Title: First freshwater member ever reported for the family Bathycoccaceae (Chlorophyta; Archaeplastida) from Argentinean Patagonia revealed by environmental DNA survey.

Authors: Enrique Lara, Leonardo D. Fernández, M. Romina Schiaffino, Irina Izaguirre

PII: S0932-4739(17)30047-0
DOI: http://dx.doi.org/doi:10.1016/j.ejop.2017.05.008
Reference: EJOP 25509

To appear in:

Received date: 28-2-2017
Accepted date: 31-5-2017

Please cite this article as: Lara, Enrique, Fernández, Leonardo D., Schiaffino, M.Romina, Izaguirre, Irina, First freshwater member ever reported for the family Bathycoccaceae (Chlorophyta; Archaeplastida) from Argentinean Patagonia revealed by environmental DNA survey.European Journal of Protistology http://dx.doi.org/10.1016/j.ejop.2017.05.008

First freshwater member ever reported for the family Bathycoccaceae (Chlorophyta; Archaeplastida) from Argentinean Patagonia revealed by environmental DNA survey.

Enrique Lara[1*], Leonardo D. Fernández[2], M. Romina Schiaffino[3], Irina Izaguirre[4]

[1]Laboratory of Soil Biology, University of Neuchâtel, Rue Emile Argand 11, Neuchâtel CH-2000, Switzerland

[2]Centro de Investigación en Recursos Naturales y Sustentabilidad (CIRENYS), Universidad Bernardo O'Higgins, Fábrica 1990, Santiago, Chile

[3] Centro de Investigaciones y transferencia del Noroeste de la Provincia de Buenos Aires (CITNOBA, CONICET-UNNOBA), 6000, Junín, Argentina.

[4]Departamento de Ecología, Genética y Evolución, IEGEBA (UBA-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

*corresponding author: enrique.lara@unine.ch. Tel: +41 32 718 23 27

Abstract

We characterized molecularly the first freshwater member ever reported for the family Bathycoccaceae in Lake Musters (Argentinean Patagonia). Members of this family are extremely numerous and play a key ecological role in marine systems as primary producers. We cloned a fragment comprising the SSU rRNA gene + ITS region from environmental DNA using specific mamiellophyte primers. The unique SSU rRNA gene sequence obtained clustered robustly with *Bathycoccusprasinos*. Analysis of the two-dimensional structure of the ITS region showed the presence of a typical supplementary helix in the ITS-2 region, a synapomorphy of Bathycoccaceae, which confirmed further its phylogenetic placement. We finally discuss the possible causes for the presence of this organism in Lake Musters.

Keywords: Mamiellophyceae; Picoplankton; Ribosomal genes; Southern Patagonia

Introduction

Photosynthetic picoeukaryotes have been recognized as essential primary producers in aquatic systems (Li, 1994). The advent of molecular methods revealed a considerable phylogenetic diversity in marine surface waters (Diez et al., 2001b; Moon-van der Staay et al., 2001) showing the existence of several new deep clades of photosynthetic organisms (Not et al., 2012) that most likely play an important functional role in these ecosystems. Amongst this diversity revealed by environmental DNA studies, mamiellophytes are particularly well represented. These tiny organisms comprise the smallest known free-living eukaryote, *Ostreococcus tauri* (>1 µm) which became famous also because of the compaction of its genome, which turned it into a model organism for studying eukaryotic genome evolution (Derelle et al., 2006). Mamiellophytes are believed to play a considerable role in the marine ecosystem. Their pivotal role as primary producers has been shown experimentally as they responded positively and strongly to CO_2 addition (Newbold et al., 2012). Indeed, they were shown to be main primary producers in cold waters such as the coastal upwelling zone off central Chile (Collado-Fabbri et al., 2011) and the High Arctic (Balzano et al., 2012). At the global scale, they compete against picocyanobacteria for this role, being more efficient under fast changing light conditions, arguably because the architecture of their eukaryotic genome is more plastic than their prokaryotic counterparts (Kulk et al., 2011). Mamiellophytes form huge populations in the ocean, reaching densities of 10^3-10^5 cells/ml (Countway and Caron, 2006). This has been confirmed also by environmental sequencing, where related sequences were very frequent, sometimes even dominating photosynthetic communities (Fuller et al., 2006; Guillou et al., 2004; Not et al., 2004; Worden, 2006).

