

Prevalence of Viral Photosynthetic and Capsid Protein Genes from Cyanophages in Two Large and Deep Perialpine Lakes

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Cyanophages are important components of aquatic ecosystems, but their genetic diversity has been little investigated in freshwaters. A yearlong survey was conducted in surface waters of the two largest natural perialpine lakes in France (Lake Annecy and Lake Bourget) to investigate part of this cyanophage diversity through the analysis of both structural (e.g., *g20*) and functional (e.g., *psbA*) genes. We found that these cyanophage signature genes were prevalent throughout the year but that the community compositions of *g20* cyanomyoviruses were significantly different between the two lakes. In contrast, *psbA*-containing cyanophages seemed to be more similar between the two ecosystems. We also found that a large proportion of *g20* sequences grouped with cyanomyophage isolates. *psbA* sequences, belonging to phages of *Synechococcus* spp., were characterized by distinct triplet motifs (with a novel viral triplet motif, EFE). Thus, our results show that cyanophages (i) are a diverse viral community in alpine lakes and (ii) are clearly distinct from some other freshwater and marine environments, suggesting the influence of unique biogeographic factors.

Viruses infecting cyanobacteria (i.e., cyanophages) have been recognized as an important player in aquatic ecosystems (1). They have been shown to influence host population mortality as well as population succession and community structure and thus are a major driving force behind biogeochemical cycles (2–4). To the best of our knowledge, most of the characterized cyanophages (largely of marine origin) are tailed double-stranded DNA viruses and belong to three families (1, 5–7): the *Myoviridae* (T4-like, TIM5-like), the *Podoviridae* (T7-like), and the *Siphoviridae* (lambda-like). The ratio of these three groups of cyanophages varies depending on both biological hosts and environmental conditions (1, 8). In surface waters of the vast open ocean, the relative proportion of cyanomyoviruses/cyanopodoviruses/cyanosiphoviruses is about 20:10:1, as revealed by BLAST searching against the Global Ocean Sampling (GOS) metagenome database using the available 29 cyanophage genomes and their TerL amino acid sequences as queries (9).

The T4-like cyanomyoviruses generally have a broad host range (10) and are abundant in various environments. The genetic diversity of this group has been examined using the T4-like portal protein-encoding gene *g20* as a molecular marker in different locations of both marine and freshwater ecosystems (11–24) and even in paddy soils (25). Distinct cyanomyoviruses could be detected in freshwater (12, 18, 21, 26) and paddy soil (25) ecosystems in comparison to their marine counterpart. Also, the diversity, abundance, or structure of cyanomyoviruses can vary between stations of the same lake (21) or ocean (15, 18, 27). When sampled from a single location, it has also been shown that abundance and community structure and/or diversity of these cyanophages can vary with time (11, 12, 16, 20, 23, 28, 29) and depth (13, 27). Together, these studies reflect distinct temporal, vertical, and/or geographical distribution patterns of cyanomyoviruses across aquatic environments. Comparatively, the cyanopodoviruses and cyanosiphoviruses are much more host specific (9, 12, 30). The occurrence and diversity of cyanopodoviruses (5, 8, 31) were investigated only in marine ecosystems, but no report has been provided so far for freshwaters. Such a lack of data is also true for cyanosiphoviruses even if several genomes of these cyanophages

have been made available recently from the Mediterranean Sea, Atlantic Ocean, and Chesapeake Bay (9, 26, 32).

Among remarkable features discovered in cyanophages are the host-derived photosynthesis reaction genes (e.g., mainly *psbA* and *psbD*) present in their genomes. These auxiliary metabolic genes are assumed to benefit the host through their expression during infection, supplying substantial energy and carbon for phage production (33–36). The *psbA* gene encodes the photosystem II D1 protein, and it has been found in approximately 2/3 of both cyanomyovirus and cyanopodovirus isolates (30) but not in cyanosiphoviruses (5, 9, 26). As the *psbA* gene may not be specific to a single cyanophage family, it represents an additional genetic marker to explore diversity and evolutionary history of cyanophages in aquatic environments. Furthermore, Sharon et al. (37) found that viral *psbA* sequences can be very abundant in the environment, and 62% of identifiable *psbA* sequences issued from the GOS metagenome database have been assigned to virus origin. PCR-based studies have also revealed that *psbA* gene sequences are diverse in both marine waters (36–39) and freshwaters (38, 40, 41).

During the last decade, previous studies have partially identified the diversity, dynamics, or role (as mortality agents of picocyanobacteria) of cyano(myo)phages in French perialpine lakes (12, 17, 23, 42, 43). This body of work has suggested that the cyano(myo)phage community is diverse, displays marked seasonal variations, and is concentrated mainly in surface waters down to 20 m deep. Furthermore, the cyanophage community could, at certain periods of the year (e.g., mainly in spring and only occasionally in summer or autumn), be an important mortality factor for the picocyanobacterial community. However, we have

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not yet provided a detailed description of the cyanophage genetic composition, and more generally such an investigation is absent from large and deep lakes. Thus, we conducted a complete annual survey of Lake Annecy and Lake Bourget in the 0- to 20-m upper lit layer with the goal of investigating cyanophage community composition. Such sampling strategy was chosen to respond to the potential high temporal, vertical, and geographical variability of cyanophages as described previously, so as to unveil a global and comprehensive image of cyanophages in perialpine lakes. Specifically, we employed two genetic markers, using PCR-denaturing gradient gel electrophoresis (DGGE) and cloning sequencing approaches, to examine part of the genetic diversity of T4-like cyanomyoviruses (*g20* gene) and *psbA*-containing cyanophages (*psbA* gene) in two contrasting ecosystems: an oligotrophic (Annecy) and an oligo-mesotrophic lake (Bourget). To the best of our knowledge, this is the first study for freshwater ecosystems with such a temporal and taxonomic resolution.

