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ORIGINAL PAPER

Phylogenetic diversity and dominant ecological traits of freshwater Antarctic Chrysophyceae

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Abstract

Previous studies conducted in summer in the lakes at Hope Bay (Antarctic Peninsula) between 1991 and 2007 showed a large numerical contribution of flagellated Chrysophyceae to the phytoplankton communities, particularly in the oligotrophic lakes, as evidenced by light microscopy observations and molecular fingerprinting. Given the ecological relevance of this group in these Antarctic microbial foodwebs, we carried out further molecular analyses (clone libraries and 18S Illumina high throughput sequencing) to characterize their phylogenetic diversity. The results of this study significantly increased the retrieved Chrysophyceae biodiversity. Clone libraries in two selected lakes (one oligotrophic and one mesotrophic) yielded 12 different chrysophycean OTUs, whereas 81 Swarm OTUs were recovered from six lakes using Illumina HiSeq. With the combination of both methods, we observed sequences of all the chrysophyte known clades, although most of the diversity belonged to Clade D, a group comprising mixotrophic and heterotrophic species. The percentage of reads for this clade in Illumina HiSeq ranged from 30% to 96% of the total Chrysophyceae reads. Based on experiments and observations, we also describe the main ecological traits of this group: the dominant taxa were small pigmented flagellates, well adapted to survive in oligotrophic systems, sometimes abundant under ice-cover subjected to low light intensities, and that have phagotrophic behavior. The used combination of methods allowed us to characterize the biodiversity and ecology of the Chrysophyceae, the dominant phytoplankton group in the oligotrophic lakes of this Maritime Antarctic region.

Keywords Chrysophyceae · Antarctic lakes · Clone library · 18S Illumina HiSeq · Molecular and functional diversity

Introduction

The success of flagellated nanoplanktonic algae in Antarctic freshwater lakes has been widely documented (e.g. Priddle et al. 1986; Laybourn-Parry et al. 1997; Butler 1999; Vinocur and Unrein 2000; Izaguirre et al. 2001). Amongst them, Chrysophyceae, Cryptophyceae, Chlorophyceae and Prasinophyceae are dominant groups in different lake ecosystems (e.g. Mataloni et al. 2000; McKnight et al. 2000; Marshall and Laybourn-Parry 2002; Bell and Laybourn-Parry 2003; Laybourn-Parry and Pearce 2007; Izaguirre et al. 2021). However, most studies lack an accurate taxonomic

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affiliation of the species that compose the assemblages due to the difficulties in identifying most chrysophytes using light microscopy. Phytoplankton taxonomy has traditionally been based on morphological characteristics, with the underlying assumption that particular morphological traits were unique to a species and physiologically meaningful (Savin et al. 2004). Nevertheless, microscopical identification in small and naked phytoplankters is often challenging because of their small cell-size and the limited number of distinct morphological characters alongside the known difficulties in cultivation (Rippka et al. 2000; Ernst et al. 2003). The introduction of molecular approaches into microbial ecology allows overcoming these biases and characterizing environmental communities to a relatively detailed taxonomic level. In particular, sequencing of the 18S rRNA gene has revealed an astounding diversity of microbial eukaryotes in every environment sampled so far (Epstein and López-García 2008; Debroas et al. 2017). However, molecular approaches targeting the ribosomal genes do not provide direct information about the function of the organisms.

Several studies have shown that many of these freshwater planktonic pigmented flagellates are usually mixotrophic, capable of ingesting bacteria, and in some cases possibly also taking up dissolved organic carbon to supplement their carbon budgets and/or gain inorganic nutrients for photosynthesis (Laybourn-Parry et al. 2000; McKnight et al. 2000; Laybourn-Parry et al. 2005; Izaguirre et al. 2021). Chrysophytes are ancestrally plastid-bearing protists, but have been transiting in the course of their evolutionary history between mixotrophy and strict heterotrophy, with progressive losses of photosynthesis-related genes and structures (Graupner et al. 2018). These phenomena occurred relatively fast in evolution, and simultaneously in several clades.

Lakes presenting different trophic status in Hope Bay (Antarctic Peninsula) have been surveyed for over 15 years during the austral summers, thus allowing the characterization of their phytoplankton communities (e.g. Izaguirre et al. 1998, 2003). Microscopical analyses showed a large contribution of flagellated Chrysophyceae, particularly in the oligotrophic lakes, which were identified as *Ochromonas* spp. and *Chromulina* spp., or were reported in the floristic lists as unidentified chrysophyceans (Allende and Izaguirre 2003; Izaguirre et al. 2003). In some occasions these taxa were completely dominant under ice cover, with peaks at the beginning of the Antarctic summer before ice melting (Izaguirre et al. 1993) and having a main role in the lakes' food webs (Allende and Izaguirre 2003). In all these surveys, the identifications of the small flagellated chrysophyceans were achieved by light microscopy that permitted only to distinguish amongst different morphotypes. Traditionally, the taxonomy of this group was based on the number of flagella, life forms and cycles and other morphological features (e.g. Nicholls and Wujek 2003; Kristiansen 2005), but after the introduction of molecular techniques, the classification experienced important changes. All non-scaled heterotrophic forms were initially named *Spumella*; this genus being paraphyletic, it became eventually splitted into several monophyletic taxa (Grossmann et al. 2016). Forms possessing siliceous scales formed a monophyletic group containing, amongst others, genera *Paraphysomonas* and *Clathromonas* (Scoble and Cavalier-Smith 2014). Altogether, phototrophic, mixotrophic and strictly phagotrophic forms were incorporated within an enlarged monophyletic class Chrysomonadea (Boenigk et al. 2005; Cavalier-Smith and Chao 2006). In addition, the internal classification based on flagella numbers had to be revised (Andersen 2007 and cites therein). A survey of environmental sequences from marine and freshwater systems, together with all cultured species at that time, provided a framework to establish the phylogenetic diversity of chrysophytes, with the identification of several clades formed only by uncultured species (del Campo and Massana 2011).

Our initial molecular study of the nanoplankton assemblages in lakes from Hope Bay based on a fingerprinting technique of the 18S rRNA gene diversity (denaturing gradient gel electrophoresis—DGGE) allowed to identify the dominant taxa, all belonging to Chrysophyceae, which were represented by five different band positions in the DGGE, corresponding to the five morphotypes previously identified by microscopy (Unrein et al. 2005). Given the dominance of this group in the nanoplankton of the oligotrophic food webs of oligotrophic Antarctic lakes, we decided to study their diversity in further detail using a polyphasic approach: we combined microscopy and ecological information obtained during successive field studies, with two different molecular techniques, one allowing the retrieval of long sequences rich in phylogenetic information (clone libraries) and the other, 18S short Illumina reads, with a more complete covering of biodiversity.

We also analyzed the ecological traits and functional diversity of the group based on two phytoplankton functional classifications used globally (Reynolds et al. 2002; Kruk et al. 2010). Moreover, in order to determine the trophic strategy of the dominant chrysophytes, we performed short-term *in situ* ingestion experiments with labelled bacteria. Combining these approaches, we described the biodiversity and the main putative ecological traits of the Chrysophyceae that inhabit the phytoplankton communities in the lakes of the Antarctic Peninsula.

