Valve morphology of *Didymosphenia geminata* (Bacillariophyceae) from Santa Cruz and Tierra del Fuego provinces, Patagonia, Argentina

Morfología valvar de *Didymosphenia geminata* (Bacillariophyceae) de las provincias de Santa Cruz y Tierra del Fuego, Patagonia, Argentina

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Summary

Background and aims: In 2013, blooms similar to those produced by *Didymosphenia geminata* (Lyngbye) M. Schmidt around the world, were detected in the Grande River basin, Tierra del Fuego province, and in 2014 in de las Vueltas River in Santa Cruz province. The aim of this paper is to analyze the valve morphology and morphometry of these materials to establish if they correspond to *D. geminata* or to other species of the genus that is producing the massive growth in southern Patagonia.

M&M*:* Samples were collected at Grande River in 2013 and 2015 and, in de las Vueltas River in 2015 and 2016. Samples were analyzed with light and electron microscopy. For morphometric analyses 100 specimens from each sample were measured and statistical analyses were carried out using the R statistical package.

Results*:* The studied populations have the same fine morphology described for other populations collected in Patagonia. Nevertheless, in some sampling sites from Santa Cruz province, we found a morphotype with a markedly smaller size, subcapitated poles, little marked constrictions of the poles, a broad central area (relative to cell size) and 1 to 3 stigmata, that was never reported in South America.

Conclusions*:* The studied populations correspond to *D. geminata* ssp. *geminata* Metzeltin & Lange-Bertalot. From a morphological point of view the smaller morphotype present in Santa Cruz belongs to the same subspecies but can be easily misidentified during the routine monitoring programs carried out with light microscope due its size and valve outline.

Key words

Diatoms, *Didymosphenia geminata,* southern Patagonia, morphology, invasion.

Resumen

Introducción y objetivos: En 2013 floraciones similares a las producidas por *Didymosphenia geminata* (Lyngbye) M. Schmidt en distintos lugares del mundo fueron detectadas en la Cuenca del Río Grande, provincia de Tierra del Fuego y, en 2014 en el río de las Vueltas en la provincia de Santa Cruz. El objetivo del trabajo fue analizar la morfología y morfometría valvar de estos materiales para establecer si corresponden a *D. geminata* o a otra especie del género que está produciendo floraciones masivas en la Patagonia sur.

M&M*:* Las muestras fueron colectadas en el Río Grande en 2013 y 2015 y en el Río de las Vueltas en 2015 y 2016. El material fue analizado con microscopía óptica y electrónica. Para los análisis morfométricos fueron medidos 100 especímenes por muestra y los análisis estadísticos fueron llevados a cabo con el programa estadístico R.

Resultados*:* Las poblaciones estudiadas presentan las mismas características morfológicas descriptas para poblaciones de distintos lugares de Patagonia. Sin embargo, en Santa Cruz observamos un morfotipo marcadamente más pequeño, con polos subcapitados, constricción suavemente marcada, un área central amplia con 1 a 3 estigmas, que no había sido descripto antes para Sudamérica.

Conclusiones*:* Las poblaciones estudiadas corresponden a *D. geminata* ssp. *geminata* Metzeltin & Lange-Bertalot. Desde un punto de vista morfológico, los especímenes pequeños presentes en Santa Cruz pertenecen a la misma subespecie pero por su tamaño y contorno valvar pueden ser confundidos con otros taxones durante los programas de monitoreo llevados a cabo con microscopía óptica.

Palabras claves

Diatomeas, *Didymosphenia geminata,* Patagonia sur, morfología, invasión.

Introduction

Didymosphenia geminata (Lyngbye) Schmidt has been extensively studied in the last years due to its invasive behavior. *D. geminata* is a freshwater diatom that forms dense soggy carpet-like layers, several inches thick, lining the bottom of the aquatic environments where it lives. During the last fifteen years, blooming reports increased and called the attention not only of scientists but also of governments all around the world, due to the serious ecological damage they caused -e.g. changes in food chain structure and rivers and streams hydraulic characteristics- and economic losses that affected tourism, fisheries and hydroelectric industries (Kilroy, 2004; Spaulding & Elwell, 2007; Brand & Grech, 2020).

Although there are some early records of the species in Chile (Asprey, 1964; Rivera & Gebauer, 1989), the first record of *D. geminata* forming blooms in South America was in Chile and Argentina in 2010 (Segura, 2011; Reid *et al*., 2012; Sastre *et al*., 2013).