The presence of mamiellophytes is believed to be marginal in freshwater systems. Amongst the five families that compose Mamiellophyceae, only one (Monomastigaceae) is common in freshwater; members of family Crustomastigaceae have been also reported, albeit rarely (Marin and Melkonian, 2010). A recent environmental DNA survey based on pyrosequencing revealed also the existence of members of a third family (Dolichomastigaceae) in lacustrine environments (Taib et al., 2013). But in spite of the accumulation of genetic environmental data produced by next generation sequencing techniques, the presence of Mamiellophyceae or Bathycoccaceae has never been reported in freshwater systems.

In the course of a molecular fingerprinting-based study on diversity of eukaryotic plankton in a series of lakes located in Southern Argentinean Patagonia (Schiaffino et al., 2016), we obtained a partial SSU rRNA gene sequence affiliated to the Mamiellaceae/Bathycoccaceae clade as determined with BLAST (Altschulet al., 1997). Based on this newly obtained sequence, we designed a PCR strategy to obtain (1) a longer SSU rRNA sequences from these lakes in order to place it into the mamiellophyte tree, as well as (2) ITS 1 and 2 sequences to confirm its position based on the presence of a taxonomically relevant supplementary helix in ITS2.

Material and Methods

In a previous study (Schiaffino et al., 2016), several lakes were sampled along a gradient stretching from Patagonia to Maritime Antarctica. Their pico-eukaryotic diversity was screened using Illumina HiSeq and denaturing gradient gel electrophoresis (DGGE) approaches. Some DGGE well-represented bands were excised and sequenced. These bands were fragments comprising the variable regions V1-V3 of the gene coding for the SSU rRNA (Diez et al., 2001a).

One of the bands (GenBank accession number KC923042) obtained from Lake Musters (Chubut Province, Argentina) was affiliated to order Mamiellales. Thus, we designed specific primers to amplify a fragment comprising a large part of the SSU rRNA gene and the complete ITS1+2 region of the new putative Bathycoccaceae from the original environmental sample. We based our primer design an alignment comprising SSU and LSU rRNA sequences of several representatives of the Bathycoccaceae/Mamiellophyceae clade, both environmental and derived from cultures. We chose the primers to be specific only to this clade and not to amplify Monomastigales, which can be common in freshwaters (Marin and Melkonian, 2010). The resulting primers were BatSSUF 5'- CCGGGCTTTTTCAAGTCTGGT-3' and BatLSUR 5'-CTTGTCTGAACTGAGGTCAAAAGG-3'. We performed a 40 cycles PCR with an annealing temperature of 55°C using Promega's GoTaq polymerase (Madison, Wisconsin, USA). Products (ca. 2kb) were cloned into apCR2.1 Topo TA cloning vector and transformed into E. coli TOP10' One Shot cells (both from Invitrogen) according to the manufacturer's instructions. Clone inserts were amplified using PCR primers. White colonies were picked and PCR was performed directly without any cultivation step of the bacteria. Sequencing was performed on right-sized clones with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Genève, Switzerland) using a BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

Phylogenetic analyses

The obtained sequence was aligned against a set of sequences of families Bathycoccaceae and Mamiellaceae plus some Dolichomastigales (Dolichomastigaceae + Crustomastigaceae) that were subsequently used to root the

tree. We used an alignment built using Clustal W (Thompson et al., 1994) and refined it manually. We built a maximum likelihood tree based on 1660 characters using a RAxML algorithm (Stamatakis et al., 2008) as proposed on the Black Box portal (http://phylobench.vital-it.ch/raxml-bb/) using the GTR+Γ+I model. Model parameters were estimated in RAxML over the duration of the tree search. Node robustness was estimated by bootstrapping (1000 replicates).