Material and methods

Studied lakes and sampling

The studied lakes are located in Hope Bay, Antarctic Peninsula, (63°23′S, 56° 59′W), which is included in the Maritime Antarctic Region (Fig. 1). As in other areas of this

region, the climate is cold moist maritime (Lewis Smith 1984). Some of the lakes are influenced by the presence of seabirds, particularly penguins. A complete description of the studied area and their lakes was presented in previous papers (e.g. Izaguirre et al. 1998; Unrein et al. 2005; Allende and Pizarro 2006). Six lakes were sampled in this region during 9 consecutive summer campaigns from 1991 to 1999, and in the austral summers 2003, 2004 and 2007: Boeckella, Esperanza, Flora, Encantado, Chico and Pingüi. Each lake was sampled at least twice each summer (mostly in January and February). In our previous published studies, we reported limnological information of the lakes and their phytoplankton communities (e.g. Izaguirre et al. 1998, 2003; Allende and Izaguirre 2003), and in this paper we include new data obtained in subsequent campaigns (2003, 2004 and 2007), in order to cover a longer period of time and thus provide ranges of the main characteristics of the lakes based on a larger data set. Table 1 summarizes the historical physical and chemical data obtained over the whole period of studies, as well as the geographical location of the lakes and their main morphometric features. The methodology employed for the measurements of the different physical and chemical variables was fully described in previous publications (e.g. Izaguirre et al. 1998, 2003; Unrein et al. 2005; Schiaffino et al. 2011).

Phytoplankton samples for microscope analyses

Samples for qualitative and quantitative phytoplankton analyses were taken from the subsurface layer (about 50 cm

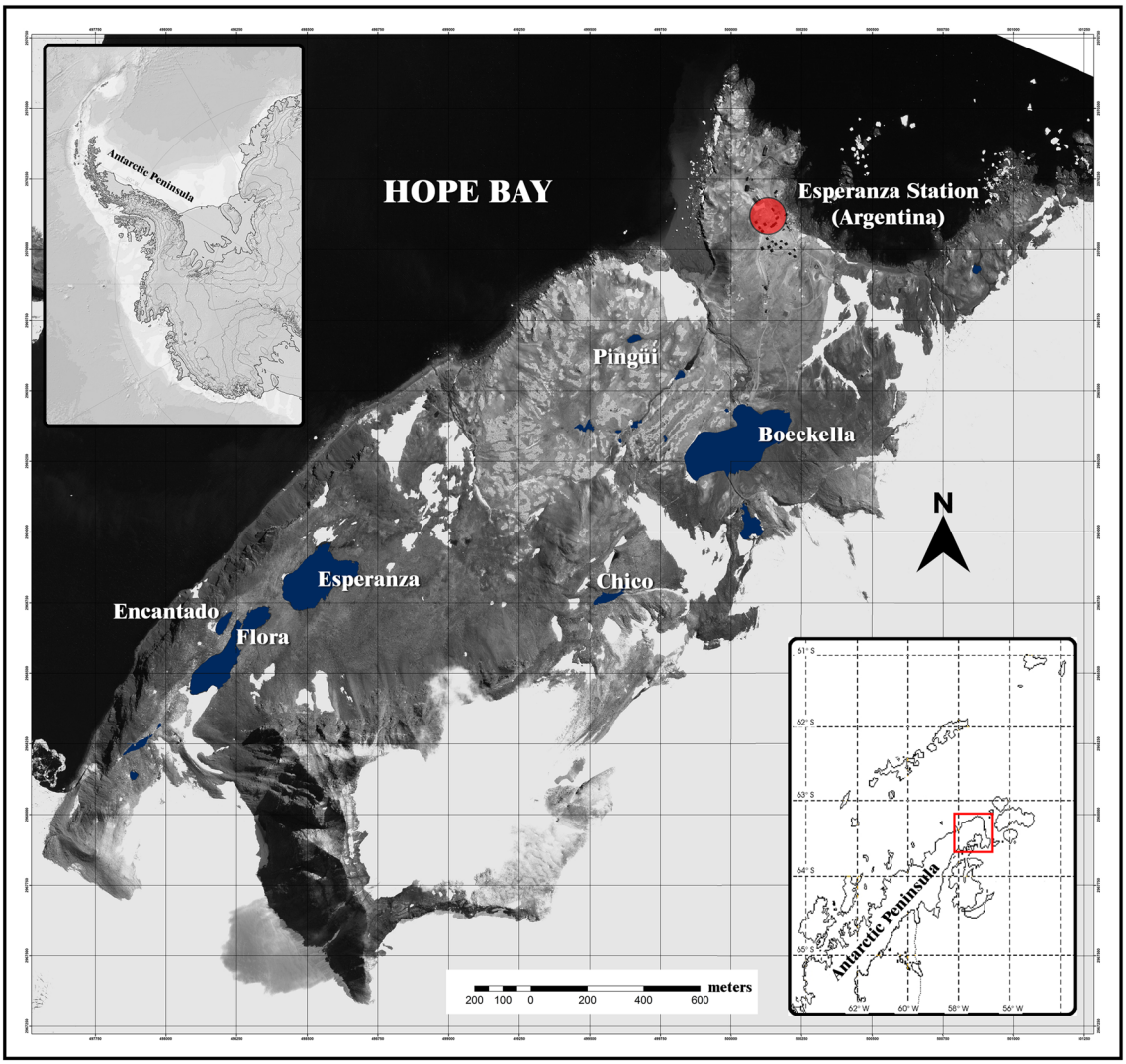

Fig. 1 Geographic location of the surveyed lakes at Hope Bay (Antarctic Peninsula). Sampled lakes: Boeckella, Esperanza, Flora, Encantado, Chico and Pingüi (unoficial names). Map redrawn from a base map kindly provided by Dr. Thiago T. C. Pereira (State University of Minas Gerais), published in Schaefer et al. (2015)

Table 1 Characteristics of the studied lakes and historical data of the main limnological features

	Lakes					
	Esperanza	Flora	Enscantado	Chico	Boeckella	Pingüi
Geographical coordinates	63.41 S; 57.0 W	63.41 S; 57.0 W	63.41 S; 57.0 W	63.41 S; 56.0 W	63.40 S; 57.0 W	63.40 S; 57.0 W
Surface Area (m^2)	27,500	6250	2500	2972	67,454	1540
Maximum depth (m)	~5	~7	~2	5.5	4	<1
Distance to the sea (m)	300	270	250	1100	650	450
Altitude (m)	52	52	55	100	49	20
Trophic condition	oligotrophic	oligotrophic	oligotrophic	oligo-mesotrophic	meso-eutrophic	hypertrophic
pH	6.20 (0.55)	6.05 (0.39)	6.13 (0.38)	6.85 (0.58)	6.57 (0.49)	7.41 (0.35)
Water temperature in Austral summer (° C)	2.96 (3.39)	1.87 (1.83)	2.62 (3.40)	0.32 (0.59)	2.44 (1.40)	5.04 (4.35)
Conductivity (µS cm^{-1})	36.84 (25.89)	24.96 (13.19)	128.0 (124.34)	52 (36)	64 (19)	2016 (1236)
P-PO_4 (mg L^{-1})	0.057 (0.113)	0.011 (0.01)	0.022 (0.045)	0.112 (0.114)	0.899 (0.391)	25.74 (32.76)
Dissolved inorganic nitrogen (mg L^{-1})	0.141 (0.103)	0.121 (0.044)		0.361 (0.314)	3.199 (2.902)	108.25 (95.98)
Chl *a* (ug L^{-1})	0.32 (0.31)	0.69 (0.61)	0.77 (0.48)	1.93 (3.29)	15.33 (9.94)	106.59 (162.48)