Valve morphology of the genus *Didymosphenia* has been well documented (Dawson 1973a, b; Antoine & Benson-Evans, 1983; Stoermer *et al.,* 1986; Metzeltin & Lange-Bertalot, 1995, 2014) but there is still no agreement among authors about the number of species (Guiry & Guiry, 2017; Metzeltin & Lange-Bertalot, 2014). Uyua and collaborators (2016a) gave the first morphological description of *Didymosphenia* populations from Argentina –located in Chubut province- and compare these materials with others around the world. Although the authors determined that these populations morphologically correspond to *D. geminata*, they considered that species differentiation merely by means of morphological characteristics is difficult. Besides, Metzeltin & Lange-Bertalot (2014) pointed out that identifications are more difficult if a small stage of the cell cycle is available. Moreover, it is also very difficult if local morphotypes of the same species are variable depending of the region of the world where they are (Stoermer *et al.,* 1986).

Since 2010, *D. geminata* has spread throughout Patagonia (Lamaro *et al.,* 2019). In the Grande River basin, Tierra del Fuego, a fisherman detected blooms similar to those produced by *Didymosphenia* in February 2013. This river is one of the most famous international fishing centers of salmonids and its presence near a "fishing lodge" suggested that it could have reached the island transported by fishermen from other regions of the world. At the same time this fact is worrisome because, this site could function as a center of anthropic dispersion. On the other hand, in March 2015 a monitoring program by the Subsecretaría de Pesca of Santa Cruz Province to evaluate the situation of the species in the province was held. In the framework of this program, 217 samples from 48 water bodies (rivers, streams and lakes) from different basins were analyzed. *Didymosphenia* was recorded only in de las Vueltas River. Samples collected in different sections of the de las Vueltas River were studied with electron microscopy to analyze the fine morphology of these populations.

Uyua (2017) held morphological and molecular analyzes from the first populations of the species that produced big blooms in rivers and streams of Argentina. These analyses made it possible to corroborate that the blooms reports from Patagonia are produced by *D. geminata.* Besides, comparisons among Argentinean populations showed the same genetic variability than those populations from others sites around the world. Similar results were obtained when fine morphology was analyzed. These results support the idea that *D. geminata* is an invasive species that recently has spread worldwide, but there are studies that show the presence of the species in Patagonia since long time ago (Asprey, 1964; Rivera & Gebauer, 1989; Jones *et. al.,* 2019). In fact, Rivera and Gebauer (1989) analyzed materials collected by Boyer in Chile in the earlier XX century deposited at the Academy of Natural Science of Philadelphia confirming the presence of the species in the northern region of the country.

The aim of this paper is to establish if the massive growths detected in Tierra del Fuego and Santa Cruz correspond to *Didymosphenia geminata* or to other species of the genus based on fine valve morphology and morphometry.

Material and Methods

Study area

The study area comprises Santa Cruz and Tierra del Fuego provinces, Argentina (Fig. 1). Samples were collected at de las Vueltas and Grande Rivers. De las Vueltas River belongs to Lake Viedma sub-basin within Santa Cruz River basin. Source waters for this river originate on the southern bank of the Desierto Lake and flows into Viedma Lake. The river flow