MATERIALS AND METHODS

Sample collection and processing. Water samples were collected once or twice each month between January and November 2011 at reference stations of Lake Annecy and Lake Bourget as detailed elsewhere (23). A few hours following sampling, 20-liter samples were first filtered through a 60- μ m-pore-size mesh and then filtered through 1- μ m-pore-size filters (Millipore, Bedford, MA). The final filtrate (i.e., <1- μ m fraction) was concentrated to a final volume of 200 to 250 ml by using a 30,000-Da-molecular-weight-cutoff, spiral-wound, Millipore ultrafiltration cartridge (regenerated cellulose, PLTK Prep/Scale TFF, 1 ft²; Millipore). To ensure that all remaining small free-living bacteria were removed, we filtered the <1- μ m concentrated fraction through 0.45- μ m-pore-size filters twice (Millipore). The absence of cellular contamination was verified using flow cytometry (not shown). This <0.45- μ m viral concentrate (VC) was stored in the dark at -20°C until further processing.

PCR amplification and DGGE. The PCR was conducted in two stages prior to the DGGE analysis (17, 23, 30). The first PCR stage on the VC, the template, was conducted with the primer set in the absence of the GC clamp. The second PCR was then performed on the product of the first-stage PCR, using the GC clamp-containing primer set (i.e., with a 40-nucleotide [nt] GC clamp attached to the 5' end of the forward primer). PCRs were performed using the DNA thermal cycler T-Professional (Biometra), whereby we amplified the T4-like portal protein-encoding *g20* gene using the primer set CPS1.1/8.1 (44) and the photosystem II D1 protein-encoding *psbA* gene using the primer set Pro-*psbA*-1F/1R (36). Briefly, for all primer sets, 25 μ l of reaction mix contained 1 \times PCR buffer, 4 mM MgCl₂, 200 μ M each deoxynucleoside triphosphate (dNTP), 0.4 μ M each primer, 0.5 U of Platinum *Taq* DNA polymerase (Invitrogen), and 1 μ l of VC. Prior to PCR with the *psbA* gene, VC was treated with DNase (0.002 units of DNase I for 1 μ l of VC; Sigma) to remove environmental free DNA and to avoid the potential amplification of *psbA* sequences from free DNA released from cellular hosts. The program for the first-stage PCR was a 15-min virion lysing and denaturation at 95°C, followed by 34 cycles of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. The program for the second-stage PCR was 5 min of denaturation at 95°C, followed by 24 cycles of denaturation at 95°C for 30 s, annealing for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. After various tests of optimization (not shown), the best annealing temperatures were 46°C and 50°C for CPS1.1/8.1 and Pro-*psbA*-1F/1R, respectively.

The DGGE was conducted as described previously (23). The linear denaturing gradient was at 25 to 50% and 40 to 60% for amplicons of CPS1.1/8.1 and Pro-*psbA*-1F/1R, respectively. Twenty microliters of PCR products was loaded into wells with 5 μ l of 5 \times loading buffer (12.5% Ficoll, 25 mM Tris, 5 mM EDTA [pH 8.0], 0.5% SDS, 0.1% [wt/vol]

xylene cyanol, and 0.1% [wt/vol] bromophenol blue). Electrophoresis was carried out for 16 h in 1 \times TAE buffer (pH 7.4; 40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA) at 120 V and a constant temperature of 60°C using the CBS-DGGE 2000 system (C.B.S. Scientific Co., Inc.). Due to the limited gel size (i.e., the number of wells) accepted by this device, we ran samples in two gels (one for Lake Annecy and the other for Lake Bourget for each marker gene) put together in the same tank. To standardize the banding pattern in each gel, a pooled sample (corresponding to equal volumes of all obtained samples from the two lakes mixed together) was loaded in the middle and at each side of the gels. To identify the DGGE bands shared by the two lakes, in addition to the pooled sample, the samples of the other lake (those possessing typical distinct banding patterns) were also loaded at the same time in the DGGE migration to serve as an extra reference. Gels were stained in a 2 \times SYBR green I (Molecular Probes, Invitrogen) solution for 45 min and were visualized on a UV transilluminator (Tex-35 M; BioBlock Scientific) and photographed with GelDoc (Bio-Rad).

DNA purification, cloning, and sequencing. Gel slices of 47 and 39 visibly different DGGE band representatives were excised from the *g20* and *psbA* DGGE gels, respectively. DNA of each DGGE band representative was eluted from the excised gel slice by adding 100 μ l of 1 \times sterile TAE buffer and heated for 15 min at 95°C. Three microliters of eluted DNA served as the template in a 22- μ l PCR mixture using the corresponding primer set. PCRs were performed under the same conditions of the first-stage PCR, as described above. The amplicons were first verified by electrophoresis in a 1.5% agarose gel, purified by the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare), and finally cloned into pCR4-TOPO vectors by using the TOPO TA cloning kit (Invitrogen). Respectively, 45 and 29 bands of *g20* and *psbA* resulted in clones, and the other bands (mainly DGGE bands with weak luminosity or fluorescent density) failed, probably due to the low DNA concentration. Eight randomly selected clones for each band representative were then sent for sequencing to GATC Biotech. After cleaning and correcting sequences by using BioEdit 7.0.5.3 (45), we obtained 110 and 62 different nonredundant sequences for *g20* and *psbA*, respectively.

