Gene Flow, Recombination, and Selection in Cyanobacteria: Population Structure of Geographically Related *Planktothrix* Freshwater Strains

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Several *Planktothrix* strains, each producing a distinct oligopeptide profile, have been shown to coexist within Lake Steinsfjorden (Norway). Using nonribosomal peptide synthetase (NRPS) genes as markers, it has been shown that the *Planktothrix* community comprises distinct genetic variants displaying differences in bloom dynamics, suggesting a *Planktothrix* subpopulation structure. Here, we investigate the *Planktothrix* variants inhabiting four lakes in southeast of Norway utilizing both NRPS and non-NRPS genes. Phylogenetic analyses showed similar topologies for both NRPS and non-NRPS genes, and the lakes appear to have similar structuring of *Planktothrix* genetic variants. The structure of distinct variants was also supported by very low genetic diversity within variants compared to the between-variant diversity. Incongruent topologies and split decomposition revealed recombination events between *Planktothrix* variants. In several strains the gene variants seem to be a result of recombination. Both NRPS and non-NRPS genes are dominated by purifying selection; however, sites subjected to positive selection were also detected. The presence of similar and well-separated *Planktothrix* variants with low internal genetic diversity indicates gene flow within *Planktothrix* populations. Further, the low genetic diversity found between lakes (similar range as within lakes) indicates gene flow also between *Planktothrix* populations and suggests recent, or recurrent, dispersals. Our data also indicate that recombination has resulted in new genetic variants. Stability within variants and the development of new variants are likely to be influenced by selection patterns and within-variant homologous recombination.

Bacteria, and cyanobacteria in particular, have been shown to form population subdivisions (1–3). How bacteria disperse and the mechanisms that cause them to genetically differentiate or to maintain homogeneity are, however, far from established. Some advances have been made in genetic subdivision of the marine cyanobacteria *Prochlorococcus* and *Synechococcus*, which show niche partitioning and thus the formation of ecotypes (1, 4). Notably, these ecotypes can be separated based on sequence differences in 16S rRNA genes (more than 95 to 96% similarity [4, 5]). The widely distributed freshwater cyanobacterial species *Planktothrix rubescens* and *Planktothrix agardhii* (6–10) share identical or nearly identical 16S rRNA gene sequences (8). *Planktothrix* strains in the Norwegian Lake Steinsfjorden have been shown to produce several different types of oligopeptides. Strains may have unique oligopeptide profiles, allowing a definition of distinct chemotypes (CHT) (2). A field study identified coexisting strains representing four chemotypes in Lake Steinsfjorden. These chemotypes differed considerably in ecological traits and had a long history of coexistence and thus were defined as ecotypes (2) sensu Koeppel et al. (11).

Cyanobacterial oligopeptides form a diverse group of compounds that are either produced by nonribosomal peptide synthetases (NRPSs; large multisubunit enzymes) (12) or via a ribosomal pathway followed by enzymatic modifications (13, 14). Large gene clusters encode the NRPSs, which for some of the most frequently occurring oligopeptides, such as microcystin (*mcy*), cyanopeptolin (*oci*), and aeruginosin (*aer*), have been sequenced from *Planktothrix* (13). Correlation between distinct phylogenetic clusters

(distinct genetic clusters are henceforth denoted as genetic variants), using *mcyC*, *mcyD*, and *ociB* genes and oligopeptide profiles suggests a link between chemotypes and genetic differentiation of *Planktothrix* in the Norwegian Lake Steinsfjorden (3). The coexistence of several types of genetic *Planktothrix* variants could imply that the oligopeptide composition of a strain may contribute to its ecological adaptation and that we may define the various variants as subpopulations of *Planktothrix* (we use the prefix "sub" since the bacteria are coexisting within the same lake).

Discussions about the processes that may lead to distinct within-species variants have focused on two main models. The first model emphasizes the importance of selective sweeps, where ecological specialization is driven by selection and a low level of gene flow. The second model explains sequence clusters as a cohesive effect of recombination event(s) (15). This second model considers no recombination barriers between clusters of sequences (16–18), except that the recombination frequency decreases with increased sequence divergence. With no barriers, recombination

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TABLE 1 Length of entire gene and of the gene used as a marker and best BLAST hit for *ociB*, *mcyC*, *glyT*, and *recA*

Region	Total length[a] of gene (bp)	Length of marker (bp)	Start/stop positions in gene	Best BLAST hit [organism] (E-value, score)
ociB	14,130	876	1238/4515	*ociB* [*Planktothrix agardhii* NIVA CYA 116] (0.0, 5405)
mcyC	3,726	982	1784/2865	Peptide synthetase [*Planktothrix agardhii* 213] (0.0, 709)
glyT	1,182	754	211/995	Glycosyltransferase, group 1 [*Lyngbya* sp. strain PCC 8106] (4e–107, 391)
recA	1,089	650	208/899	Recombination protein A [*Lyngbya* sp. strain PCC 8106] (4e–110, 400)

[a] That is, the total length of gene in the *Planktothrix* genome NIVA CYA 98 according to RAST annotation.

and gene flow between species may have a diversifying effect, promoting a network of species (for a review, see the study by Fraser et al. [19]). However, a third model and perhaps the most likely explanation for structure of distinct variants is a balance between recombination and positive selection (20). Recombination has been shown to occur in cyanobacteria (21), also involving NRPS genes in strains of both *Microcystis*, *Anabaena*, and *Planktothrix* (22–27). These recombination events are often associated with changes in the produced microcystin isoforms. Findings of positive selection suggest an important function for a given cyanopeptolin isoform (25).

