Radio Faro 50, 36390 Vigo, Spain.*

Abstract. Sunesen, I.; F. Rodríguez, J. A. Tardivo Kubis, D. Aguiar Juárez & E. A. Sar. 2023. First record of *Biecheleria brevisulcata* (Suessiaceae, Dinophyceae) from Argentina. *Darwiniana*, nueva serie 11(1): 337-346.

In the framework of a phytoplankton and biotoxin monitoring program implemented since 2008 in marine coastal waters of the Buenos Aires Province (Argentina), the strain LPCc022 of a nanoplanktonic, thin-walled woloszynskioid dinoflagellate, has been isolated. LSU rDNA-based phylogeny showed that the isolated strain belonged to a molecular clade corresponding with of *Biecheleria brevisulcata*. *B. brevisulcata* can be differentiated by light microscopy from the species *B. baltica*, *B. halophila*, *B. cincta* and *B. tirezensis* based on the location of the nucleus in the cell, the size range of the cells, and the environments they inhabit. This is the first report of the genus *Biecheleria* and the species *B. brevisulcata* in Argentina and overall South-western Atlantic waters.

Keywords. *Biecheleria*; molecular characterization; morphological characterization; phylogeny; Suessiaceae.

Resumen. Sunesen, I.; F. Rodríguez, J. A. Tardivo Kubis, D. Aguiar Juárez & E. A. Sar. 2023. Primer reporte de *Biecheleria brevisulcata* (Suessiaceae, Dinophyceae) para Argentina. *Darwiniana*, nueva serie 11(1): 337-346.

En el marco de un programa de monitoreo de fitoplancton y biotoxinas implementado desde 2008 en aguas marinas costeras de la Provincia de Buenos Aires (Argentina), ha sido aislada la cepa LPCc022 de un dinoflagelado woloszynskioide, nanoplanctónico, de paredes delgadas. La filogenia basada en LSU rDNA mostró que la cepa aislada pertenecía a un clado molecular correspondiente a *Biecheleria brevisulcata*. *B. brevisulcata* puede ser diferenciada mediante microscopía óptica de las especies *B. baltica*, *B. halophila*, *B. cincta* y *B. tirezensis* sobre la base de la ubicación del núcleo en la célula, el rango de tamaño de las células y los ambientes que habitan. Este es el primer reporte del género *Biecheleria* y la especie *B. brevisulcata* en Argentina y en general en aguas del Atlántico sudoccidental.

Palabras clave. *Biecheleria*; caracterización molecular; caracterización morfológica; filogenia; Suessiaceae.

INTRODUCTION

Dinoflagellates have a cell covering, the amphiesma, formed by a single layer of flattened amphiesmal vesicles placed beneath the cell membrane. These vesicles can contain cellulosic plates in the case of armored/thecate dinoflagellates (absent in unarmored/athecate dinoflagellates), or can contain very thin plates in the so-called "thin walled" dinoflagellates (Fensome et al., 1993). The latter, also known as woloszynskioids (Moestrup et al., 2009a, b; Takahashi et al., 2014), include diverse genera such as *Protodinium* Lohmann (Lohmann, 1908),

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Symbiodinium Freudenthal (Freudenthal, 1962), *Polarella* M. Montresor, G. Procaccini & D. K. Stoecker (Montresor et al., 1999), *Biecheleria* Moestrup, Lindberg & Daugbjerg (Moestrup et al., 2009a), *Biecheleriopsis* Moestrup, Lindberg & Daugbjerg (Moestrup et al., 2009b), *Pelagodinium* Siano, Montresor, Probert & de Vargas (Siano et al., 2010), *Ansanella* H. J. Jeong, S. H. Jang, Moestrup & N. S. Kang (Jeong et al., 2014), *Yihiella* S. H. Jang, H. J. Jeong, Moestrup & N. S. Kang (Jang et al., 2017), among others.

Most woloszynskioids inhabit marine or brackish waters but remain frequently overlooked in plankton samples. In that sense, morphological and molecular information are available for many species, given their relatively recent descriptions. Their identification with light microscopy is arduous due to their small size and morphological resemblance (Moestrup et al., 2009a, b; Ruiz-de la Torre et al., 2022), while their morphological examination with scanning and transmission electron microscopy (SEM and TEM) requires laborious treatment (Balzano et al., 2012; Raho et al., 2018). Thus, molecular analysis using ribosomal and mitochondrial marker regions is often needed to reveal the genetic diversity of woloszynskioids and distinguish them at species level (Benico et al., 2019; Jang et al., 2022).