Morphometric data obtained from Drago and Paira (1987), Izaguirre et al. (1998). Mean values of the physical and chemical variables and standard deviation (between brackets) were calculated from own data obtained in different Austral summer campaigns since 1991 until 2007

depth) in each lake. Living specimens were examined at the Antarctic Esperanza station using light microscopy for their identification based on morphology and consulting specialized taxonomical literature for each algal group (e.g. Ettl 1983; Komárek and Fott 1983; Starmach 1985; Komárek and Anagnostidis 1999, 2005). Samples for quantitative analyses were fixed with 1% acidified Lugol's iodine solution, and counts were performed with an inverted microscope following the Utermöhl (1958) method. After 1998, samples for the quantification of pico and nanoplankton by epifluorescence were also collected and fixed with filtered 10% cold glutaraldehyde (1% final concentration) and immediately filtered through 0.2 µm and 0.6-µm pore-size polycarbonate filters, respectively. Counts were carried out in Zeiss Axioplan or Olympus BX40 microscopes following the procedure described in previous publications (e.g. Allende and Pizarro 2006; Izaguirre et al. 2016). Specific biovolumes were calculated using appropriate geometric formulae according to Hillebrand et al. (1999).

Grazing experiments

To evaluate the phagotrophic behavior of the dominant chrysophytes, during January and February 2004 we carried out in *in situ* grazing experiments using fluorescently labelled bacteria (FLB) as tracers. FLBs were prepared from a culture of *Brevundimonas diminuta* (syn. *Pseudomonas diminuta*) following the technique detailed in Unrein et al. (2007). For each experiment we collected 2.5 L of lake water in an acid-washed plastic bottles thoroughly rinsed with distilled water. Water samples were previously filtered through a 55-µm zooplankton net to exclude zooplankton. Experiments were run in triplicate and the bottles were incubated *in situ* in each lake 30 cm beneath the water surface. The concentration of FLB used was about 20% of the natural abundance of heterotrophic bacteria *in situ*. Subsamples of each bottle were taken at three times: t0 (immediately after the addition of the FLB tracers); t1 (1 h after addition) and t2 (after 2 h). From each bottle 100 ml were fixed with pre-filtered cold glutaraldehyde at 1% final concentration. Then, 30 ml to 80 ml of each fixed experimental sample were filtered through 0.8-µm pore-size polycarbonate black filter (Millipore), and examined at 1000 × magnification under an epifluorescence microscope. Chrysophyceae were identified by their morphology and by their red autofluorescence (Chlorophyll) under blue light excitation, that are easily differentiated from that of the other abundant flagellates *Chlamydomonas* (Chlorophyceae) and *Pseudopedinella* (Dictyochophyceae). Tracer particles (FLB) within each organism were enumerated at the same time. More details of the experiments performed were published in Gerea et al. (2016).

Molecular biodiversity analyses

We sampled each of the six lakes during the austral summers 2003 and 2004 (in January and February) for molecular analyses. Samples were collected at the upper layer of the water column (about 50 cm depth). Depending on the trophic status of the lake, between 1 L and 2.5 L of sample were first filtered through a 50-µm net to remove zooplankton, and then sequentially filtered through 20 µm, 3 µm and 0.2-µm

pore-size polycarbonate filters for community DNA extractions. For molecular analyses we used the filters of 3 µm and 0.2-µm pore-size that should retain nano- and picoplankton. Details of the procedures for DNA extraction (phenol/chloroform) and touchdown polymerase chain reaction (PCR) amplifications were published in Unrein et al. (2005). Previously to the molecular sequencing analyses we run several DGGE analyses comparing the band patterns corresponding to the filters of 3 µm and 0.2-µm pore-size, that proved a very similar band pattern for both filters. The methodology of the DGGE was fully detailed in Unrein et al. (2005). Photographs of two gels obtained comparing both size fractions are shown in Online Resource 1.

Clone libraries were carried out for two selected lakes (Boeckella and Esperanza) that exhibit contrasting trophic status (meso-eutrophic and oligotrophic, respectively); this selection was made based on the results obtained in the DGGE patterns. For each lake we mixed DNA samples collected in the austral summers of 2003 and 2004, using the 3 pore-size polycarbonate filters. The two clone libraries, one for each lake (named Boeck and Esp), were performed following the procedure described by Massana et al. (2004). The 18S rRNA gene was amplified by PCR using 10 ng of DNA template and the primers Euk1F (5′- AAC CTGGTTGATCCTGCCAGT- 3′) and EukR (5′- TGATCC TTCTGCAGGTTCACCTAC- 3′) as described by Medlin et al. (1988). The amplified rRNA gene product (50 µl) from three PCR individual reactions was pooled and cleaned using Qiagen PCR purification kit and subsequently cloned with TOPO TA cloning kit (Invitrogen) according to manufacturer's instructions. Ninety-six putative positive colonies were picked and transferred to a multi-well plate with LB medium and 7% glycerol and stored at −80 °C. The presence of the 18S rDNA insert was checked in fifty colonies per sample by PCR with the same primers and using 1 µl of the culture as template. The colonies having the insert (34 clones for Boeck and 37 clones for Esp) were chosen for partial sequencing using the internal primer Euk528f (Elwood et al. 1985) at the Macrogen Europe sequencing services. Sequences were subjected to a BLAST search (Altschul et al. 1997) to obtain a first indication of their phylogenetic affiliation. Based on these results, 5 clones from Boeck and 7 clones from Esp that affiliated to Chrysophyceae were selected for complete sequencing of the 18S rRNA gene using a combination of internal primers. Sequences from the separate partial reactions were overlapped using software Geneious (Biomatters), manually inspected, and yielded a resulting alignment of 1700 bp to 1800 bp. The consensus sequence for each clone was submitted again to a BLAST search for phylogenetic assignment. For the selection of the clones, we checked which sequences were repeated in the library.

In order to generate a global picture of the eukaryotic diversity in the six water bodies, we also applied a metabarcoding approach (Illumina HiSeq technology) to explore the diversity of the Chrysophyceae. In this case we analyzed the DNA obtained from the 0.2 pore-size filters. We amplified extracted DNA using primers specific to the V9 variable region of the 18S rRNA gene (135 bp) using the protocol as in Amaral-Zettler et al. (2009) and adapted after Lara et al. (2015). Sequencing was carried by the Fasteris company (Geneva, Switzerland) using Illumina HiSeq 2500 technology.

Quality check (removal of reads without perfect forward and reverse primers, chimera removal and phred score filtering) of the sequences was performed following the pipeline used in Schiaffino et al. (2016) and adapted after de Vargas et al. (2015). Sequences were then clustered into OTUs (operational taxonomic units) using the SWARM algorithm (V2, Mahé et al. 2014). We randomly selected 14,437 sequences from each lake, which corresponds to the total number of sequences in the sample with the lowest number of reads (i.e. lake Pingui). Then, the affiliation of the OTUs was done using the software GGSearch (McWilliam et al. 2013) against the curated ribosomal eukaryotic database PR^2 (Guillou et al. 2013). We extracted the Chrysophyceae, and the taxonomic affiliation of each Swarm OTU was verified against the NCBI database.

To determine if the sequencing depth was enough to cover the major diversity of the Chrysophyceae, we performed rarefaction curves with the rarefaction function of the R vegan package (v.2–3, 2).