Kurmayer and Gumpenberger (6) were the first to reveal homogeneous and more heterogeneous populations of *Planktothrix* in geographically close but spatially isolated lakes. In order to generalize the findings of differentiation of *Planktothrix* variants in Lake Steinsfjorden (2, 3), we have here gathered a data set comprising *Planktothrix* strains from four freshwater localities in southeast of Norway: Lake Steinsfjorden, Lake Kolbotnvannet, Lake Gjersjøen, and Lake Lyseren. In addition to the NRPS genes, *ociB* and *mcyC*, we have included two non-NRPS genes, *recA* (recombination protein A) and *glyT* (glycosyl transferase 1). The *recA* and *glyT* genes encode proteins not associated with NRPS functions. Analyses of phylogenetic patterns and genetic diversity reveal that differentiation into several distinct *Planktothrix* variants is not confined to Lake Steinsfjorden only, nor is the differentiation NRPS dependent. Regardless of the lake affiliation, our data indicate gene flow both within variants but also between populations in the different lakes. The biological implications of these results are discussed.

MATERIALS AND METHODS

***Planktothrix* cultures and study sites.** In all, 82 *Planktothrix* strains (*P. rubescens* and *P. agardhii*) from four lakes in southeastern Norway were investigated: Lake Steinsfjorden, Lake Kolbotnvannet, Lake Gjersjøen, and Lake Lyseren. Because of freshwater flow from Lake Kolbotnvannet to Lake Gjersjøen, these two lakes share the same drainage system. Otherwise, the water bodies belong to separate drainage systems and are therefore not in direct contact. They also differ with regard to size, phosphorus and nitrogen concentrations, and maximum depths. Both Lake Steinsfjorden and Lake Lyseren are nutrient-poor and has maintained an almost stable trophic status throughout the last decades. Nutrient levels in Lake Kolbotnvannet and Lake Gjersjøen, on the other hand, have decreased from extremely high (ca. year 1960 to 1980) to moderately high within the last 50 years. During this time period, the nutrient concentration in Lake Kolbotnvannet has been approximately twice as high as in Lake Gjersjøen (Thomas Rohrlack, unpublished data). The phosphor levels in Lake Gjersjøen have been limited since the 1980s.

Monitoring of Lake Kolbotnvannet and Lake Gjersjøen provide evidence for *Planktothrix* occurrence in these lakes since 1962. *Planktothrix* blooms were first observed in 1964 (28). No monitoring has been conducted for Lake Steinsfjorden and Lake Lyseren. However, the oldest included sample from Lake Steinsfjorden in this study was isolated in 1964.

All strains included were isolated in the period from 1964 to 2008 as described previously (7) and were cultured nonaxenically in Z8 medium (29). Previously, Rohrlack et al. determined the oligopeptide profile and chemotype affiliation of *Planktothrix* strains. Strains and chemotypic classifications are described in Table SA1 in the supplemental material (7).

DNA isolation, amplification, and sequencing. DNA was isolated by using an EZNA plant DNA minikit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions. Variable regions in the NRPS genes—*ociB* from the cyanopeptolin synthetase (*oci*) gene cluster and *mcyC* from the microcystin synthetase (*mcy*) gene cluster—were utilized as markers (3). In addition, two non-NRPS markers—*recA* and *glyT*—were introduced. These gene regions were chosen based on a comparison of the completely sequenced *Planktothrix* NIVA CYA 98 (13) genome (CHT 1) with sequences from two other genomes in progress (NIVA CYA 407 and 405), representing CHT 5 and CHT 9, respectively. Regions within the *recA* and *glyT* genes (i.e., the amino acid coding regions; see Table 1 for specific sites and details) were chosen as markers since they met certain requirements (100 to 300 bp length, at least 30 variable sites, and conserved regions on both sides for primer annealing). The *recA* gene has been used to study phylogenetic and nucleotide diversity in *Microcystis* genotypes (30). Putative functions of the proteins encoded by the two non-NRPS genes were identified by using BLAST and InterProScan. The length, the start and stop position in the gene, and the putative functions of all genes are listed in Table 1 and in Table SA3 in the supplemental material. The non-NRPS genes have no known functional connection to the NRPS genes.

PCR and sequencing primers were designed using Vector NTI (Invitrogen, Carlsbad, CA) and CLC DNA Workbench 5 (CLC Bio, Aarhus, Denmark). Primers used for amplification and sequencing are listed in Table SA4 in the supplemental material. All PCRs were performed using BD Advantage 2 buffer and enzyme. Sanger sequencing was performed using an ABI 3730 DNA analyzer and BigDye Terminator v3.1 solution (Applied Biosystems, Foster City, CA). All sequences are accessible in GenBank under the accession numbers JN563931 to JN563953, JN597239 to JN597260, JN563954 to JN563979, and JX967756 to JX967995.