Phylogenetic analysis. For the analysis of *g20*, all sequences we obtained were translated into amino acids and aligned using MAFFT version 7 (46), using reference sequences of both environmental and culture representative cyanophages (Table 1). Multiple alignments were then cured using Gblocks (47) with a stringent option by not allowing a contiguous unconserved position inside the final blocks. Phylogenies were reconstructed using Bayesian inference and maximum likelihood methods. Bayesian inference was carried out using MrBayes 3.2.1 (48) with two runs, four chains, 1.5 million generations, sampling every 1,000 generations, mixed models of amino acid substitution, and a burn-in value of 25%. The maximum likelihood phylogeny was constructed using PhyML 3.0 (<http://www.atgc-montpellier.fr/phyml/>) (49) with 100 bootstrap replicates, the rtREV (general reverse transcriptase) model, and gamma-distributed rate heterogeneity among amino acid substitutions (i.e., the best model for this aligned *g20* sequence data set determined by using MEGA5) (50).

Since a high level of conservation exists among *psbA* sequences, DNA sequences were used for phylogenetic reconstruction instead of the deduced amino acid sequences as for *g20* (30, 51, 52). All obtained *psbA* nucleotide sequences were also aligned using MAFFT with culture representatives of cyanophages, cyanobacteria, and eukaryotic microalgae, as well as environmental sequences from other studies (25, 36–39). The multiple alignments were again cured by using Gblocks, and phylogenies were reconstructed as described above. The general time-reversible (GTR) model and gamma-distributed rate heterogeneity among nucleotide substitutions was for the maximum likelihood phylogeny. The Bayesian phylogeny employed the codon model of nucleotide substitution and allowed different rates for transitions and transversions.

Statistical analysis. To evaluate whether the (*g20* or *psbA*) cyanophage community composition of Lake Annecy differed from that of Lake Bour-

TABLE 1 Origins of *g20* environmental sequences used for the phylogenetic tree shown in Fig. 2

Environment	Prefix of sequence label	Location(s)	No. of sequences obtained	GenBank accession no.	Reference
Freshwater	LAB_g20	Lake Annecy and Bourget (HS, France)	110	KC626330 to KC626439	This study
	LB	Lake Bourget (HS, France)	47	AY426128 to AY426174	12
	LAC95	Lake Constance (Germany)	6	AY705091 to AY705145	18
	CUL	Cultus Lake (BC, Canada)	9	AY705091 to AY705145	18
	CHL	Chilliwack Lake (BC, Canada)	1	AY705091 to AY705145	18
	CAT	Catfish Pond	1	AY705091 to AY705145	18
	SPM	Shore pond mat (Arctic mat)	8	AY705091 to AY705145	18
	LE	Lake Erie (North America)	45	DQ318388 to DQ318432	21
	PFW	Paddy field floodwater (Japan)	77	AB471562 to AB471638	20
Marine	BES	Beaufort Sea (Arctic Ocean)	9	AY705091 to AY705145	18
	CHS	Chuckchi Sea (Arctic Ocean)	3	AY705091 to AY705145	18
	ANT	Antarctic Peninsula (Southern Ocean)	4	AY705091 to AY705145	18
	GOM	Gulf of Mexico (NW Atlantic Ocean)	5	AY705091 to AY705145	18
	MAL	Malaspina Inlet (NE Pacific Ocean)	1	AY705091 to AY705145	18
	SAI	Salmon Inlet (NE Pacific Ocean)	2	AY705091 to AY705145	18
	PES	Pendrell Sound (NE Pacific Ocean)	2	AY705091 to AY705145	18
	COL	Coast of Colombia (SE Pacific Ocean)	1	AY705091 to AY705145	18
	CHI	Coast of Chile (SE Pacific Ocean)	1	AY705091 to AY705145	18
	SE	Skidaway Estuary (Savannah, GA)	29	AY027985 to AY028013	24
	GS	Gulf Stream (along edge of Sargasso Sea)	36	AY027938 to AY027973	24
	SS	Sargasso Sea (NW Atlantic Ocean)	65	AY028014 to AY028078	24
	CB	Chesapeake Bay (United States)	15	AY152732 to AY152746	19
Soil	AnCf	Paddy soil of Anjo (Japan)	25	AB560191 to AB560215	25
	KuCf	Paddy soil of Kuroishi (Japan)	26	AB560146 to AB560171	25
	OmCf	Paddy soil of Omagari (Japan)	19	AB560172 to AB560190	25

get, we carried out statistical analyses using the UniFrac distance metric statistical tools available at <http://bmf.colorado.edu/unifrac/> (53, 54). We used the unweighted UniFrac option in order to compare community composition based on presence/absence importance (i.e., on qualitative data). This tool measures the distance between two communities by calculating the fraction of the branch length in a phylogenetic tree (54). In brief, we used the Bayesian phylogenetic tree and a file mapping sequence labels to their habitats as input for each analysis (*g20* or *psbA*). The P test was conducted based on the UniFrac distance matrix generated for each cyanophage assemblage.

Nucleotide sequence accession numbers. Sequences were deposited in GenBank under the accession numbers KC626330 to KC626439 for *g20* and KC626440 to KC626501 for *psbA*.

RESULTS

Photosynthetic gene *psbA* sequences. From the 29 visibly different representative bands excised, we obtained 62 nonredundant sequences for both lakes, with nucleotide similarity varying between 72.9% and 99.9% and GC content varying between 43.3% and 48.1% (Fig. 1). Among the eight sequenced clones analyzed for each band, between 1 and 6 different sequences were obtained, with nucleotide dissimilarity up to 21.7%. Eleven out of the 29 *psbA* bands contained a single sequence.