Complete rDNA sequences were aligned with MAFFT v6.903b (Katoh et al. 2002) and Maximum likelihood trees were done using RAxML (v7.2.8; Stamatakis 2006) with the GTR + G evolutionary model under rapid hill climbing mode. Alternative trees were run to select the best likelihood tree (out of 1000 trees) and bootstrap was calculated with 1000 pseudoreplicates. Bootstrap values were added to the best tree with RAxML. The shorter Illumina sequences were added to the original tree using the EPA (Evolutionary Placement Algorithm) algorithm (Berger et al. 2011).

Results

Microscopical analyses

Phytoplankton quantitative analyses performed by inverted microscopy showed a high contribution of Chrysophyceae to total phytoplankton biovolume in all lakes, except in the hypertrophic pond Pingüi, varying between 59% and 96%, with the highest values in the most oligotrophic lakes (Fig. 2). Mean total phytoplankton abundances for the field campaigns 2003 and 2004 ranged from 2930 ind. ml^{-1} to

12,066 ind. ml^{-1}, with mean Chrysophyceae abundances between 954 ind. ml^{-1} and 5093 ind. ml^{-1}. In terms of biovolume, the average values of pigmented Chrysophyceae ranged from 19,213 ind. ml^{-1} to 98,362 μm^3 ml^{-1}. *Pseudopedinella* spp. (Dictyochophyceae) was also well represented in the most oligotrophic lakes (biovolumes 172–26,280 μm^3 ml^{-1}). Green algae, represented mainly by *Chlamydomonas* spp. (Volvocales), were more abundant in the mesotrophic and eutrophic water bodies, attaining very high biovolumes in Pingüi pond (1.19×10^6 μm^3 ml^{-1}). Using light microscopy examinations only 7 different chrysophycean morphotypes (differing in shape and in the presence of 1 or 2 visible flagella) were identified in the six lakes; all had small sizes (2–10 µm), and in almost all samples (92%) organisms of 3–5 µm constituted more than 90% of the total chrysophytes. Two morphotypes were attributed to *Ochromonas*-like species and the others remained as unidentified Chrysophyceae. One of the most abundant morphotypes was a small species (3–4 µm) with one visible flagellum, whose abundance varied from 100 ind. ml^{-1} to 9770 ind. ml^{-1}, whereas one of the *Ochromonas*-like species (3–5 µm) was also relatively abundant, varying between 140 ind. ml^{-1} and 9120 ind. ml^{-1}. In some lakes and on some sampling dates, we also recorded a relatively high abundance of Chrysophyceae cysts.

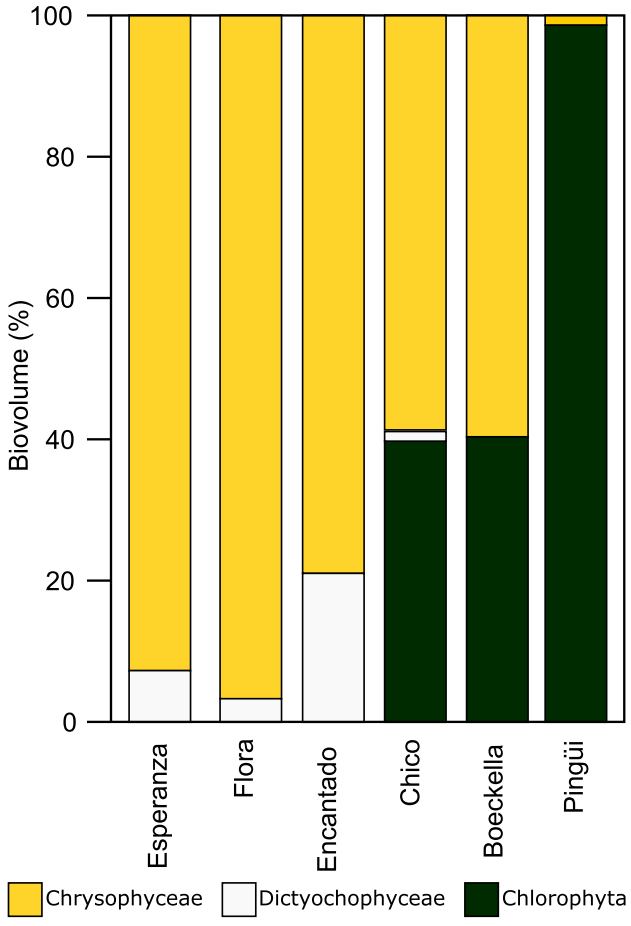

Fig. 2 Contribution to total phytoplankton biovolume by different phytoplankton groups in the studied lakes. Average data corresponding to campaigns 2003 and 2004 ($n = 4$)

We classified the dominant phytoplankton taxa following two widely used functional classifications. According to the scheme proposed by Reynolds et al. (2002), the oligotrophic lakes of Hope Bay are dominated by the functional group or "codon" X3 that includes chrysophytes with tolerance to low nutrient content. Following the classification of Kruk et al. (2010), the algal assemblages are dominated by the morphology-based functional group (MBFG) II, which is represented by species of small size, with flagella and siliceous exoskeletal structures (resting cells). On the other hand, in lakes of a higher trophic status (lakes Boeckella and Pingüi) the phytoplankton assemblages include also a relatively high proportion of green volvocalean algae (*Chlamydomonas* spp.), which belong to codon X2 (according to Reynolds et al. 2002) and to MBFG V (according to Kruk et al. 2010).

Trophic mode

Grazing experiments performed *in situ* using FLB as prey allowed to confirm the phagotrophic behavior of the most abundant pigmented Chrysophyceae species in all lakes, except Pingüi. Accordingly, ingested FLBs were observed inside the previously characterized dominant morphotypes (Fig. 3 shows an example of *Ochromonas*-like cells). However, it was not possible to calculate reliable grazing rates due the low proportion of cells with ingested FLBs, and the low abundances of some morphotypes and ambiguous identification. With the experiments performed we also proved the phagotrophic activity of the mixotrophic algae *Pseudopedinella,* belonging to Dictyochophyceae, which was previously published (Gerea et al. 2016).

Molecular biodiversity analyses

Twelve 18S rRNA gene complete sequences corresponding to different taxa of Chrysophyceae were obtained in the clone libraries of the two selected Antarctic lakes (Boeckella and Esperanza) and have been deposited in the GenBank database with accession numbers MG674904 to MG674915 (Table 2). Five of them showed the closest culture match with *Hydrurus foetidus,* two with Chrysophyceae sp. CCMP2296 and two with *Mallomonas annulata*. The other three sequences showed closest matches with *Ochromonas tuberculata*, *Ochromonas* sp. CCMP1899 and *Spumella*-like JBNZ40 (Table 2). Environmental sequences yielding higher similarity were also found (such as clone Ant_26a that was always closer than *H. foetidus*), but the similarity increase