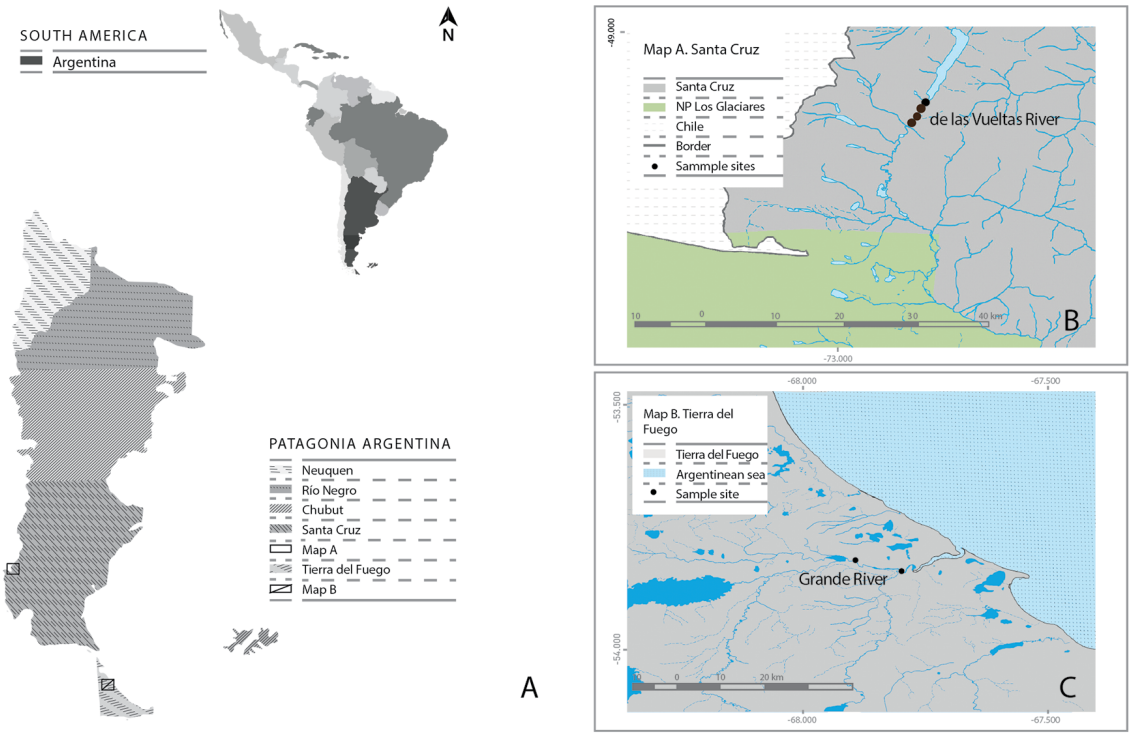

Fig. 1. Map of study area. **A:** Map of sample locations in south Patagonia Argentina. **B**: Map of sample sites of de las Vueltas River in Santa Cruz province. **C**: Map of sample sites of Grande River from Tierra del Fuego province.

average is between 47-86 m^3/s in spring-summer and between 19-40 m^3/s in autumn-winter (Díaz, 2005). The rivers Toro, Cóndor, Eléctrico, Milodón and Fitz Roy are tributaries on the right margin and del Bosque River is tributary on the left margin.

The Grande River belongs to Grande River basin. This river has its springs in Chilean territory and carries the waters of various courses. Some tributary rivers in Argentine territory are Bella Vista and Herminita rivers on the right and left margin respectively and, Turba and Mac Lennan Rivers downstream. The Grande River basin has 8580 km^2, corresponding to the Argentine territory 3780 Km^2, and its average flow is 40-45 m^3/s (Urciuolo *et al.,* 2009).

Methods

Sampling was held at Grande River in Tierra del Fuego province and de las Vueltas River in Santa Cruz province. Two samples collected in December 2013 by Ben Pascal and in March 2015 by the Secretaría de Desarrollo Sustentable de la Provincia de Tierra del Fuego, were analyzed from Grande River. Four samples collected in March 2015 and another one in September 2016 by the Subsecretaría de Pesca de la Provincia de Santa Cruz were analyzed from de las Vueltas River (Fig. 1). Samplings were carried out following international security recommendations (Duncan *et al*. 2007). The six sampling sites were located with GPS. Periphyton was collected by brushing a variable surface from different rocks and from submerged and emergent plants and macroalgae. Samples were preserved in 4% formaldehyde or alcohol 70%.

Materials were treated to eliminate organic matter following the method described in Hasle and Fryxell (1970). For light microscopy (LM) analyses samples were mounted in Naphrax® and, for scanning electron microscopy (SEM) they were deposited on 1 cm^2 pieces of glass mounted on metal stubs and then coated with gold. LM analyses were held with an Olympus CKX41 microscope and photographed using

an Olympus Evolt E-330 camera. SEM analyses were held with a Jeol JSM-6360 LV SEM at the Electron Microscopy Service of the Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Argentina. Uncleaned, cleaned subsamples and permanent slides were deposited at the Herbario of the División Ficología, Museo de La Plata under the following numbers:

ARGENTINA. Provincia de Tierra del Fuego. Río Grande, Estancia Menéndez, Latitud. 53° 50' S y Longitud 67° 47' O. Enero 2013. Colector: Ben Pascal. 13420 (LPC).

ARGENTINA. Provincia de Santa Cruz. Río de las Vueltas, Latitud 49° 05' 46.1" S y Longitud 72° 53' 56.2" O. Marzo 2015. Colector: Subsecretaría de Pesca de la provincia de Santa Cruz.13421 (LPC).

ARGENTINA. Provincia de Santa Cruz. Río de las Vueltas, Latitud 49° 06' 43.9" S, Longitud 72° 54' 44.3" O. Marzo 2015 Colector: Subsecretaría de Pesca de la provincia de Santa Cruz. 13422 (LPC).

ARGENTINA. Provincia de Santa Cruz. Río de las Vueltas, Latitud 49º 05' 50.1" S, Longitud 72º 53' 55.3" O. Marzo 2015. Colector: Subsecretaría de Pesca de la provincia de Santa Cruz. 13423 (LPC).

ARGENTINA. Provincia de Santa Cruz. Río de las Vueltas, Latitud 49º 06' 15.2" S, Longitud 72º 54' 23.8" O. Marzo 2015. Colector: Subsecretaría de Pesca de la provincia de Santa Cruz. 13424 (LPC).

