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Unexpected Importance of Potential Parasites in the Composition of the Freshwater Small-Eukaryote Community▽

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The diversity of small eukaryotes (0.2 to 5 μm) in a mesotrophic lake (Lake Bourget) was investigated using 18S rRNA gene library construction and fluorescent in situ hybridization coupled with tyramide signal amplification (TSA-FISH). Samples collected from the epilimnion on two dates were used to extend a data set previously obtained using similar approaches for lakes with a range of trophic types. A high level of diversity was recorded for this system with intermediate trophic status, and the main sequences from Lake Bourget were affiliated with ciliates (maximum, 19% of the operational taxonomic units [OTUs]), cryptophytes (33%), stramenopiles (13.2%), and cercozoa (9%). Although the comparison of TSA-FISH results and clone libraries suggested that the level of Chlorophyceae may have been underestimated using PCR with 18S rRNA primers, heterotrophic organisms dominated the small-eukaryote assemblage. We found that a large fraction of the sequences belonged to potential parasites of freshwater phytoplankton, including sequences affiliated with fungi and Perkinsozoa. On average, these sequences represented 30% of the OTUs (40% of the clones) obtained for each of two dates for Lake Bourget. Our results provide information on lacustrine small-eukaryote diversity and structure, adding to the phylogenetic data available for lakes with various trophic types.

It has been recognized that the eukaryotic component of picoplankton plays a critical role in the functioning of aquatic ecosystems (29, 34). However, numerous members of this community possess very few morphological characteristics that can be used for identification by traditional microscopic methods. Thus, molecular techniques offer a very useful alternative for the elucidation and description of picoeukaryotic plankton diversity in aquatic environments. Numerous studies have revealed new lineages and unexpected diversity of picoeukaryotes in the open ocean (7, 29, 37), coastal areas (35, 46), anaerobic sediments (5), acid rivers (2), and deep-sea vents (11, 30). However, the diversity, distribution, and natural abundance of small-eukaryote taxa in freshwater systems are still poorly known. Nevertheless, a few recent studies have reported high diversity of 18S rRNA sequences in this community (<5 μm) in lakes and general dominance of heterotrophic cells in the small-eukaryote assemblage (25, 27, 45). A salient feature of the first findings is the presence of sequences affiliated with parasitic groups in lakes with different trophic types (25). This suggests that the ecological impact of parasitism may have been underestimated in marine systems (31) and also in freshwater systems. However, the data on lacustrine small-eukaryote diversity are still largely incomplete, and additional investigation of the composition and organization of freshwater small-eukaryote assemblages is essential; in particular, no data are available for mesotrophic lakes.

To draw up an inventory of lacustrine picoplankton (<5 μm) over a full trophic status range (from oligotrophic to hypereutrophic), we set out to extend the currently available data by characterizing the small-eukaryote community in mesotrophic Lake Bourget, using both fluorescent in situ hybridization (FISH) coupled with tyramide signal amplification (TSA) and a cloning-sequencing method. The results enabled us to identify the main phylogenetic groups present on two different sampling dates in the epilimnion of this lake and to describe the structure of the complex assemblage.

MATERIALS AND METHODS

Study site and sampling. The study was conducted in mesotrophic Lake Bourget (45°44′N, 05°51′W; altitude, 231 m), France's largest natural lake. Lake Bourget is a warm, meromictic lake located in eastern France on the edge of the Alps. It suffered from eutrophication until the mid-1980s, before an important program of restoration began. This ecosystem is now considered a mesotrophic lake. The mean annual phosphorus concentration was 28 μg liter^{-1} in 2005. The transparency values (secchi depths) for the two sampling dates in May and August were 9.6 and 5.2 m, respectively, while the concentrations of dissolved total P at a depth of 2 m were 13 and 8 μg/liter. The lake is elongated (18 by 3.5 km) and orientated north-south; it has an area of 42 km^2, a total volume of 3.5 × 10^9 m^3, maximum and average depths of 145 and 80 m, respectively, and a water residence time of approximately 10 years. It has a catchment area of about 560 km^2 with maximum and average altitudes 184 m and 700 m, respectively. Sampling was carried out 2 m below the surface at a permanent station, which is the reference station for water quality surveys and is located in the deepest zone of the water column.

A previous exploratory analysis of small-eukaryote diversity was conducted for the epilimnion of Lake Bourget using terminal restriction fragment length polymorphism (RFLP). Terminal RFLP revealed that the greatest contrasts in the diversity profiles of the small-eukaryote community composition occurred in May (199 terminal restriction fragments) and August (130 terminal restriction fragments) (results not shown). Also, the abundance and composition of biological variables, such as heterotrophic bacteria and picocyanobacteria, flagellates, or ciliates, were also markedly different in May and August (4; S. Personnic and S. Jacquet, unpublished data). We therefore chose to carry out one cloning-sequencing analysis in May (16 May 2005) and one cloning-sequencing analysis in August (10 August 2005).