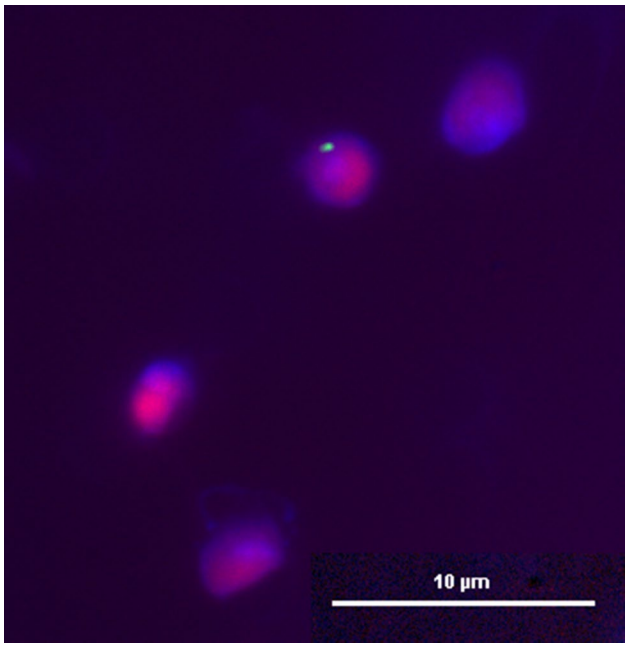

Fig. 3 Epifluorescence micrograph of Antarctic Chrysophyceae (*Ochromonas*-like sp.) with an ingested fluorescently labelled bacteria (FLB). The image is an overlay of the cells observed under UV radiation showing the nucleus in blue after DAPI staining, and the chloroplast in read and the FLB in yellow-green under blue light excitation. The photograph corresponded to the experiment carried out in situ after 2 h hours of incubation in the oligotrophic lake Esperanza (Hope Bay) in the austral summer 2004

was moderate, implying that the sequences obtained here were indeed new and not yet sampled in other environmental surveys. Our complete sequences were added to a tree of Chrysophyceae 18S rDNA sequences to visualize the relationships amongst them (Fig. 4). According to the last phylogenetic reconstruction of the group (del Campo and Massana 2011) most clones affiliated to Clade D, whilst four clones retrieved from Lake Esperanza were related to other clades (2 with Clade B2, 1 with Clade E and 1 with Clade F).

A total of 44,861 reads assigned Chrysophyceae were recovered from all the studied lakes by Illumina HiSeq, and clustered in 81 different Swarm OTUs (Online Resource 2), with a range in per lake OTU number ranging from 24 to 58. As depicted by the rarefaction curves performed for each water body (Fig. 5), the sequencing coverage was adequate since Swarm OTUs richness tended to reach a plateau; this analysis was carried out excluding Pingüi pond because this hypertrophic water body presented an extremely low richness and relative abundance of Chrysophyceae sequences (0.9% of the sequences). Nine Swarm OTUs were dominant considering their number of reads and, except in the hypertrophic pond Pingüi, which accounted from 86% to 99% of the total chrysophytes read abundance (Fig. 6).

The short Illumina reads were incorporated into the phylogenetic tree of Chrysophyceae shown in Fig. 4 using the Evolutionary Placement Algorithm (Online Resource 3). We observed a good correspondence between clone libraries and short sequence metabarcoding analyses, since most of the Illumina OTUs with high number of reads corresponded to the same clades than the retrieved clones. Clade D, with 21 OTUs and accounting for about 72% of the total reads, was the dominant one. Indeed, 4 of the 9 dominant OTUs belonged to this Clade D. Amongst them, OTU 5 (97.8% similar to clone Esp29) presented a high number of reads in all lakes except in Pingüi, whilst OTUs 16 (99.3% similar to clone Esp21) and 2 (identical to Esp16) were also generally important in all lakes except Pingüi. Clade B was the second in importance: subclade B2 included 20 OTUs and 13% of the signal, whilst B1 included a single OTU and 7% of the reads. Three OTUs within clade B were amongst the most dominant, and two of them were virtually identical to sequenced clones: 3 was 99.3% similar to Esp10, and 29 was identical to Esp17. The remaining clades detected by Illumina accounted for a smaller fraction of reads (less than 3.5% each), even though they contributed substantially to the number of OTUs: 22 in clade C, 13 in clade E, 3 in clade F and one in clade A. Two of the dominant OTUs belonged to clades E (21, identical to Esp24 clone) and C (148) (Fig. 6 and Online Resource 2).

Discussion

Chrysophyceae biodiversity

The combination of different methods used throughout several years of studies of Hope Bay's lakes allowed us to extensively characterize the biodiversity and ecology of the Chrysophyceae, one of the dominant phytoplankton groups in the oligotrophic ecosystems of this region. The present study increased the retrieved biodiversity of Chrysophyceae; with the use of the two molecular techniques 12 different OTUs were obtained in the clone libraries, whereas 81 different Swarm OTUs were recovered by Illumina HTS. These results constitute a significant advance in the knowledge of the biodiversity of this algal group in the studied Antarctic lakes, since our first molecular studies using DGGE had revealed only five dominant Chrysophyceae OTUs, in coincidence with the five different morphotypes that had been observed by light microscopy (Unrein et al. 2005).

It is important to mention that our previous molecular analyses in these lakes (DGGE) indicated that we recovered very similar band patterns (see Online Resource 1) with both filters used for the DNA extractions (3 µm and 0.2-µm pore-size), as more than 90% of the chrysophycean cells had a size in the range of 2–5 µm as was determined by

Table 2 Phylogenetic affiliation of the sequences retrieved from clone libraries performed with samples of lakes Boeckella (Boeck) and Esperanza (Esp)

Clone name	AN (clone)	Length (bp)	Closest Cultured Match (CCM)	Similarity (%)	AN (CCM)	Source of the CCM	Closest Match (CM)	Source of the CM	Similarity (%)	AN (CM)
Boeck 01	MG674904	1716	*Hydrurus foetidus*	98.8	FM955256	Algal collection at Natural History Museum, University of Oslo, Norway. Source: cold water stream	clone Ant_26a (1580 bp)	Seasonal snowdrift in Antarctica	99.9	HE820739
Boeck 02	MG674905	1725	*Hydrurus foetidus*	97.7	FM955256	Algal collection at Natural History Museum, University of Oslo, Norway. Source: cold water stream	clone Ant_26a (1580 bp)	Seasonal snowdrift in Antarctica	98.0	HE820739
Boeck 04	MG674906	1715	Chrysophyceae sp. CCMP2296	99.5	EU247834	National Center for Marine Algae and Microbiota, Bigelow Laboratory, US. Source: Arctic seawater	–	–	–	
Boeck 27	MG674907	1790	*Hydrurus foetidus*	99.0	FM955256	Algal collection at Natural History Museum, University of Oslo, Norway. Source: cold water stream	clone Ant_26a (1580 bp)	Seasonal snowdrift in Antarctica	99.2	HE820739
Boeck 31	MG674908	1790	*Hydrurus foetidus*	98.7	FM955256	Algal collection at Natural History Museum, University of Oslo, Norway. Source: cold water stream	clone Ant_26a (1580 bp)	Seasonal snowdrift in Antarctica	99.1	HE820739
Esp 10	MG674909	1719	*Mallomonas annulata*	94.9	KM817862	National Center for Marine Algae and Microbiota, Bigelow Laboratory, US. Source: European lake	clone SA2_2F3 (1552 bp)	Seawater in the Framvaren Fjord (Norway)	95.2	EF527170
Esp 16	MG674910	1708	Chrysophyceae sp. CCMP2296	97.5	EU247834	National Center for Marine Algae and Microbiota, Bigelow Laboratory, US. Source: Arctic seawater	clone LG18-10	Oligotrophic lake: Lake George, Adirondack Park (US)	99.2	AY919744

Table 2 (continued)