The triplet peptides associated with the D1 protein motif (R/K)ETTXXXS(Q/H) were also analyzed, and we detected three triplets, referred to as ETE, ESE, and EFE, which accounted for 75.8%, 17.7%, and 6.5% of obtained sequences, respectively. The phylogenetic analysis of these sequences with phytoplankton and cyanophage culture representatives showed that all obtained *psbA* sequences were grouped together with viruses infecting *Synechococcus* spp. (Fig. 1), of which 82.3% were more closely related to cyanopodoviruses (S-CBP1 and S-CBP3) rather than to cyanomyoviruses. The other 17.7% (11 sequences) had the closest homologue to the freshwater *Synechococcus* myovirus S-CRM01.

The UniFrac analysis failed to detect any significant differences between Lake Annecy and Lake Bourget *psbA* sequences ($P = 0.15$).

Capsid assembly gene *g20* sequences. From the 45 visibly different representative bands excised, we obtained 110 nonredundant sequences, with nucleotide similarity varying between 66.3% and 98.9%. Among the eight sequenced clones analyzed for each band, between 1 and 6 different sequences were obtained, with nucleotide dissimilarity up to 33.7%. Fourteen out of the 45 *g20* bands contained a single sequence.

The phylogenetic analysis of these sequences with both cyanomyovirus culture representatives and other environmental sequences (Table 1) allowed us to discriminate 17 clades (Fig. 2). Five of them were initially identified by Sullivan et al. (44) as the culture-containing clusters I, II, and III, cluster 3 (P-SSM9/11/12 cluster), and the environmental sequence-only cluster 2. Another one was the cluster A previously defined by Zhong et al. (24). We redefined the other 11 clusters as environmental sequence-only clusters Env 1 to 10 and cluster 4. Overall, our global clustering pattern agreed with the analysis of Sullivan et al. (44). However, the previously defined environmental sequence-only clusters (like cluster 1 but not cluster 2) were reorganized when sequences of a paddy field (20, 25) and alpine lakes (this study) were introduced, for which sequences were distributed in cluster Env 1 and 3 to 9.

