Application of cryopreservation to genetic analyses of a photosynthetic picoeukaryote community

Masanobu Kawachi^{ab*}, Takafumi Kataoka^{ab}, Mayumi Sato^a, Marie-Hélène Noël^a, Akira Kuwata^{bc}, Mikihide Demura^d, Haruyo Yamaguchi^{ab}

^a Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

^b JST, CREST, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan

^c Tohoku National Fisheries Research Institute, Shinhamma-cho 3-27-5, Shiogama, Miyagi 985-0001, Japan

^d Faculty of Life and Environmental Sciences, University of Tsukuba 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

*Corresponding author:

E-mail address: kawach9i@nies.go.jp (M. Kawachi)

Abstract

Cryopreservation is useful for long-term maintenance of living strains in microbial culture collections. We applied this technique to environmental specimens from two monitoring sites at Sendai Bay, Japan and compared the microbial diversity of photosynthetic picoeukaryotes in samples before and after cryopreservation. Flow cytometry (FCM) showed no considerable differences between specimens. We used 2,500 cells sorted with FCM for next-generation sequencing of 18S rRNA gene amplicons and after removing low-quality sequences obtained 10,088–37,454 reads. Cluster analysis and comparative correlation analysis of observed high-level operational taxonomic units indicated similarity between specimens before and after cryopreservation. The effects of cryopreservation on cells were assessed with representative culture strains, including fragile cryptophyte cells. We confirmed the usefulness of cryopreservation for genetic studies on environmental specimens, and found that small changes in FCM cytograms after cryopreservation may affect biodiversity estimation.

Keywords

18S rRNA gene; 454 pyrosequencing; Cryopreservation; Photosynthetic picoeukaryotes

1. Introduction

Photosynthetic picoeukaryotes have a cell diameter of less than 2–3 μm (Vaulot et al., 2008). Many recent genetic studies have indicated the existence of a large number of unknown organisms in the pico-size fractions of ocean specimens, suggesting high biodiversity (Massana, 2011). In the open ocean, photosynthetic picoeukaryotes are typically much less abundant than photosynthetic pico-sized prokaryotes such as *Prochlorococcus* and *Synechococcus* (Worden and Not, 2008). However, small eukaryotic phytoplankton, including photosynthetic picoeukaryotes, contributes up to 44% of total CO_2 fixed in the oceans because of their large cell volume and thus higher cell-specific CO_2 fixation rates than those of *Prochlorococcus* and *Synechococcus* (Jardillier et al., 2010). The high biodiversity may indicate the diversified, but as yet poorly understood, ecological roles of photosynthetic picoeukaryotes (such as mixotrophic ability, symbiotic relationships with other microorganisms, nutritional assimilation and so on). In contrast to the fast accumulation of sequencing data on unknown unculturable microorganisms, there are very few culture strains of photosynthetic picoeukaryotes (Vaulot et al., 2008; Not et al., 2009).

Flow cytometry (FCM) is a useful and powerful tool for distinguishing microorganisms according to their cell sizes and the presence or absence of photosynthetic pigments. FCM cell sorting allows the separation of photosynthetic and non-photosynthetic organisms to better assess the diversity of photosynthetic eukaryotes (Marie et al., 2010). The efficiency of this approach depends on the concentration of the target cells. Sample concentration before sorting—especially in the case of ocean specimens with low cell density—is essential (Marie et al., 2010). The morphology of FCM-sorted cells from ocean specimens has been studied with scanning electron microscopy (Gómez-Pereira et al., 2013; Hamdi et al., 2015). FCM sorting, especially in combination with other techniques, may lead to breakthroughs in our understanding of the biodiversity and ecological roles of microorganisms including photosynthetic picoeukaryotes.

Techniques for cryopreservation of cultivated cells have been developed for microbial culture collections to achieve stable and long-term preservation of the strains (Morris, 1981; Mori et al., 2002; Day, 2004). Once viability during cryopreservation is achieved, storage in liquid nitrogen allows preservation of morphological, physiological, biochemical, and genetic properties. Although not all species can be cryopreserved at present, this approach may be applicable to environmental specimens containing diverse microorganisms and intended for genetic studies or establishment of culture strains. Biodiversity of microorganisms in environmental specimens changes quickly after sample collection (Mangot et al., 2013); therefore, cryopreservation would be useful for studies of microorganism biodiversity. The aim of this study was to assess the feasibility of cryopreservation for genetic studies of environmental specimens.

