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Nuisance *Didymosphenia geminata* blooms in the Argentinean Patagonia: Status and current research trends

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Large nuisance blooms of Didymosphenia geminata *have become increasingly widespread in Patagonia. Although the first published account for South America was in 1964, reports of large growths in Chile and Argentina commenced around 2010. Since then, these blooms have been observed all along the Andes region to the south of parallel 42°S. General surveys are needed to help provide an explanation. Possibilities include one or more new genetic variants or responses of local populations to global environmental changes. Electron microscopy of material from the Argentinean Patagonia revealed marked differences between regions, though it is unclear how much local factors and/or variations in life cycle contribute. Thus, we are approaching the problem from a molecular perspective, which we hope will help to overcome this limitation. Initial studies showed that* D. geminata *seems to be highly recalcitrant to DNA extraction, thus hindering the survey of molecular markers. We have now developed an improved DNA extraction technique for* Didymosphenia *mats, which markedly outperforms other techniques. However, endpoint polymerase chain reaction analyses suggest the persistence of polymerase chain reaction inhibitors in the samples, highlighting the need of further improvements for quantitative studies.*

Keywords: diatom, invasions, Argentina, phylogeography

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Introduction

Didymosphenia geminata (Lyngbye) Schmidt is an epilithic and epiphytic diatom in the Cymbellales (Bacillariophyta) that has shown great increases in many countries in recent years (Blanco and Ector, 2009; Whitton et al., 2009). This diatom is characterized by its ability to develop massive colonies that can cover large stretches of river bed and submerged vegetation and sometimes the marginal zones of lakes, thus jeopardizing the natural communities at those environments (James and Chipps, 2016; Segura, 2011; Whitton et al., 2009). Although they pose no threat to human health, *D. geminata* blooms can harm recreational and industrial activities, pose problems for water management and have a negative impact on the invaded ecosystems (Watson et al., 2015).

Different explanations for the mass growths have been proposed and the evidence up to early 2015 was reviewed by Watson et al. (2015), which provides a source for recent literature. Based on their study of an upland stream draining peaty moorland in England, Ellwood and Whitton (2007) concluded that the ability to make effective use of the relatively high concentrations of organic phosphate which occurred in spring was important for the diatom. Colonies showed very high phosphomonoesterase and phosphodiesterase activity and staining indicated that the upper part of the stalks was the main site for the phosphomonoesterase activity. This was studied further by Aboal et al. (2012). Where measurements have been made in other studies of phosphate in the water of rivers with *D. geminata*, the predominant form of phosphate has usually been organic. This and a range of different types of information about phosphate release from peat led Ellwood and Whitton (2007) to suggest that the widespread increases in *D. geminata* may be associated with climatic change leading to increased phosphate release in spring. A link between *D. geminata* blooms and climatic warming has been also highlighted by Taylor and Bothwell (2014). Several studies have indicated the importance of the dense mass of long stalks for retaining as well as accumulating nutrients, especially phosphate, within the colonies. In addition, Kilroy and Bothwell (2011) observed a relationship between nutrients availability and *D. geminata* division rates, and two recent papers have revealed a significant, negative relationship between P availability and *D. geminata* thriving (Montecino et al., 2016; Jackson et al., 2016). Together, these results suggest the hypothesis that *D. geminata* makes very effective use of relatively high concentrations of organic P in late winter and spring. Subsequently it is then well adapted to maintain high populations for some months when P concentrations are much lower.

The intense interest in *D. geminata* which occurred as a result of its discovery in New Zealand in 2004 and the suggestion that it was invasive there (Biggs et al., 2006) has subsequently led to the assumption that it is an invader elsewhere. Currently this is far from being resolved unambiguously, but what is clear is that several rivers have been studied sufficiently carefully to be certain that there have been major increases. The following provides a brief account of the situation for Argentina and Chile.

Reports in Chile and Argentina become frequent in about 2010

The first historical report of *D. geminata* in South America is that of Asprey et al. (1964). It was included among the "very rare species," having been observed at only two out of eight sites surveyed, Sarmiento Lake and Los Cisnes River. Samples were, however, only taken from the plankton caught with a very coarse net, which did not survey the typical environment of *D. geminata*. Material was subsequently shown to one of the present authors (BAW) and positively identified. Apart from a single report for the Mejillones River in Chile (Rivera and Gebauer, 1989), no further mention of its occurrence appeared until massive blooms were reported to occur during 2010 in the rivers Futaleufú and Espolón and the Cea Creek in Chile (Segura, 2011). Subsequently, its presence has been reported for the Chilean rivers of Risopatrón, Figueroa, Pico, Ñirehuao, Emperador Guillermo, Simpson, Aysén, D. Lago Monreal, Cochrane, Baker, Yelcho, Espolón, Bellavista, Noroeste, Llanquihue and Bío Bío (Khan-Bureau et al., 2016; Rivera et al., 2013).

