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Genetic Diversity of Eukaryotic Plankton Assemblages in Eastern Tibetan Lakes Differing by their Salinity and Altitude

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Abstract

Eukaryotic plankton assemblages in 11 high-mountain lakes located at altitudes of 2,817 to 5,134 m and over a total area of ca. one million square kilometers on the Eastern Tibet Plateau, spanning a salinity gradient from 0.2 (freshwater) to 187.1 g l⁻¹ (hypersaline), were investigated by cultivation independent methods. Two 18S rRNA gene-based fingerprint approaches, i.e., the terminal restriction fragment length polymorphism and denaturing gradient gel electrophoresis (DGGE) with subsequent band sequencing were applied. Samples of the same lake type (e.g., freshwater) generally shared more of the same bands or T-RFs than samples of different types (e.g., freshwater versus saline). However, a certain number of bands or T-RFs among the samples within each lake were distinct, indicating the potential presence of significant genetic diversity within each lake. PCA indicated that the most significant environmental gradient among the investigated lakes was salinity. The observed molecular profiles could be further explained (17–24%) by ion percentage of chloride, carbonate and bicarbonate, and sulfate, which were also covaried with change of altitude and latitude. Sequence analysis of selected major DGGE bands revealed many sequences (largely protist) that are not related to any known cultures but to uncultured eukaryotic picoplankton and unidentified eukaryotes. One fourth of the retrieved sequences showed 97% similarity to the closest sequences in the GenBank. Sequences related to well-known heterotrophic nanoflagellates were not retrieved from the DGGE gels. Several groups of eukaryotic plankton, which were found worldwide and detected in low land lakes, were also detected in habitats located above 4,400 m, suggesting a cosmopolitan distribution of these phylotypes. Collectively, our study suggests that there was a high beta-diversity of eukaryotic plankton assemblages in the investigated Tibetan lakes shaped by multiple geographic and environmental factors.

Introduction

Saline lakes at high altitudes are often productive [52] and represent an important and extreme ecosystem harboring many novel prokaryotic microorganisms [21, 31, 34]. Yet, almost nothing is known about the diversity and dynamics of eukaryotic plankton assemblages in such environments. Both salinity and elevation can be expected to have a fundamental impact on microbial communities of lakes. Salinity has been found to be an important factor influencing the microbial food web [37] and shaping prokaryotic communities, resulting in only little overlap of bacterial communities between freshwater and saline lake ecosystems [54]. Similarly, solar ultraviolet radiation (UVR; 290–400 nm) is a crucial environmental factor in high-mountain lakes because of the natural increase of UVR flux with elevation and the usually higher water transparency of high-mountain lakes [43]. Solar UV radiation is harmful to various aquatic microorganisms [43, 44] and has been shown to alter both planktonic and benthic communities [38, 49].

In contrast to the many efforts of exploring prokaryotic diversity in such aquatic habitats (e.g., [2, 34, 54]), our knowledge about the genetic diversity of eukaryotic plankton assemblages is very scant [10]. Recent application of molecular approaches to eukaryotic plankton assemblages (largely protists) using the 18S ribosomal RNA (rRNA) gene as phylogenetic marker has revealed high levels of protistan diversity in marine ecosystems (e.g., [12, 13, 15, 29, 32, 33, 35]). On the contrary, only few studies have investigated the genetic diversity of eukaryotic plankton assemblages in inland freshwaters [17, 23, 40, 42], which were found different from those in marine ecosystem. Interestingly, it is still not known whether slow evolution of saline lakes from freshwater lakes may allow eukaryotic plankton originally adapted to freshwater conditions to adapt to saline conditions. The increase of UVR with altitude [4] may, in addition, influence the distribution and structure of eukaryotic plankton assemblages in saline high-mountain lakes.

The present study aims at (1) revealing the influence of salinity or ion concentration on eukaryotic plankton assemblages in habitats with stable salinity conditions, (2) investigating if typical groups of eukaryotic plankton assemblages, which were frequently detected in lowland habitats, are also present in high-mountain lakes located at extreme altitudes, and finally (3) exploring if there are yet undetected eukaryotic microorganisms in these underexplored environments. We, therefore, investigated the overall community structures based on the 18S rRNA genes in 11 lakes located on the East Tibetan Plateau by means of cultivation-independent terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) analyses. Subsequent DGGE band sequencing allowed a phylogenetic affiliation of dominant community members. Both fingerprinting methods have been applied for diversity estimation and community profiling of eukaryotic assemblages (largely the protistan assemblages and picoeukaryotic communities) in marine (e.g., [8, 9, 12–14, 25]) inland water ecosystem [10, 24, 48], and other habitat [41]. The investigated lakes are characterized by a broad salinity gradient from freshwater (0.2 g l^{-1}) to hypersaline (187 g l^{-1}), as well as by an altitude gradient ranging from 2,817 to 5,134 m. The set of investigated lakes includes Lake Qinghai, which is the seventh largest saline lake in the world, as well as five lakes located at altitudes above 4,400 m, which are among the most elevated lakes in the world.