In the framework of a phytoplankton and biotoxins monitoring program in marine coastal waters of the Buenos Aires Province, a nanoplanktonic, thin-walled woloszynskioid dinoflagellate, was isolated in 2018 and established into culture. The aim of the present study was to provide the specific identification of strain LPCc022, using molecular (LSU rDNA sequencing) and morphological analyses.

MATERIAL AND METHODS

Strain isolation and culture

The clonal strain of a planktonic woloszynskioid (LPCc022) was established from a surface sample collected with a 30 µm mesh net hauls in marine coastal waters of Mar Azul at about 10 to 20 m from the shoreline.

Examined material: ARGENTINA, Buenos Aires Province, Villa Gesell District, Mar Azul Locality (MAZ) (37° 20' 38" S; 57° 01' 31" W), 9 January 2018, Strain LPCc022, isolated from field material label LPC 11524. The water temperature and salinity of Mar Azul was estimated based on averages of seven values of each variable taken between December and February (unpublished data) obtained with an multiparameter probe Hanna HI 9828 (Hanna, USA), temperature 23.7 °C (±1.94) and salinity 33.57 (±0.55).

Single cells were isolated the day after sampling by micropipette using a Zeiss Axiovert 40 CFL inverted microscope with phase contrast and differential interference contrast (DIC) (Zeiss Microimaging, Goettingen, Germany). Individual cells were washed several times in local filtered seawater and when free of contaminants they were transferred into 6-well tissue culture plates containing 10 ml natural seawater enriched with Guillard's f/2 medium (Sigma-Aldrich, Saint Louis, USA). Cells were incubated at 16°C, at salinity of 30, and under light supplied by cool-white fluorescent tubes with irradiance of 100-125 µmol photons m^{-2} s^{-1} on a 12:12 light:dark regime, in a growth chamber (SEMEDIC I-290F, SEMEDIC SRL, CABA, Argentina). After successful isolation, culture was scaled up to 40 ml medium in 100 ml flasks and incubated in the described conditions.

Microscopy

For light microscopy (LM) analyses, live cells of strain LPCc022 were observed using a Leica DMLA microscope (Leica Microsystems, Wetzlar, Germany) equipped with DIC. For the study of the thecal plate arrangement, specimens were stained with calcofluor white (Fluorescent Brightener 28, Sigma, St. Louis, MO, USA) following Fritz & Triemer (1985). Photographs were taken with the digital camera AxioCam 2008 (Carl Zeiss Microscopy GmbH, Jena, Germany). Unfortunately, the strain LPCc022 was lost while adjusting the procedure for scanning electron microscope (SEM) analysis, precluding its examination with this technique.

DNA extraction, amplification, and sequencing

An aliquot of 1.5 ml of late exponential growing culture of strain LPCc022 was taken and concentrated by centrifugation, washed in two drops of milli-Q water, placed in 200 µl microtubes, cold

shocked in liquid nitrogen and kept at -20°C until further analysis. DNA extraction used Chelex® chelating resin (Bio-Rad, Hercules, California, USA), following Richlen & Barber (2005). DNA extracts were kept at -20°C before PCR analyses. The D1-D2 domains of the LSU rRNA gene regions were amplified using the pair of primers D1R/D2C (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3', Lenaers et al., 1989).

The amplification reaction mixtures (20 µl) were performed using Horse-Power™ Taq DNA Polymerase MasterMix (Canvax, Spain) following manufacturer's instructions. DNA was amplified in an Eppendorf Mastercycler EP5345 (Eppendorf AG, New York, USA). PCR reactions were checked by agarose gel electrophoresis (1% TAE, 80 V) and GelRed™ nucleic acid gel staining (Biotium, Hayward, CA, USA). PCR products were purified with ExoSAP-IT™ (USB Corporation, Cleveland, Ohio, USA). Sequencing reactions were performed using the Big Dye Terminator v3.1 reaction cycle sequencing kit and migrated in a SeqStudio genetic analyzer (both at Applied Biosystems, Foster City, CA, USA) at the CACTI sequencing facilities (Universidade de Vigo).