Sequence analyses and phylogeny. Sanger sequence reads were edited and assembled using Vector NTI and Sequencher 4.8 software. All sequences were aligned using CLUSTAL W (31) in the MEGA 4.0 software (32). Concatenated alignments were made for all four gene regions, using MacClade (33). Maximum-likelihood (ML) and Bayesian topologies were constructed for all four gene regions separately and for the concatenated alignment. Bayesian analyses were run using Mr.Bayes 3.1 (34). GTRCAT was chosen as a model (identified as the best model by Modeltest [35]); the MCMC procedure was set to run for 3 million generations with trees sampled every 100th generation, and the first 10% of all trees were removed. ML analyses were carried out by RAxML (36) with 100 starting trees and GTRCAT as a substitution model. Air-Appender, ML, and Bayesian analyses were performed at UiO Bioportal (www.bioportal.uio.no).

Putative recombination sites were identified utilizing SplitsTree 4 (37) with GTR as an evolutionary model and 1,000 bootstrap resamplings. The statistical significance of recombination was evaluated using the phi test.

Selective forces were investigated by analyzing the proportion of nonsynonymous (Ka) and synonymous (Ks) substitutions using DnaSP (38)

with a sliding window, window size of 50, and step size of 10. We also identified positively selected sites using CODEML from PAML v3.15 (39). The models used for likelihood ratio tests in this study were M1a (neutral), M2a (selection), M7 (beta), and M8 (beta and ϖ), with M1a and M7 as null models. PAML was run utilizing AIR-Identifier at UiO Bioportal.

DnaSP was also used to calculate nucleotide diversity within and between phylogenetic clusters, and to compare between lakes differences (strains belonging to same cluster, but derived from different lakes). Nucleotide diversity was calculated as the average number of nucleotide substitutions per site (Pi).

RESULTS

Similar *Planktothrix* variants in several Norwegian lakes. Phylogenetic analyses of 82 *Planktothrix* strains from four Norwegian lakes—Lake Steinsfjorden, Lake Kolbotnvannet, Lake Gjersjøen, and Lake Lyseren—showed highly similar and well-supported topologies for both the (*ociB* and *mcyC*) and non-NRPS genes (*recA* and *glyT*) (see Fig. SA1a to d in the supplemental material). Overall, the topologies divided the *Planktothrix* strains into either three or four main phylogenetic clusters. Notably, nearly identical clustering was found for both NRPS and non-NRPS genes. The structures of the genetic clusters are in high accordance with the oligopeptide chemotypes defined by Rohrlack et al. and we defined the distinct clusters as variants 1, 5, 7, and 9, according to the chemotypic classifications (2, 7). Deviations were found in the *recA* and *mcyC* topologies, where some of the variant 5 strains formed unresolved clusters with respect to variant 1 and/or variant 7 strains. These incongruent signals are likely due to lower resolution in the *mcyC* and *recA* genes or recombination between variant 1 and 5. In *glyT*, the topology split variant 7 in two clusters, although the split has low statistical support. Despite several attempts, the NIVA-CYA 604 strain is missing in *mcyC* due to poor PCR performance. All PCR-amplified strains are listed in Table SA1 in the supplemental material. Incongruent topologies were also detected for strains NIVA-CYA 15, NIVA-CYA 604, NIVA-CYA 86, NIVA-CYA 10, NIVA-CYA 117/4, and NIVA-CYA 597.

The concatenated data set reveal four main variants, however, as a result of incongruence for some strains, as mentioned above, and low resolution in *recA* and *mcyC*; variant 5 strains are split into two distinct clusters in the concatenated topology (Fig. 1). All variants contain strains from at least two lakes (see Fig. 1 and 2). NRPS and non-NRPS topologies, together with chemotypic classifications, indicate that NIVA-CYA 15 (variant 14) and NIVA-CYA 86 (variant 12) represent phylogenetically novel and distinct *Planktothrix* variants.

Recombination events between genetic variants. To investigate possible recombination events, we performed split decomposition analyses (Fig. 2). The analyses revealed reticulated topologies for NIVA-CYA 15, NIVA-CYA 604, and NIVA-CYA 86 in *ociB*, *recA*, and *glyT*, indicating a possible history of recombination, as already suggested by the phylogenetic analyses. *P* values below 0.05 in *ociB*, *recA*, and *glyT* show that the results are highly significant. Recombination was indicated for NIVA-CYA 597 in the *ociB* and *mcyC* genes. Split decomposition analysis and phi testing did not indicate recombination within the *mcyC*. However, a split decomposition analysis is unable to identify recombination that spans the entire sequence region. Recombination in NIVA CYA 597 is therefore likely to be a recombination of the whole A domain in *mcyC* or the entire gene. Visual inspection of variable sites in the alignment reveals a mosaic pattern in several strains (100% similarity to different *Planktothrix* variants in different

parts of the gene). Figure 3 illustrates an example of this mosaic pattern in comparison of NIVA-CYA 15 with representative strains from the other variants.

Genetic diversity within and between variants. By nucleotide diversity analyses, we calculated the average number of nucleotide substitutions per site (Pi, Fig. 4). Within-variant calculations (including strains from all four lakes) showed almost no nucleotide diversity (on average 0.00021 nucleotide substitutions per site for all genes; Fig. 4). Strains were also divided by their lake affiliations, and genetic diversity was calculated between groups of strains, in a between-lake comparison. Pi values showed very low diversity (on average for all between-lakes measures; 0.00008 average nucleotide substitutions per site). In comparison, significantly higher nucleotide diversity was found between variants (average for all markers of 0.0273) when strains from all lakes were included.