Materials and Methods

Study Sites and Sampling

Eleven lakes located on the East Tibetan Plateau at altitudes ranging from 2,817 to 5,134 m above sea level were investigated (Fig. 1). The lakes were chosen in order to cover a salinity

gradient from 0.2 to 187.1 g l⁻¹ [50]. Water samples were collected not only from surface waters (top 50 cm) with a 5-l Schindler sampler but also from different water depths in the large, deep lakes Qinghai and Namocuo (Table 1). Fifty-milliliter water samples were fixed with 2% formaldehyde (final concentration) on location and stored at 4°C in dark for subsequent quantification of heterotrophic nanoflagellates (HNF) and bacteria and were analyzed within 2 months. About 300-ml water samples for determination of phytoplankton, and ciliates were preserved with 1.5% Lugol's Iodine. Plankton samples (250–500 ml water) for DGGE and T-RFLP analyses were collected on 0.2-μm pore size Isopore filters using a hand pump at a pressure less than 15 mm Hg. Filters for extraction of DNA were stored during the field campaign and during the transport to the laboratory in liquid nitrogen. Untreated water samples of 2 to 3 l were transported to the laboratory for immediate chemical analysis. Water temperature, pH, conductivity, and Secchi-depth were measured on location (Table 1). Concentrations of the eight major ions potassium (K⁺), sodium (Na⁺), calcium (Ca²⁺), magnesium (Mg²⁺), chloride (Cl⁻), sulfate (SO₄²⁻), carbonate (CO₃²⁻), and bicarbonate (HCO₃⁻), as well as the concentration of total nitrogen (TN) and total phosphorus (TP) were measured according to the standard methods [19] after transportation of samples to the laboratory. The salinity (salt concentration) of the investigated habitats was determined by summing up the concentrations of the eight major ions [52] (Table 1).

Microbial Counts

Total bacteria and HNF were counted on the same filters by epifluorescence microscopy after 4,6-diamidino-2-phenylindole (DAPI) staining as described previously [39, 54]. In brief, subsamples (1–2 ml) were stained with DAPI (0.1% w/v, final concentration) for 10 min [54], filtered onto black 0.2 μm pore size membrane filters (Millipore) at low vacuum pressure, and counted at ×1,250 magnifications by using an epifluorescence microscope (Zeiss, Germany) equipped with a BP 365, FT 395, and LP 397 filter set. Individual autofluorescence was measured under green excitation using a BP 510–560, FT 580, and LP 590 filter set and was excluded for calculation of total bacteria. HNF were counted and measured in the same way as bacteria on the same filters. Phytoplankton and ciliates were counted in a sedimentation chamber with 50–200 ml water samples at ×200 or ×400 magnification on an inverted microscope. Ciliates were only counted as in total abundance (Table 1). Phytoplankton species were identified to genus or species level.

DNA Extraction and Purification

DNA was extracted from biomass collected on filters by the standard phenol-chloroform extraction, precipitation with ethanol and purification with a Wizard DNA Cleanup kit (Promega), and subsequently concentrated to a volume of 50 μl.

PCR and DGGE Analysis

Two to 10 ng of extracted DNA was used as template for PCR amplification of partial 18S rRNA genes by using primers EukA1 (5'-CCT ACG GGA GGC AGC AG-3') and Euk516r (5'-CCG TCA ATT CMT TTG AGT TT-3') with a 40 bp GC-clamp [14]. The PCR protocol included a 130-s denaturing step at 94°C followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 40 s and extension at 72°C for 130 s, with an additional 7 min of final extension at 72°C. PCR products were examined by gel electrophoresis on 1.0% agarose gel. For a few samples with only low PCR products, reamplification was performed using 5 μl of the respective PCR products as templates. DGGE was carried out with a DCode system (Bio-Rad) by using a 6% polyacrylamide gel with a 40% to 70% gradient of a DNA-denaturant agent (100% denaturant agent is 7M urea and 40% deionized formamide). Eight hundred nanograms of PCR products were loaded, and the gel was run at 100 V for 16 h at 60°C in 1×TAE buffer. The gel was stained with the nucleic acid stain

SybrGold (Molecular Probes) for 45 min, rinsed with 1×TAE buffer, and visualized under UV.

PCR and T-RFLP Analysis

PCR amplification for T-RFLP was performed as described before [26] using a eukaryote-specific primer set, i.e., the labeled forward primer EK-82f 5'-GAA ACT GCG AAT GGC TC-3' [16] and the unlabeled universal reverse primer TGT ACA CAC CGC CCG TC. Amplicons were visualized by standard gel electrophoresis and purified with commercially available purification kits (Promega). Purified PCR products (15 ng) were digested with 10 U MspI for 3 h and were loaded on an ABI3100 genetic analyzer (Applied Biosystems). T-RFLP profiles were analyzed using GENESCAN V 3.1 software (Applied Biosystems). T-RFLPs smaller than 50 bp were excluded from further analysis. Statistical analysis of T-RFLP was done as described in [1] by using *R* [20]. Briefly, T-RFLP data were normalized by dividing all peak areas by the total area of all peaks in a sample profile. Then, rather than defining a threshold below which all peaks are considered to be noises, we separated background “noise” from real signals by a filtering algorithm that uses the variability in the data to identify “true” peaks [1]. Functions that implement the procedure for identifying the “true” peaks and binning the different fragment lengths are available at <http://www.webpages.uidaho.edu/~joyce/Lab%20page/TRFLP-STATS.html>.

Cluster Analysis of DGGE Band Patterns and T-RFLP Profiles

High-resolution digitized DGGE gel images were processed with BioNumerics 4.5 (Applied Maths, St-Martens-Latem, Belgium) software. The analysis parameters included 14% background subtraction, cut off below 0.62% in least square filtering, 0.40% optimization of band positions, and 1.0% tolerance of band positions. Bands migrating to the same position in different lanes were identified. A binary matrix with presence or absence data was built separately for DGGE band patterns and TRFLP profiles. The unweighted pair-group method with arithmetic averages (UPGMA) dendrogram for DGGE patterns and T-RFLP profiles were obtained using the respective Jaccard's coefficient similarity matrixes.