Clone name	AN (clone)	Length (bp)	Closest Cultured Match (CCM)	Similarity (%)	AN (CCM)	Source of the CCM	Closest Match (CM)	Source of the CM	Similarity (%)	AN (CM)
Esp 17	MG674911	1726	*Mallomonas annulata*	95.7	KM817862	National Center for Marine Algae and Microbiota, Bigelow Laboratory, US. Source: European lake	–	–	–	
Esp 21	MG674912	1789	*Hydrurus foetidus*	98.8	FM955256	Algal collection at Natural History Museum, University of Oslo, Norway. Source: cold water stream	clone Ant_26a (1580 bp)	Seasonal snowdrift in Antarctica	98.9	HE820739
Esp 24	MG674913	1783	*Ochromonas tuberculata*	96.7	AF123293	The Culture Collection of Algae and Protozoa, Scottish Association for Marine Science	clone Ch8A2mF9 (1683 bp)	High Arctic lake: Char Lake (Canada)	97.4	JF730788
Esp 29	MG674914	1713	*Ochromonas* sp. CCMP1899	99.9	EF165133	National Center for Marine Algae and Microbiota, Bigelow Laboratory, US. Source: Antarctic seawater	–		–	
Esp 38	MG674915	1780	*Spumella*-like JBNZ40	98.6	AY651091	Small lake near Mandeville, New Zealand	clone 270C03	Columbia River coastal margin (US)	98.7	KJ925238

AN Accession number, *CM* closest match (when larger than the closest cultured match CCM)

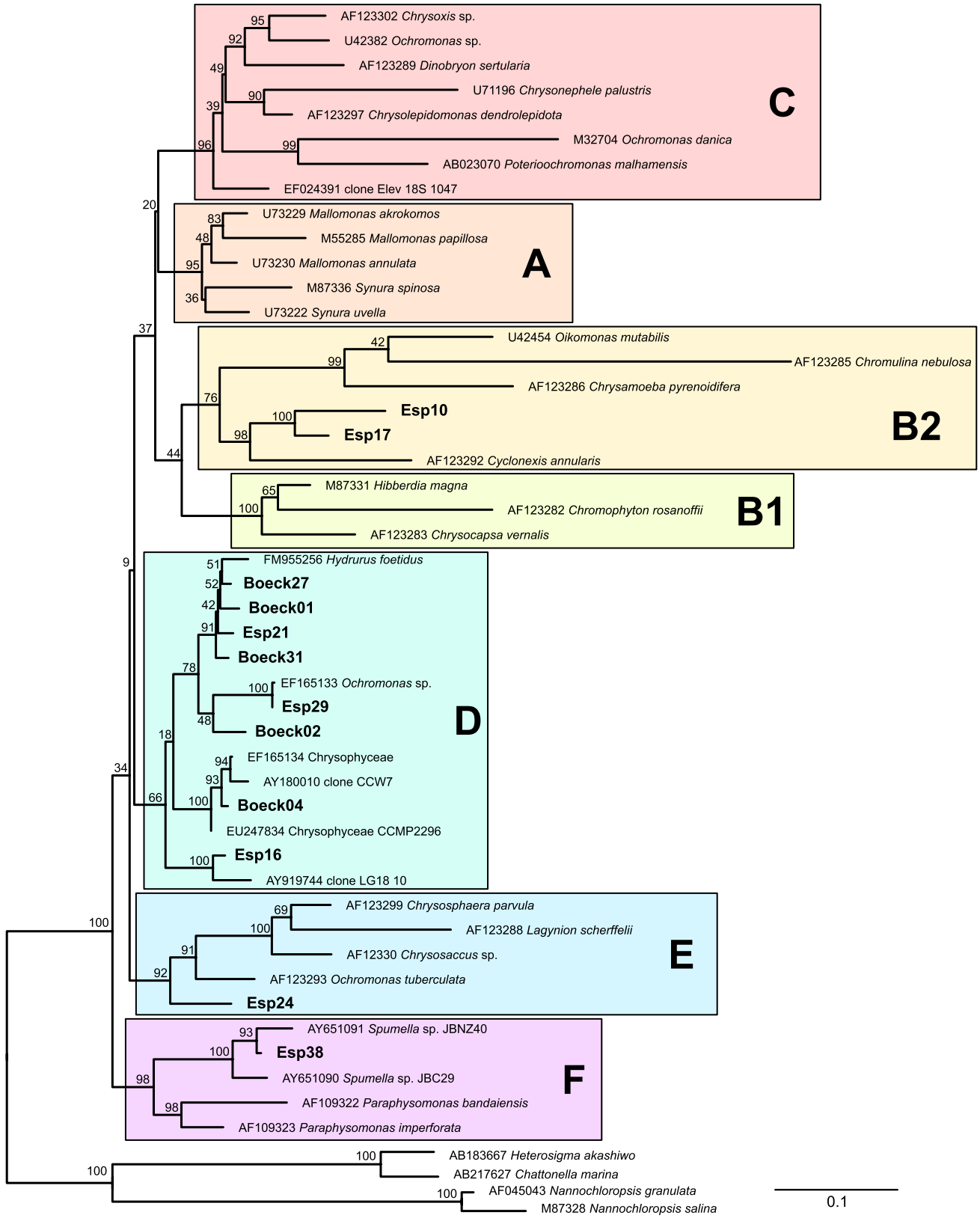

Fig. 4 Phylogenetic tree constructed by maximum-likelihood with complete 18S rDNA sequences including the 12 clones of Chrysophyceae from lakes Boeckella (Boeck) and Esperanza (Esp) (indicated in bold) together with reference sequences. Labelling of the clades as proposed by del Campo and Massana (2011)

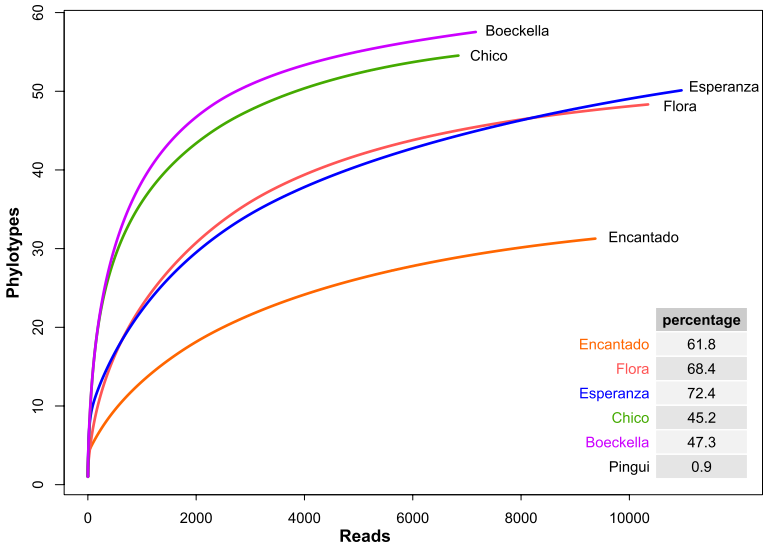

Fig. 5 Rarefaction curves for each water body using Chrysophyceae sequences from the Illumina HiSeq dataset. Percentages correspond to the proportion of Chrysophyceae in all 18S rRNA reads