Secondary structure analyses

ITS sequences were aligned against each other and against *Bathycoccus* and *Ostreococcus* sequences to identify the parts corresponding respectively to ITS1, 5.8S and ITS2. The secondary structure model of ITS2 was generated with the program MFOLD (Zuker, 2003) which can be used online on the site http://www.bioinfo.rpi.edu/applications/mfold/. Results were compared with other models of secondary structure developed for other members of Bathycoccaceae (Subirana et al., 2013).

Results and discussion

Identity and phylogenetic position of the new phylotype

Our phylogenetic analysis placed the new SSU rRNA gene sequence within family Bathycoccaceae with a total support (100% bootstrap support). Furthermore, it branched robustly (93% bootstrap support) together with *Bathycoccus prasinos*. The relatively long branch suggests that our freshwater Bathycoccaceae (hereinafter referred as freshwater Bathycoccaceae: FB) and *B. prasinos* have been separated since a relatively long time (Figure 1). All obtained SSU rRNA gene sequences from

clones were identical. The merged sequence has been deposited in GenBank under accession file XXXXX (will be provided upon manuscript acceptance for publication).

The most stable energetically model for FB ITS2 secondary structure included all universal 4 helices, and presented also a typical supplementary helix between universal helices 3 and 4 (Figure 2). This is reported as a synapomorphy of the family (Marin and Melkonian, 2010). The other four canonical helices present bi-dimensional structures that resemble that of *Bathycoccus prasinos* and *Ostreococcus tauri* (clade D) in their global shape; they possess, however, enough differences that should grant FB its own generic status once the species will be isolated (Figure 1; Subirana et al., 2013). Attempts at isolating FB have not been successful yet, possibly due to the likely small size of the organism. The remote location of Lake Musters is also a major concern for organizing sampling campaigns.

FB is the only reported case of freshwater inhabiting Bathycoccaceae. In spite of the wealth of environmental DNA surveys on freshwater planktonic eukaryotic diversity based on traditional cloning-sequencing/fingerprinting approaches (Lefranc et al., 2005; Lepere et al., 2010; Richards et al., 2005; Slapeta et al., 2005; Wu et al., 2009; Zhao et al., 2011) or massive (next generation) sequencing (Charvet et al., 2012; Mangot et al., 2013; Taib et al., 2013), no single sequence related to these organisms has been detected yet before the publication of (Schiaffino et al., 2016). However, all mentioned studies were undertaken in the Northern Hemisphere. Conversely, Patagonia has undergone a very particular geological history in isolation from other regions (Nullo and Combina, 2011), and has been mentioned as an endemism hotspot also for micro-organisms (Fernández, 2015; Woelfl, 2006). Marine

transgressions occurred periodically in the Mesozoic and Cenozoic, as well as volcanic uprisings (Nullo and Combina, 2011) that may have isolated populations of the ancestors of FB, which then adapted progressively to freshwater. Capturing the diversity of these organisms in surrounding freshwater systems may allow inferring phylogeographical hypothesis on their origin.

FB is possibly more than a simple curiosity of Patagonian microbiota. While its marine relatives are key species in oceanic systems, FB seems to be also highly abundant as indicated by the intensity of the corresponding DGGE band (Schiaffino et al., 2016), which suggests an important role as primary producers. It represents also a unique case of adaptation of a marine clade to freshwater systems, which deserves to be studied with the appropriate (genomic) tools once the original organism will be isolated and described formally.

Acknowledgments

We would like to express our gratitude to Amandine Pillonel for her help at the molecular biology laboratory, and David Singer for the help with the figures. This study was financed by grants of the FONCYT of Argentina (PICT 32732 and 0794), CONICET of Argentina (PIP 498); and the Swiss National Science Foundation project FN 31003A-143960/1. We express our gratitude to our colleagues Fernando Unrein, Patricia Rodríguez, Rodrigo Sinistro, M. Laura Sánchez, Guillermo Tell, Adrián Rua for their help with sampling. L.D.F. is supported by CONICYT from Chile (doctoral scholarships N° 21110037 and 78130011).

References

Balzano, S., Marie, D., Gourvil, P., Vaulot, D., 2012. Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples. Isme J. 6, 1480-1498.