Phylogenetic analyses

Partial LSU rRNA gene sequences obtained were inspected and aligned using MEGA X software (Kumar et al., 2018). Sequences from *Yihiella yeosuensis* (Jang et al., 2017) were used to root the tree. The original alignments for the LSU rDNA phylogeny (including gaps) consisted of 599 bp. Best evolutionary models for maximum likelihood (ML) phylogenetic analyses were estimated using the model selection tool in MEGA X software, and Tamura-Nei model (Tamura & Nei, 1993) was selected. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories ($\gamma = 0.4116$)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 46 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). The final dataset contained 492 positions.

Additionally, a Bayesian inference (BI) phylogenetic analysis was carried out by sampling across the entire GTR model space using Mr. Bayes v3.2 (Huelsenbeck & Ronquist, 2001). The program parameters were statefreqpr, dirichlet (1,1,1,1); nst, mixed; and rates, gamma. Phylogenetic analyses involved two parallel analyses, each with four chains. Starting trees for each chain were selected randomly using the default values in Mr. Bayes. The number of generations used in these analyses was 1,000,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization (burn-in phase). All final split frequencies were < 0.02. The two methods rendered similar topologies. Phylogenetic trees used ML/BI for the LSU rDNA, with bootstrap values (indicated as percentages) and posterior probabilities, in each case. Net mean p-distances between clades were calculated using MEGA X. Thus, no corrections for multiple substitutions at the same site, substitution rate biases (e.g. differences in the transitional and transversional rates), or differences in evolutionary rates among sites were considered (Nei & Kumar, 2000).

RESULTS

Molecular analysis

Phylogenetic analyses based on LSU rDNA (D1-D2 regions; Fig. 1) confirmed that strain LPCc022 belonged to a well-supported clade including *Biecheleria brevisulcata* K. Takahashi & Iwataki (ML: 83%, BI: 0.96). However, relationships among other *Biecheleria* species and several genera of the family Suessiaceae (such as *Ansanella, Biecheleriopsis* and *Pelagodinium*), were only partially resolved in some cases. For instance, the clade corresponding with *Biecheleria baltica* Moestrup, Lindberg & Daugbjerg displayed low statistical support (BI method) regarding *B. cincta* (Siano, Montresor & Zingone) Siano and *B. pseudopalustris* (J. Schiller) Moestrup, Lindberg & Daugbjerg.

Furthermore, these species were unresolved in the partial LSU phylogeny and belonged to the same clade, which only showed strong statistical support with BI posterior probabilities. Other Suessiaceae species were split in distinct clades with moderate to strong statistical support, both with ML bootstrap values (74-100) and BI posterior probabilities (0.86-1.00). *Biecheleria baltica* and *B. cincta* were the closest sequences to *B. brevisulcata* (based on net mean p-distances) ordered as follows: *B. baltica* (0.004) and *B. cincta* (0.006).