2. Material and Methods

2.1. Sampling and sample treatments

Samples were collected at two sites, C5 (38°04'N, 141°08'E) and C12 (38°11'N, 141°32'E), in Sendai Bay (Miyagi Prefecture, Japan) on 7 October and 1 December 2013. Seawater was collected with a Niskin bottle from the sea surface (1 m) and pre-filtered through a plankton net (5 μm mesh size) to remove large plankton. Cells in the filtrate were concentrated 100 $\times$ by tangential flow filtration (Vivaflow 200 cartridge equipped with a 100,000 MWCO RC membrane; Sartorius, Germany).

Dimethyl sulphoxide (DMSO; Nakarai Techs Co., Ltd., Japan) as a cryoprotectant was filter-sterilized in sterile sea water to a final concentration of 10% using 0.20 µm sterile syringe filter (Millex-LG; Millipore, USA). An aliquot (1 ml) of each sample prepared in 10–20 cryovials was mixed with 10% (v/v) DMSO solution (final concentration 5%) and cooled from room temperature to –35 °C at a rate of –1 °C min^{–1} in a programmable freezer (Bio-Cool IV; FTS Systems, USA). This was followed by cryopreservation at –196 °C in liquid nitrogen (2-step freezing method; Morris, 1981; Mori et al., 2002). Another aliquot was immediately analyzed by flow cytometer (see detail in the next section). In another day, the cryopreserved samples were thawed at 40 °C in a water bath and analyzed immediately. Those samples were named C05NF and C12NF (non-frozen, fresh), and C05F and C12F (frozen, cryopreserved). The procedures from sample collection to amplicon analysis are summarized in Fig. 1.

2. 2. Flow cytometry and photosynthetic picoeukaryote cell sorting from cryopreserved and fresh samples

A flow cytometer (EPICS ALTRA; Beckman Coulter, Germany) equipped with a cell sorter and an argon laser (excitation at a wavelength of 488 nm) was used to analyze specimens. For each cell, three optical parameters were detected by photo-multiplier tubes (PMT): two diffraction parameters, forward-angle scatter (FS, related to the particle size) and side-angle scatter, measured with the PMT1 (SS/PMT1, related to the granularity); and a fluorescence parameter, namely emission in the red (675 ± 30 nm band pass filter), measured with the PMT5 (PMT5, detection of chlorophyll *a* fluorescence). The picoplankton fraction (< 3 µm) was selected by comparing the SS of the PMT5-positive cells with that of fluorescent beads (3-µm diameter, Flow-Set Pro, A63942; Beckman Coulter, Germany) on the cytogram (Fig. 2), and 2,500 cells were sorted into a 0.2-ml PCR tube. Flow cytometric data were recorded as listmode files and analyzed by using EXPO32 v1.2 Analysis software.

2. 3. Whole-genome amplification, construction of 18S rRNA gene library and sequencing

Each sample was subjected to alkaline lysis and neutralization (Zhang et al., 1992) and an aliquot was amplified with Genomiphi V2 (GE Healthcare Life Sciences, USA), which uses Phi29 DNA polymerase, known for its extremely low error rate (1×10^{-5} after 1,000-fold amplification; Paez et al., 2004). The amplified genomic DNAs were purified by ethanol precipitation and dissolved in TE buffer (Nippon Gene, Japan). For amplification of eukaryotic 18S rRNA genes, we designed a set of primers: 545F (5'-AGGCGCGTAAATTACCCAATC-3') and 1119R (5'-TCCCCTAACTTTCGTTCTTG-3'). PCR was performed with Phusion High-Fidelity DNA polymerase (New England BioLabs, USA) and a set of fusion primer attached to a multiplex-identifier (MID) sequence. Specific amplification was confirmed by agarose gel electrophoresis with a molecular size marker. Amplicons of ca. 580 bp were extracted from agarose gel and purified with a QIAquick Gel Extraction Kit (Qiagen, Germany). The quality and fragment length of amplicons were analyzed with a Bioanalyzer (Agilent, USA) using a High sensitivity DNA kit (Agilent, USA), and DNA was quantified by using Quant-iT[™] PicoGreen dsDNA Assay Kit (Life Technologies, USA). DNA was used for 454 pyrosequencing at the Gene Research Center at the University of Tsukuba on a GS Junior System (Roche Diagnostics, USA) in accordance with the manufacturer's instructions. A GS Junior Titanium emPCR Kit (Lib-L; Roche Diagnostics, USA) was used for library

construction. The raw sequences were deposited in the Sequence Read Archive at the DNA Database of Japan (DDBJ) under accession no. DRA003055.