DGGE Bands Sequencing

DGGE bands were excised, transferred in 20 µl of MilliQ water, and incubated overnight for elution of DNA at 4°C. Two microliters of the supernatant were used for reamplification with the original primer pair (without GC clamp). PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen) and sequenced with primer EukA1 (5'-CCT ACG GGA GGC AGC AG-3') by MWG (MWG Biotech AG, Martinsried, Germany).

Phylogenetic Analysis

All obtained unambiguous sequences with a size of approx. 500 bp were aligned manually and phylogenetically analyzed by using the ARB software package [30]. To determine the first phylogenetic affiliation, each sequence was compared with sequences available in databases using BLAST from the National Center for Biotechnology Information (NCBI). The sequences were aligned with complete sequences available in the ARB database. The resulting alignments were checked and corrected manually, considering the secondary structure of the rRNA molecule. The aligned sequence were exported and again blasted into the NCBI in order to search for the closest sequences in NCBI GenBank.

Statistics

For statistical analysis, the environmental parameters were transformed to avoid skewed data distributions: ion concentrations were arcsine transformed, other chemical parameters were log10 transformed; pH, altitude, and latitude were not transformed. We used principal

component analysis (PCA) on chemical parameters (ion concentrations, pH, and conductivity) to display the main gradients in chemical parameters (Table 2). Significant marginal effects were analyzed by running separately a canonical correspondence analysis (CCA) [46] on the DGGE and the T-RFLP patterns using square root transformation for each of the environmental factors separately (i.e. ion percentages, conductivity, pH, latitude, altitude, lake area, TP, TN, salinity, Ca+Mg+CO₃, and Na+SO₄+Cl (Table 3).

We ran a CCA on the DGGE pattern and selected the three most important factors based on automatic forward selection (i.e., %Cl, %(CO₃ + HCO₃), SO₄) of the CANOCO program [46]. The geographic parameters latitude, altitude, lake area as well as the lake type sum parameters (Ca+Mg+CO₃) and (Na+SO₄+Cl) were included as passive parameters for comparison. Both CCAs were repeated using the T-RFLP data. Even though some chemical parameters had a slightly higher marginal effect (explanatory power, see Table 3), we used the above parameters in the analysis for two reasons. Firstly, the selected factors are not only the most important for the DGGE data but have also been reported as the most important in a similar study focussing on diatoms [55]. Secondly, the marginal effects were only slightly different between the factors and the selection of the above parameters allowed for a direct comparison between DGGE and T-RFLP data.

Nucleotide Sequences

The 18S rRNA gene sequences obtained from DGGE bands have been deposited in the GenBank database under the accession numbers AM709506 to AM709534.

Results

Environmental Characteristics of the Investigated Lakes

The major geographical and physiochemical characteristics of the investigated lakes have been presented elsewhere [54] and are briefly summarized in Table 1. Most of these lakes except for Erhai Lake were oligotrophic as indicated by phosphorus and nitrogen concentrations. There was a pronounced salinity gradient among the lakes (Table 1) as expressed by the high correlations between PCA axis 1 (Fig. 2) and the chemical parameters (Table 2).

Microbial Counts

Bacterial abundance ranged from 0.4 to 13.9×10⁶ cells ml⁻¹ with the highest numbers observed in the shallow eutrophic Erhai Lake (Table 1). Abundances of HNF in the investigated freshwater lakes were in general low ranging from 45 to 1,088 cells ml⁻¹. HNF were not detectable in the three hypersaline lakes (Table 1). The abundance of ciliates was between 0 (below detection limit in our sample volume) to 102 cells l⁻¹. The phytoplankton abundances ranged from 1 to 18471×10² cells l⁻¹. The dominant phytoplankton groups were *Cryptophyta*, *Chlorophyta*, and *Bacillariophyta*, while *Euglenophyta* and *Dinophyta* were present only in very low abundances. Significant numbers of cyanobacteria were only observed in Lake Erhai and in 10-m depth of Lake Namucuo representing 69% and 16% of total phytoplankton abundance, respectively. The diversity of phytoplankton in the hypersaline lakes Xiaochaidan and Gahai 2 was much lower as compared to that of other lakes (3–5 genera in the hypersaline lakes versus 7–18 genera in the other lakes) and was characterized by *Chlamydomonas* sp., *Tetraedron* sp., *Oocystis* sp., *Scenedesmus* sp., *Glenodinium* sp., and *Mougeotia* sp. The most common species of flagellated algae were *Chroomonas acuta* Hansg and *Cryptomonas erosa* Eer, which were only found from freshwater to polysaline lakes with maximum abundance of 43 cells ml⁻¹ in the eutrophic Lake Erhai.