Negative and positive selection. Analyses using PAML indicated that >87% of all sites in *ociB* and >97% of all sites in *mcyC*, *recA*, and *glyT* were subject to negative selection. Also, DnaSP analyses of *ociB* and *mcyC* essentially indicated negative selection (Ka/Ks < 1), but in addition also indicated sites with strong positive selection (Ka/Ks values ≫ 1) (Fig. 5 and see Table SA2 in the supplemental material).

For *ociB*, PAML analyses detected positive selection in amino acid 204, which corresponds to nucleotide (nt) 612 (95% confidence). In addition, DnaSP showed positive selection in a window between nt 161 and 210 (between variant 1 versus variant 5 and variant 5 versus variant 12) and nt 741 and 810 (between variant 1 versus variant 14 and variant 1 versus variant 9). Several sites in the DnaSP *ociB* graph displayed peaks with Ka/Ks values approximately equal to 1, indicating relaxed (neutral) selection.

PAML showed no significant positive selection in *mcyC*, whereas DnaSP indicated positive selection in several regions (between variant 1/5 versus variant 9, variant 7 versus variant 9, and variant 9 versus variant 12). The investigated region includes an A-domain. Previous liquid chromatography-tandem mass spectrometry analyses have shown that the McyC A-domain incorporate arginine in all microcystin isoforms produced by the investigated *Planktothrix* strains (2, 7), indicating that the putative positive selection does not result in an amino acid change at this position of the isoform.

DnaSP showed signs of relaxed selection between several of the variants in the *glyT* gene in a window between amino acids 131 and 180. PAML also indicated selection, in amino acid 29, which corresponds to nt 87 (LTR value indicating high significance). Neither PAML nor Ka/Ks analyses showed sign of positive selection in the *recA* gene.

DISCUSSION

Eighty-two *Planktothrix* strains sampled from four freshwater lakes in the southeast of Norway (Lake Steinsfjorden, Lake Kolbotnvannet, Lake Gjersjøen, and Lake Lyseren) have been investigated utilizing NRPS genes (*ociB* and *mcyC*) and non-NRPS genes (*recA* and *glyT*) as markers. In the present study we show that the distinct and coexisting *Planktothrix* variants present in Lake Steinsfjorden also are present in other freshwater bodies in southeastern Norway. A structure of distinct variants is further supported by nucleotide diversity analyses of all genes, revealing low diversity within the genetic variants regardless of lake origin.

Importance of gene flow for genetic structure and presence of similar variants in several lakes. As the phylogenetic and ge-
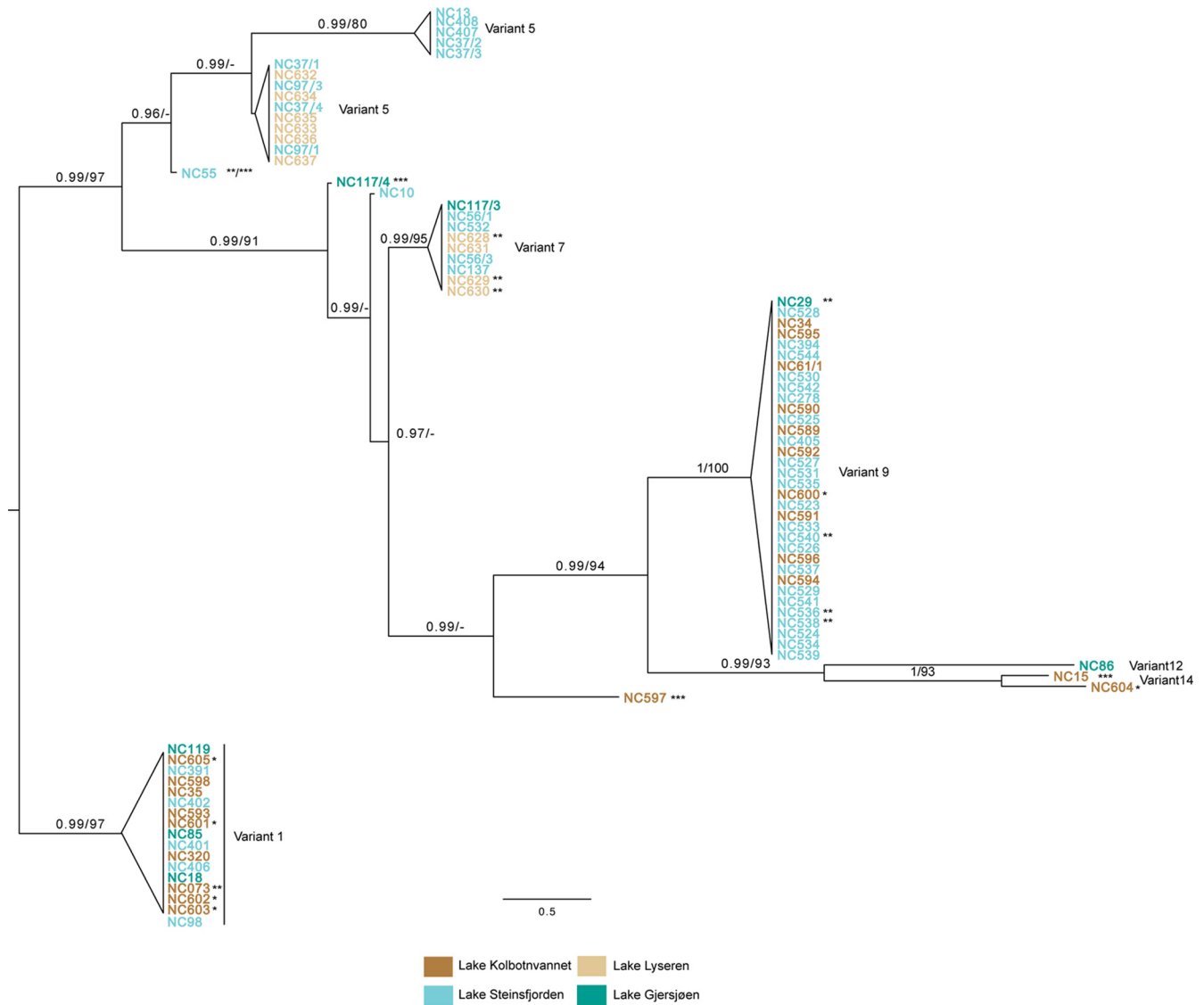
FIG 1 Phylogenetic analyses of *Planktothrix* strains: concatenated data set. Bayesian topologies of a concatenated data set of both NRPS and non-NRPS genes are shown. Bayesian probability and ML bootstrap values >80% are shown on all branches. Colors: Lake Kolbotnvannet, dark brown; Lake Steinsfjorden, turquoise; Lake Lyseren, light brown; Lake Gjersjøen, green. *, missing chemotypic classification; **, disagreement between genetic clustering pattern and chemotype classification by Rohrlack et al. (2); ***, incongruent genetic topologies (see Fig. S1 to S4 in the supplemental material).