2. 4. 1. Processing of pyrosequencing reads

The pyrosequencing reads were processed with the software package Claident ver. 0.1.2014.02.06 (Tanabe and Toju, 2013). Raw reads were cleaned with trimming low-quality 3' tail by using a minimum quality value of 27 (Kunin et al., 2010) and sorted by using sample-specific MID. The MID and forward primer (545F) sequences attached to the 3'-end were removed and the reads were filtered on the basis of length (≥ 350 bp). Then clustering was conducted within each sample on the basis of identity (cut-off of 97%) by using the built-in assembler Assams v0.1.2013.08.10 (<http://www.fifthdimension.jp/products/assams/>; Toju et al., 2013). Next, low-quality and potentially chimeric reads were eliminated by using UCHIME v0.1.2013.0810 (Edgar et al., 2011) with the default settings. Cleaned reads were first clustered into contigs within each sample and then clustered into contigs across-sample; at both steps, an identity cut-off of 95% was used. The consensus sequence of each operational taxonomic unit (OTU) was assigned to taxonomic groups. Although the rarefaction curves drawn with the vegan package ver. 2.0 (Oksanen et al., 2008) in R (<http://cran.r-project.org>) showed that the OTU number was saturated for each sample (Fig. 3), the OTU number was affected by the total read number. To avoid this bias, 10,000 reads were randomly selected in triplicates. In each sample, singleton in each library was removed as artifactual sequences (Tedersoo et al., 2010).

2. 4. 2. Taxonomic assignment of picoeukaryotes

The consensus sequence of each OTU was aligned to non-redundant alignment of 18S rRNA genes (SSU Ref_Nr_99 ver. 115, SILVA database, Quast et al., 2013) by using the First Aligner tool in the ARB software package (Ludwing et al., 2004); the alignment was corrected manually where necessary. The aligned sequences were added to a guide tree without allowing change in the overall topology using the ARB Parsimony tool, and taxonomy was determined according to the classification ranks maintained by the Eukaryotic Taxonomy Working Group of the SILVA project. Representative nucleotide sequences of OTUs were also compared with known nucleotide sequences of cultured eukaryotes in the non-redundant (nr) database by using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997; <http://blast.st-vi.ncbi.nlm.nih.gov/Blast.cgi>).

2. 4. 3. Diversity and OTU composition analysis

Diversity was evaluated by the index of the inverse of Simpson's index (D_s^{-1}) using the vegan package (Oksanen et al., 2008). The OTUs richness was evaluated by both nonparametric richness indices (ACE and Chao1) and parametric richness estimation (Bunge et al., 2012). The patterns of OTU composition were compared between treatments and between sites on the basis of the abundance of 10,000 reads. The distance of the OTU pattern between samples was measured by their correlation and clustered by Ward's minimum variance with multiscale bootstrap resampling (2,000 replications) using the package pvclust ver. 1.2 (<http://www.sigmath.es.osaka-u.ac.jp/shimo-lab/prog/pvclust/>).

2. 5. Comparison of culture strain cells before and after cryopreservation

Skeletonema menzelii NIES-2842 (Heterokontophyta), *Karlodinium veneficum* NIES-1966 (Dinophyta), and *Rhodomonas duplex* NIES-765 (Cryptophyta) were obtained from the Microbial Culture Collection, National Institute for Environmental Studies (NIES), Japan (<http://mcc.nies.go.jp/>). Each strain was cultured in a 10 ml medium and an aliquot containing intact cells was analyzed by FCM as described in section 2.2, and by light microscope (BX51; Olympus, Japan). The rest of the culture (ca. 6 ml) was cryopreserved and thawed as described in section 2.1. Optical characteristics of the cells were analyzed by FCM and their condition and morphology were analyzed by light microscopy as above. Images were captured with a digital camera (DP72; Olympus, Japan) and saved as digital files.

3. Results and discussion

3.1. Estimation of diversity of picoeukaryotic assemblages

Four amplicon libraries were constructed, and a total of 116,806 reads were obtained in our pyrosequencing runs (Table 1). After the removal of low-quality and potentially chimeric sequences, we obtained 10,088–37,454 reads in each library. 10,000 reads were randomly selected from each library and pooled for subsequent analysis. After singleton removal, 39,689 reads were grouped into 72 eukaryotic OTUs. Of these OTUs, 26 were found in C05F, 29 in C05NF, 36 in C12F, and 38 in C12NF (Table 2). The rarefaction curves of those OTUs plateaued in all the samples (Fig. 3), indicating that the number of reads was sufficient for analyzing the diversity of picoeukaryotes in samples containing 2,500 cells.