Between 100 and 120 ml of lake water from the initial water samples was prefiltered through 5-μm-pore-size polycarbonate filters (Millipore) at a pressure of <20 × 10^5 Pa in order to eliminate larger cells. It is well known that whatever the aquatic ecosystem, the prefiltration process allows passage of cells

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TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′–3′)	Source or reference
EUK 1209R	Eukarya	GGG CAT CAC AGA CCT G	13a
NCHLO01	Non-Chlorophyta	GCT CCA CTC CTG GTG GTG	49a
CHLO02	Chlorophyta	CTT CGA GCC CCC AAC TTT	49b
CRYPT13	Cryptophyceae	CGA AAT ATA AAC GGC CCC AAC	Designed by F. Not

larger than the nominal pore sizes and can retain smaller cells if the filters become clogged (7). Although we found that the filtration step slightly lowered the total abundance (about 10 to 15%), it did not modify the relative abundance of different morphotypes (27). The microbial biomass present in the filtrate (<5-μm size fraction) was then collected (pressure, $<100 \times 10^5$ Pa) on 0.2-μm-pore-size polycarbonate filters (Millipore) and stored at −80°C for nucleic acid extraction. In addition, 100 ml from the initial samples was collected and fixed immediately with 4% (final concentration) formaldehyde for counting total bacteria and with 1% glutaraldehyde for protist identification. Samples of the the metazooplankton were obtained from the 0- to 50-m water column and fixed in a sucrose/formaldehyde solution (final concentrations, 6 and 4%, respectively) (42). Microphytoplankton and metazooplankton counts were determined at the INRA Thonon station by J. C. Druart and L. Lainé in the course of water quality monitoring for Lake Bourget.

Molecular analysis. (i) Nucleic acid extraction. Nucleic acids were extracted as described previously by Lefranc et al. (25).

(ii) Eukaryotic rRNA genetic library. Environmental DNA extracts (16 May 2005 and 10 August 2005) were used to construct the 18S rRNA gene clone libraries. The eukaryote-specific primers Ek-82F (GAAACTGCGAATGGC TC), Ek-1F (CTGGTTGATCCTGCCAG), and Ek-1520R (CYGCAGGTTCA CCTAC) were used for PCR amplification (29). Clone libraries were constructed using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) with PCR vector 2.1 according to the manufacturer's instructions.

Clones were randomly picked from different plates. The presence of the target small-subunit rRNA gene insert in positive colonies was checked by PCR amplification using flanking vector primers (M13f and M13r). Amplicons that were the expected size were subsequently digested with restriction enzyme HaeIII, and the resulting RFLP products were separated by electrophoresis in a 2.5% low-melting-point agarose gel (NuSieve) at 60 mV for about 3 h. Clones from the same library that produced the same RFLP pattern were grouped together and considered members of the same operational taxonomic unit (OTU). At least one clone of each OTU was selected and extracted with a QIAprep Spin miniprep kit (Qiagen, Courtaboeuf, France). Euk-1F was used for partial sequencing. Sequencing reactions were performed by MWG (Roissy, France) (http//www.mwg-biotech.com).

(iii) Phylogenetic analysis. To determine the first phylogenetic affiliation, each sequence was compared with sequences available in databases using BLAST from the National Center for Biotechnology Information (1). Six sequences affiliated with *Pinus luchuensis* were removed from the data set. The sequences were aligned with complete sequences from an ARB database using the ARB automatic alignment tool (www.arb-home.de) (32). The resulting alignments were checked and corrected manually in view of the secondary structure of the rRNA molecule. Sequences were inserted into an optimized tree according to the maximum parsimony criteria without allowing any changes to the existing tree topology (32). The resulting tree was pruned to retain the closest relatives, sequences representative of eukaryotic evolution, and our clones. Chimeras were checked by constructing alternative phylogenetic trees using 350-bp pieces from the 5′ and 3′ ends. According to Zwart et al. (60), an environmental clade comprises at least two sequences that are at least 95% identical and originate from at least two different aquatic sites.

Rarefaction analysis was performed using analytic rarefaction software (version 1.3) (www.uga.edu/~strata/software/Software.html) based on the analytic solution presented by Raup (43) and Tipper (52). The relative distribution of OTUs in the library was used to calculate coverage values (Good's coverage) and the nonparametric richness estimator S_{chao1}, which was the most appropriate estimator for microbial clone libraries (17).

TSA-FISH. A 50- to 65-ml portion of prefiltered water was fixed with paraformaldehyde (final concentration, 1%) for 1 h at 4°C and then filtered through a 0.2-μm-pore-size Anodisc filter (Whatman, Versailles, France). The cells were then dehydrated using an ethanol series (50, 80, and 100% ethanol for 3 min each). Filters were stored at 4°C in the dark.

Hybridization conditions described by Not et al. (40) were used. In brief, for hybridization with fluorescein isothiocyanate (FITC)-labeled probes, filters were covered with a hybridization buffer (40% deionized formamide, 0.9 M NaCl, 20 mM Tris-HCl [pH 7.5], 0.01% sodium dodecyl sulfate), 10% blocking reagent [Roche Diagnostic Boehringer, Basel, Switzerland]) and oligonucleotide probes labeled with horseradish peroxidase (50-ng μl^{-1} stock). The mixture was left to hybridize at 35°C for 3 h. After two washing steps consisting of 20 min at 37°C with wash buffer (56 mM NaCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.5]), samples were equilibrated in TNT buffer (approximately 7% Tween 20, 150 mM NaCl, 100 mM Tris-HCl [pH 7.5]) at room temperature for 15 min. TSA (PerkinElmer, Waltham, MA) was performed for 30 min at room temperature in the dark using a TSA mixture (dextran sulfate and amplification diluent at a ratio of 1:1; FITC-tyramide and the dextran sulfate-amplification diluent mixture at a ratio of 1:50). The filters were then incubated in two successive 5-ml TNT buffer baths at 55°C for 20 min each time to halt the enzymatic reaction and to remove dextran sulfate. The filters were mounted in a mixture containing antifading reagent AF1 (Citifluor; Biovalley, Conches, France) and propidium iodide (10-μg/ml stock solution, stored at 4°C). The probes used in this study are listed in Table 1. Theas designed to target the non-Chlorophyta algae, and the CHLO02 probe, which was designed more recently, targets more numerous Chlorophyta species than CHLO01 and has the advantage of not targeting any non-Chlorophyta taxa (40). CRYPT13 targets all Cryptophyceae except one colorless clade (designed by F. Not [unpublished data]). Hybridized cells were examined with a Leica DM IRB epifluorescence microscope. The excitation and emission filters were 490- and 517-nm filters for FITC and propidium iodide. For each sample 10 to 15 randomly chosen microscopic fields were analyzed and counted manually.