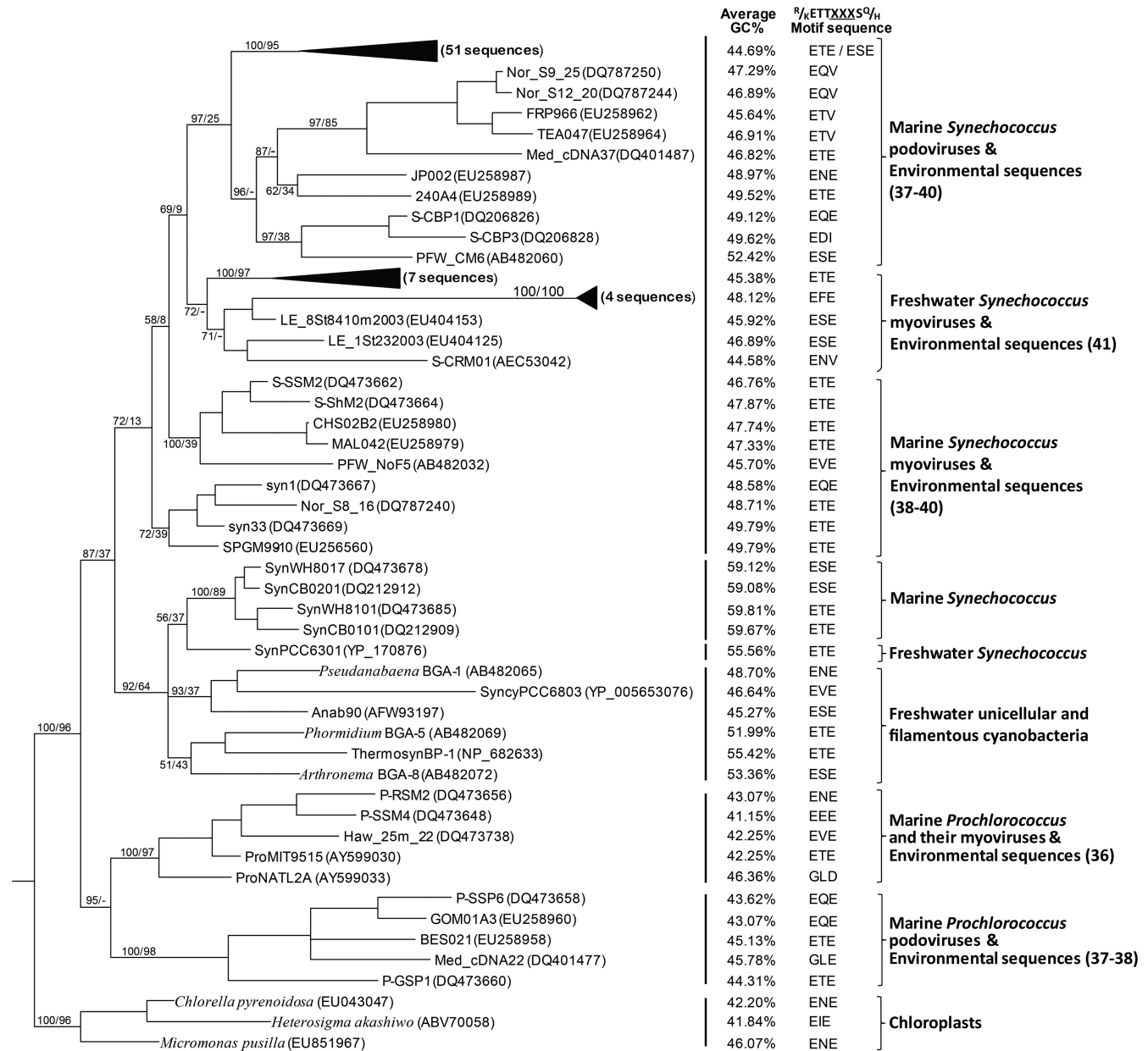


FIG 1 Phylogenetic tree, GC contents, and triplet sequences from D1 protein motifs of a partial *psbA* gene from the culture representatives of cyanophages, cyanobacteria, and eukaryotic microalgae and from the environmental samples of this study and others (36–41). A Bayesian phylogenetic tree was constructed based on the alignment of 611 homologous nucleotides of the *psbA* gene. Values at nodes as shown on the main branches are the Bayesian inference (BI) posterior probability and the maximum likelihood (ML) bootstrap value in the order of BI/ML. With the clade support value of both BI and ML of $\geq 90\%$, the sequences of Lake Annecy and Bourget are grouped into black triangles. The number of sequences is given in parentheses and written in bold.

In the color ring in the phylogenetic tree of Fig. 2, cluster A contains exclusively *g20* sequences from the marine field, whereas only freshwater-originated sequences could be detected in clusters Env 6, 7, and 9. No cluster contained exclusively sequences from the paddy field soil, which were clustered with other water-originated sequences in clusters Env 1, 2, 4, and 10. Sixty-three percent of the sequences obtained from Lake Annecy and Lake Bourget were grouped into culture-containing cluster II, whereas the others were in different environmental sequence-only clades, like Env 1, 2, 3, 6, and 8 and cluster 4. No sequences were in the culture-containing clusters I and III, clusters 2 and 3, or cluster A (Fig. 2).

When the *g20* sequences were pooled over the year, the Uni-Frac analysis revealed significant differences between Lake Annecy and Lake Bourget ($P = 0.03$).

DISCUSSION

Methodological considerations. DGGE allows fingerprinting communities based on the fractionation of amplicon sequences (55). In our study, we observed that the cyanophage community composition (either from *g20* or *psbA*) varied throughout the year (23) (data not shown), so considering a sample(s) at a single time point or at selected periods of the year was likely to miss some

limited to major taxa. Minor populations may produce no visible bands or bands insufficiently visible to be cut from gels. Also, when cut, low-density bands can result in too-low concentrations of purified DNAs to be cloned. This was observed for *psbA*: 10 out of 39 band types failed to produce clones. Second, a single band type can contain multiple sequences whose composition may vary with time. As we selected only one or two bands for each band type for sequencing, it is possible we missed some sequences (also typical of the same band type) that could occur during periods other than the one analyzed.

All in all, our DGGE-based approach was likely to provide a rough minimum estimate of cyanophage community composition. Considering these viral populations followed the seed bank model (i.e., only a few members of viruses were actively abundant at any given time, and most of them were rather rare and/or inactive) (29, 51, 56), this 1-year investigation could at least reveal some of these annually actively abundant populations. Hence, our approach provided a solution to identify rapidly and at reduced cost the actively abundant viral fraction occurring at some periods of the year in response to environmental and/or biological factors.

Cyanophage *g20* diversity. Our analysis, using PCR-DGGE with primers CPS1.1 and CPS8.1 to target the *g20* gene in Lake Annecy and Lake Bourget, first revealed the prevalence of cyanomyoviruses in these perialpine lakes. Cyanomyoviruses of Lake Bourget have been previously investigated by Dorigo et al. (12) by using a PCR-DGGE-based approach between September 2002 and January 2003 and by Roux et al. (57) through a metagenomic-based approach in July 2008. When we blasted against the virome using our *g20* sequences as queries (E value of ≤ 0.01), only 0.004% (24 read sequences) of reads were detected to have similarity to *g20*. After tentative assembly of these reads to contigs, only two of them overlapped with our *g20* sequences and could be recruited for the phylogenetic analysis. This earlier metagenomic study generated relatively short reads (on average 427 bp in length), and the sequencing depth was likely not sufficient to allow the construction of longer contigs of rare sequences (cyanomyoviruses accounted for $< 0.1\%$ of total viruses as revealed by flow cytometry and qPCR) (23). However, the two *g20* contig sequences obtained from the virome were detected to be different from those obtained by Dorigo et al. (12) and from the present study. One clustered in the culture-containing cluster II and the other was in cluster Env 7. A significant difference of *g20* sequences in Lake Bourget was also detected between this study and the one of Dorigo et al. (12) ($P = 0$), and only one nucleotide sequence was identical. Our sequences were located mainly in the culture-containing cluster II, whereas no sequence obtained by Dorigo et al. (12) clustered with cyanomyovirus isolates. Most of them were in clusters Env 1, 3, 4, 5, and 7, where our sequences were rarely present (Fig. 2). Such a difference between these three studies, for the same lake, may be due to the technique employed (PCR-DGGE versus metagenomic), the sampling strategy (water at 5 m versus integrated water at 0 to 20 m), the period of investigation (July versus September to January versus January to November, moreover, in different years), or the difference in primer specificities (primers CPS1/8 used by Dorigo et al. [12] versus CPS1.1/8.1 in our study). It is noteworthy here that Sullivan et al. (44) tested CPS1/8 and CPS1.1/8.1 over a wide range of marine cyanomyovirus isolates and revealed that CPS1/8 could amplify only 56% of cyanomyoviruses, whereas such a percentage reached 100% when using CPS1.1/8.1. This could explain the observation