The inverse of Simpson's index (D_s^{-1}) was calculated for each data set and their combinations to compare the photosynthetic picoeukaryote diversities (Table 2). The highest index value (14.6) was obtained for a combination of all 39,689 sequences and 72 OTUs. The diversity indices were 11.0 in C5 and 8.8 in C12, followed by the indices of individual samples, which ranged from 6.0 to 8.6. In the case of parametric richness and nonparametric richness (ACE and Chao1), similar to the number of OTUs, the values of fresh samples were higher than those of cryopreserved samples. Nonparametric richness indices has been used in a limited number of studies to assess picoeukaryotic diversity (Cheung et al., 2010; Massana et al., 2004). Chao1 and ACE values, which estimate the total number of OTUs, are usually 2–5 times the number of OTUs observed (Countway et al., 2007; Cheung et al., 2010). However, both of our values were similar to, or slightly higher than, the number of OTUs. This also confirms that the size of our samples was sufficient to assess biodiversity. Marie et al. (2010) examined sequence diversity in 18S rRNA clone libraries constructed from sorted populations of picoeukaryotes and nanoeukaryotes. The percentages of sequences from both size fractions increased with the number of sorted cells and suggested that the OTUs of dominant groups started to saturate between 1,000 and 10,000 cells, whereas samples with less than 1,000 picoeukaryotes or 100 nanoeukaryotes were contaminated with fungi. A dominant species of *Bathycoccus* was detected in a sorted sample of 1,000 cells but disappeared in sorted samples with larger cell numbers. This result may indicate that certain dominant species with low affinity for the primer set can be amplified only under noncompetitive conditions. Samples with larger cell numbers may not always provide accurate results in covering the whole biodiversity. For comparative analysis of biodiversity, the number of sorted cells needs to be optimized.

3. 2. Taxonomic composition of amplicon libraries

Trimmed contigs were grouped into at least 9 supergroup or division level (Fig. 4). The data on BLAST top hits (83%–99% identity) for the 72 OTUs are summarized in Table 3. Possible photosynthetic groups corresponded to 40 OTUs (81% reads), whereas the rest of the reads and OTUs corresponded to possible colorless groups which devoid their chloroplast. The most representative photosynthetic groups were Heterokontophyta (possible photosynthetic stramenopiles; 20 OTUs, 17,118 reads), followed by Cryptophyta (9 OTUs, 11,498 reads), Chlorophyta (5 OTUs, 1,245 reads), Dinophyta (5 OTUs, 2,234 reads), and Haptophyta (1 OTU, 65 reads). The colorless protist groups were Syndiniales of the Dinophyta (13 OTUs, 4,397 reads), Opisthokonta (3 OTUs, 11 reads), Rhizaria (7 OTUs, 2,891 reads), possible colorless chrysophytes (2 OTUs, 32 reads), and other stramenopiles (7 OTUs, 198 reads). Some phytoplankton could be engulfed by colorless protists and maintain similar fluorescence signals even inside predator cells during FCM sorting.

More than half of Heterokontophyta were diatoms, including small chain-forming species such as *Skeletonema* and *Chaetoceros*. These are typical phytoplankton species in Japanese coastal waters (Yamada et al., 2014), and *Skeletonema* reads were the most abundant in all samples. Colony and cell sizes are variable in such diatoms, and possible colony breakdown may have resulted in large numbers of individual cells in our samples. *Bolidomonas* and *Pelagomonas*—well-known picophytoplanktonic heterokontophytes (Vaulot et al., 2008)—were detected more sporadically. *Phaeoplaca* (contig 27; Table 3) was also found in the C5 sample with relatively high read numbers. *Paraphysomonas* species (contigs 31 and 55) are heterotrophic chrysophytes characterized by secondary loss of chloroplasts (Presig and Hibberd, 1983). Stramenopiles, including Labyrinthulomycetes, Pseudofungi, and marine stramenopile (MAST), were sporadically found in all samples.

Cryptophyta were the second dominant group. *Geminigera* (contigs 19 and 84) and *Teleaulax* (contigs 7 and 8) were identified in all samples. Although chlorophyte reads were not abundant, 5 distinct OTUs were reliably identified. Among chlorophyte OTUs, contig 26 showed 99% identity with *Bathycoccus prasinos*, a well-known widespread picophytoplankton species (Vaulot et al., 2008); this is the first record of this species from the Japanese coast. Photosynthetic dinophytes were sporadically recognized in samples. Contig 5 (98% identity to *Karlodinium*) showed high read numbers in C05F and C12NF. Colorless dinoflagellates belonging to the genus *Amoebophrya* (87–99% identity, Syndiniales, Dinophyta) were also found in C5 and C12 with high read numbers in fresh samples. The members of Syndiniales may have been sorted by FCM because they are well-known parasites of photosynthetic organisms (Guillou et al., 2008).