Coupled with TSA-FISH (EUK 1209R), the filters were stained with calcofluor white (23). Calcofluor white binds to chitin, the major component of the cell wall of chytrids, and allows targeting of some stages of the development cycle (53). Filters were incubated with a mixture containing a 10% KOH solution and 0.1% calcofluor white (1:1) for 10 min. The filters were then incubated in two successive distilled water baths for 10 min each time at room temperature in the dark. Marked cells were counted using a fluorescence microscope with UV excitation.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers EF196680 to EF196802.

RESULTS

Phytoplankton and metazooplankton. Differences in the structure of nano- and microphytoplankton were observed between the two dates for which clone libraries were constructed (Fig. 1). In May, the community was dominated by Chlorophyceae (64%), including *Choricystis minor*, numerous undetermined Chlorophyceae, and diatoms (*Fragilaria* sp., *Asterionella* sp., and *Cyclotella* sp.) (22%). In August, cyanobacteria accounted for 39% of the phytoplankton population, and there was a *Planktothrix rubescens* bloom. The other phytoplankton belonged to the Chlorophyceae (34%) and Chrysophyceae (14%).

Cladocerans dominated the zooplankton community in May (74.4%) and August (59%). The maximum density of the genus *Daphnia* was recorded in the summer, whereas the rotifer density was very low at this time. Indeed, rotifers represented only 2% of the zooplankton community in August and 19% in

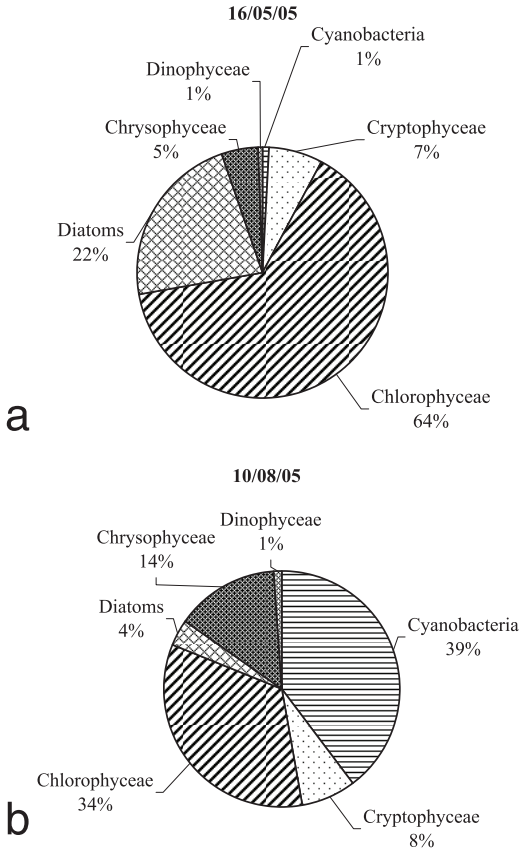


FIG. 1. Relative levels of nano- and microalga groups and microcyanobacteria on 16 May 2005 and 10 August 2005 in Lake Bourget (depth, 0 to 20 m).

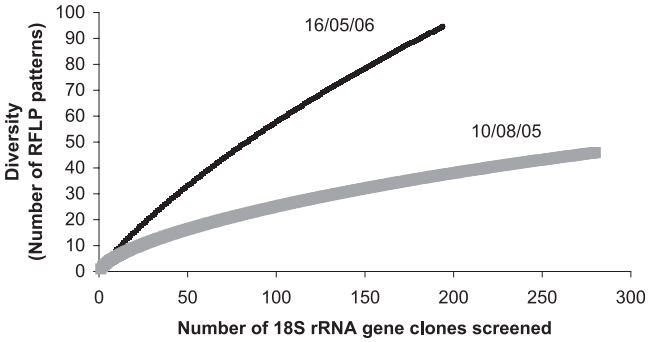


FIG. 2. Rarefaction curves determined for the two 18S rRNA gene libraries generated for Lake Bourget. The number of different RFLP patterns was determined after digestion with restriction endonuclease HaeIII.

May. The proportions of copepods were 21 and 41% in May and August, respectively.

Composition of the small-eukaryote community. (i) Microscopic observations. We used the TSA-FISH method to identify and quantify total small eukaryotes and specific groups of the pigmented organisms. In samples collected in May and August 2005, the densities of small eukaryotes targeted by probe EUK 1209R were 2,053 and 1,272 cell ml^{-1} (Table 2).

In May the numbers of small-eukaryote cells that hybridized with the CHLO02 and CRYPT13 probes were similar (359.6 and 548.2 cells ml^{-1}, respectively), whereas the number of small-eukaryote cells that hybridized with NCHLO01 was 913 cells ml^{-1}. NCHLO01 and CHLO02 targeted about 72% of the total eukaryotes in May and approximately 60% in August. The CHLO02 probe targeted over 27.2% of the eukaryotes detected by EUK 1209R, and CRYPT13 targeted twice as many cells as the CHLO02 probe in August (Table 2). Also, microscopic observations after primuline coloration showed that cryptophytes (*Cryptomonas* and *Rhodomonas*) were the dominant pigmented flagellates.