ARGENTINA. Provincia de Santa Cruz. Río de las Vueltas, Latitud 49º 05' 46.1" S, Longitud 72º 53' 56.2" O. Septiembre 2016. Colector: Subsecretaría de Pesca de la provincia de Santa Cruz. 13425 (LPC).

ARGENTINA. Provincia de Tierra del Fuego, Latitud 53° 48' 51.6" S, Longitud 67° 55' 01.5" O. Marzo 2015. Colector: Dirección General de Recursos Hídricos 13425 (LPC).

The terminology used to describe the morphology of the studied materials is that proposed in Anonymous (1975), Ross *et al*. (1979) and Barber & Haworth (1981). For morphometric analyses 100 specimens from each sample were measured considering maximum length and maximum width, and in 30 of the 100 also were measured apical pole width, foot pole width, apical pole constriction, foot pole constriction, maximum width/head-pole width ratio, number of striae in 10 µm, number of areolae in 10 µm, and number of stigmata, features considered as diagnostic characters.

A non-parametric Kruskal-Wallis test was performed to verify the significance of possible differences in length and width of *D. geminata* among the different rivers (populations) analyzed in this study and with others previously studied from Chubut province (Uyua *et al.,* 2016b). To evaluate the differences between the groups, a *post hoc* Dunn (1964) multiple comparisons test (dunn.test) was performed using the Bonferroni adjustment. Statistical analyses were carried out using the R statistical package version 2.15.1 (R development Core Team, 2013).

In de las Vueltas River, it was possible to measure *in situ* parameters as temperature, pH, dissolved oxygen (DO), electrical conductivity and turbidity in March 2015 with Horiba U10 equipment.

Results

Light microscopy analyses of untreated materials producing large masses of stalks, brown dense soggy carpet-like layers confirmed the presence of the species *Didymosphenia geminata* in Tierra del Fuego and Santa Cruz (Fig. 2).

Didymosphenia geminata from Tierra del Fuego

The cells have heavily silicified and slightly cuneate frustules (Fig. 2A-F). Valves are strongly heteropolar, symmetric or slightly asymmetric respect the apical plane with capitate apical and basal poles. Valve face is flat, with a marginal rib at the junction of the valve face and mantle that ends in spines at the apical pole (Figs. 3B, C). Some specimens show a smooth spine while others have a serrated spine. Uniseriate striae are strongly radial all alongside and near the apical pole the striae are parallel (Figs. 3A-E). Internally, the valve face presents branched transapical ribs at valve center and at apical pole. Areolae are externally surrounded by small papillae. The basal pole presents a wide pore field, with small pores aligned in longitudinal rows. The *raphe-sternum* that presents "ghost areolae" is narrow, widening abruptly to an elliptic central area that shows 2-5 stigmas with an oval external opening and globular and spongy occlusion in the internal side (Fig. 3A, D, F-G). Raphe is lateral, slightly curved; terminal fissures are differently curved at each pole. Internal distal ends slightly bent towards the same side, ending in small *helictoglossae* (Figs. 3F-H). External proximal raphe