DGGE Analysis

The analysis of 19 samples from the 11 lakes by DGGE resulted in six (pond) to 22 (lake Namocuo 0.5 m depth) bands per sample; 49 bands were identified as unique (Fig. 3A, Table 1). No single band was found common to all samples. The most abundant band (as shown by band Qinghai-5-2) was present in seven out of the 11 lakes. In general, we could successfully directly sequence the excised dominant bands from the DGGE gels (Fig. 3A). Sequencing of 51 DGGE bands resulted in 30 sequences of unambiguous quality (approx. 500 bp length) and 21 sequences with many ambiguous positions, which were not analyzed further. BLAST analysis of the 30 excised bands showed that most of these sequences were affiliated with *Chlorophyta*, *Dinophyceae*, *Ciliophora*, and metazoa (Table 4). Eleven sequences were closely related (higher than 97%) to sequences from described organisms, whereas another 11 sequences had a similarity of <97% to environmental clone sequences from uncultured or unidentified eukaryotes (Table 4). All DGGE band sequences obtained from freshwater and most sequences from oligosaline habitats were affiliated with sequences and organisms which so far have exclusively been found in freshwater habitats, while only a few of the sequences appeared in lakes over wide salinity ranges (Table 4). For instance, band Namocuo-67-3 retrieved from oligosaline Namocuo Lake (salinity 2 g l⁻¹) and band Qinghai-5-9 retrieved from polysaline Qinghai Lake (salinity 23 g l⁻¹) are identical to the 18S rRNA gene sequence of *Mastigodiptomus nesus*, which was regarded as an exclusively freshwater copepod [7, 9]. A band migrating to the same band position (Fig. 3A) was also found in samples from the hypersaline Lake Gahai 1 (salinity 67 g l⁻¹). Band Yanghu-05-9 from oligosaline Lake Yanghu (salinity 2 g l⁻¹) and bands Qinghai-05-5, Qinghai-5-1, Qinghai-5-2 from polysaline Lake Qinghai were closely related with sequences (>97% identity) obtained from eusaline habitats (salinity 30–40 g l⁻¹). At the highest salinity range, band Gahai2-05-10 retrieved from hypersaline Lake Gahai 2 (salinity 99.8 g l⁻¹) and band Xiaochaidan-05-10 retrieved from hypersaline Lake Xiao-chaidan (salinity 187.1 g l⁻¹) shared the same sequence, which showed only low similarity (89%) to the closest database sequence.

T-RFLP Analyses

In total, 197 T-RFs were included in the 18S rRNA gene based T-RFLP analysis from all 19 samples. Twenty-nine of the T-RFs were detected in relative fluorescent intensities of at least 10%. Samples taken from freshwater lakes generated T-RFLP profiles with 26–32 T-RFs (with the exception of High lake 3), whereas richness in samples taken from oligo- (43–59 T-RFs, with the exception of Yanghu and Erhai), hyper- (21, 57, and 70 T-RFs, respectively), and polysaline lakes (39–44, with the exception of the deepest sample from Qinghai) were in general higher (Table 1). The most abundant of these dominant peaks included the T-RF 298 which was present in Lake Namocuo (30 m depth), Lake Yanghu, Lake Qinghai (5 and 10 m depth), Lake Gahai 1, and Lake Gahai 2. Interestingly, communities from Lake Namocuo (67 m depth), Lake Qinghai (5, 10, and 17 m depth), Lake Gahai 1, and Pond were dominated by a single peak reaching relative abundances between 43% and 88%.

Cluster Analysis

Dendrograms generated by comparisons of the DGGE banding patterns indicated that irrespective of the sampling depth communities from the same lake (i.e., Lake Qinghai and Lake Namocuo) always clustered together and shared more bands than community profiles obtained from different lakes. Furthermore, most samples from freshwater and oligosaline lakes tended to form a separate group from those obtained from poly- and hypersaline lakes. Some exceptions were the eukaryotic assemblages of hypersaline Lake Gahai 2 and oligosaline Lake Erhai, whereas the freshwater Pond assemblage grouped separately from all other samples. However, cluster formed by different lakes shared rather low similarities

and usually not a large number of bands (Fig. 3A). Dendrograms generated by comparisons of the T-RFLP profiles displayed in general similar patterns with distinct clusters for the different lake types, e.g., most oligosaline lakes grouped together as well as most poly- and hypersaline lakes (Fig. 3B). In contrast to the DGGE pattern, however, much lower similarities were observed for T-RFLP profiles grouping together.

Correspondence Analysis

DGGE and T-RFLP yielded in general similar estimates of the significance of environmental factors, although, the environmental factors explained more of the variance of the DGGE pattern than of the variance of the T-RFLP profiles (Table 3). As geographical and chemical variables largely covaried, the CCA yielded in a similar pattern (Fig. 4). For instance, in general, CCA analysis of chemical variables resulted in five clusters (Fig. 4B and D) separated mainly by percentage of chloride and carbonate (saline lakes contain high concentration of chloride, while freshwater lakes contain high concentration of carbonate). The first cluster contained hypersaline lakes (Gahai 1, Gahai 2, and Xiaochaidan), the second contained polysaline (Qinghai), the third contained large oligosaline lakes (Namocuo and Yanghu), the fourth cluster contained freshwater and oligosaline lakes (High Lake 1, High Lake 3, and Pond), and the fifth contained only the freshwater High lake 2. The cumulative percentage for the first two axes explaining the species-environment relationship was accordingly similar for geographical (latitude, altitude, lake area) and chemical variables (Table 5). Regarding the DGGE pattern, CCA for the selected geographical variables and for chemical variables explained 19.0% and 21.4% of the data, respectively. Somewhat lower values (18.4% and 17.4%) were obtained analyzing the T-RFLP profiles together with the geographical and chemical variables (Table 5). In sum, the selected variables explained the molecular profiles (i.e., eukaryotic plankton assemblage).

Discussion

Currently almost nothing is known about the genetic diversity of eukaryotic plankton assemblages and their controlling factors in high mountain lakes, particularly in hypersaline lakes. These habitats represent important and interesting microbial ecosystems because both salinity and elevation may have fundamental impacts on structure and evolution of eukaryotic plankton assemblages of lakes. We investigated the genetic diversity of eukaryotic plankton assemblages in 11 high-mountain lakes differed by their salinity and altitude using two cultivation-independent methods.