Coupled with TSA-FISH, calcofluor white showed that a fungus stage with chitin accounted for 19.2% of the cells hybridizing with the EUK 1209R probe in May and 6.9% of such cells in August.

(ii) 18S rRNA libraries. The results of clone library construction offer a precise taxonomic vision of the small-eukaryote community composition. For a total of 486 clones obtained from two libraries, 129 OTUs could be identified after the analysis of RFLP profiles. The S_{chao1} index was 179.7, and the coverage value for both genetic libraries was 91.4%. The total diversity of the small-eukaryote assemblage was estimated by constructing rarefaction curves using the number of clones obtained for the clone libraries (Fig. 2). Rarefaction curves provide an estimate of phylotype diversity relative to sampling effort (i.e., number of clones sequenced). The highest diversity observed with the terminal RFLP analysis (results not shown) was confirmed by the rarefaction curve results; the diversity obtained on 16 May 2005 was higher than that obtained on 10 August 2005.

The sequencing of clones showed that small-eukaryote populations belonged to various phylogenetic groups. Cryptophyta represented 30% of the sequences (14.6% of the clones) in both libraries (Table 3), and four groups can be distinguished in the tree (Fig. 3). Fungus sequences (23.3% of the sequences and 21.2% of the clones) exhibited the highest diversity after Cryptophyta. Most of these sequences fell into the *Rhizophydium* clade belonging to chytrids (Fig. 4). The sequences of Perkinsozoa, Ciliophora, Cercozoa, Bicosoecida, and Cho-

TABLE 2. Small-eukaryote levels and percentages of organisms targeted by fluorescent probes in TSA-FISH and by calcofluor white in natural samples[a]

Date	Concn (cells ml^{-1}) with probe EUK 1209R	% of organisms targeted by:			% of calcofluor white-stained cells
		NCHLO01	CHLO02	CRYPT13	
16 May 2005	2,053.4 ± 288.6	44.4 ± 1.9	27.2 ± 2.9	26.6 ± 4.1	19.2 ± 0.3
10 August 2005	1,272.6 ± 0.186	43.1 ±1.2	17.2 ± 5.9	33.5 ± 10.1	6.9 ± 2.5

[a] The values are means ± standard deviations.

TABLE 3. Numbers of OTUs and clones identified in phylogenetic groups in the two genetic libraries

Taxon	No. of OTUs (no. of clones) in Lake Bourget on:	
	16 May 2005	10 August 2005
Bicosoecida	8 (17)	1 (13)
Cercozoa	5 (5)	3 (5)
Choanoflagellida	7 (10)	1 (2)
Chrysophyceae	8 (15)	
Ciliophora	3 (10)	6 (112)
Fungi	24 (88)	3 (15)
LKM11	2 (2)	
Perkinsozoa	5 (5)	5 (84)
Acantharea		1 (25)
Hyphochytriomycetes	1 (1)	
Chlorophyta	4 (5)	1 (1)
Cryptophyta	29 (40)	11 (31)
Haptophyceae		1 (1)
Total	96 (198)	33 (288)

anoflagellida were less diverse than the sequences of the two groups cited above (approximately 6% of the OTUs) (Fig. 5). Bicosoecida and Acantharea represented 6.2 and 5.2% of the sequences, while the relative proportions of Chlorophyta, Cercozoa, Chrysophyceae, and Choanoflagellida were approximately the same and these groups were represented by five to eight sequences (on average, 5.7% of the OTUs) (Table 3).

The results for the two libraries constructed (16 May 2005 and 10 August 2005) showed some variations in the compositions and proportions of small-eukaryote groups. Acantharea and Haptophyceae were found only on 10 August 2005, whereas sequences affiliated with the Chrysophyceae class, which formed a single clade, were totally absent (Fig. 6). Cryptophyta, which was the main photosynthetic group, Chlorophyceae, and Cercozoa were present at almost the same proportions in May and August (Table 3).

In August the Ciliophora group represented 18% of the OTUs (six sequences), but the proportion of this group in May was low. Similarly, fungi were represented by 29 sequences (44.5% of the clones) in May but by only 3 sequences in August. Sequences affiliated with the Perkinsozoa group accounted for 15.2% of the OTUs (29.2% of the clones) in August, whereas in May the proportion was 5% of the sequences and clones (Table 3).