netic diversity analyses indicate, strains from all lakes fall within the cluster of variant 1, 5, 7, or 9 with high support and reveal that *Planktothrix* forms a structure of distinct variants in all four lakes. Except for strains from Lake Lyseren, which were sampled in 2008, the investigated strains were sampled repeatedly between 1965 and 2008. Considering that *Planktothrix* has probably been present in the water bodies between 50 and 100 years, a likely explanation for the stability for over 40 years may be a high frequency of gene flow. A possible interpretation of the high between-variant/within-variant genetic diversity ratio (see Fig. 4) is that there is more frequent recombination within than between variants. The majority of sites in all of the investigated genes are under negative selection, which is likely to influence the maintenance of this structuring of distinct variants. Nevertheless, the presence of positively selected sites indicates possibilities for new variation within the *Planktothrix* community. This combination of positive and

purifying selection has been detected earlier in the microcystin and cyanopeptolin gene clusters (3, 6, 26, 40, 41) and has been suggested to be the driving force in differentiation of *Planktothrix* variants (3).

Although the results show that the variants are present in several lakes, not all variants are present in all lakes. Which mechanisms are involved in this structuring of the *Planktothrix* metapopulation? Analyses of genetic diversity indicate high gene flow both within the genetic variants and between the study lakes. The presence of similar variants in different lakes may be due to dispersal between the lakes via recreational activity, birds, or other animals; it could also have been due to recent events or even recurrent events.