Diversity of Eukaryotic Plankton in Tibetan Lakes

In the present study, nearly one fourth of the retrieved DGGE band sequences showed less than 97% identity to their closest relatives. In particular for hypersaline lakes, band Gahai2-05-10 and Xiaochaidan-05-10 had a similarity of 89% to their closest relative. Half of the obtained sequences are related to unidentified environmental sequences. In some cases, bands migrating to the same gel position yielded different sequences, such as Gahai2-05-7, High lake 2-05-7, and Yanghu-05-7. It should be noted that only 18S rRNA gene fragments of approximately 500 bp were amplified in our study, due to the limitations of the DGGE analysis. In comparison to clone library analyses, DGGE profiling and subsequent sequencing of distinct bands do not allow a thorough coverage of the present diversity in a sample [36]. Usually, not all DGGE bands are reliably recoverable from the gels, and faint bands in principle constrain the sequence analysis to the most dominant and abundant ones.

It is of interest that the copepod sequences obtained from poly- and oligosaline lakes were identical to the sequences of the copepods *Mastigodiatomus nesus* and *Leptodiatomus*

moorei. *M. nesus* has been considered to be distributed only in the freshwater habitats of the West Indies [7]. Recently, it was also found in a freshwater Mexican karstic sinkhole [11]. *L. moorei* has also been regarded to be endemic to freshwater habitats in North America based on 18S rRNA gene phylogeny [45, 47]. Currently, it is not known if this ecological plasticity is an intrinsic trait of all members belonging to this group or due to specific ecophysiological adaptations among members of this group. Further experiments on isolated strains are needed and would also allow us to elucidate how well 18S rRNA gene phylogenies might reflect ecophysiological differences.

Collectively, our results seem to support the presence of a considerable number of uncultured, and potentially not yet described, microeukaryotic taxa in inland water ecosystems [23]. However, even for those taxa detected both in Tibetan lakes and lowland aquatic habitats, it is not known if all members of such taxa are characterized by uniform ecological traits. Recent results from ecophysiological investigations on protistan morphospecies such as the morphospecies *Spumella* spp. demonstrated pronounced and ecologically relevant intraspecific differences in thermal adaptation [5, 6]. There are increasingly data available which show that considerable ecophysiological diversity among protist morphospecies may be present beneath the level of 18S rRNA gene diversity as well [5, 27, 53].

Influence of Salinity and Altitude on Eukaryotic Plankton Assemblage

The most important environmental gradients among the investigated lakes were salinity, latitude, and altitude. Further analyses of ion composition suggested that ion percentage of chlorine and carbonate were the most important chemical variables potentially structuring the diversity pattern of eukaryotic plankton assemblages in the investigated lakes. The salinity and ion percentage of the investigated lakes are strongly regulated by altitude and latitude. The elevation of the Himalaya Mountains in the southern part of Tibet has resulted in a gradual decrease of annual precipitation from south (Fig. 1: high altitude and low latitude) to north (Fig. 1: low altitude and high latitude) [56]. Most Tibetan lakes at high altitude and low latitude were freshwater or oligosaline lakes characterized by relatively high concentration of carbonate, while lakes at low altitude and high latitude regions were mostly saline or hypersaline characterized by high concentration of chloride and sodium. Thus, the geographical and chemical variables largely covaried. Both CCA and UPGMA analysis indicated that samples of the same type (e.g., hypersaline) were generally more similar to each other than samples of different types (e.g., freshwater versus saline and hypersaline). This is also supported by the sequence analysis of excised DGGE bands indicating that sequences retrieved from freshwater and oligosaline habitats clustered with sequences also obtained from freshwater habitats. In addition, the microscopy data indicated that the phytoplankton composition was completely different between freshwater and hypersaline lakes. Obviously, there is a general separation of eukaryotic plankton assemblages between freshwater and saline lakes even if the slow evolution of the salinity has lasted for thousands of years in the investigated region.

According to general ecological principles, more extreme environments are expected to be inhabited by less diverse communities [17]. The genetic diversity of eukaryotic plankton assemblages, as resolved by DGGE and T-RFLP analysis, along the investigated salinity gradient, does not seem to follow this principle (Table 1). The number of DGGE bands or T-RFs per sample did not decrease with increasing salinity. For instance, the average numbers of DGGE bands and T-RFs of the three hypersaline lakes (Lake Gahai 1, salinity 66 g l⁻¹; Lake Gahai 2, salinity 99.8 g l⁻¹; and Lake Xiaochaidan, salinity 187.1 g l⁻¹) are 16.7 (SD, 3.2) and 49.3 (SD, 25.4), respectively, while the average numbers of DGGE bands and T-RFs of all other samples are 16.6 (SD, 3.9) and 41.8 (SD, 14.1), respectively. The same phenomenon has been observed in investigations on the bacterial community composition

along a salinity gradient in the investigated lakes [54] and solar salterns [10] in which high microdiversity of bacteria were found in hypersaline lakes.

However, other factors related to elevation may also influence the eukaryotic plankton structure as well. According to the estimates [4, 38], the potential UVR impact on the investigated lakes may differ due to altitude by 20–40%; the UVR impact differences between the investigated Tibetan lakes and lowland lakes strongly exceeds this range. HNF have been considered abundant in aquatic ecosystem and a numerically major component of aquatic microbial food webs [3, 51]. In our study, the average abundance of HNF in the investigated lakes was only 209 cells ml⁻¹. The average abundance ratio of HNF to bacteria was less than 1:10,000, which is much lower than the documented ratio in lowland lakes [3, 51]. It is well possible that we may have underestimated the abundance of HNF because the fixed HNF cells tend to stick to the walls of the sampling bottle or form aggregates and are, therefore, lost for enumeration. However, this may only explain part of the observed phenomenon of HNF in low abundance. The low number of DGGE sequences related to heterotrophic nanoflagellates seemed to support the microscopy observation that HNF probably constitute a minor group of protists in the investigated Tibetan lakes (Table 1). Thus, it is possible that the strong elevation may have subsequent influences on eukaryotic community composition and microbial food web structure.