In Argentina, the alga was first recorded in 2010 in the Futaleufú River, five months after its

discovery in Chile downstream in the same river basin. It was sparse from late August – early September (austral winter), but developed massive blooms covering up to 30% of the river bed from about December to April (austral spring and summer) (Sastre et al., 2013). Since then, the alga appears to have undergone a very rapid expansion north to Neuquén and Río Negro Provinces (Abelli Bonardi et al., 2012; Beamud et al., 2013) and south to the southern end of the continent, the Río Grande in Tierra del Fuego by 2013. *D. geminata* also formed mass growths on the shore of Lake Nahuel Huapi in 2013 (Beamud et al., 2013). By 2015 nuisance blooms were recorded in five Argentinean provinces, including Neuquén, Chubut, Tierra del Fuego, Río Negro and Santa Cruz. Unfortunately, historical data on benthos are almost absent for these provinces of Argentina, making it difficult to assess whether the species was already part of the native populations and has developed the invasive behavior only in recent times, or if its current presence responds to a recent introduction. Native or exotic invader, one of the main questions is to establish whether blooms are due to the overall dispersion of a new genetic variant/s or to the increase of local populations.

Frustules from the Argentinean Chubut Province blooms were analyzed morphologically and compared with published data from Chile and other countries (Rivera et al., 2013; Uyua et al., 2016). Morphological differences were found between locations. Further genetic studies are needed to assist understanding of morphological differences and hence also the alga's ecology. This in turn will aid in dealing with economic and ecological risks associated with the alga. Improved molecular detection methods should enhance the early detection of the diatom for better monitoring and management.

Current status of genetic studies in Argentina

Genetic studies of populations of *Didymosphenia* are still in a developing phase, with very few worldwide and a limited number of sequences in public databases (Cary et al., 2014; Jaramillo

Table 1. Sampling locations and gene markers analyzed in *Didymosphenia geminata* samples from Argentina. Please see also Figure 1.

River	Lat, Long	MPI[1]	S-18S[2]	U-18S[3]	rbcl[4]	COX[5]
Azul Pasarela	−42.03708, −71.60347	Yes	+	+	+	−
Azul Pasarela	−42.03708, −71.60347	No	−	−	N/A	−
Rivadavia	−42.67785, −71.70151	Yes	+	+	+	−
Quemquemtreu	−40.22314, −70.73296	No	−	−	N/A	−
Quilaquina	−40.32000, −71.37077	No	−	−	N/A	−
Grande	−53.82377, −67.75871	Yes	+	+	+	−
Futaleufú (A)	−43.13694, −71.60442	No	+	+	N/A	−
Futaleufú (B)	−43.13694, −71.60442	Yes	+	+	+	−
Futaleufú (C)	−43.16710, −71.58847	Yes	+	+	+	−
Futaleufú (D)	−43.17303, −71.59444	Yes	+	+	+	−
Futaleufú (E)	−43.17208, −71.65081	Yes	+	+	+	−
Futaleufú (F)	−43.17767, −71.63106	Yes	+	+	+	−
Chubut	−42.33946, −70.86890	Yes	+	+	+	−
De las Vueltas (A)	−49.26792, −72.87361	Yes	−	−	−	−
De las Vueltas (B)	−49.31466, −72.89750	Yes	N/A	N/A	−	−
Toro	−49.13204, −72.94325	No	−	−	−	−

[1]MPI: Mouth pipeting isolation.
[2]18S rDNA gene, amplified by *D. geminata* specific primers (Cary et al., 2006, 2007).
[3]18S rDNA gene, amplified by universal primers (Moon-van der Staay et al., 2001).
[4]Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (Jones et al., 2005).
[5]Cytochrome c-oxidase subunit 1 (Evans et al., 2007).