Similar selective pressure regardless of ecological conditions. The presence or absence of variants in a given lake are likely to be influenced by factors that control growth and/or loss pro-
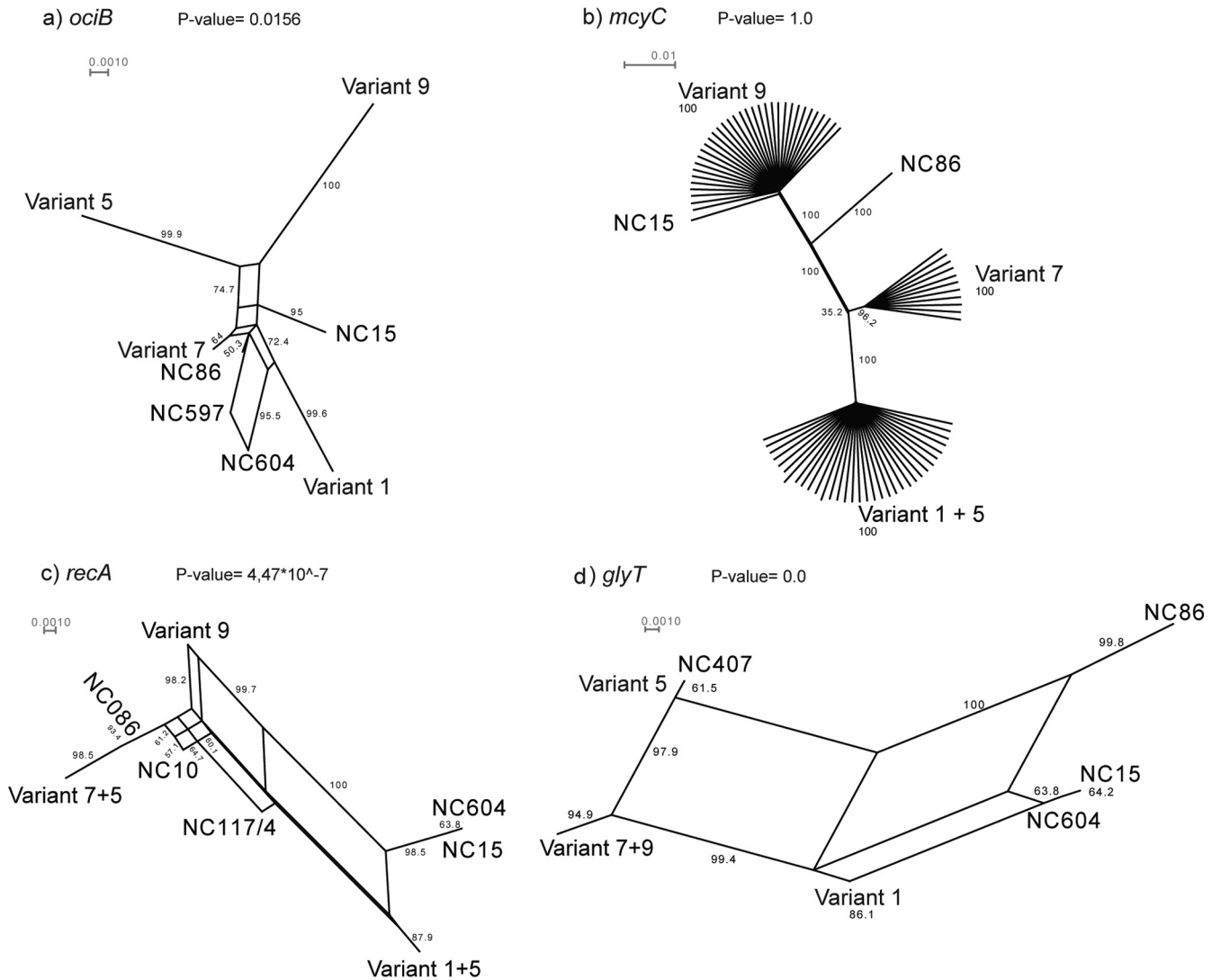
FIG 2 Split decomposition analyses of *Planktothrix* strains from the four investigated lakes. Split network analyses are shown for *ociB* (a), *mcyC* (b), *recA* (c), and *glyT* (d). Bootstrap values are shown. Strains are denoted by their variant classification. Strains with incongruent topology or shown as single nodes in Fig. 1 are indicated as NC numbers (NIVA-CYA 15, NIVA-CYA 86, NIVA-CYA 604, NIVA-CYA 10, and NIVA-CYA 117/4). Phi test *P* values are also shown.

cesses. A pattern of variant structure seems to be present in all four lakes, regardless of the ecological conditions in these lakes (see Table SA5 in the supplemental material for more details). Further, due to the differences in ecological conditions, we might expect differences in selective pressures acting on the various gene vari- ants; however, all variants seem to be predominantly under nega- tive selection. A previous study (2) found no correlation between the success of *Planktothrix* CHTs and water temperature or the availability of major nutrients. Other abiotic factors, such as light, may, on the other hand, play a role in maintaining these variants.
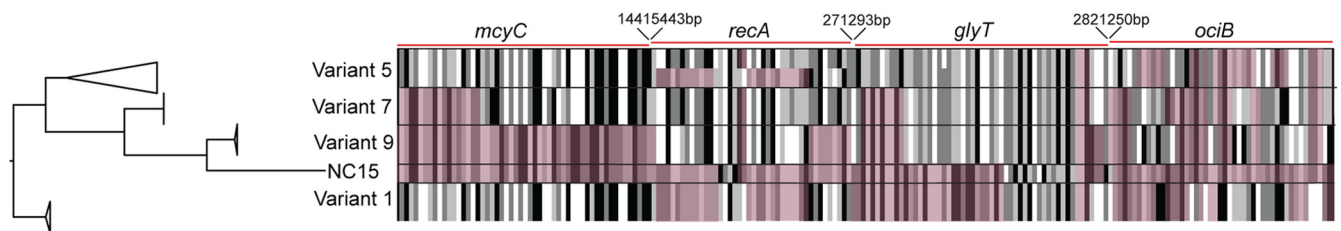