The relatively low percentage of the above factors in explaining the molecular profiles suggested that more biological factors might also be involved in structuring the eukaryotic plankton assemblages among the investigated lakes. For instance, the fish communities between freshwater and saline lakes are largely different [50]. Usually, fishes cannot thrive in lakes with salinity higher than 60 g l⁻¹ [50]. The fish compositions between lakes at high and low altitude are also significantly different. There are almost no planktivorous fishes in lakes at altitude above 4,000 m [50]. Thus, different fish communities driven by elevation may have different top-down effects on the structure of eukaryotic plankton assemblages.

The Strength of Molecular Fingerprinting Methods for Environmental Community Analyses

Recent application of molecular approaches have been successfully applied for diversity estimations of eukaryotic plankton assemblages (largely the protistan assemblages and picoeukaryotic communities) in marine ecosystem and revealed a large and previously undescribed diversity of protists and other eukaryotic microorganisms (e.g., [12, 13, 15, 32, 35]). We employed a culture independent approach (DGGE, T-RFLP) to assess the genetic composition and distribution of eukaryotic plankton in Tibetan lakes spanning salinity and altitude gradient. This study represents one of the few studies applying genetic fingerprinting methods for analyses of eukaryotic plankton communities in different inland waters [17, 23, 40, 42].

PCR-based analyses using primers targeting all *Eukarya* cover in principle only the dominant populations in complex communities and do by far not allow an absolute measure for the overall diversity present. In our study, many of the retrieved 18S rRNA gene sequences from the DGGE bands belonged to protist and other eukaryotic microorganisms. This was similar to those studies in marine ecosystems (e.g., [15, 32, 35]). However, we also retrieved some metazoan sequences from the dominant DGGE bands although we did not find the adult metazoan in our lugol-fixed samples by microscopy. Our microscopy data indicated low abundance of ciliates in comparison to the high abundance of phytoplankton. Yet, we still uncovered many ciliate sequences among the DGGE bands. These discrepancies might be largely related to the 18S gene copy numbers of different organisms [28] and, thus, do not allow an absolute quantitative interpretation of such community profiles. Furthermore, using group-specific primers, e.g., for diatoms [18] or ciliates [22],

would increase the retrieval of underrepresented sequences within a sample and allow resolving the community structures of eukaryotic plankton important for the ecosystem.

The present study demonstrated that DGGE and T-RFLP are applicable for comparison of eukaryotic plankton assemblages (largely protist) among diverse inland water ecosystems. The two approaches were performed separately in different labs using two different primer pairs. Both approaches indicated low similarity among samples from different lakes and tended to separate lakes with low and high salinity values. Given that the design and application of group-specific primers will further increase resolution, PCR-based fingerprint techniques will prove a useful tool for assessing eukaryotic microorganisms and relating their overall community structure to environmental variables.

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Figure 1.

Locations of investigated lakes in Tibetan Plateau. The numbers 1 to 11 refer to investigated lakes Pond, Erhai, Qinghai, Gahai1, Gahai2, Xiaochaidan, high lake1, high lake 2, high lake 3, Namocuo, and Yanghu, respectively (see Table 1 for lake details)

Figure 2.
PCA analyses on conductivity, pH, and ion percentages of investigated Tibetan lakes

Figure 3. Dendrogram obtained by UPGMA clustering of DGGE patterns (*left side, A*) and T-RFLP patterns (*right side, B*) of 19 samples from 11 Tibetan lakes. Similarity is expressed as a percentage value of Jaccard's coefficient. Samples from Lakes Qinghai and Nomocuo were taken from different depths. The *numbered dots* (*left side, A*) refer to the excised and sequenced bands as shown in Table 4

Figure 4.

CCA biplots based on either DGGE (**A** and **B**) or T-RFLP (**C** and **D**) and either geographical (**A** and **C**) or selected chemical parameters (**B** and **D**). Chemical parameters yield in a very similar spreading of sampling sites; for geographical parameters, the axes are switched, i.e., latitude and altitude correlate with the first axis in the DGGE analysis, whereas they correlate with the second axis in the T-RFLP analysis. Except for this difference, both analyses yield in very similar results. In **A** and **B**, *NM* and *QH* referred to Lake Namucuo and Lake Qinghai, respectively

Geographical, physical, chemical, and biological characteristics of the investigated lakes