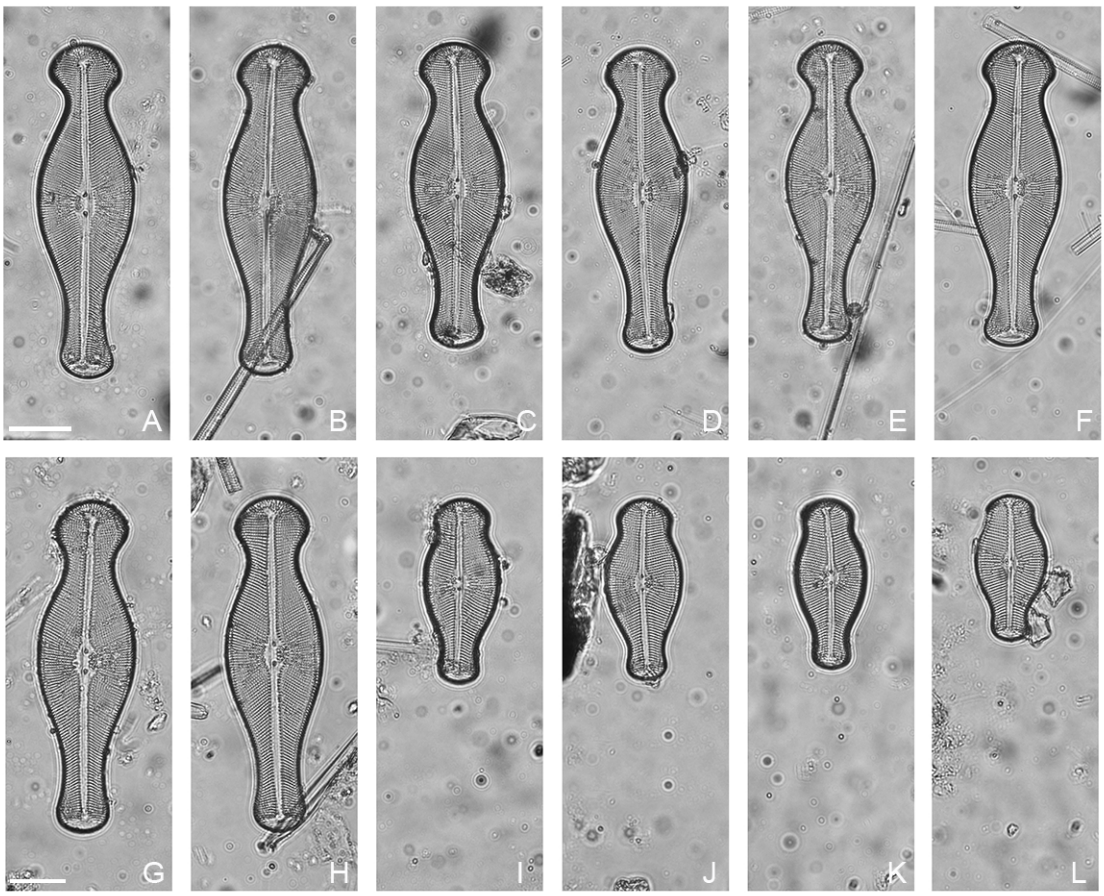

Fig. 2. LM. *Didymosphenia geminata* from de las Vueltas River (Santa Cruz province) and Grande River (Tierra del Fuego province). **A-F:** Río Grande River, Tierra del Fuego. **G-L**: de las Vueltas River, Santa Cruz. Scale bars = 20 µm.

endings simple teardrop shaped. Internal proximal raphe endings are located below the central nodule that presents a medium depression (Figs. 3F-H).

Morphometric parameters: length: 125-146 µm; width 37-42.5 µm; l/w: 2.9-3.5; striae: 8-11 in 10 µm; areolae: 10-12 in 10 µm; stigmas: 2-5; 40-44 rows of areolae in the pore fields in 10 µm and 14 areoles in 10 µm in the girdle bands.

Didymosphenia geminata from Santa Cruz

Morphotype #1: Specimens with similar characteristics to those from Tierra del Fuego. Frustules are slightly cuneate. Apical and basal poles capitate; apical pole larger than basal pole (Fig. 2A-F). Central area almost elliptical, asymmetric with 2 to 4 stigmata (Fig. 3D). External stigma openings rounded to elongated transversely (Fig. 3D). Raphe lateral with expanded central areas ends, distal raphe ends long and abruptly bent –in some cases slightly obtuse- to the opposite side of the stigmata (Fig. 3A-C, E). Radial striae alternating longer and shorter in the central area becoming slightly divergent towards poles, almost parallel at basal pole and radial at the apical pole (Fig. 3). The striae are extended over the valve surface reaching the middle of the mantle, leaving a hyaline area on the edge of the valve mantle.

Morphometric parameters: length: 126.8-133.6 µm; width: 36.6-39.6 µm; l/w: 3.3-3.5; striae: 9-11 in 10 µm; areolae: 10-12 in 10 µm; stigma: 2-4.

Morphotype #2: The fine morphology of these specimens is similar to that of the specimens above described (Fig. 4E-H). This morphotype differs from

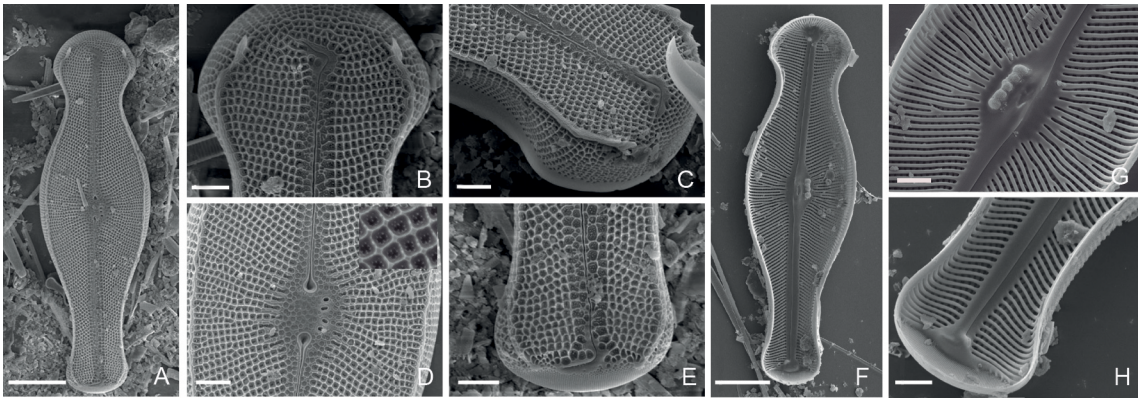

Fig. 3. SEM. *Didymosphenia geminata* from Grande River. **A-E**: Valves in external view. **A**: general valve view. **B-C**: detail of the apical pole. **D**: Valve center. **E**: Detail of the basal pole. **F-H**: Valves in internal view. **F**: general valve view. **G**: Detail of the central area. **H**: Detail of the basal pole. Scale bars= A, F: 20 µm; B-E; G-H: 5 µm.