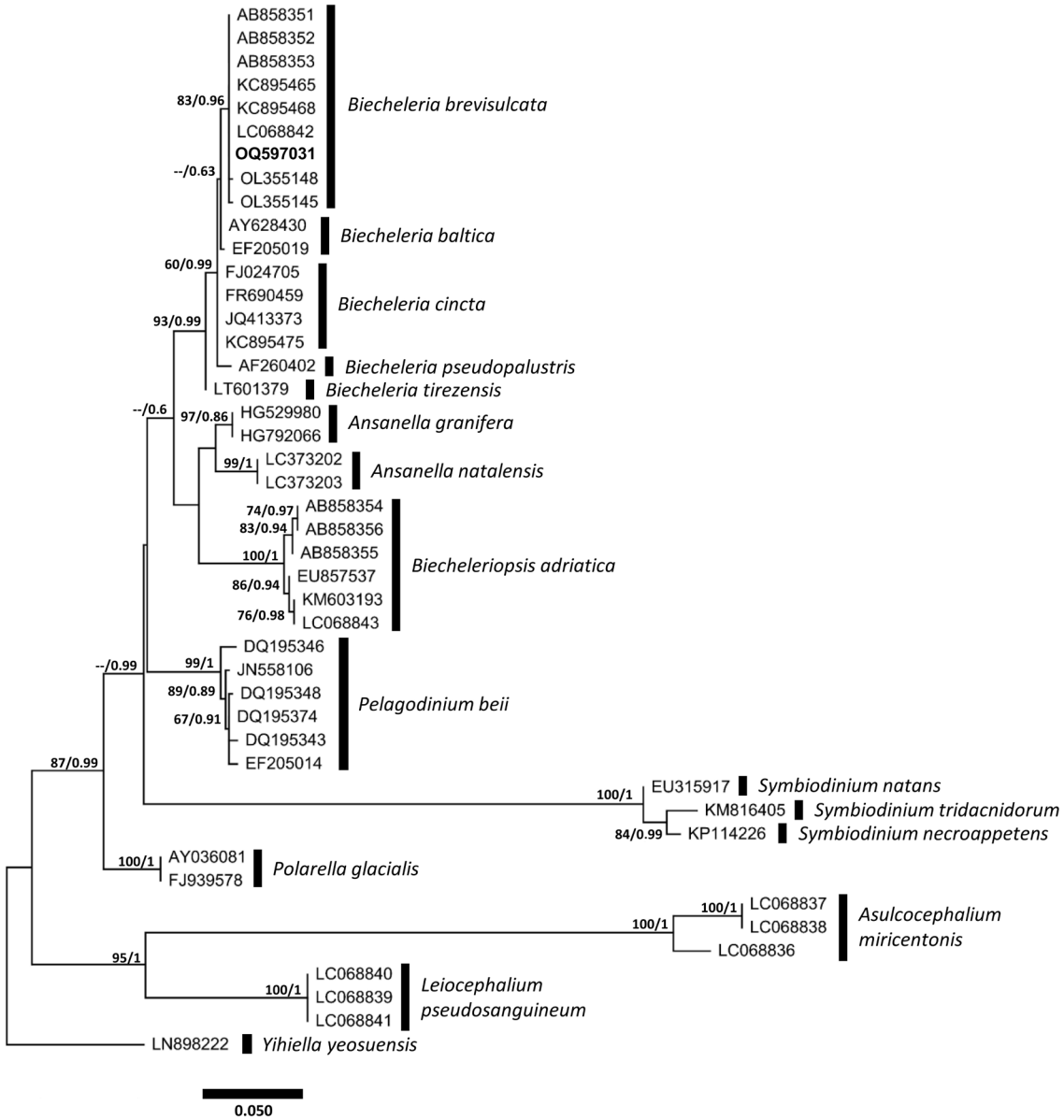

Fig. 1. Phylogenetic tree of the D1-D2 LSU rDNA obtained by BI model showing the relationships among the *Biecheleria brevisulcata* strain from Buenos Aires coastal waters and strains from other places around the world. Numbers on branches are bootstrap percentages (n = 1,000) and posterior probabilities (n = 1,000,000) after ML and BI analyses, respectively. Values lower than 60%/0.60 were not considered representative in these analyses and shown by hyphens, respectively.

Morphological analysis

Biecheleria brevisulcata K. Takahashi & Iwataki (Fig. 2A-I).

Reference. Takahashi et al., 2014: 56, figs 1-24; Choi et al., 2021: 578, fig. 1U-Y.

Cells were spherical to ellipsoidal, mushroom-shaped, dorsoventrally slightly flattened, 8.2-15.2 µm length (average 11.4 ± 2.2), 6.4-13.7 µm width (average 9.8 ± 2.0), 1.2-1.4 length/width (average 1.2 ± 0.1) (n=30). The epicone was slightly conic and wider than the antapically bilobed hypocone (Fig. 2A, C-E, G-I). Cingulum was placed in the equatorial part of the cell, descendent by about more than one cingular width (Fig. 2D), and sulcus was deep, wider towards the distal end (Fig. 2A, C, D). Chloroplasts were yellow-brown, peripherally located (Fig. 2A-E), with 2 to 6 pyrenoids (Fig. 2A, E black arrowhead). The nucleus was large, situated in the epicone and reaching the cingulum (Fig. 2E, F, n). Stigma was large, placed in the anterior end of the sulcus (Fig. 2G, I white arrowheads). The thecal plates were subtly reveled by calcofluor staining (Fig. 2H, I).