Table 1

Lakes	Sampling depth (m)	Longitude (E)	Latitude (N)	Altitude (m)	Area (km ²)	Sampling date	pH	Salinity (g l ⁻¹)	Lake type	Temperature	Total nitrogen (mg l ⁻¹)	Total phosphorus (mg l ⁻¹)	Bacterial abundance (10 ⁶ ml ⁻¹)	HNF abundance (10 ² ml ⁻¹)	Phytoplankton abundance (10 ² l ⁻¹)	Ciliate abundance (cells l ⁻¹)	T-RF number	DGGE band number
High Lake 1	0.5	91.966	32.916	5134	6.7	23-Jul-04	8.2	0.2	Freshwater	11.0	0.20	0.025	1.7	2.72	1316	27	26	18
High Lake 2	0.5	91.522	32.405	4987	0.1	23-Jul-04	9.2	0.3	Freshwater	10.9	0.87	0.045	5.6	4.54	139	n.d.	29	20
High Lake 3	0.5	91.464	31.144	4548	15.0	24-Jul-04	8.6	0.5	Freshwater	15.1	0.77	0.045	2.5	2.27	1782	23	51	19
Pond	0.5	100.740	36.577	3210	0.5	15-Jul-04	8.7	1.0	Freshwater	15.7	1.09	0.035	2.6	1.81	ND	ND	32	6
Xianghu	0.5	90.435	29.117	4462	638.0	02-Aug-04	9.2	1.9	Oligosaline	ND	ND	ND	ND	ND	ND	ND	71	17
Manocuo	0.5	90.869	30.707	4710	1864.0	26-Jul-04	9.4	2.0	Oligosaline	10.8	0.31	0.025	1.2	1.81	190	38	59	13
	5						9.4	2.0		10.8	0.23	0.045	1.4	1.36	149	83	46	16
	10						9.4	2.0		10.0	0.24	0.045	1.7	3.63	349	91	43	16
	20						9.4	2.0		6.0	0.26	0.036	1.6	1.36	399	66	51	22
	30						9.4	2.0		6.0	0.25	0.036	2.0	1.81	488	n.d.	49	15
	67						9.4	2.0		4.6	0.31	0.036	0.9	1.36	366	102	46	18
Yarhai	0.5	100.739	36.576	3203	5.0	15-Jul-04	8.4	2.0	Oligosaline	16.5	2.3	0.165	13.9	10.88	18471 ^b	76	14	21
Xinghai	0.5	100.509	36.607	3203	4340.0	16-Jul-04	9.3	22.6	Polysaline	13.5	0.94	0.015	0.4	0.45	1082	22	44	18
	5						9.3	22.4		13.0	0.90	0.006	0.5	0.91	1212	25	39	16
	10						9.3	23.0		13.0	0.95	0.006	0.6	1.36	18	7	44	18
	17						9.3	22.9		12.0	0.97	0.015	0.8	1.36	1	29	25	15
Yahai 1	0.5	100.580	37.006	3211	47.2	15-Jul-04	9.2	66.7	Hypersaline	15.9	2.05	0.036	0.6	n.d.	ND	ND	21	13
Yahai 2	0.5	97.550	37.150	2849	32.0	19-Jul-04	8.3	99.8	Hypersaline	21.0	2.7	0.045	3.5	n.d.	134	5	70	18
Xiaochaidan	0.5	95.170	37.480	3172	71.5	20-Jul-04	9.2	187.1	Hypersaline	21.0	3.8	0.086	2.3	n.d.	277	7	57	19

The geographical, physical, and chemical characteristics of the investigated lakes have been presented as supplements in a previous study by Wu et al. [54]

ND not determined, n.d. not detectable

^aSum of the ions potassium, sodium, calcium, magnesium, chloride, sulfate, carbonate and bicarbonate

^bCyanobacteria make up 69% of total phytoplankton abundance

Table 2

Correlation coefficients of chemical variables with PCA axis 1

Chemical parameter	PCA axis 1 score
Na	0.999
Conductivity	0.998
Mg	0.996
Cl	0.992
SO ₄	0.987
K	0.979
Total nitrogen	0.757
CO ₃ + HCO ₃	0.691
Ca	0.503
pH	0.239
Total phosphorous	0.186

Table 3

Significant marginal effects

Environmental parameter	DGGE		T-RFLP	
	Eigenvalue	Variance explained	Eigenvalue	Variance explained
% (CO ₃ + HCO ₃)	0.239	12.3	0.342	8.9
Latitude	0.237	12.2	0.325	8.4
%(Na++SO ₄ +Cl)	0.229	11.8	0.349	9.0
Altitude	0.222	11.4	0.314	8.1
%(Ca++Mg+CO ₃)	0.221	11.4	0.339	8.8
%Cl	0.215	11.1	0.338	8.7
Conductivity	0.188	9.7	0.352	9.1
Salinity	0.187	9.6	0.356	9.2
TN	0.186	9.6	0.341	8.1
%SO ₄	0.183	9.4	0.324	8.4
%K	0.165	8.5	0.379	9.8
pH	ns		0.347	9.0
Area	ns		0.387	10.0
TP	ns		ns	ns
%Ca	ns		0.350	9.1
%Mg	ns		0.310	8.0
%Na	ns		0.342	8.8