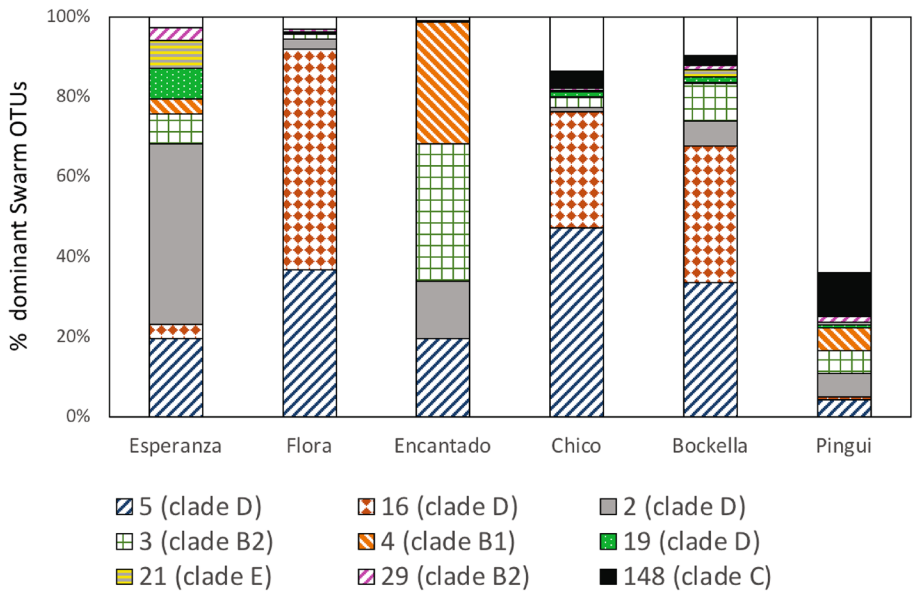

Fig. 6 Percentage of the nine dominant Swarm OTUs belonging to Chrysophyceae obtained by Illumina HiSeq in the six studied lakes

our microscopical examinations. Moreover, it is well known that the sequential filtration process is not very effective to separate fractions because it allows the passage of cells larger than their nominal pore sizes (and fragments), and can lead to the retention of smaller cells when filters are clogged (Díez et al. 2001; Schiaffino et al. 2016). This was especially true in our case, since the dominant chrysophytes in these lakes have virtually the same size than the pore size used to separate pico and nanoplankton.

Our phylogenetic analysis based on clone libraries showed that most of the clones obtained from Hope Bay lakes could be assigned to Clade D of the phylogenetic reconstruction published by del Campo and Massana (2011). In line, the most abundant OTUs retrieved by Illumina HiSeq also corresponded to this clade, which includes mostly pigmented species, but also some heterotrophs (Boenigk et al. 2005).

Five sequences of the clone library (Boeck01, Boeck02, Boeck27, Boeck31 and Esp21) were closely related (similarities between 97.7% and 99.0%) to *Hydrurus foetidus* (and these were a bit closer to the environmental clone Ant_26a obtained from seasonal snowdrift in Antarctica). Interestingly, related *Hydrurus* spp. morphotypes had been reported as very abundant in one of our previous studies based on microscope examinations conducted in a lotic ecosystem in Hope Bay, Maritime Antarctica (Izaguirre and Pizarro 1998), but in the epilithon community, where they usually formed macroscopic thalli. Nevertheless, this species can release swimming spores, a phenomenon that can easily

be induced by disturbances (Klaveness 2017). A molecular analysis of 18S rDNA sequences carried out by Remias et al. (2013) revealed that polar *Ochromonas*-like populations from snowfields collected in Maritime Antarctica and the High Arctic shared an unexpected close phylogenetic relatedness with *H. foetidus*, suggesting a large morphological variability in that morphospecies. Klaveness et al. (2011) performed phylogenetic analyses of 18S and 28S rRNA genes to elucidate the position of *H. foetidus* and placed this taxon in a well-defined clade (namely the *Hydrurus*-clade).

Two clones found in our library (Boeck04 and Esp16) presented the closest cultured match with Chrysophyceae sp. CCMP2296 (99.5% and 97.5%), originating from Arctic seawater, and also showed highest closest match with the environmental clone LG18-10 found in an oligotrophic lake. Two other clones (Esp10 and Esp17) showed the closest cultured match with *Mallomonas annulata,* although with relatively low percentage of similarity (95.7% and 94.9%); according to the phylogenetic reconstruction of del Campo and Massana (2011), these clones would be placed within Clade B2. Clone Esp24, moderately related to *Ochromonas tuberculata* (96.7% similarity), was associated with Clade E; in three high Arctic lakes, Charvet et al. (2012) also found some sequences similar to this species, and also reported a large dominance of chrysophytes in the protist communities of these lakes. Clone Esp29 was almost identical (99.9%) to *Ochromonas* sp. CCMP1899, whose source was Antarctic seawater, and it is associated with clade D.

Although most of the Swarm OTUs obtained by Illumina HiSeq were associated to a few clades (mainly Clade D, followed by Clade B2), the other OTUs are distributed within several other clades (Online Resources 2 and 3), but were poorly represented according to the number of reads. The most abundant OTUs can be considered as the "core chrysophyte species" whereas those poorly represented (infrequent and with low abundance) would belong to the "rare or occasional" biosphere (Magurran and Henderson 2003). As discussed by Schiaffino et al. (2016), these two components are not independent from each other, and rare taxa can become core species when the environmental conditions become favourable. As in other studies (e.g. Nolte et al. 2010; Debroas et al. 2015; Schiaffino et al. 2016; Velasco-González et al. 2020), the rare OTUs obtained by Illumina HiSeq represented a high proportion of total richness.

Considering both the core and the rare species, our studies show that chrysophytes are considerably diverse in the Antarctic lakes from this region. Indeed, the high throughput sequences cover all the chrysophytes clades described in del Campo and Massana (2011). In general, the biodiversity of polar limnetic systems had been regarded as low due to the extreme climatic conditions and to the isolation of the Antarctic continent (Ellis-Evans 1996; Wall and Virginia 1999; Vincent 2000). However, Pearce and Galand (2008) pointed out that the extent of the biodiversity uncovered is most often determined by the sample effort and, as many more studies appear, a relatively higher microbial biodiversity becomes apparent. In addition, the recovered biodiversity is also dependent on the methodological approaches used as demonstrated here for chrysophytes in the comparison between DGGE bands (5), microscopy (5–7 morphotypes), clones (12) and high throughput sequencing (81).

Ecological insights

The phytoplankton analyses of the samples collected in 2003 and 2004 (data published here) confirmed the clear dominance of Chrysophyceae in the algal assemblages of the oligotrophic lakes of Hope Bay. Throughout more than 15 years of studies in the lakes of this region during the austral summers we also observed peaks of members of this group in some particular occasions; e.g. *Ochromonas*-like species can achieve great abundances under the ice-cover before melting (Izaguirre et al. 1993, 2003; Allende and Izaguirre 2003). Usually, these peaks were not accompanied by high concentrations of chlorophyll-a (Izaguirre et al. 1996), probably because in darkness *Ochromonas* reduce their chloroplasts considerably (Kristiansen 2005). Our previous studies also indicated that in the meso-eutrophic lake Boeckella a summer succession of chrysophytes and chlorophytes (volvocaleans) occurred, with prevalence of chrysophytes at the beginning and at the end of the austral summer when the lake usually is covered with ice, and dominance of chlorophytes during the open water season (Allende and Izaguirre 2003). The only water body that contained few chrysophytes both in terms of diversity and biomass was the hypertrophic pond Pingüi. In general, flagellated algae, including Chrysophyceae, Chlorophyceae, Cryptophyceae and Prasinophyceae have been reported to be very abundant in many Antarctic lakes (Laybourn-Parry and Pearce 2007; Butler 1999; Mataloni et al. 2000; McKnight et al. 2000; Izaguirre et al. 2001; Marshall and Laybourn-Parry 2002; Bell and Laybourn-Parry 2003). Motility, pigment adaptation to low light intensity and mixotrophy are adaptive advantages that allow them to dominate the plankton community of Antarctic freshwater ecosystems (Priddle et al. 1986). A phytoplankton community dominated by chrysophytes has largely been documented as typical of lakes with a combination of cold summer water temperatures, low or moderate productivity, low nutrient availability, low alkalinity and conductivity and neutral or slightly acidic pH (Sandgren 1988 and cites therein), conditions that coincide with those of the Antarctic lakes from Hope Bay. Similarly, chrysophytes were very abundant and diversified in another type of oligotrophic environment, such as *Sphagnum* bogs, as shown in a recent study (Singer et al. 2019). Chrysophytes have also been reported to be well adapted to low