The remaining relatively minor groups were Opisthokonta, Rhizaria, and Haptophyta. Opisthokonta OTUs had a small number reads and were related to metazoans (Hydrozoa), colorless protists (Ichthyosporea), and fungi (Basidiomycota). The Rhizaria includes three groups of amoeboid organisms, namely Cercozoa, Radiolaria, and Foraminifera. Six OTUs belonged to Cercozoa and one to Radiolaria. Interestingly, the haptophyte OTU (contig 23) showed 99% identity with a symbiont of radiolaria. Host radiolarian cells can break and release symbiont cells during filtration through a plankton net and FCM analysis. Broken cells of either Cercozoa or Radiolaria may contaminate samples for DNA analysis. We confirmed that FCM cell sorting allows efficient genetic analysis of target picophytoplankton groups even in cryopreserved specimens, as it reduced contamination with heterotrophic protists in 18S rRNA gene amplicon libraries in the previous papers (Shi et al., 2009;

Marie et al., 2010).

3. 3. Comparison of picoeukaryote diversity in samples before and after cryopreservation

Cluster analysis of eukaryotic OTUs for each library indicated significant differences ($P < 0.05$) in OTU composition between the C5 and C12 sites with high bootstrap values (Fig. 5). The results indicated that the OTU compositions were more similar between cryopreserved and fresh samples from the same site than between similarly treated C5 and C12 samples. We also compared the read numbers of major taxonomic groups for both cryopreserved and fresh samples, in which OTUs presenting more than 100 reads were selected as major groups (Table 3). 9 OTUs (contigs 1, 7, 8, 11, 12, 19, 82, 83, 84) belonging to photosynthetic stramenopiles (Bacillariophyceae), Cryptophyta, Dinophyta and Chlorophyta were common in both treatments.

A significant correlation between cryopreserved and fresh treatments was also suggested in correlation analysis for 6 major taxonomic groups, Alveolata, Chlorophyta, Cryptophyta, Rhizaria, photosynthetic stramenopiles (Heterokontophyta) and non-photosynthetic stramenopiles (Fig. 6, correlation coefficient: 0.81). The read numbers of photosynthetic stramenopiles and Alveolata were slightly higher in fresh samples than in cryopreserved samples, whereas those of non-photosynthetic stramenopiles, Cryptophyta, Chlorophyta, and Rhizaria were slightly lower. A significant correlation in major taxonomic groups between cryopreserved and fresh samples suggests the usefulness of cryopreservation for molecular genetic surveys of microbial biodiversity in environmental samples. Microbial biodiversity changes quickly after sample collection (Mangot et al., 2013). Preparing cryopreserved specimens would facilitate studies of the biodiversity of microorganisms. Our data indicate that cryopreservation followed by FCM cell sorting allows accurate and efficient identification of photosynthetic picoeukaryotes in environmental specimens. This would be a notable advantage comparing usual sample preparation for NGS. Filtrated samples stored in the deep freezer have been used for NGS analysis of aquatic microbial diversity. In such method, microorganisms on the filter were analyzed all together, while cell suspensions thawed from cryopreserved specimens can be used for FCM cell sorting with particular targets, and possibly morphological observations and culturing.

3. 4. Flow cytometric and light microscopic comparison of culture strain cells before and after cryopreservation

Although the bias in OTU composition between cryopreserved and fresh samples was small in the major taxa (Fig. 4), there was a bias of read numbers in some OTUs (Table 2). We assumed that this result would be caused by treatment of cell cryopreservation as a reason. It is known that the efficiency of cryopreservation varies between algal species (Rhodes et al., 2006, Day, 2004): Cyanophyta and Chlorophyta are generally cryopreserved with high viability (Mori et al., 2002), whereas Heterokontophyta, Cryptophyta, and Dinophyta are hardly cryopreserved with low or no viability (Rhodes et al. 2006; Day, 2004). Thus, we compared cryopreservation experiments using culture strains not natural seawater samples to assess the effects of cryopreservation. We chose two culture strains, *Skeletonema menzelii* NIES-2842 (Heterokontophyta) and *Karlodinium veneficum* NIES-1966 (Dinophyta), because closely related OTUs with these two species were included and have great differences of the sequence read number in our analyses (e. g. contigs 5 and 18, respectively)(Table 3). Some OTUs belonging to Cryptophyta also had a strong read number bias

(Table 3). However, appropriate strains were not available in the world culture collections and we therefore chose *Rhodomonas duplex* NIES-765 as a representative of the Cryptophyta.