that numerous *g20* sequences were closely related to cyanomyovirus isolates in the present study compared to the relatively few reported by Dorigo et al. (12). Sixty-three percent of *g20* sequences (69 out of 110) obtained from Lake Annecy and Lake Bourget were in the culture-containing cluster II, which is in agreement with the distribution pattern in the GOS metagenome database (i.e., 65.6%) (44). However, we did not have sequences in the culture-containing clusters I and III, while 12.7% (65 out of 512) of sequences in GOS field samples were in these clusters. This was the first application of the revised primers CPS1.1 and CPS8.1 for environmental samples, and our results seem to support their improvement in targeting the *g20* gene of cyanomyoviruses.

The *g20* homologue is ubiquitous among T4-like myoviruses, with hosts ranging from proteobacteria to cyanobacteria (14, 44). While using the primer set CPS4/G20-2, which targets the same region of the *g20* gene, Short and Suttle (18) obtained sequences (BES02B-27 and BES02B-28) from waters at a 3,246-m-deep station of the Arctic Beaufort Sea, where cyanobacteria and their phages are not supposed to be found. The two authors speculated that the environmental *g20* sequences out of clusters containing cyanomyovirus isolates were not of cyanophage origin. The *g20* sequence of the clone BES02B-27 grouped with sequences obtained from alpine lakes (this study) (12) and also with those from a paddy field floodwater (20) in the environmental sequence-only cluster Env 6 (Fig. 2). In contrast, the BES02B-28 clustered with *g20* sequences obtained from paddy soil (25) in cluster Env 10. Although the *g20* sequences of BES02B-27 and BES02B-28 clones were generated by another primer set and the CPS1.1/8.1 could not amplify some noncyanomyovirus isolates (44), we might not exclude the possibility that CPS1.1/8.1 could amplify *g20* sequences of T4-like viruses other than cyanomyoviruses for which the sequences of the environmental sequence-only cluster Env 6 may be suspect (see above). This is a common issue of diversity studies through the culture-independent approach by using PCR, since the validity of the primers depends on a limited number of isolates. Recently, Clasen et al. (11) employed the isolation-based/culture-dependent approach to examine marine cyanomyoviruses infecting *Synechococcus* sp. WH7803 and revealed important cooccurring cyanomyovirus genetic diversity. Such an approach could be used for cyanomyoviruses (because they can have a wide host range), and it displays advantages, compared to the culture-independent primer assays, for a robust evaluation of host-virus dynamics in response to environmental factors and changes. However, the isolation-based/culture-dependent approach is likely to explore only a small portion of the cyanomyoviruses and has limitations in terms of gaining access to nonviable viruses as well as those that cannot cross-infect *Synechococcus* sp. WH7803.

Cyanophage *psbA* diversity. Lake Annecy and Lake Bourget are two perialpine lakes located in the same ecoregion and are both dominated by *Synechococcus* spp. (43). It is thus not surprising that all *psbA* sequences obtained from these ecosystems were grouped with viruses infecting *Synechococcus* spp. in the phylogenetic reconstruction, since the viral *psbA* gene was initially acquired from a host, and its transfer was largely limited by host range (36). Viruses infecting *Synechococcus* can be discriminated based on their families and environmental origins in *psbA* phylogenetic trees as clades containing marine podoviruses, freshwater myoviruses, or marine myoviruses (Fig. 1). It is noteworthy that such clustering still deserves validation because of the lack of freshwater podoviruses and the insufficient representation of

freshwater myoviruses and marine podoviruses in phylogenetic analyses that have been proposed until now. Moreover, the intra-genic recombination of the *psbA* gene within cyanophages as well as between hosts and cyanophages has been reported as a common event (36), which could possibly cause phylogenetic confusion if viral *psbA* genes of different cyanobacterial genera and/or different phage families within the same cyanobacterial genus are involved in genetic exchanges. For example, S-ShM1, S-SSM1, and P-SSM1 cluster differently with the myoviruses of the same cyanobacterial genus, and they have been suspected of intragenic *psbA* gene exchanges between marine myoviruses of *Synechococcus* and *Prochlorococcus* (36). The phage-to-phage intragenic exchange of the *psbA* gene among cyanophage families via host intermediates during infection might also be possible in the case of broad-host-range myoviruses (e.g., S-CBM2), which may share the same host (i.e., *Synechococcus* CB0101) with podoviruses (e.g., S-CBP1 and S-CBP3).

The GC content of the *psbA* nucleotide sequences and triplet peptides associated with the D1 protein motif (R/K)ET TXXXS(Q/H) (where XXX marks the position of the triplet peptides) have been proposed or used to distinguish viral *psbA* sequences from the host's in marine ecosystems (36, 38, 39, 52). Although this approach has not been widely tested for freshwater systems, we noticed that the GC content of *Synechococcus* viruslike *psbA* sequences of Lake Annecy and Lake Bourget ranged between 43.3% and 48.2%. In fact, we found that 90% of these *psbA* sequences (56 out of 62) had a GC content below 46% and were thus out of the percentage range (46 to 51%) found in marine *Synechococcus* phages (36). Our results echo the other freshwater studies (11, 41), where low GC content has also been detected in some *Synechococcus*-virus-like *psbA* sequences and in the only known characterized freshwater *Synechococcus* phage, S-CRM01 (44.6%), at the same 729-nt region. Together, these results suggest that freshwater *Synechococcus* phages might have a lower GC content in *psbA* sequences than their marine counterparts, and this may be a result of a lower GC content in their *Synechococcus* hosts. However, one may argue that this conservative *psbA* fragment (729 bp) is too short to predict for the complete *psbA*-coding sequence (approximately 1.1 kb) and to support the above-given assumption. This result will deserve further investigation with increasing available sequences.