FIG 3 Use of multiple alignment to visualize an example of mosaic pattern and recombination. The concatenated alignment shows only variable sites. The four genes are placed in a correct order, as they are located on the NIVA-CYA 98 genome, and distances between genes are indicated. Representatives from the four genetic major variants and NIVA-CYA 15 are aligned, and areas marked with a red shade indicate identical regions between NIVA-CYA 15 and the variants.
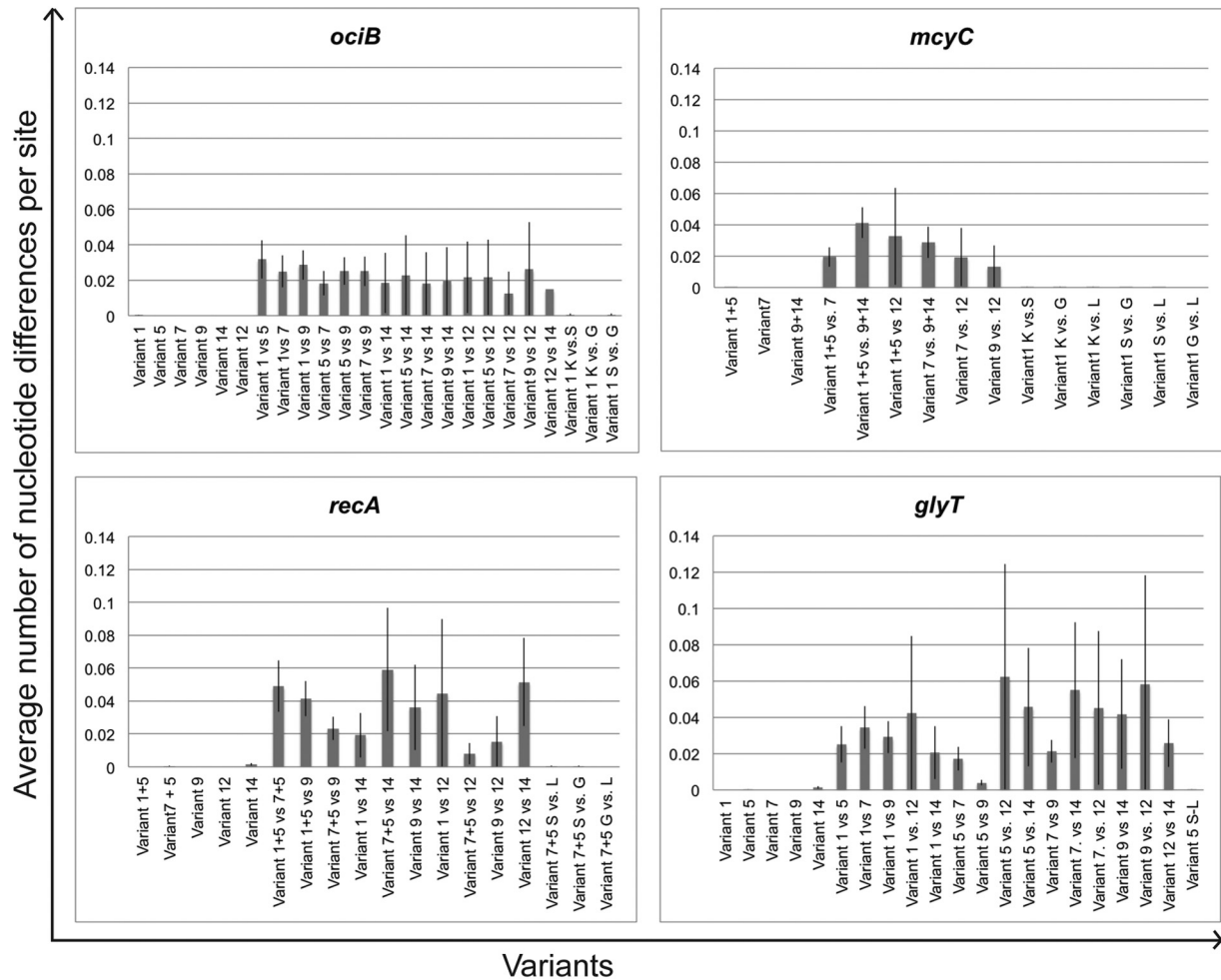
FIG 4 Histograms showing genetic diversity between and within the *Planktothrix* variants. Genetic diversity was measured by average number of nucleotide substitutions per site (Pi). Error bars indicate the standard deviations of Pi. Abbreviations: G, Lake Gjersjøen; S, Lake Steinsfjorden; K, Lake Kolbotnvannet; L, Lake Lyseren.

Another possibility is that the variants we demonstrated here may be the result of biological factors that control their presence, such as pathogens, parasites, and grazers. In this respect, it is of interest to note that the oligopeptides have been suggested to be involved in the defense against predators (42, 43) and fungal parasites (44).

Recombination, gene flow, and formation of new genetic variants. Our results also show the presence of other, less abundant variants. For example, several analyses indicate that NIVA CYA 15-K might be a recombinant of variants 1 and 4. Variants 1 and 4 produce microcystins with an unmethylated amino acid at position 7 or 3, respectively, whereas NIVA CYA 15-K produces the unusual microcystin [Asp3][Dha7]Mcyst-RR with unmethylated amino acids in both positions. This could possibly be a result of a recombination in the microcystin operon (17). Split decomposition analysis supports this hypothesis. Based on the genetic analyses, we regard NIVA CYA 15-K as a true variant. NIVA-CYA 604, isolated from Lake Kolbotnvannet in 2007, clusters close together with NIVA-CYA 15 in *recA* and *glyT* and shows a mosaic pattern indicating recombination. No oligopeptide profile is available for NIVA-CYA 604. Several other strains also show signs of recombination (see Fig. 2). In the chemotypic classifications of

Rohrlack et al. (7), NIVA-CYA 10 and NIVA-CYA 117/4 were both classified as CHT 7. A visual inspection of the alignment indicates that NIVA-CYA 117/4 is a true recombinant with a mosaic pattern that switches between variant 1-5-1 (data not shown). However, NIVA-CYA 10 cannot be excluded as a chimeric sequence. Our data indicate that recombination is an ongoing process in the *Planktothrix* population.