Charvet, S., Vincent, W.F., Lovejoy, C., 2012. Chrysophytes and other protists in High Arctic lakes: molecular gene surveys, pigment signatures and microscopy. Polar Biology 35, 733-748.

Collado-Fabbri, S., Vaulot, D., Ulloa, O., 2011. Structure and seasonal dynamics of the eukaryotic picophytoplankton community in a wind-driven coastal upwelling ecosystem. Limnol. Oceanogr. 56, 2334-2346.

Countway, P.D., Caron, D.A., 2006. Abundance and distribution of Ostreococcus sp in the San Pedro Channel, California, as revealed by quantitative PCR. Appl. Environ. Microbiol. 72, 2496-2506.

Derelle, E., Ferraz, C., Rombauts, S., Rouze, P., Worden, A.Z., Robbens, S., Partensky, F., Degroeve, S., Echeynie, S., Cooke, R., Saeys, Y., Wuyts, J., Jabbari, K., Bowler, C., Panaud, O., Piegu, B., Ball, S.G., Ral, J.P., Bouget, F.Y., Piganeau, G., De Baets, B., Picard, A., Delseny, M., Demaille, J., Van de Peer, Y., Moreau, H., 2006. Genome analysis of the smallest free-living eukaryote Ostreococcus tauri unveils many unique features. Proc. Natl. Acad. Sci. U. S. A. 103, 11647-11652.

Diez, B., Pedros-Alio, C., Marsh, T.L., Massana, R., 2001a. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl Environ Microbiol 67, 2942-2951.

Diez, B., Pedros-Alio, C., Massana, R., 2001b. Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl. Environ. Microbiol. 67, 2932-2941.

Fernández, L.D., Lara, E., Mitchell, E. A. D. , 2015. Checklist diversity and distirbution of testate amoebae in Chile. European Journal of Protistology 51, 409-424.

Fuller, N.J., Campbell, C., Allen, D.J., Pitt, F.D., Zwirglmaierl, K., Le Gall, F., 2006. Analysis of photosynthetic picoeukaryote diversity at open ocean sites in the Arabian Sea using a PCR biased towards marine algal plastids. Aquat Microb Ecol 43, 79-93.

Guillou, L., Eikrem, W., Chretiennot-Dinet, M.J., Le Gall, F., Massana, R., Romari, K., 2004. Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems. Protist 155, 193-214.

Kulk, G., van de Poll, W.H., Visser, R.J.W., Buma, A.G.J., 2011. Distinct differences in photoacclimation potential between prokaryotic and eukaryotic oceanic phytoplankton. Journal of Experimental Marine Biology and Ecology 398, 63-72.

Lefranc, M., Thenot, A., Lepere, U., Debroas, D., 2005. Genetic diversity of small eukaryotes in lakes differing by their trophic status. Appl. Environ. Microbiol. 71, 5935-5942.

Lepere, C., Masquelier, S., Mangot, J.F., Debroas, D., Domaizon, I., 2010. Vertical structure of small eukaryotes in three lakes that differ by their trophic status: a quantitative approach. Isme J. 4, 1509-1519.

Li, W.K.W., 1994. Primary production of Prochlorophytes, cyanobacteria, and eukaryotic ultraphytoplankton. Measurements from flow cytometric sorting. Limnol Oceanogr 39, 169-175.

Mangot, J.F., Domaizon, I., Taib, N., Marouni, N., Duffaud, E., Bronner, G., Debroas, D., 2013. Short-term dynamics of diversity patterns: evidence of continual reassembly within lacustrine small eukaryotes. Environmental Microbiology 15, 1745-1758.

Marin, B., Melkonian, M., 2010. Molecular Phylogeny and Classification of the Mamiellophyceae class. nov (Chlorophyta) based on Sequence Comparisons of the Nuclear- and Plastid-encoded rRNA Operons. Protist 161, 304-336.

Moon-van der Staay, S.Y., De Wachter, R., Vaulot, D., 2001. Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409, 607-610.

Newbold, L.K., Oliver, A.E., Booth, T., Tiwari, B., DeSantis, T., Maguire, M., Andersen, G., van der Gast, C.J., Whiteley, A.S., 2012. The response of marine picoplankton to ocean acidification. Environmental Microbiology 14, 2293-2307.