We compared cells from cryopreserved and fresh samples by using FCM (Fig. 7) and light microscopy (Fig. 8). Eukaryotic cells were distinguished from bacterial contamination by comparing side-angle scatter (SS) and forward-angle scatter (FS) (data not shown). Cryopreservation of *S. menzelii* did not considerably affect the intensity of SS and chlorophyll autofluorescence (Figs. 7a, b), and no significant difference in cell morphology was detected (Figs. 8a, b, Table 4). Cryopreservation of *K. veneficum* slightly decreased the intensity of SS and did not change chlorophyll autofluorescence (Figs. 7c, d), but it deformed cells, which became smaller, possibly because of cryoprotectant effects (Fig. 8d, Table 4). Cryopreservation of *R. duplex* slightly decreased the intensity of SS, but increased chlorophyll autofluorescence (Figs. 7e, f). Although cryopreservation altered cell morphology beyond recognition (Fig. 8f), individual cells became large and rounded. Cells ranged from 12.7–16.7 μm in length and 6.0–8.0 μm in width before cryopreservation (Fig. 8e, Table 4) and from 17.3–31.0 μm in length and 13.7–25.3 μm in width after cryopreservation (Fig. 8f, Table 4).

After cryopreservation, the cell size and morphology of the three species did not change uniformly, but even *R. duplex* showed only a small difference in FCM cytograms despite drastic changes in cell appearance. Most cells looked as though they were close to bursting, but they did not disintegrate and their chlorophyll fluorescence resembled that of living cells in cytograms. The cell density of *R. duplex* was not affected by cryopreservation. If other members of the Cryptophyta also do not disintegrate upon cryopreservation, this may explain why we detected them in all samples. However, it should be noted that a small shift in cytograms may generate a species (OTU) composition bias. Because environmental specimens contain diverse microorganisms with different cell densities and in different cell states (such as at different stages of the cell cycle), small changes in cytograms may affect the results of genetic studies. Accumulating data on cytogram shifts before and after cryopreservation would help to reduce such bias. Another way to reduce bias would be to increase the number of cells for sorting or enlarge the gate setting for FCM sorting, or both, although this might increase the number of contaminating cells. We need more reciprocal trials with different culture strains and environmental specimens to optimize the settings.

4. Conclusions

We applied cryopreservation to environmental specimens from the Japanese coast and investigated the diversity of picophytoplankton. Significant differences in the diversity of major OTUs were not observed between specimens before and after cryopreservation. By using three representative culture strains, we confirmed that the cytograms before and after cryopreservation showed only small differences. Genetic analysis of cryopreserved environmental specimens should allow repeated experiments for the same specimens, facilitating a more detailed investigation of microbial biodiversity.

Funding

This study was supported by JST, CREST and partly supported by NIES.

Author's contributions

MK and HY conceived and designed the study. AK took seawater samples. MK, HY, and MHN performed cryopreservation. MS, HY and MD analyzed cells and conducted flow cytometric cell sorting. HY and TK performed molecular experiments for 454 pyrosequencing. TK analyzed the sequence data. HY took photos of the cells. MK, TK, MS and HY wrote and approved the paper.

Acknowledgments

This study was supported by JST, CREST and partly supported by NIES. Our use of the GS Junior System was supported by the Japan Advanced Plant Science Network and Gene Research Center, the University of Tsukuba. We would like to thank Daniel Vaultot for comments and discussions on the initial stage of our works.

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Figure legends

Fig. 1. Flowchart illustrating the major steps of genetic analysis of photosynthetic pico- and nanophytoplankton with cryopreservation.

Fig. 2. Cytograms (side scatter vs. chlorophyll autofluorescence) of samples before and after cryopreservation. (a, b) Samples collected at C5. (c, d) Samples collected at C12. (a, c) Samples before cryopreservation; (b, d) samples after cryopreservation. Green color dots, sorted cells.

Fig. 3. Rarefaction curves of eukaryotic OTUs of the selected 10,000 reads.

Fig. 4. Taxonomic composition of eukaryotic OTUs assigned to supergroup or division level. Randomly selected 10,000 reads were used.

Fig. 5. Cluster analysis of dissimilarity among samples based on eukaryotic OTUs. Numbers on the nodes represent *p*-values computed with multiscale-bootstrap resampling (2,000 replications).

Fig. 6. Taxonomic differences (A) between samples before and after cryopreservation; (B) between C5 and C12.

Fig. 7. Cytograms (side scatter vs. chlorophyll autofluorescence) of three algal strains before and after cryopreservation. (a, b) *Skeletonema menzelii* (NIES-2842); (c, d) *Karlodinium veneficum* (NIES-1966); (e, f) *Rhodomonas duplex* (NIES-765). (a, c, e) Samples before cryopreservation; (b, d, f) samples after cryopreservation. Sensitivity was adjusted by using standard beads, Flow-Set Pro (Beckman Coulter). Red, eukaryotic algae; blue, contaminating bacteria.