DISCUSSION

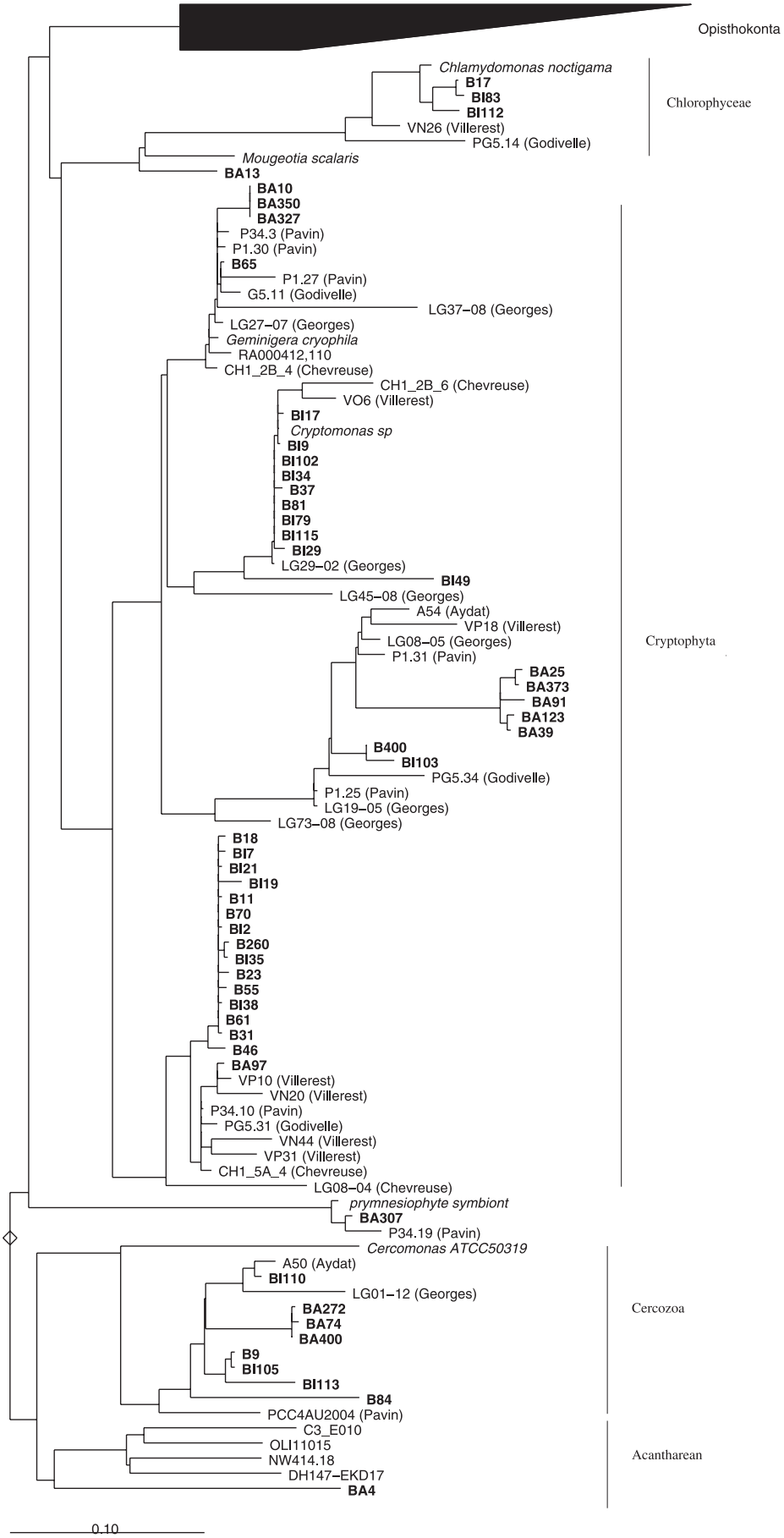
Small-eukaryote diversity in the Lake Bourget system. The 18S rRNA sequences showed that richness changed with the sampling date, with the greatest richness recorded in May (Fig. 2). These results are similar to those obtained by a terminal RFLP analysis of the epilimnion of Lake Bourget (see Materials and Methods). Diversity rarefaction curves constructed from samples collected in this mesotrophic lake did not reach clear saturation; however, the coverage value for our genetic library was high (91.4%). Thus, we believe that a satisfactory inventory was made of most of the small-eukaryotic diversity in our targeted samples. On the other hand, a comparison with rarefaction curves of Lefranc et al. (25) showed that the highest diversity was observed in the mesotrophic lake. The intermediary trophic status of the system might explain these results, but the size of the lake must also be considered. Indeed, Lake Bourget is larger than the other lakes studied. Reche et al. (44) showed that there was a positive relationship between lake area and bacterial richness, consistent with the first prediction of the "island biogeography" theory, which proposes that the size and remoteness of an island determine its species richness. So far, studies of protist diversity have not produced strong evidence concerning the link between the diversity of the microorganisms and the trophic state of the system. However, studies of phytoplankton have reported that oligotrophic and eutrophic lakes have lower diversity than mesotrophic lakes (9, 16). Moreover, Horner-Devine et al. (16) observed that productivity could influence the composition and richness of bacterial communities. Thus, the richness of members of the *Cytophaga-Flavobacterium-Bacteroides* group exhibited a significant hump-shaped relationship with productivity. It was also possible to demonstrate under experimental conditions that algal diversity followed a hump-shaped progression along a eutrophication gradient and that this progression resulted from a compromise between competition, predation, accessibility of nutrient resources, and many other ecological processes (26, 51). We recently reported that the development of small eukaryotes could also depend on these factors (27). It is thus not surprising that the diversity of these organisms fits the same pattern as the diversity of bacteria, microphytoplankton, and metazooplankton.

Cryptophyta, Chlorophyta, and Haptophyta. On average, the clones affiliated with pigmented organisms accounted for 34.9% of the OTUs and 17% of the clones in both libraries. These low proportions of pigmented organisms agree with molecular studies conducted for other lacustrine environments (25, 27, 45), and the pigmented taxa were Cryptophyta, Chlorophyta, and Haptophyta.

According to all the methods (microscopy, cloning, and FISH), Cryptophyta is an important group within the small pigmented lacustrine eukaryotes. In Lake Bourget samples, Cryptophyta, which on average represented 30% of the total small eukaryotes detected by TSA-FISH, dominated the typical small-organism photosynthetic groups. This dominance agrees with previous results obtained for other lacustrine systems by microscopic observations (21, 47) and by molecular methods (45, 50). Our sequences and those obtained from other lacustrine studies revealed four clades. Sequences in the first clade (from sequence BA10 to sequence CH1_2B_4) seem to be restricted to oligo- and mesotrophic systems.

The cloning method showed that Haptophyceae, a photosynthetic class of small eukaryotes, was always present at very low proportions in lakes and marine environments (31, 45, 55). Only one sequence of Haptophyceae was retrieved from Lake Bourget.