morphotype #1 in the markedly smaller size and in the valve outline (Figs. 2; 4) with subcapitate poles and slightly marked constriction (2I-L). Central area wide with 1 to 3 stigmata on the primary side (Figs. 2G-L; 4).

Morphometric parameters: length: 54.9-74.4 µm; width: 26.2-32.3 µm; l/w: 1.92-2.67; striae: 9-11 in 10 µm; areolae: 10-12 in 10 µm; stigma: 1-3.

Comparisons among specimens within each population (rivers) show morphological variations in the raphe terminal fissures of the apical pole, being in some cases abruptly bent and in others much less marked, and the morphology of spines with three points in some specimens and very poorly developed in others.

Statistical analyses of morphometric parameters show that specimens from Grande River are larger than those at de las Vueltas River ($p<0.05$, Kruskal Wallis test). Besides, a comparison of the length of the cells with different populations from Patagonia previously studied (Uyua *et al*., 2016b) shows that Grande y Chubut rivers present the largest specimens from Patagonia ($p<0.05$, Kruskal Wallis test) and de las Vueltas River has the greatest range of size variation (Fig.5; Table 1). On the other

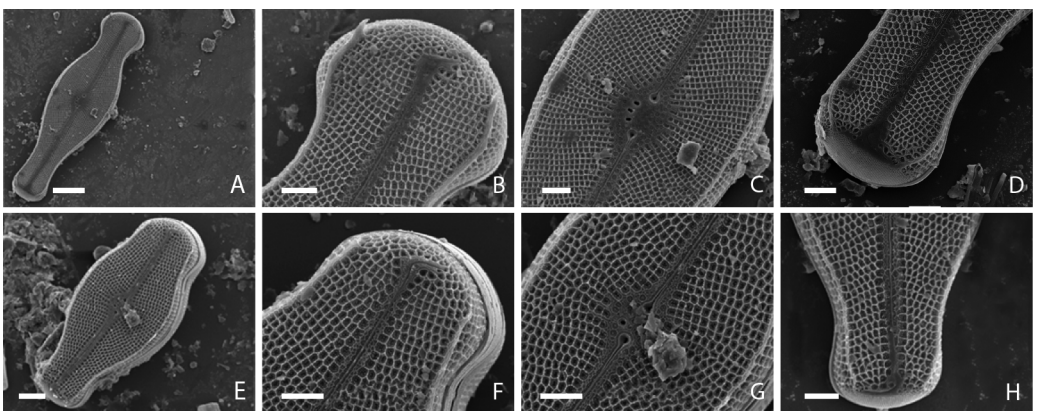

Fig. 4. SEM. External valvar morphology of the two morphotypes found in de las Vueltas River, Santa Cruz province. **A, E:** Valve view. **B, F:** Detail of apical pole. **C, G:** Detail of the central area. **D-H:** Detail of the basal pole. Scale bars = A: 20 µm; E: 10 µm; B-D, F-H: 5 µm.