Fig. 8. Light micrographs of three culture strains before and after cryopreservation. (a, b) *Skeletonema menzelii* (NIES-2842). (c, d) *Karlodinium veneficum* (NIES-1966). (e, f) *Rhodomonas duplex* (NIES-765). (a, c, e) Samples before cryopreservation; (b, d, f) samples after cryopreservation. Photos were taken within 1 h after thawing of samples. Bars = 10 μ m.

Table 1. Treatment conditions and sequence summary.

	C05NF	C05F	C12NF	C12F
Treatment	None	Cryopreservation	None	Cryopreservation
Final amplicon length (bp)	675	753	689	662
Total reads	31,414	13,525	33,116	38,751
Reads after trimming	23,561	10,088	30,123	37,454
OTUs in total reads	40	26	41	48
Reads after removal of singletons from subsets of # in 10,000 reads	9,870	9,997	9,845	9,977
OTUs in 10,000 read subsets	29	26	38	36

Table 2. Diversity of picoeukaryote OTUs constructed by using a cut-off identity of 95% for each library.

Analyzed library	No. of OTUs*	D_s^{-1} **	Richness			
			Parametric		Non-parametric	
			Best model	SE	Chao1	ACE
C05NF	29	6.0	30.6	1.4	29	29
C05F	26	8.1	26.8	1.0	26	26
C12NF	38	8.6	41.7	2.3	38	38
C12F	36	6.0	38.8	2.1	36	36
C05NF+C05F	43	11.0	46.3	2.9	43	43
C12NF+C12F	50	8.8	51.6	1.3	50	50
Total	72	14.6	76.1	2.7	72	72

*After singleton was removed from each sample.

** D_s^{-1} , the inverse of Simpson's index.

SE indicates standard error.

Table 3. List of OTUs and their affiliations to supergroups; top BLAST hits with their accession numbers and affiliations at the division/phylum and class level; and read numbers in each sample.

OTUs		BLAST top hit					Read number			
				Accession		Identity				
	Supergroup*	Division/Phylum	Class (Order)	no.	strain	(%)	C05NF	C05F	C12NF	C12F
contig_15	Alveolata	Dinophyta	Dinophyceae	HM066998	<i>Brachidinium capitatum</i>	94	0	43	0	0
					<i>Gymnodinium</i> sp. QZ-2012					
contig_11	Alveolata	Dinophyta	Dinophyceae	JQ639761	voucher HBI:HB201110a	98	0	12	161	43
					<i>Heterocapsa pygmaea</i> isolate					
contig_58	Alveolata	Dinophyta	Dinophyceae	EF492500	CCMP1322	91	0	0	0	9
contig_5	Alveolata	Dinophyta	Dinophyceae	JF790988	<i>Karlodinium micrum</i> clone 1129	98	0	1398	547	9
contig_68	Alveolata	Dinophyta	Dinophyceae	JF790988	<i>Karlodinium micrum</i> clone 1129	93	0	0	12	0
			Dinophyceae							
contig_29	Alveolata	Dinophyta	(Syndiniales)	AF239260	<i>Amoebophrya</i> sp. 'Dinophysis'	96	665	0	0	0
					<i>Amoebophrya</i> sp. ex					
			Dinophyceae		<i>Alexandrium affine</i> strain					
contig_22	Alveolata	Dinophyta	(Syndiniales)	AY775284	JH0210a	89	528	0	4	4
			Dinophyceae		<i>Amoebophrya</i> sp. ex					
contig_63	Alveolata	Dinophyta	(Syndiniales)	JX173254	<i>Alexandrium</i> sp. isolate MS2	99	0	0	1811	0
					<i>Amoebophrya</i> sp. ex					
			Dinophyceae		<i>Cochlodinium polykrikoides</i>					
contig_77	Alveolata	Dinophyta	(Syndiniales)	KF791347	isolate Yeosu small	92	0	0	6	23
					<i>Amoebophrya</i> sp. ex					
			Dinophyceae		<i>Cochlodinium polykrikoides</i>					
contig_45	Alveolata	Dinophyta	(Syndiniales)	KF791347	isolate Yeosu small	87	0	2	2	0
			Dinophyceae		<i>Amoebophrya</i> sp. ex <i>Gonyaulax</i>					
contig_30	Alveolata	Dinophyta	(Syndiniales)	AY775285	<i>polygramma</i> strain GS0209a	87	259	0	0	0