light intensities and being highly versatile in relation to their nutrition type (Kristiansen 2005).

Our grazing experiments with FLB showed that the dominant planktonic chrysophytes ingested bacteria (Fig. 3), confirming their mixotrophic behavior. A previous study carried out in the same Antarctic lakes (Gerea et al. 2013), which was based on the analysis of the digestive vacuole content by CARD-FISH (catalyzed reporter deposition-fluorescent *in situ* hybridization), evidenced some selectivity by *Ochromonas*-like cells and *Pseudopedinella* sp. (Dictyochophyceae) over particular groups of heterotrophic prokaryote phylotypes. Most pigmented chrysophytes are actually mixotrophic, capable of ingesting bacteria (Sanders and Porter 1988; Schmidtke et al. 2006; Unrein et al. 2010; Saad et al. 2016); phagotrophy contributing in a variable but relatively high extent to their growth (e.g. Sanders et al. 1990; Olrik 1998; Rottberger et al. 2013; Wilken et al. 2014). Although grazing rates could not be inferred from our results, the *in situ* cell-specific grazing rates assembled from different published studies (Table 3) indicate that mixotrophic chrysophytes have rates of grazing comparable to those of purely heterotrophic flagellates. Therefore, at the ecosystem level, the grazing impact on the bacterial community can be high, particularly in oligotrophic systems (e.g. Bird and Kalff 1987; Unrein et al. 2010). Besides the potential of being phagotrophic, the Antarctic Chrysophyceae exhibit other relevant morphological and functional traits: they are unicellular flagellated organisms, have usually small sizes (maximum linear dimension generally < 6 µm), require silica for the formation of statospores, can migrate vertically in the water column, and due to their small size have high susceptibility to be grazed by zooplankton (mainly copepods). These attributes correspond to some of the main categories of traits that have been proposed for describing functional diversity in phytoplankton ecology (Weithoff 2003; Litchman and Klausmeier 2008; Litchman et al. 2010). Taking into account their main morphological and functional traits, different functional classifications have been proposed, amongst which that of Reynolds et al. (2002) constitutes a milestone in the definition of phytoplankton functional groups (Salmaso et al. 2015). According to this classification, functional group X3, which includes chrysophytes with tolerance to low nutrient content, dominates in the oligotrophic lakes of the region, whereas taking into account the classification proposed by Kruk et al. (2010) these assemblages fit in the MBFG II category (organisms of small size, with flagella and siliceous exoskeletal structures). Moreover, in the oligotrophic lakes of Hope Bay, chrysophytes are usually accompanied by organisms belonging to the Dictyochophyceae, represented by genus *Pseudopedinella* (Gerea et al. 2016), another mixotrophic flagellate included in the same functional groups. In turn, the mesotrophic and eutrophic lakes (Boeckella and Pingüi, respectively) are dominated by green algae (mainly *Chlamydomonas* spp.) placed in categories X2 (according Reynolds et al. 2002), and MBFG V (according Kruk et al. 2010). Other organisms observed in the studied lakes, like diatoms and cyanobacteria, are mostly benthic species (e.g. Izaguirre et al. 2003). These results

Table 3 Main ecological features of the Chrysophyceae from the studied Antarctic lakes

Feature	Description in chrysophyceae
Morphological and functional traits (main traits proposed by Weithoff 2003; Litchman and Klausmeier 2008)	Flagellated; small sizes (mostly nanoplanktonic forms < 10 µm); unicellular; Si demand for statospores (resting stage cells); phagotrophy; low to moderate sink velocity; high vulnerability to grazing
Basic ecological strategy according Grime's (1977) scheme CSR (Reynolds 2006)	C-strategists (10^{-1}–10^{3} µm^3; highly effective dispersal; *r* selection; opportunistic demographic patterns)
Phytoplankton functional classification (Reynolds et al. 2002 updated by Padisák et al. 2009)	Mainly codon X3 (shallow, well mixed oligotrophic environments; codon X2 (shallow, meso-eutrophic nvironments)
Morphology-based functional groups (MBFG) according to Kruk et al. (2010)	Group II: small flagellated organisms with siliceous structures (in Antarctic species, siliceous-walled cysts)
Máximum linear dimension (MLD)—own data	3–10 µm
Cell biovolumes—own data	7–113 µm^3
Phagotrophy—Cell-specific grazing rates (Bird and Kalff 1986, 1987, 1989; Sanders et al. 1989; Epstein and Shiaris 1992; Havskum and Riemann 1996; Isaksson et al. 1999; Thouvenot et al. 1999; Hitchman and Jones 2000; Domaizon et al. 2003; Laybourn-Parry and Marshall 2003; Pålsson and Granéli 2003; Kamjunke et al. 2007; Unrein et al. 2010; Saad et al. 2016; Gerea et al. 2016)	0–103.8 bact. flag.$^{-1}$ h^{-1} (avg. 9.1 bact. flag.$^{-1}$ h^{-1})
Maximal potential growth rates (Sandgren 1988; Reynolds 1997; Kruk 2010)	0.3–0.9 day^{-1}
Typical habitats (Sandgren 1988; Nicholls and Wujek 2003; Kristiansen 2005)	Soft-water lakes and ponds with moderate productivity; waters with low alkalinity and conductivity; usually pH 6–7; mostly in environments with low nutrient conditions

indicate that the phytoplankton of these Antarctic lakes is characterized by a low ecological functional diversity as summarized in Table 3.

The studies conducted in freshwater lakes of Hope Bay over many years showed that despite the overall species-poor assemblages typical of Antarctic freshwater environments, chrysophytes are diverse in the oligotrophic water bodies of this region. The two molecular methods used here allowed to obtain a greater diversity than that previously registered from microscopy and fingerprinting analyses. However, only a few OTUs were dominant, whilst most of the diversity can be considered as rare species, which suggests that these organisms might have been overlooked by microscopical surveys. Both molecular approaches indicated that most chrysophyte taxa belonged to Clade D. On the other hand, both diversity and abundance dropped drastically in the hypertrophic lake, thus confirming the typical ecological requirements of the group previously discussed. Likewise, the phytoplankton ecological studies carried out in successive summer's campaigns showed that the nanoplankton of the oligotrophic lakes of the region has an important contribution of chrysophytes in terms of abundance, whose main ecological traits allow them to be successful in these Antarctic lakes.

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Declarations

Conflict of interest There are not any conflict of interest.

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