Similarly, a low number of Chlorophyta sequences (three sequences affiliated with Chlamydomonadales) were found in this lake. This group was found in only three lakes with different trophic levels (25). Chlorophyta accounted for 22% (on average, for the two sampling dates) of the small eukaryotes targeted by the probe EUK 1209R in the epilimnion of Lake Bourget. The TSA-FISH results differed from the results obtained with 18S rRNA clone libraries, strongly suggesting that



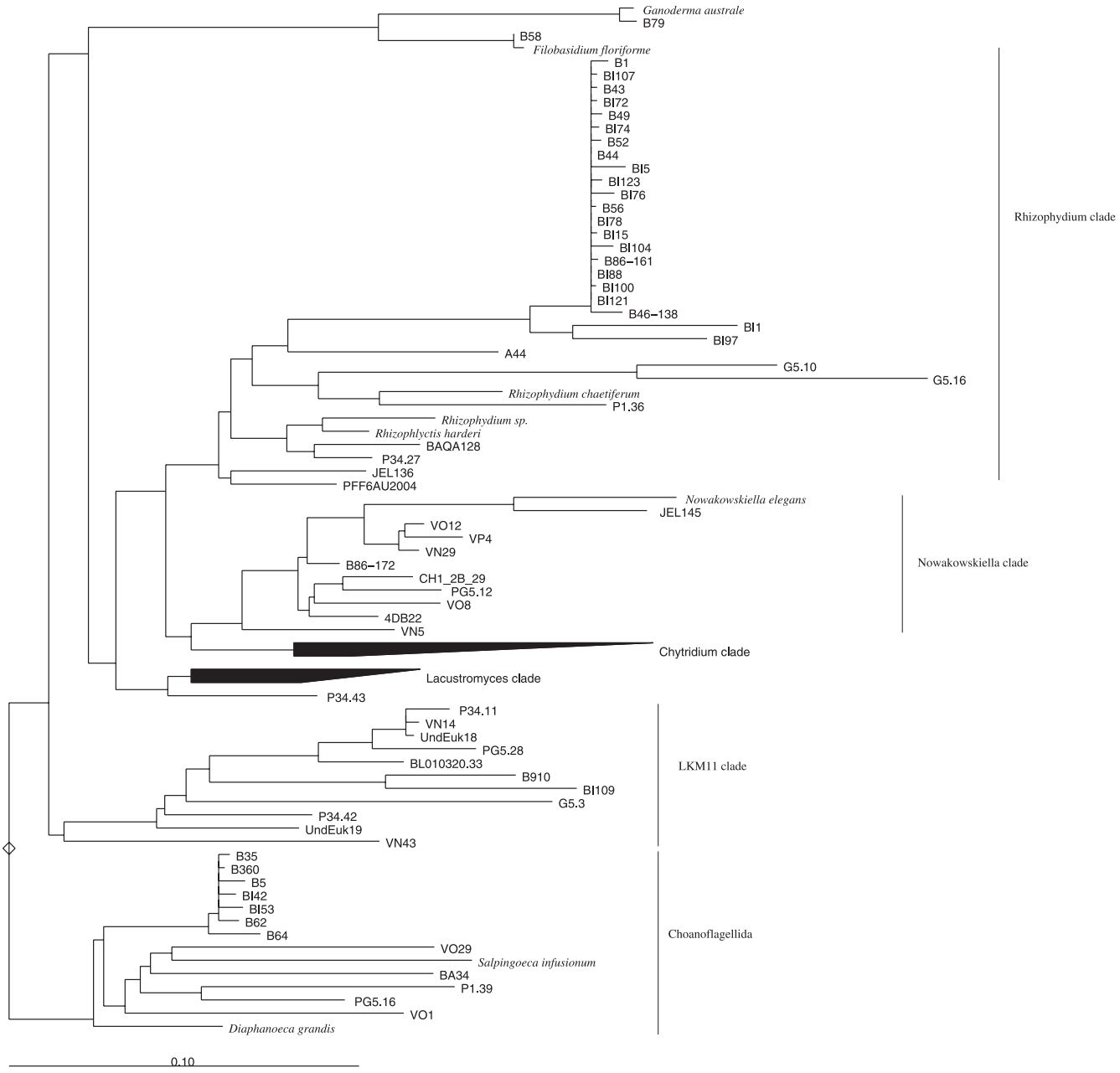


FIG. 4. Phylogenetic tree for opisthokonts. See the legend to Fig. 3 for details.

the Chlorophyceae was underestimated using PCR with 18S rRNA primers of eukaryotes. Although 18S rRNA gene-based studies are promising for describing small eukaryotes, this technique may underestimate plastidic forms in samples (25, 49). The low levels of these organisms may be explained by a difference in the number of 18S rRNA gene copies or by some PCR biases (59). Amplification of ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) genes (12) or *psbA* (57) seemed to offer an interesting additional method for better analysis of the pigmented small-eukaryote community. We therefore experimented with RuBisCO gene amplification in this study. However, our analysis showed that for a total of 141 clones obtained from RuBisCO gene (*cbbL*) amplification, only sequences affiliated with cyanobacteria were obtained in

FIG. 3. Phylogenetic tree for small-subunit rRNA gene sequences covering the diversity of Chlorophyceae, Cryptophyta, and Cercozoa. Sequences derived in this study are indicated by bold type. The names of the lakes in which sequences were identified are indicated in parentheses.

FIG. 5. Phylogenetic tree for alveolates. See the legend to Fig. 3 for details.

the clone libraries. This result was certainly due to the high abundance of cyanobacteria in our environmental samples. Fuller et al. (13) recently developed a nondegenerate marine algal plastid-biased PCR primer to minimize amplification of picocyanobacteria. Our results highlight the utility of designing primers for algal plastids as a valuable method to study picophytoplankton diversity specifically.