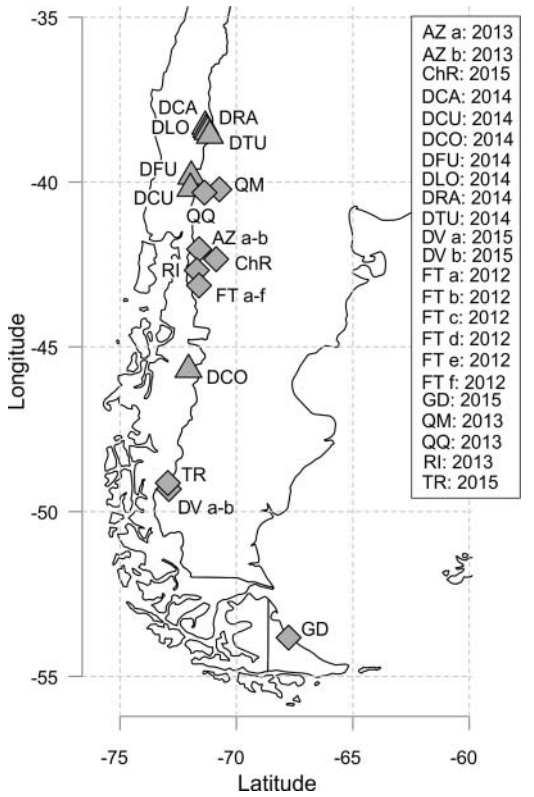

Figure 1. Origins of the South American samples studied here. *AZ* Azul River, *RI* Rivadavia River, *QM* Quemquemtreu River, *QQ* Quilaquina River, *GD* Grande River, *FT* Futaleufú River, *CH* Chubut River, *DV* De las Vueltas River, *TR* Toro River, *DCA* Caracoles River, *DRA* Rahue River, *DLO* Lollen River, *DTU* Tucapel River, *DFU* Fuy River, *DCU* Curriñe, *DCO* Pollux. The Chilean samples' (triangles) sequences were kindly provided by Dr. Leyla Cardenas. Please see also Table 1.

et al., 2015; Kermarrec et al., 2011; Khan-Bureau et al., 2016). A key requirement of any molecular study is the availability, or correct design, of a suitable method to obtain genetic material in adequate amounts and of high purity. *D. geminata* mats pose a challenge due to the low cellular content relative to extracellular matrix, and its chemical complexity, which results in low nucleic acids yields and high levels of enzymatic inhibition (Jones et al., 2015). We have compared five DNA extraction protocols in terms of yield and purity of the DNA extracts, along with the efficacy and efficiency of the DNA suspensions in Polymerase Chain Reaction (PCR) amplifications. Low and highly variable DNA yields were observed, similar to those for other recalcitrant materials like mummified plant tissues, leaves with high contents of secondary metabolites and different types of microbial mat (Aljanabi and Martinez, 1997, Bey et al., 2010; Cota Sanchez et al., 2006; Jackson et al., 1990; Porebski et al., 1997; Rogers and Bendich, 1985). As for the enzymatic assays, PCR amplification of the 18S rDNA gene revealed that inhibition was frequent for the majority of the studied protocols. Moreover, even for DNA preparations for which PCR was not completely inhibited, the amplification product yields did not correlate with the corresponding template amounts, indicating the persistence of PCR inhibitors (Jones et al., 2015). Our results suggest that DNA extraction protocols that incorporate: lysis buffers containing anionic surfactants, such as cetyltrimethyl ammonium bromide (CTAB), along with high salt concentrations to facilitate the removal of polysaccharides; washing steps after DNA precipitation; and the incorporation of chelating agents, may overcome or ameliorate the problems sufficiently for downstream molecular applications (Jones et al., 2015; Uyua et al., 2014).

Inter-sample variability and the performance of the available PCR primers can also hinder *D. geminata* molecular studies. We collected benthic samples between April 2012 (austral autumn) and November 2015 (austral spring) from different sites in Chubut, Neuquén, Santa Cruz and Tierra del Fuego Provinces (Table 1, Figure 1 MAP). These samples were processed by our optimized DNA extraction method (Uyua et al., 2014), and the DNA suspensions obtained were used to amplify sequences of the 18S, *rbcl* and COX genes. Although several genes could be amplified in many samples; it was impossible to amplify some in some samples (Table 1), in some cases even when DNA extracts were obtained from cells isolated by mouth pipeting (Table 1). This suggests the presence of inhibitors in or attached to *D. geminata* cells and/or a suboptimal primer behavior, possibly due to sequence variability. The need for improved PCR primers was also reflected by gradient PCR assays, in which we observed a range of problems such as primer dimer formation, non-specific bands and the need for too low an annealing temperature to generate adequate amounts of DNA (Figure 2). Figure 2 includes a gradient PCR analysis of primers F27 and R1492 directed against the 16S rRNA gene (Weinbauer et al., 2002), which shows that a temperature of 53.9°C resulted in very few primer dimers, large amounts of amplified DNA and no non-specific