Distribution. *B. brevisulcata* was found in marine coastal waters of Mar Azul (Buenos Aires Province), this is the first record of the species from Argentina and from South-western Atlantic waters.

GenBank accession number. OQ597031.

DISCUSSION

Molecular comparison

The molecular data obtained in the present study confirmed the presence of *B. brevisulcata* in the Argentine Sea based on partial LSU rDNA sequencing and the phylogenetic analysis of strain LPCc022. Genetic distances among the different species of *Biecheleria* are rather small, and their phylogenetic relationships are better resolved using ITS rDNA regions than LSU or SSU rDNA (e.g. Moestrup et al., 2009a; Raho et al., 2018). Nevertheless, in the case of *B. brevisulcata*, its molecular identification just employed partial LSU rDNA sequences (D1-D3 regions; Takahashi et al., 2014). Moreover, these authors could only distinguish the suessioid genera *Biecheleria* and *Biecheleriopsis* using molecular data (LSU rDNA; 518 unambiguously aligned positions), given their strong morphological similarity except for subtle differences in the third row of cingular vesicles at the cingular margin in dorsal view, zigzag line in *Biecheleria*, and straight line in *Biecheleriopsis* (Takahashi et al., 2014). Therefore, the LSU rDNA based-phylogeny elaborated in the present work followed a similar approach to these authors and other studies (Takahashi et al., 2014, 2015; Jang et al., 2017; Raho et al., 2018), to identify the studied strain as *B. brevisulcata* and depict its relative position to other *Biecheleria* species and several members of the family Suessiaceae. Finally, it worths to be mentioned that among the nine currently accepted species of *Biecheleria* (Guiry & Guiry, 2022), only five of them were available for the phylogenetic analyses. Three of the missing taxa are those of freshwater nature (*B. aesculus* (Baumeister) Moestrup, *B. cestocoetes* (R.H. Thompson) Moestrup and *B. ordinata* (Skuja) Moestrup), excluded in the morphological comparisons and also absent in the GenBank genetic database.

The species *B. halophila* (Biecheler) Moestrup, Lindberg & Daugbjerg (=*Gymnodinium halophilum* Biecheler) erected by Biecheler (1952) and transferred by Moestrup et al. (2009a), could not be included in the phylogeny. Its molecular characterization is still pending this given that it has not been isolated again since its original description (see commentary given by Moestrup et al., 2009a: 203). The sequence EF205019 of *Wolozynskia halophila* (Biecheler) Elbrächter & Kremp (sensu Kremp et al., 2005) corresponds to *Biecheleria baltica*, not to *B. halophila* (Moestrup et al., 2009a).

Comparison of morphometric and morphologic features

The genus *Biecheleria* was characterized based on the following morphological data: apical elongated vesicle (AEV), several chloroplasts, eyespot type E (Moestrup & Daugbjerg, 2007),