Stramenopiles. Among the small eukaryotes, some groups, such as Chrysophyceae, were present on only one sampling date. Members of the Chrysophyceae, which includes autotrophic, mixotrophic, and heterotrophic taxa (3), were found in May, with eight sequences affiliated with the heterotrophic organism *Oikomonas mutabilis*. Lake Bourget sequences and the results of other studies revealed a large clade formed by freshwater ecosystem sequences. This group was retrieved at all the trophic levels studied with the exception of the hypereutrophic level. The Chrysophyceae class is well recognized as an important component of freshwater assemblages (25, 48), but as in marine studies, cloning of 18S rRNA showed that these organisms frequently belong to heterotrophic lineages (8, 45). Only one study revealed a high proportion of 16S rRNA gene clones phylogenetically related to photosynthetic chrysophytes in marine environments (13).

Most of the sequences of the Bicosoecida lineage which were present on the two sampling dates were affiliated with *Cafeteria roenbergensis*. Sequencing results of several studies revealed a wide distribution of Bicosoecida in freshwater environments (25, 45, 50). Bicosoecida seem to be separated into two major groups, a freshwater cluster and a marine cluster (J. del Campo and R. Massana, presented at the 11th International Symposium on Microbial Ecology, Vienna, Austria, 20 to 25 August 2006). Recent observations using FISH probes specific for *Cafeteria* sp. showed that this genus was not quantitatively important in the sea.

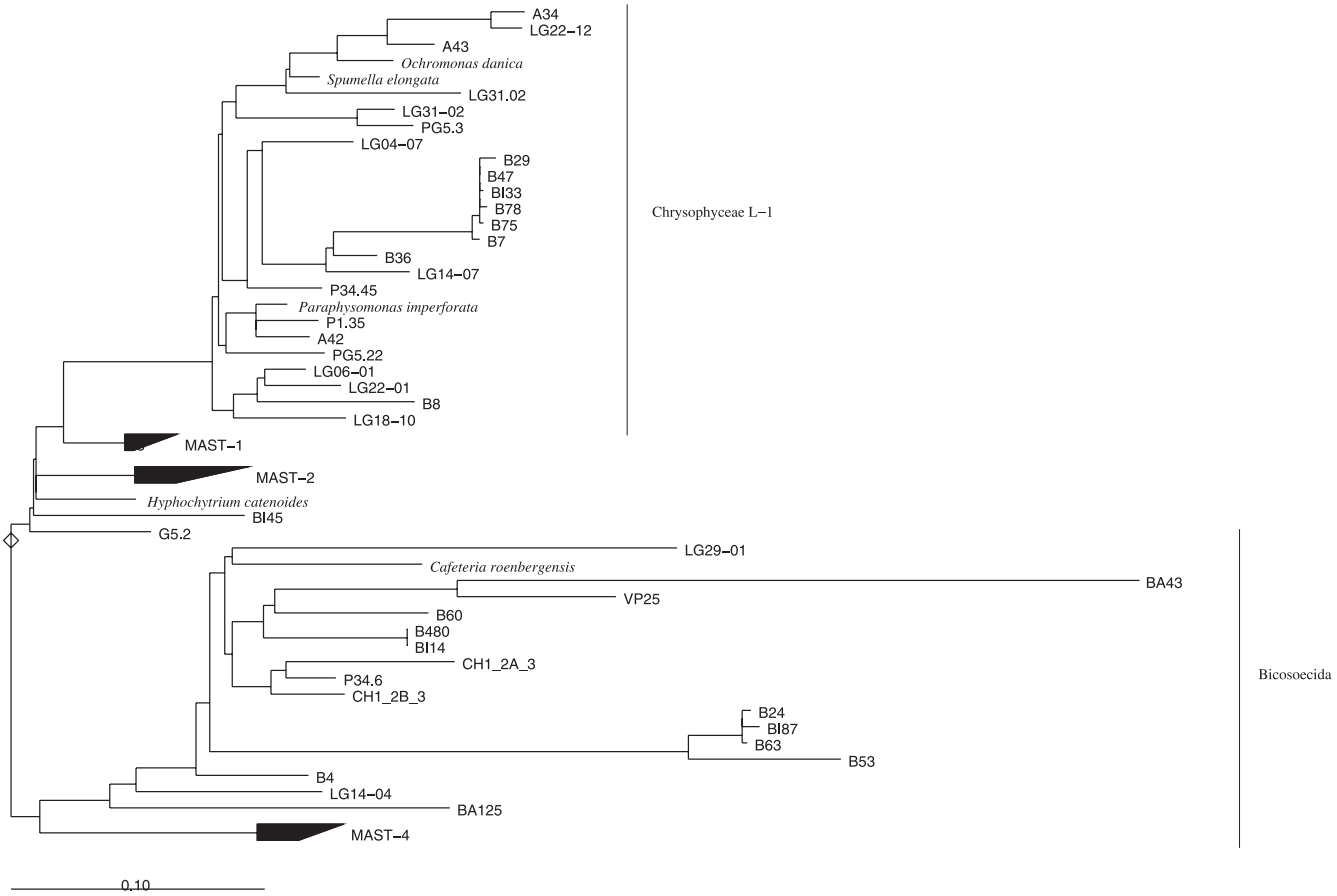


FIG. 6. Phylogenetic tree for stramenopiles. See the legend to Fig. 3 for details.