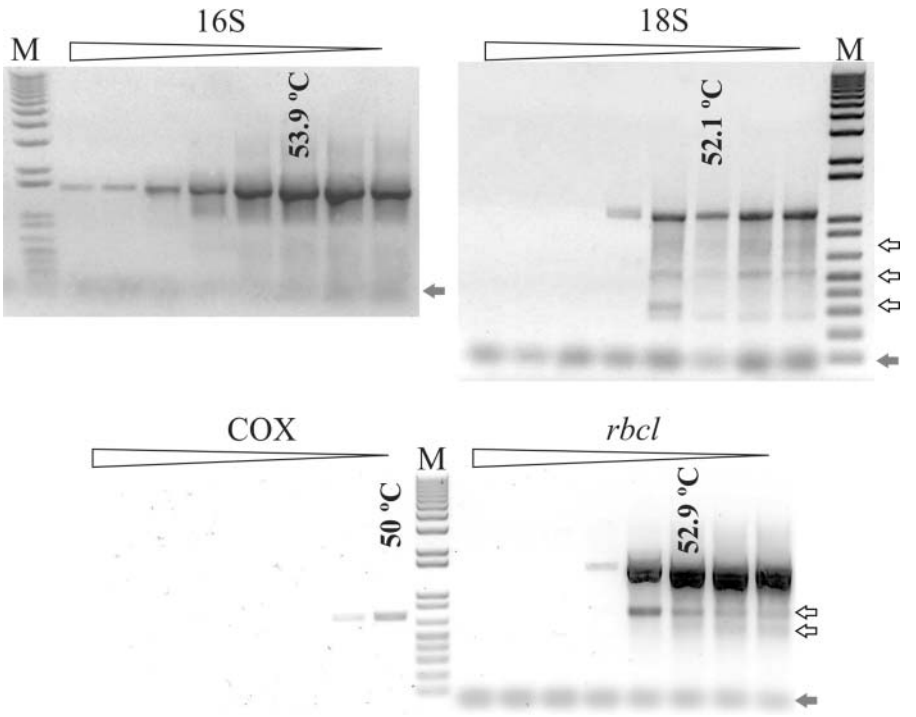

Figure 2. Performance of different PCR assays, as revealed by gradient PCR. Each panel has the name of the gene amplified (16S, 18S, COX, *rbcl*). 16S analyses were performed using metagenomic DNA as template and primers F27 and R1492 as described elsewhere (Manrique et al., 2012). Cycling conditions for the rest of genes were: a 2′ denaturation at 95°C; 35 cycles of 95°C 15″, gradient of 50 to70°C for 15″, 72°c for 50″; and a final extension step at 72°C during 5′. All reactions were performed in a BioRad My Cycler thermal cycler (Bio-Rad Laboratory, Inc.). Amplification primers were D602F – D1670R for the 18S gene (Cary et al., 2014, 2006), GAZF2-GAZR2 for COX (Evans et al., 2007) and DPrbcL1 – DPrbcL7 for *rbcl* (Jones et al., 2005). Twenty percent of the PCR reactions were run in 2% agarose gels, revealed with GelRed Nucleic Acid Gel Stain (Biotum) and observed in a UV transilluminator. M: 1Kb Plus DNA ladder (Invitrogen). Open arrows indicate the position of non-specific bands. Closed gray arrows display the location of primer dimers. Triangles above the gels indicate the direction of the gradient from higher (left) to lower (right) temperature. The temperatures that worked best are indicated on the corresponding gel lanes.

PCR bands. Conversely, amplifications of the *D. geminata* 18S gene sequences presented substantial amounts of primer dimers and either too many non-specific bands or very little specific DNA. Both the COX and *rbcl* primers required very low annealing temperatures to produce acceptable amounts of amplification product (COX) or to diminish the production of non-specific bands. Moreover, the COX PCR produced very little DNA, which can resulted in a negative impact when used to amplify the gene from the natural samples (Table 1).