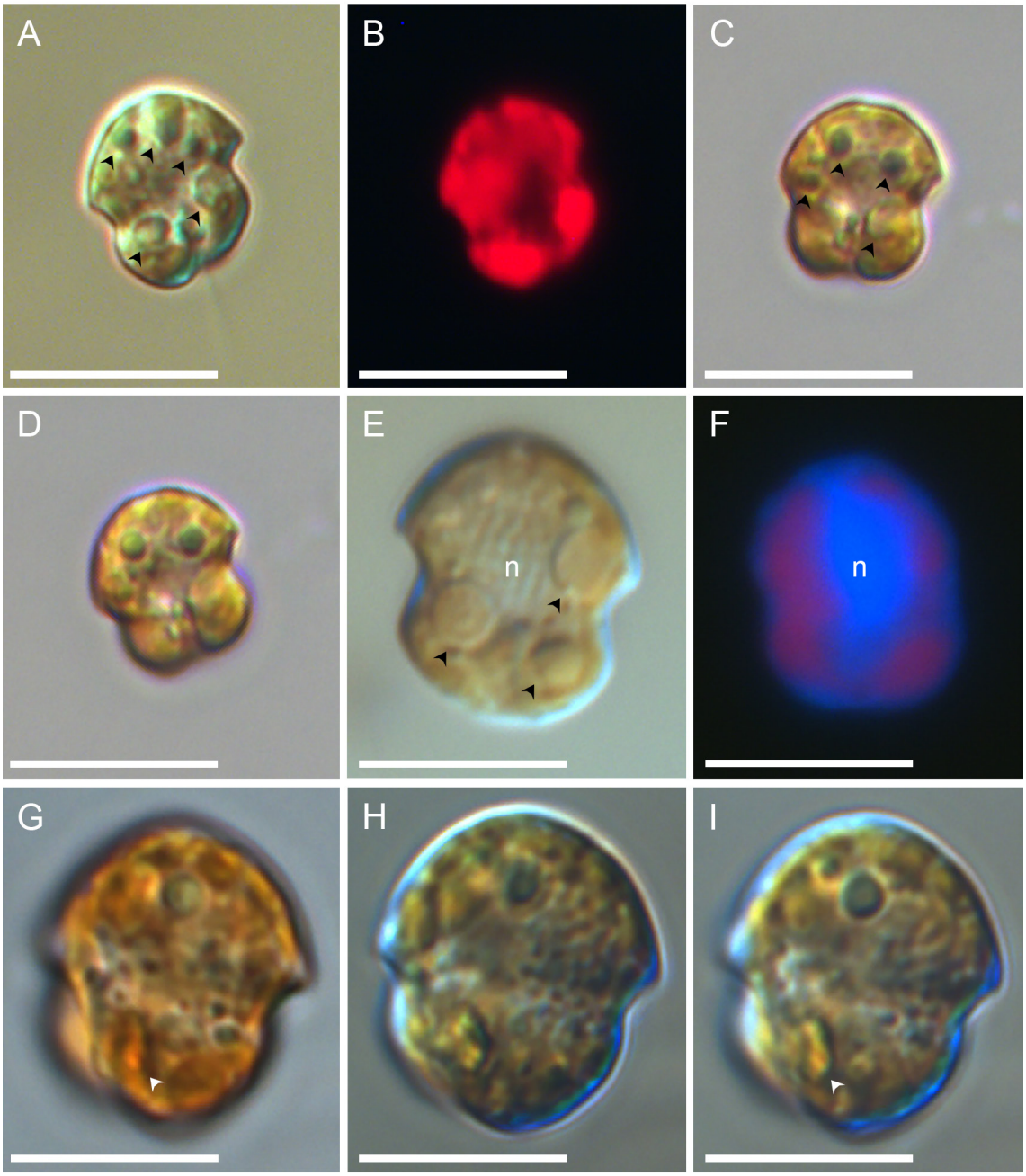

Fig. 2. Light and fluorescence microscopy of *Biecheleria brevisulcata*. Strain LPCc022. Scale bars = 10 µm. **A,** cell in ventral view, note posterior flagellum. Black arrowheads show pyrenoids. **B,** fluorescent micrographs of the same cell, showing the chloroplasts. **C, D,** cells in ventral view showing cingular displacement and sulcus wider towards the distal end. **E,** cell in ventral view in deep focus showing a large nucleus (n) and pyrenoids (black arrowheads). **F,** fluorescent micrographs of a cell stained with calcofluor, showing the nucleus (n) and chloroplasts. **G,** cell showing a large stigma towards the anterior end of the sulcus (white arrowhead). **H, I,** different foci of the same cell in ventral tilted view revealing presence of thecal plates. Note stigma (white arrowhead). Color version at https://www.ojs.darwin.edu.ar/index.php/darwiniana/article/view/1138/1304

more than six latitudinal series of amphiesmal vesicles, and resting cyst spherical with numerous spines or bristles, and molecular data (Moestrup et al., 2009a). Unfortunately, in the present work SEM examination could not be carried out due to the loss of the studied strain of *B. brevisulcata*.

Up to date there are 9 recognized species of *Biecheleria* (Guiry & Guiry, 2022). Of these *B. halophila*, *B. cincta* and *B. brevisulcata* are marine (Moestrup et al., 2009a; Balzano et al., 2012; Takahashi et al., 2014, respectively), *B. baltica* is brackish (Moestrup et al., 2009a), and *B. tirezensis* S. Fraga, N. Raho, J. P. Abad & I. Marín is athalassic (Raho et al., 2018). The four other species, *B. pseudopalustris* (Moestrup et al., 2009a), *B. aesculus*, *B. cestocoetes* and *B. ordinata* (Moestrup & Calado, 2018) are freshwater and will be excluded of the comparison with the target species.