ns not significant

Table 4

Phylogenetic affiliations of 18S rRNA gene sequences obtained from DGGE bands

Taxon	DGGE bands	DGGE bands origin (lake, depth, and lake type)	Closest relatives (accession number)	Similarity (%)	Origin
Dinophyceae	Qinghai-05-5	Qinghai, 0.5 m, polysaline	Clone FV23_2H12G4 (DQ310254)	99	Marine, anoxic fjord water
Unknown picoeukaryote ^a	Qinghai-5-1	Qinghai, 5 m, polysaline	Clone BB01_69 (AY885060)	98	Marine
Unknown picoeukaryote ^a	Qinghai-5-2	Qinghai, 5 m, polysaline	Clone TAGIRI-27 (AB191435)	97	Marine
Copepoda, Animalia	Qinghai-5-9	Qinghai, 5 m, polysaline	<i>Mastigodiptomus nesus</i> (AY339156)	100	Freshwater
Chlorophyta	Gahai1-05-3	Gahai 1, 0.5 m, hypersaline	<i>Picochlorum</i> sp. RCC115 (AY526738)	99	Marine
Ciliophora	Pond-05-2	Pond, 0.5 m, freshwater	clone PAF2AU2004 (DQ244040)	99	Freshwater
Diphyllia, Colodictyonidae	Erhai-05-1	Erhai, 0.5 m, oligosaline	<i>Diphyllia rotans</i> (AF420478)	99	Freshwater
Dinophyceae	Erhai-05-13	Erhai, 0.5 m, oligosaline	clone G5.7 (AY642722)	96	Freshwater
Dinophyceae	Erhai-05-18	Erhai, 0.5 m, oligosaline	<i>Woloszynskia leopolensis</i> (AY443025)	95	Freshwater
Unknown picoeukaryote ^a	Gahai2-05-7	Gahai2, 0.5 m, hypersaline	Isolate DGGE band 20 (AM084289)	97	Hypersaline
Stramenopiles ^a	Gahai2-05-10	Gahai2, 0.5 m, hypersaline	<i>Wobblia lunata</i> (AB032606)	89	Marine
Chlorophyta	Xiaochaiddan-05-7	Xiaochaiddan, 0.5 m, hypersaline	<i>Dunaliella</i> sp. CCMP 367 (DQ009769)	99	Hypersaline
Stramenopiles	Xiaochaiddan-05-10	Xiaochaiddan, 0.5 m, hypersaline	<i>Wobblia lunata</i> (AB032606)	89	Marine
Dinophyceae	High lake1-05-1	High lake 1, 0.5 m, freshwater	<i>Amoebophrya</i> clone F (AY829526)	98	Freshwater
Ciliophora	High lake2-05-4	High lake 2, 0.5 m, freshwater	clone VN2 (DQ409092)	99	freshwater
Unknown picoeukaryote ^a	High lake2-05-6	High lake 2, 0.5 m, freshwater	clone VP25 (DQ409124)	97	Freshwater
Stramenopiles	High lake2-05-7	High lake 2, 0.5 m, freshwater	clone P34.19 (AY642708)	99	Freshwater
Rotifera	High lake2-05-10	High lake 2, 0.5 m, freshwater	<i>Keratella quadrata</i> (DQ297697)	100	Freshwater
Ciliophora	High lake3-05-3	High lake 3, 0.5 m, freshwater	<i>Obertrunia Georgiana</i> (X65149)	99	Freshwater
Oomycetes	High lake3-05-5	High lake 3, 0.5 m, freshwater	<i>Pythium insidiosum</i> (AY486144)	100	Freshwater
Chlorophyta	High lake3-05-6	High lake 3, 0.5 m, freshwater	DGGE band 4DB40 (AJ862466)	99	Freshwater
Ciliophora	Namocuo-5-4	Namocuo, 5 m, oligosaline	clone PAA2AU2004 (DQ244028)	95	Marine

Taxon	DGGE bands	DGGE bands origin (lake, depth, and lake type)	Closest relatives (accession number)	Similarity (%)	Origin
Unknown picoeukaryote ^a	Namocuo-5-2	Namocuo, 5 m, oligosaline	Clone BS_DGGE_Euk-2 (DQ234282)	96	Holocene marine sediments
Dinophyceae	Namocuo-05-10	Namocuo, 5 m, oligosaline	clone G5.7 (AY642722)	96	Freshwater
Copepoda	Namocuo20-2	Namocuo, 20 m, oligosaline	<i>Leptodiaptomus moorei</i> (AY339154)	100	Freshwater
Copepoda	Namocuo-67-3	Namocuo, 67 m, oligosaline	<i>Mastigodiptomus nesus</i> (AY339156)	100	Freshwater
Ciliophora	Yanghu-05-5	Yangu, 0.5 m, oligosaline	clone VNP11 (DQ409125)	98	Freshwater
Chlorophyta	Yanghu-05-6	Yangu, 0.5 m, oligosaline	<i>Hagniononas turbinea</i> (AB248252)	99	Freshwater
Ciliophora	Yanghu-05-7	Yangu, 0.5 m, oligosaline	<i>Ophryoglena catenula</i> (U17355)	90	Fish parasite,
Cryptophyta	Yanghu-05-9	Yangu, 0.5 m, oligosaline	clone AD101 (DQ781322)	96	Marine

^aNo sequence from known organism with similarity >91% in NCBI GenBank (only environmental sequences)

Table 5

Results of CCA for selected chemical variables (%($\text{CO}_3 + \text{HCO}_3$), %Cl, % SO_4) based on forward selection and for selected geographical variables (latitude, altitude, lake area)

	DGGE			T-RFLP		
	CCA for chemical variables			CCA for geographic variables		
	Axis 1	Axis 2		Axis 1	Axis 2	m
Sum of all canonical eigenvalues	1.945			1.945		0.936
Significance of first canonical axis (P value)	0.004			0.008		0.002
Eigenvalue	0.272	0.143		0.246	0.124	
Species-environment correlations	0.930	0.921		0.911	0.930	
Cumulative percentage of species data explained	14.0	21.4		12.6	19.0	
Cumulative percentage of species-environment relationship explained	53.0	80.9		52.5	79.1	
Correlation of environmental parameters to CCA axis						
%($\text{CO}_3 + \text{HCO}_3$)	-0.836	0.103				
%Cl	0.753	0.246				
%(SO_4)	0.528	-0.730				
Latitude				0.831	0.202	0.237
Altitude				-0.883	0.129	-0.041
Lake area				-0.025	-0.639	-0.942
						-0.072