Regarding genetic variability, we have sequenced about 950 bp of the 18S rDNA gene of some of our samples, and preliminary analyses of these sequences suggest that there is a considerable variation among the *D. geminata* populations studied. We combined our sequences with sequences for several *Didymosphenia* and other Cymbellales sequences available in public databases. Phylogenetic analysis strongly supported the monophyly of *Didymosphenia* (Figure 3a), but also revealed intra-specific variation that was in many cases correlated with geographic origin (Figure 3b). A group of sequences from Argentina were very close to the Chilean strains described previously (Jaramillo et al., 2015; Nakov et al., 2014). Likewise, sequences from Italy and some from the USA clustered according to their corresponding geographic origin. A statistical parsimony analysis of these sequences revealed similar patterns, with the South American strains grouped in three closely related clusters and the European strains in four close clusters, but separated from the American or New Zealand sequences (Figure 3c). Thus, our preliminary analyses support the concept that geographically close strains seem to be phylogenetically

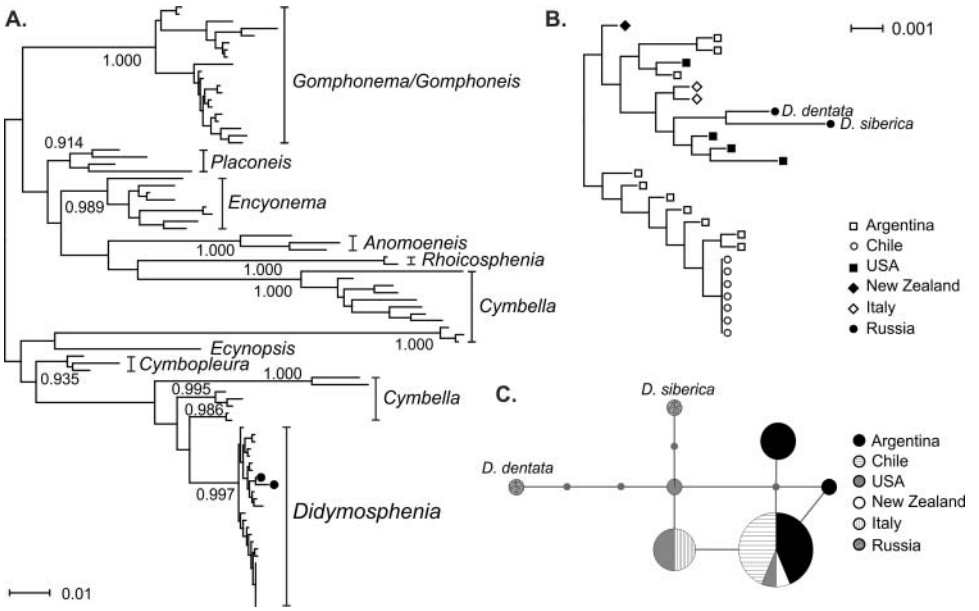

Figure 3. Analysis of South American and worldwide 18S *D. geminata* sequences. (a) Maximum likelihood phylogenetic tree of the Cymbellales, including the worldwide *D. geminata* sequences. The analysis was performed with the program PhyML (Guindon et al., 2010), using an evolutionary model (JC-69) inferred by MrAIC (Nylander, 2004). Tree searches consisted of 10 random addition sequences followed by Nearest-Neighbor-Interchanges (NNI) and Subtree-Pruning-Regrafting (SPR) tree rearrangements. Numbers close to branches correspond to *aLTR* supports. Branch lengths are proportional to the number of nucleotide substitutions (scale bar units are substitutions per aligned position). *Didymosphenia* sequences indicated by circles correspond to *D. dentata* and *D. siberica*. (b) Detail of the *Didymosphenia* branch, with the origins of the sequences indicated by symbols (please see legend). (c) Gene genealogies obtained by the TCS program (Clement et al., 2000). Radius of circle is proportional to number of accrued sequences. The origin of the sequences in each cluster are indicated in the legend. The smaller gray dots indicate missing haplotypes.

closer than remote ones. These observations must be corroborated by data from other genes and extended phylogeographic analyses.

Conclusions and remarks

D. geminata has expanded its range in an alarming and very rapid way since its first report in 2010. The molecular and phylogeographic studies that are underway should help clarify the relationship of the nuisance blooms with the global evolutionary history of the alga. Preliminary data presented here indicate that the nuisance blooms are probably not due to the expansion of a single genetic variant, but to the sum of local responses to factors, possibly including global change. There is an urgent need of complementary information, encompassing deeper phylogeographic studies and detailed ecological surveys including the interaction of the alga with other macro- and microorganisms.

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