B. brevisulcata was compared with other marine, brackish and athalassic species (Table 1). The analysis with light microscopy allowed to determine that all the species of the latter group are similar or almost similar in cell-shape, morphological relation epicone/hypocone and sulcus/cingulum, morphology and localization of the chloroplasts and general aspect. *B. brevisulcata* presented more similarity in cell size with *B. cincta* and *B. tirezensis*, the smaller species, than with *B. baltica* and *B. halophila*, the larger species (Table 1). A differential feature between *B. brevisulcata* and the other species of this group was the location of the nucleus (Table 1). The nucleus was situated in the epicone, reaching the cingulum, in *B. brevisulcata* (this study, fig. 2E, F, H; Takahashi et al., 2014, fig. 2; Choi et al. 2021, fig. 1v), while it occupied most of the cell (slightly displaced towards the epicone) in *B. cincta* (Siano et al., 2009, fig. 36), or was situated in the dorsal side of the epicone in *B. tirezensis* (Raho et al., 2018: fig. 2). The position of the nucleus of the specimens of strain GSND01 of *B. cincta* from East China Sea showed by Luo et al. (2013, fig. 1 a) better corresponds to *B. brevisulcata* than *B. cincta*. Consistently, Raho et al. (2018: 110) pointed out in their analysis about secondary structures of nuclear ITS2 rDNA, that the strain GSND01 of *B. cincta* (Luo et al., 2013) probably should be considered a *B. brevisulcata* strain.

In the case of *B. baltica* (=*Woloszynskia halophila* (Biecheler) Elbrächter & Kremp according to Moestrup et al., 2009a) the nucleus was located in the center of the cell, often appearing slightly displaced towards the apex (Kremp et al., 2005, fig. 1a as *Woloszynskia halophila*) and in case of *B. halophila* (=*Gymnodinium halophilum*) was located in the centre towards dorsal side (Moestrup et al., 2009a: 217, fig. 44, reproduced from the protologue of *G. halophilum*).

Additionally, species are different according to the environments where they inhabit. *B. tirezensis* was isolated from Tirez pond, Spain, an athalassic hyperhaline environment with quite different properties from those from which all the other *Biecheleria* species live in (Raho et al., 2018). *B. baltica* and *B. halophila* were isolated from the Baltic Sea and characterized by pertaining to cold waters, but *B. baltica* was typically present in full-strength seawater at 7-14°C while *B. halophila* was typically found in brackish water at 0-6°C (Moestrup et al., 2009a). The marine *Biecheleria* species with the widest known distribution are *B. brevisulcata* and *B. cincta*. The former was reported from Tsuruoka and Sakata, Yamagata, Sea of Japan, and Nagayo, Nagasaki, East China Sea, Japan (Takahashi et al., 2014) and Yongho, Bay of Busan, Strait of Korea, South Korea (Choi et al., 2021), East China Sea (Hu et al., 2022) previous to the current report in temperate coastal waters of Mar Azul (Argentine Sea, Argentina). Instead, *B. cincta* has been found in temperate waters of Mare Chiara, Tyrrhenian Sea, Italy (Siano et al., 2009), Shiwha Bay, Yellow Sea, Korea (Kang et al., 2011), and from cold polar waters of Beaufort Sea, Artic Ocean (Balzano et al., 2012).

In conclusion, molecular and morphological characterization of strain LPCc022 confirmed the first record of the genus *Biecheleria* and the species *B. brevisulcata* in the Argentine Sea, and as far as we can determine, for the Southwest Atlantic Ocean. The current knowledge about woloszynskioid dinoflagellates will benefit from ongoing sampling efforts and the isolation of new strains, to better understand their relevance and diversity in the region.