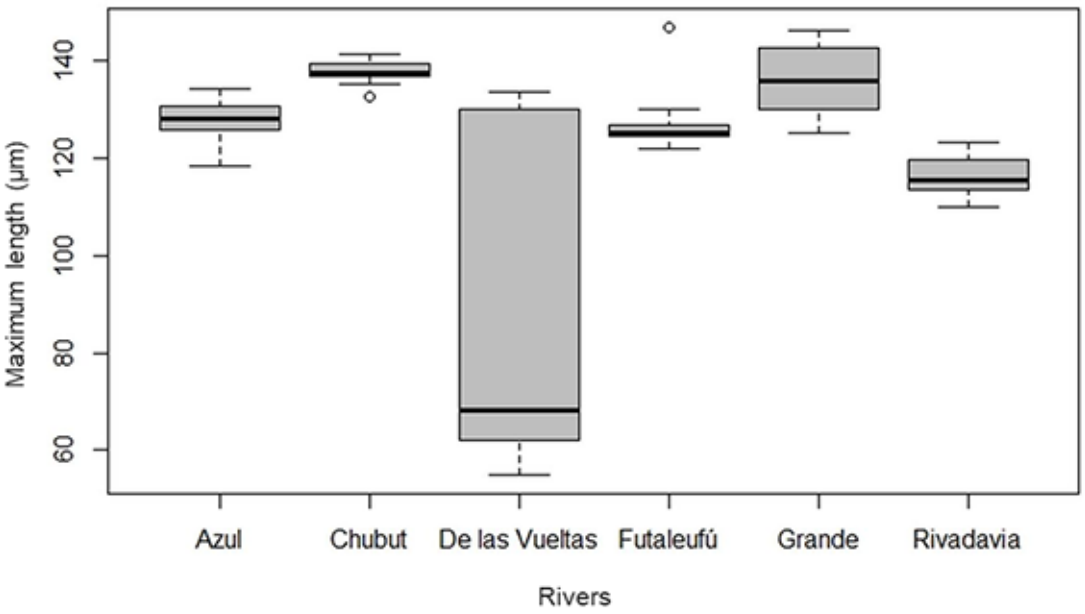

Fig. 5. Boxplot of maximum length of populations from different rivers from Patagonia. Azul, Chubut, Futaleufú and Rivadavia rivers belong to Chubut province (data by Uyua *et al.,* 2016b), Grande River belongs to Tierra del Fuego province and de las Vueltas River belongs to Santa Cruz province.

hand, only one sample collected in March 2015 (LPC 13422) showed a similar proportion of the two morphotypes while the others showed a high proportion (94 %) of cells with 54-74 µm length range (morphotype #2). A comparison between samples collected in different seasons (March 2015 and September 2016) but in the same site in de las Vueltas River showed differences between the proportions of morphotypes present (Fig. 6).

On the other hand, physicochemical parameters measured in de las Vueltas River showed that the water body had low temperature (6.5 °C), high DO concentrations (16.18 mg/l), low conductivity (0.094 mS /cm), low turbidity (3 ppm) and pH 8.86.

Table 1. Comparison of morphometric data of *D. geminata* from Patagonia Argentina

	Author	Length (µm)	Width (µm)	Striae/ 10 µm	Stigma	Areolae/ 10 µm
D. geminata from Chubut (Argentina)	Uyua *et al.,* 2016b	109-147	30-42	8-11	2-5	10-12
D. geminata from Río Negro (Argentina)	Beamud *et al.*,2013	107-133	34-41			
D. geminata from Santa Cruz (Argentina). Morphotype #1	This study	126-134	36-39	10-12	2-4	10-12
D. geminata from Santa Cruz (Argentina). Morphotype #2	This study	54-75	26-33	9-11	1-3	10-12
D. geminata from Tierra del Fuego (Argentina)	This study	125-146	37-42.5	8-11	2-5	10-12

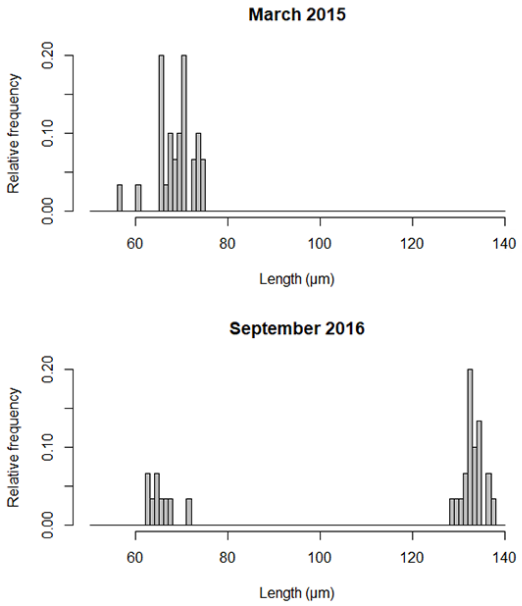

Fig. 6. Histograms of length variation of *Didymosphenia geminata* cells from the same location in de las Vueltas River in different seasons of the year.