Cloning-sequencing techniques showed that members of the MAST clade (new stramenopiles), which are particularly frequent in clone libraries of surface marine picoplankton (36), are not detected in freshwater environments, whatever the trophic status of the lake (25, 27, 28). These results thus highlight differences in the stramenopile lineage between freshwater and marine environments.

Rhizaria. Molecular biology techniques enabled us to demonstrate the occurrence of the Rhizaria group, which is not usually identified by microscopy in such environments but which is probably classified as unidentified flagellates in many studies because of the general lack of distinct morphological features of the small cells (24). This group was associated with environmental sequences from lakes having various trophic levels.

Acantharea, found only in May, are heterotrophic typical blue-water organisms with a cosmopolitan distribution. They have been found in coastal waters (56), in the Arctic Ocean (31), in hydrothermal vents (11), and in the Mediterranean Sea (33), suggesting that there has been wide adaptation or long-distance transport of these organisms. However, until now no sequence affiliated with Acantharea was recorded for lacustrine systems (25, 45). The Acantharea sequence found in Lake Bourget occurs near marine sequences. The organisms appear to take up species-specific positions in the water column, often at considerable depth, and it has been reported that strontium sulfate is necessary for the formation of all Acantharea skeletons (58).

Ciliates. Lake Bourget sequences include two alveolate clades belonging to the ciliates formed by sequences obtained from a wide range of lakes (25, 50). Ciliates were the main group in the August clone library (39% of the clones), whereas they represented only 5% of the clones in May. The detection of these sequences in this picoplankton fraction may have been the result of methodological effects, but the occurrence of unidentified small ciliates seems to be the most probable explanation (25, 31, 35). Future research comparing the diversity of the larger size fraction and the application of specific probes combined with microscopy may help to resolve the origin of seemingly large-celled organisms in the smallest size fractions.

Like new stramenopiles, novel alveolate groups have been identified from sequences retrieved from the marine environment. Novel marine alveolates belong to two independent and well-supported lineages, groups I and II. Group I is composed entirely of environmental sequences, whereas group II also includes the parasite *Amoeobophrya* (15). Although widespread in marine waters, these alveolate lineages have not been reported for Lake Bourget or in other freshwater ecosystems, suggesting once again that a specific group occurs according to the type of aquatic environment.

Fungi and Perkinsozoa: unexpected importance of parasitism. On average, fungi and Perkinsozoa represented about

30% of the OTUs (40% of the clones) obtained on each of the two dates for Lake Bourget. In the present study, fungi dominated the 16 May 2005 library, with 24 sequences (45% of the clones) mostly affiliated with chytrids characterized by free-swimming zoospores (size, 2 to 5 μm) in the reproductive stage (22). Also, results of calcofluor white staining confirmed their significant level in the small-eukaryote fraction, notably in May. Calcofluor white is a fluorescent dye that has binding affinity specific for both cellulose and chitin. Even though calcofluor white is not specific for chytrids or other fungi, we can estimate the presence of these organisms by coupling these staining and morphological features (23). Of the four clades defined by James et al. (19), chytridial sequences fell mostly within the *Rhizophidium* clade and *Nowakowskiella* clade and never in the *Lacustromyces* clade. Some sequences are included in the LKM11 group (25) defined by Van Hannen et al. (54). Thus, fungi were found in all lakes with the exception of Lake George (in northeastern New York) (45) even though the number of clones studied by the authors was equivalent to the number that we analyzed here. However, the primers used by Richards (45) were not similar to those used in the other studies (25, 27, 28, 50), which might explain the difference. Another explanation might be the spatial diversification (i.e., biogeography) of microbial eukaryotes. For example, microbial eukaryotes, such as Ascomycetes, are not randomly distributed but instead exhibit spatially predictable patterns on local to regional scales (14). However, little is known to date about the taxon-area relationships of protists on a continental scale.

Chytrids are parasitic fungi with a wide range of hosts, including diatoms, Chlorophyceae, Chysophyceae, and cyanobacteria, such as *Planktothrix* (18). Thus, they can be involved in ecological processes leading to the succession of phytoplankton communities and can reduce algal bloom densities. We found that there was strong development of *Pankthotrix* in Lake Bourget in August, when the level of chytrids was low, while in May the dominance of fungi in the library was concomitant with the absence of cyanobacteria but a high abundance of Chlorophyceae in the microphytoplankton. It was previously reported by Lepère et al. (27) that fungi were associated with Chlorophyceae, which dominated phytoplankton in May especially in the oligotrophic Lake Pavin. Conversely, no clear relationship between diatoms and fungi could be discerned.

The second parasitic group was Perkinsozoa, especially on 10 August 2005. These parasitic protists affiliated with the alveolates form a recently established phylum at the base of Apicomplexa and dinoflagellates. The entire group of the Perkinsozoa is probably parasitic with a zoospore life stage (10, 20). *Perkinsus marinus* is known to parasitize a variety of bivalves (38), and *Parvilucifera infectans* parasitizes other protists, such as dinoflagellates (39, 41). All the sequences obtained from Lake Bourget showed one clade including some marine sequences (Fig. 5).

This work provided an assessment of small-eukaryote diversity in the epilimnic zone of mesotrophic Lake Bourget, but it also allowed us to extend the studies recently conducted for lacustrine systems. The results obtained with molecular approaches led to the first characterization of lacustrine clades and showed that the main new clades defined in the oceanic ecosystem (stramenopiles and alveolates) are not present in lakes. In addition, while the most common consumer strategy, parasitism (6), is usually left out of lake food web functioning, the results highlight the importance of the parasitic group in the small-eukaryote fraction and suggest that further investigation of parasitic groups is needed to assess their ecological relevance and their impact on microbial population dynamics and planktonic food web structure.

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