Table 1. Comparison of *Biecheleria brevisulcata* with species described for marine, brackish and athalasic environments based on characters observed with light microscopy. References: **a,** this study; **b,** Takahashi et al. (2014); **c,** Choi et al. (2021); **d,** Hu et al. (2022); **e,** Moestrup et al. (2009a); **f,** Siano et al. (2009); **g,** Balzano et al. (2012); **h,** Kang et al. (2011); **i,** Raho et al. (2012); nd, no data; * measured data from the quoted literature.

	B. brevisulcata **a, b, c, d**	***B. baltica*** **e**	***B. halophila*** **e**	***B. cincta*** **f, g, h**	***B. tirezensis*** **i**
Cell length (µm)	8.2-15.2 **a** 10.5-18.0 **b** 11.2-15.2 **c** 7.2-9.2 **d**	17.0-35.0	10.0-27.0	9.0-15.0 **f** 8.2-13.8 **h**	9.0-15.0
Cell width	6.4-13.7 **a** 9.0-16.5 **b** 9.7-13.9 **c** 5.8-8.9 **d**	12.0-32.0	13.0-22.0	9.0-12.5 **f** 6.9-13.2 **h**	9.0-12.5
Length/width	1.2-1.4 **a**	nd	nd	nd	nd
Cell shape	spherical to ellipsoidal **a**, **b**, **c, d** mushroom shape **a**, **b, d**	spherical or ellipsoid	globular or ellipsoid	almost spherical **f**, **h**	almost spherical
Epicone / hypocone relation	epicone wider than bilobed hypocone **a**, **b**, **c, d**	epicone almost equal or slightly larger than hypocone	epicone equal to larger, rarely smaller than hypocone	epicone hemispherical, hypocone is slightly bilobed **f**	epicone slightly taller and wider than the hypocone
Cingulum	median, descendent by more than one cingular width **a** median, descendent by one and a half cingular width **b** median or slightly lower, descendent by one and a half cingular width **c, d***	descendent, displaced approximately one cingular width, with a short, finger-like extension projects from the right-hand side of the epicone at the confluence of the cingulum and the sulcus	descendent with a long finger-like projection extending from the right-hand side of the epicone at the junction of the cingulum and the sulcus	descendent, displaced about once its width **f** descendent, displaced by 0.2-0.4 cell length **h**	deep and descending, displaced by about one width
Sulcus	wider towards the posterior end **a**, **b**, **c, d**	extended to the antapex, markedly concave in this region.	nd	deep, wider at the antapex **f**, **h**	deep and wider at the antapex narrowing towards the cingulum
Chloroplasts	yellow-brown, located towards periphery **a**, **b**, **c, d**	golden-brown	golden-brown, located towards periphery*	yellow-brown, placed towards periphery **f**, **g, h**	yellow-brown peripherally distributed
Pyrenoids	2 to 6 pyrenoids **a**, **b**, **c,** mainly below the nucleus **b**	nd	nd	several, visible in the cytoplasm **f**	Several, visible in the cytoplasm
Nucleus	situated in the epicone and reaching the cingulum **a** occupying most part of the epicone and reaching the cingulum*, **b**, **c** middle or slightly upper part of the hypocone, not visible* **d**	located centrally, often appearing slightly anterior	central and dorsal	occupying most of the cell, slightly displaced in the epicone **f** central part of the cell **h**	situated in the dorsal side of the epicone
Stigma	large, placed in the posterior end of the sulcus **a** placed near the sulcus **b**, **c**	nd	nd	bright-orange, positioned in the ventral side of the hypocone **f**	bright-orange, positioned in the ventral side of the hypocone along the right side of the sulcus
Environment	marine **a**, **b**, **c**, **d**	brackish, cold waters 0-6°C	marine, cold waters 7-14°C	marine **f**	athalassic, euryhaline and eurythermal
Strain from	Mar Azul, Buenos Aires Province, Argentine Sea **a** Tsuruoka, Yamagata, Sea of Japan, Nagayo, Nagasaki, East China Sea **b** Yongho, Bay of Busan, Strait of Korea **c** East China Sea **d**	Baltic Sea	Tvärminne, Baltic Sea Finland	MareChiara (MC), Tyrrhenian Sea (Mediterranean Sea), Italy **f** Beaufort Sea, Artic Ocean **g** Shiwha Bay, Yellow Sea, Korea **h**	Tirez pond, Castilla-La Mancha, Spain

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