Only one virus-specific triplet (EDV) could be detected in alpine lakes and was obtained from the virome of Lake Bourget. The *psbA* sequence to which it belongs is closely related to the freshwater cyanomyovirus S-CRM01 (data not shown). Our study revealed the existence of three triplets (ETE, ESE, and EFE) possessed by the *psbA*-containing cyanophages from Lake Annecy and Lake Bourget, and EFE has never been described before. The *psbA* sequences containing EFE triplets belonged to a single DGGE band and were also closely related to the freshwater cyanomyovirus S-CRM01 (Fig. 2). These sequences occurred in spring in Lake Bourget, when a previous study reported important viral lysis induced by cyanophages (30). ESE and ETE triplets were comparatively more frequently observed in both viruses and hosts (Fig. 1) (37). The ETE triplet was detected in 20 out of 33 currently known marine *psbA*-containing *Synechococcus* myoviruses, in one *Synechococcus* podovirus (*syn26*), in 3 *Prochlorococcus* myoviruses (P-SSM1/9/12), and one podovirus (P-GSP1). Approximately 17% of viruslike *psbA* sequences obtained from the GOS metagenomic data set (37) contained this ETE triplet, and only 5.3% were found

in *psbA*-containing cyanophages from Lake Erie (41). Interestingly, the majority of *Synechococcus* viruslike *psbA* sequences (75.3%) with ETE triplets were from Lake Annecy and Lake Bourget, which may reflect the unique features of their viral *psbA* sequences or their common *Synechococcus* hosts in alpine lakes. The ESE triplet signature was rarely detected in viruslike *psbA* sequences from marine environments (37, 38). It was initially thought to be issued from cyanobacteria exclusively (37). However, the ESE triplet could be found in two freshwater cyanophages obtained from Lake Erie (MC15 and MC17) (41) and three marine cyanophages: S-RSM4 (58) and S-PWM2 and S-PWM3 (38). Our analysis revealed that 17.7% of *Synechococcus* viruslike *psbA* sequences from alpine lakes, 25% from paddy field floodwater (40), and 39.5% from Lake Erie were with ESE. Therefore, it seems that the ESE triplet was preferentially observed in freshwaters, suggesting also perhaps a unique community of potential hosts.

The ecological relevance of the existence of a particular motif found in perialpine lakes is in question. The D1 protein motif (R/K)ETTXXXS(Q/H) is a variable region within the PEST-like domain located in the loop between transmembrane helices D and E. It is implicated as the site of initial cleavage of the D1 protein and initiates the protein turnover from one isoform to another (37, 59, 60). There are two isoforms for D1 protein detected in cyanobacterial hosts (61). Isoform I is constitutively expressed, whereas isoform II is upregulated in response to high light and UV stress (36, 62–64). Sharon et al. (37) analyzed the triplet signatures of D1 protein motifs from the GOS metagenomic database and found that some viruses contain unique viral triplets (virus-specific triplet), while some others share the same motif as the host (host-like triplet). They hypothesized that the unique viral triplets may make D1 protein less susceptible to photodamage and turnover than the host D1, by conferring to the phage a feature similar to isoform II of host D1 protein, which could be important for life support during short periods of phage morphogenesis. Since viral *psbA* sequences were initially acquired from hosts through horizontal gene transfer, the interpretation for the difference of these unique viral triplets could be that, throughout their evolution, viruses modified the D1 protein motif in order to (i) facilitate adaptation to harsh light conditions or (ii) alter its role for their selfish benefits (e.g., by replacing the host light-sensitive D1 protein by the more stable viral D1 protein during infection with the goal to supply continuous energy for viral replication) (37, 60). Interestingly, as described above for our lakes, most viral D1 proteins possess host-like triples (e.g., ETE/ESE, which are detected in 94% of *psbA* sequences, most of which are related to *Synechococcus* podoviruses) and only a few contained virus-specific triplets (e.g., EDV and/or EFE, which are of *Synechococcus* myovirus origin and detected only occasionally in Lake Bourget). These results could thus suggest that the modification of the D1 protein motif by viruses in response to high light and/or stress conditions has not been a frequent event in perialpine lakes, compared to in the ocean, where virus-specific triplets account for up to 30% of GOS viral D1 proteins. This agrees with the finding that D1 proteins of most freshwater *Synechococcus* strains are more resistant to high light and stress (37, 62, 65), so the modification of the D1 protein for their cooccurring viruses may not be required. The sporadic occurrence of unique viral triplet-containing cyanomyoviruses could thus indicate (i) the existence of only a few *Synechococcus* spp. containing light-sensitive D1 proteins, so their viruses needed

to produce their own stable D1 to replace the host's during replication in Lake Bourget, (ii) underwater light intensity in Lake Bourget could be reduced compared to that in Lake Annecy because of the trophic state and higher biomass levels in surface waters of the former (66). All above-given assumptions need a deeper analysis, experimental tests, and verifications to examine how the triplet divergence of viruses affects D1 protein structure and thereby photosynthesis, the difference between the D1 proteins of virus and host in terms of structure and function and between freshwater and marine environments, and if virus-specific D1 is restricted to specific depths or displays a narrow vertical distribution pattern in Lake Bourget.