Not, F., Latasa, M., Marie, D., Cariou, T., Vaulot, D., Simon, N., 2004. A single species, Micromonas pusilla (Prasinophyceae), dominates the eukaryotic picoplankton in the western English channel. Appl. Environ. Microbiol. 70, 4064-4072.

Not, F., Siano, R., Kooistra, W., Simon, N., Vaulot, D., Probert, I., 2012. Diversity and Ecology of Eukaryotic Marine Phytoplankton, in: Piganeau, G. (Ed.), Genomic Insights into the Biology of Algae. Academic Press Ltd-Elsevier Science Ltd, London, pp. 1-53.

Nullo, F., Combina, A., 2011. Patagonian continental deposits (Cretaceous-Tertiary). Biol. J. Linnean Soc. 103, 289-304.

Richards, T.A., Vepritskiy, A.A., Gouliamova, D.E., Nierzwicki-Bauer, S.A., 2005. The molecular diversity of freshwater picoeukaryotes from an oligotrophic lake reveals

diverse, distinctive and globally dispersed lineages. Environmental Microbiology 7, 1413-1425.

Schiaffino, M.R., Lara, E., Fernández, L.D., Balagué, V., Singer, D., Seppey, C.C.W., Massana, R., Izaguirre, I., 2016. Microbial eukaryote communities exhibit robust biogeographical patterns along a gradient of Patagonian and Antarctic lakes. Environmental Microbiology 18, 5249-5264.

Slapeta, J., Moreira, D., Lopez-Garcia, P., 2005. The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. Proc. R. Soc. B-Biol. Sci. 272, 2073-2081.

Stamatakis, A., Hoover, P., Rougemont, J., 2008. A Rapid Bootstrap Algorithm for the RAxML Web Servers. Syst. Biol. 57, 758-771.

Subirana, L., Pequin, B., Michely, S., Escande, M.L., Meilland, J., Derelle, E., Marin, B., Piganeau, G., Desdevises, Y., Moreau, H., Grimsley, N.H., 2013. Morphology, Genome Plasticity, and Phylogeny in the Genus Ostreococcus Reveal a Cryptic Species, O. mediterraneus sp nov (Mamiellales, Mamiellophyceae). Protist 164, 643-659.

Taib, N., Mangot, J.F., Domaizon, I., Bronner, G., Debroas, D., 2013. Phylogenetic Affiliation of SSU rRNA Genes Generated by Massively Parallel Sequencing: New Insights into the Freshwater Protist Diversity. PLoS One 8.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Woelfl, S., 2006. Notas sobre protozoos ciliados dulceacuicolas de Chile. Gayana 70, 24-26.

Worden, A.Z., 2006. Picoeukaryote diversity in coastal waters of the Pacific Ocean. Aquat. Microb. Ecol. 43, 165-175.

Wu, Q.L.L., Chatzinotas, A., Wang, J.J., Boenigk, J., 2009. Genetic Diversity of Eukaryotic Plankton Assemblages in Eastern Tibetan Lakes Differing by their Salinity and Altitude. Microb. Ecol. 58, 569-581.

Zhao, B.Y., Chen, M.J., Sun, Y., Yang, J.X., Chen, F.Z., 2011. Genetic diversity of picoeukaryotes in eight lakes differing in trophic status. Canadian Journal of Microbiology 57, 115-126.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406-3415.

Figures

Figure 1

Maximum likelihood tree based on SSU rRNA gene sequence data, including sequences from all Bathycoccaceae species plus some Mamiellaceae. Some Dolichomastigales sequences were used to root the tree. The phylogeny was constructed using the algorithm RAxML under the GTR+Γ+I model. Node robustness was estimated by bootstrapping.

Figure 2

Reconstruction of the secondary structure of the internal transcribed spacer 2 (ITS2) of FB, taking into account the energetically most favorable configuration, built using MFold (Zuker, 2003). Each helix of FB (c) is compared to its equivalent in *Osterococcus tauri* clade D (a) and *Bathycoccus prasinos* (b), re-drawn after Subirana et al., 2013.