Low diversity of Norwegian variants compared to other *Planktothrix* communities in Fennoscandia and Central Europe. Our findings of frequent dispersal between lakes are supported by an investigation of *Planktothrix* strains isolated from Fennoscandian lakes (7). Here, CHT 1 was shown to be present in lakes all over Fennoscandia. However, several additional CHTs were found in only 1 or 2 of the 13 lakes investigated. Further, genetic data from a larger geographic study from northern and mid-European lakes indicate similar genetic structure of *Planktothrix* variants (H. Sogge et al., unpublished data).

Compared to 15 oligopeptide chemotypes identified in Lake Maxsee, Germany (9), and 25 genetic variants from different Central European lakes (6), the *Planktothrix* communities in the present study have a substantially lower diversity of variants. A possi-
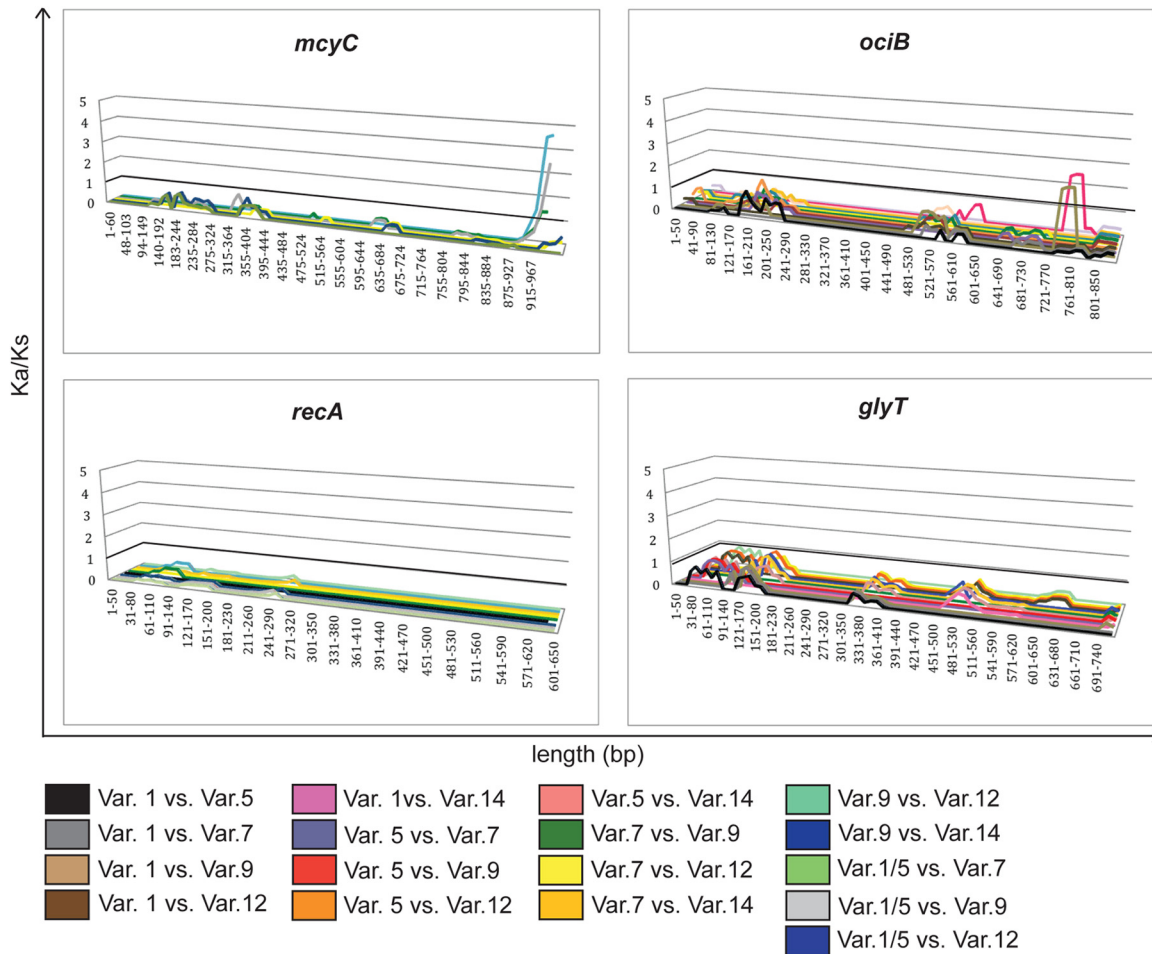
FIG 5 Analyses of nonsynonymous/synonymous (Ka/Ks) substitutions between *Planktothrix* variants. Positive selection is indicated in sites with Ka/Ks values of >1 while purifying selection in sites with a Ka/Ks score of <1.

ble explanation is that the *Planktothrix* communities in Middle European lakes are far older and thus have had more time to diverge or simply that the ecosystems in Central Europe are more complex. It should be noted that the area around Lake Kolbotn-vannet and Lake Gjersjøen has been a center of urbanization for the last 50 to 60 years, resulting in a complete change of the ecological conditions in Lake Kolbotnvannet and allowing *Planktothrix* to colonize this habitat. However, genetic differences between variants shown for all genes support the hypothesis that the variants are older than the populations in the four investigated lakes and may have been separated over a longer period of time.

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