The majority of *psbA* sequences (51 out of 62) obtained in our study had phylogenetic proximity to estuarine *Synechococcus* podoviruses. These results may indicate that these *psbA* sequences could be of *Synechococcus* podovirus origin and that the perialpine lakes may thus sustain diverse *psbA*-containing cyanopodoviruses. The cyanopodoviruses were also investigated for the same samples by targeting the DNA polymerase gene *polA*, using the primers designed by Chen et al. (8). However, the obtained sequences did not seem to be *polA* sequences and did not belong to cyanopodoviruses (data not shown). When blasted against the virome of Lake Bourget (57) using *polA* sequences of currently available cyanopodovirus isolates (P60, S-CBP1, S-CBP2, S-CBP3, S-CBP42, P-SSP7, syn5, Pf-WMP3, and Pf-WMP4) as queries, no hits were obtained, even when setting the E value at ≤ 0.1 . These results suggest that in perialpine lakes, (i) the *polA* sequences of cyanopodoviruses may be very different from known cyanopodovirus isolates and (ii) the primers used might not be suitable for freshwater cyanopodoviruses, as they were designed based on marine cyanophages. To examine freshwater cyanopodovirus diversity, it is now crucial to obtain sequences from cyanophage isolates since, to the best of our knowledge, no *Synechococcus* podovirus genome has been reported from continental waters so far.

Contrasted cyanophage community compositions among environments. For *g20* sequences, significant differences were detected between Lake Annecy and Lake Bourget ($P = 0.03$) from the pooled 1-year data. This difference could reflect the difference of trophic states between the two lakes resulting in population shifts of the potential hosts in response to environmental changes and/or biological impact, i.e., grazing, predation, or the viral lysis by itself (23). In contrast, however, the *psbA*-containing cyanophages did not show significant composition differences between the two lakes ($P = 0.15$) when considered over the year. This could be explained by the fact that (i) the *psbA*-containing cyanophages contain both cyanomyoviruses and cyanopodoviruses, the majority of which being podoviruses, and (ii) not every cyanophage possesses the host-derived *psbA* gene (5, 30). These results are consistent with a parallel analysis where we observed that *psbA*-containing cyanophages display seasonal dynamic patterns in community structure in both lakes, while it was not the case for *g20* cyanomyoviruses (X. Zhong, F. Rimet, and S. Jacquet, unpublished data). The detection of similar *psbA*-containing phages in Lake Annecy and Lake Bourget may suggest similar annual host populations in these ecosystems, since cyanopodoviruses are generally host specific. Beyond this local aspect, it is of note that possessing *psbA* in cyanophage genomes may be beneficial for the hosts, the photosynthesis productivity being indifferently maintained or enhanced in Lake Annecy and Lake Bourget

(34, 67). The selection pressure might in priority accommodate the limited nutrients for hosts of *psbA*-containing phages to sustain an annually constant population for which the viral lysis could help in nutrient recycling. This will need further investigation.

At last, we examined whether cyanomyoviruses of perialpine lakes differed from other freshwater and marine environments. Previous studies used the UniFrac analysis (25, 44) to statistically infer community composition dissimilarity of *g20* cyanomyoviruses between environments. However, because the sequences recruited for phylogenetic reconstruction of each environment (Table 1) were from different studies for which the sample(s) was either from a single time point or period or from water issued from different depths and treated using different primers (CPS1/8, CPS1.1/8.1, and CPS4/G20-2) and with different sequencing depths of coverage, the method could not be used. Indeed, such an unbalanced data set to compare environmental traits of *g20* sequences using UniFrac would generate here an erroneous view, particularly for Chilliwack Lake, Catfish Pond, Malaspina Inlet, Pendrell Sound, Coast of Colombia, and Coast of Chile, for which only one sequence was available. Nevertheless, our phylogenetic reconstruction was sufficient to show that *g20* cyanomyoviruses are clearly different between the subalpine lakes and the other environments, especially the freshwaters. Among the four clusters containing cyanomyovirus isolates (i.e., culture-containing clusters I, II, and III and cluster 3), the culture-containing cluster II had no freshwater *g20* sequences other than ours. In contrast, the other three clusters contained *g20* sequences from other freshwaters (Lake Erie, Lake Constance, Cultus Lake, paddy field floodwater, and shore pond mat) but none from our lakes (Fig. 2). This could reflect the unique environment of perialpine lakes, where a distinct viral community exists. This is not surprising when one knows that both Lake Annecy and Lake Bourget have the same geological and water origin and also a unique host community (66).

Conclusion. As in other investigated ecosystems, cyanophages seem very diverse in (peri)alpine lakes, and we found new sequences that seem to be unique to these ecosystems. In contrast to the only available and comparable study for Lake Bourget conducted a few years ago (12), a large proportion of *g20* sequences obtained in 2011 were grouped with cyanomyovirus culture isolates. This result was likely due to the improvement of primers CPS1.1/8.1 on targeting cyanomyoviruses. For the first time, *psbA*-containing cyanophages were also investigated, and our results revealed that (i) they were all of *Synechococcus* phage origin, most of which were likely podoviruses, and that (ii) freshwater *psbA* sequences displayed distinct viral triplet D1 protein motifs compared to their marine counterparts, and a novel one was discovered (e.g., EFE). The cyanomyovirus community compositions were significantly different between the two lakes, while it was not the case for *psbA*-containing cyanophages, suggesting that this last group differed from the former and that all cyanophages could respond differently to their surrounding biology and/or environment within each lake.

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