Discussion

The specimens from South Patagonia here studied show the same fine valve morphology of the specimens from Chubut and Río Negro provinces (Beamud *et al.,* 2013, Uyua *et al.,* 2016b). The size variation of Santa Cruz populations agrees with the length range of *D. geminata* reported for different populations around the world (Krammer & Lange-Bertalot, 1986; Whitton *et al*., 2009; Metzeltin & Lange-Bertalot, 2014; Bishop, 2014; Khan-Bureau *et al.,* 2016) although is the only river with the presence of cells smaller than 100 µm in length in south Patagonia Argentina. The smaller specimens (morphotype #2) from de las Vueltas River are similar to those described from Isar River in Germany, Sachalin Island in Russia (Metzeltin & Lange-Bertalot, 2014) and the Himalayan rivers in India (Bhatt *et al.*, 2008). Bhatt *et al*. (2008) found that in India cell size varied in a continuum while in de las Vueltas River we found two different groups of cells size: cells with length from 54 to 74 µm and cells from 120 to 140 µm. On the other hand, our small specimens (morphotype #2) present the same morphologic and morphometric features described for the species *Didymosphenia hulli* (Khan-Bureau *et al.* 2016) from Farmington River in United States of America. The authors show that the main differences between *D. geminata* and *D. hulli* are at genetic level. Previous genetic studies from Argentinean populations from Chubut and Tierra del Fuego provinces were carried out using three different molecular markers (Uyua, 2017). The results showed that the same genetic variability exists among the Argentinian populations studied as observed globally (Uyua, 2017). Based on these and results obtained by different authors (Kelly, 2009; Cardenas pers. comm.) we considered that it is possible that the variability found by Khan-Bureau *et al.* (2016) is the same that we found for the species *D. geminata*. More studies are necessary to resolve the presence or not of new species around the world and their phylogenetic relationship.

It is possible that these two morphotypes represent different stages of the life cycle of the species but until now the life cycle of *D. geminata* is undescribed. Bishop (2014) showed that restoration dynamics and size distribution depend on habitat demonstrating the importance of spatial variability, although he could not describe the specific mechanism of *D. geminata* cell cycle. This could explain the morphometric differences observed among the rivers from Patagonia. Besides, the results obtained by Stoermer *et al*. (1986), evidenced that *D. geminata* comprises several geographically differentiated morphotypes.

De las Vueltas River does not have easy public accesses and belongs to a basin shared between Argentina and Chile, like Futaleufú River (Chubut province) and Grande River (Tierra del Fuego province). The river is characterized by shallow waters, with rapids, rocky bottoms and dense riparian vegetation (*Notophagus antartica* forest). The parameters measured in this river and others from Patagonia such as Grande River (Urciuolo & Taier, 2011), Futaleufú River (Uyua, 2017), Limay River and in the Lake Nahuel Huapi among others (Beamud *et al.,* 2013; Beamud *et al.,* 2016) and where *Didymosphenia* form dense blooms, show that the species has a wide tolerance range. Historically, *D. geminata* was considered to be restricted to oligotrophic (low nutrient) and low temperature waters, and a broad range of conductivity in the European Alps (Krammer & Lange-Bertalot, 1986).

However, Spaulding & Elwell (2007) showed that *D. geminata* is present in waters from 4 to 27 °C. Besides, the relationship of the presence of *D. geminata* and pH is narrow: Kilroy *et al.* (2005) pointed out that most part of the literature stated a pH between 7 and 8.5 as the optimal range. Although the pH value in de las Vueltas River is higher than the optimal, it is near to the maximum and, besides, the value is within the range established by Kawecka & Sanecki (2003). *D. geminata* requires high light intensity (Kilroy *et al.,* 2005) that is one of the characteristics of this river in terms of water turbidity. Chemical parameters of de las Vueltas River are close to the "optimal" characteristics to be susceptible to be invaded by *D. geminata*.

On the other hand, an important observation in this study was the presence of different proportions of small specimens (morphotype #2) in different seasons and in different places of the same river. We consider that this is related to the life cycle but we still have not been able to solve it. Periodic samplings over time are necessary to understand the population's behavior. Besides, it is necessary to study the possible interactions with others parameters (phosphate, nitrate, calcium, etc.) as possible phenotypic response of the species to environmental characteristics.

Recently *D. geminata* was detected in the middle stretch of the Santa Cruz River (Sturlese comm. pers.). This river crosses the Santa Cruz province from the Andes to the Atlantic Ocean. Preliminary analyses show populations similar to those of Patagonia but the small specimens observed in de las Vueltas River (tributary of Santa Cruz River) were not found (Sturlese comm. pers.).

Conclusions

Based on morphological characteristics, all the Patagonian materials belong to *D. geminata* ssp. *geminata* Metzeltin & Lange-Bertalot. Although our results do not allow explaining the presence of cells in one river of Santa Cruz smaller than those cells collected from Chubut and Tierra del Fuego provinces, we consider that it is important to report this morphotype since it could be misidentified during the routine monitoring programs carried out by different governmental agencies. Up to this moment, the length variation range for *D. geminata* from Argentina is from 54 µm to 147 µm. These results show the phenotypic plasticity of the species.

Author Contributions

NMU, SES, NHS and AVS conceived the study. BR and PDC collected samples. NMU and SES prepared and analyzed the field material. JIC collaborated in statistical analysis. NMU prepared the first manuscript draft. NMU, SES, NHS y AVS finished the last manuscript version.

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