contig_21	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AY775285	<i>Amoebophrya</i> sp. ex <i>Gonyaulax polygramma</i> strain GS0209a	91	49	0	0	65
contig_44	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AF472553	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	97	0	0	909	5
contig_57	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AF472553	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	94	0	0	0	34
contig_36	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AF472553	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	95	13	0	0	0
contig_43	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AY208893	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	88	10	0	0	0
contig_41	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AY208893	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	92	4	0	0	0
contig_48	Alveolata	Dinophyta	Dinophyceae (Syndiniales)	AY208893	<i>Amoebophrya</i> sp. ex <i>Karlodinium micrum</i>	98	0	0	2	2
contig_6	Archaeplastida	Chlorophyta	Chlorophyceae	AB701508	<i>Chlamydomonas parkeae</i>	99	0	59	8	16
contig_26	Archaeplastida	Chlorophyta	Mamiellophyceae	JX625115	<i>Bathycoccus prasinos</i> isolate CCMP1898	99	51	0	4	39
contig_61	Archaeplastida	Chlorophyta	Mamiellophyceae	X73999	<i>Mantoniell squamata</i>	99	0	0	0	14
contig_9	Archaeplastida	Chlorophyta	Prasinophyceae	AB017127	<i>Pterosperma cristatum</i>	97	16	620	0	0
contig_83	Archaeplastida	Chlorophyta	Prasinophyceae	JF794047	<i>Pyramimonas</i> sp. RCC2009	99	9	94	260	55
contig_49		Cryptophyta	Cryptophyceae	AM396375	<i>Cryptomonas</i> sp. M2195	86	0	0	3	2
contig_52		Cryptophyta		EF195737	nucleomorph	99	0	0	0	0
contig_50		Cryptophyta		EU024984	Cryptophyta sp. CR-MAL03	91	0	0	42	16
contig_19		Cryptophyta	Cryptophyceae	DQ452092	Cryptophyta sp. FU44-30	91	2841	727	634	843
contig_84		Cryptophyta	Cryptophyceae	DQ452092	<i>Geminigera cryophila</i>	91	461	1979	327	427
contig_33		Cryptophyta	Cryptophyceae	AM901023	<i>Hemiselmis cryptochromatica</i>	99	2	0	0	0
					nucleomorph					

					<i>Rhinomonas reticulata</i> var.					
contig_13		Cryptophyta	Cryptophyceae	HF952608	<i>reticulata</i> nucleomorph	93	0	493	8	2
contig_8		Cryptophyta	Cryptophyceae	AJ421146	<i>Teleaulax amphioxeia</i>	99	41	830	68	175
					<i>Teleaulax gracilis</i> strain					
contig_7		Cryptophyta	Cryptophyceae	JQ966995	Cr7EHU	99	83	709	260	523
contig_59	Opisthokonta	Cnidaria	Hydrozoa	AF358073	<i>Muggiaea</i> sp. AGC-2001	99	0	0	0	2
					<i>Ichthyosporea</i> sp. ATCC PRA-					
contig_54	Opisthokonta	Amoebidiozoa	Ichthyosporea	GU810144	279	97	0	0	0	3
					<i>Leucogyrophana mollusca</i> strain					
contig_69	Opisthokonta	Basidiomycota	Agaricomycetes	DQ534684	DAOM138006	86	0	0	6	0
			Prymnesiophyceae		Haptophyte symbiont of					
contig_23		Haptophyta	e	AB180202	<i>Dictyocoryne truncatum</i>	99	0	0	63	2
contig_65	Rhizaria	Cercozoa	Imbricatea	AF411264	<i>Allas</i> sp. (3108W2)	84	0	0	70	0
contig_2	Rhizaria	Radiozoa	Polycystinea	HQ651802	<i>Lithomelissa setosa</i> isolate 2	99	0	13	0	0
contig_20	Rhizaria	Cercozoa	Thecofilosea	DQ303923	<i>Ebria tripartita</i> isolate 2	83	0	1788	0	0
contig_70	Rhizaria	Cercozoa	Thecofilosea	DQ303923	<i>Ebria tripartita</i> isolate 2	83	0	0	9	0
contig_75	Rhizaria	Cercozoa	Thecofilosea	AB558956	<i>Mataza hastifera</i>	92	0	0	7	0
contig_39	Rhizaria	Cercozoa		FJ824128	<i>Cercozoa</i> sp. CC-2009d	84	973	0	0	0
					<i>Cercozoan amoeba</i> sp. ex					
contig_67	Rhizaria	Cercozoa		DQ666485	<i>Porpyra yezoensis</i>	83	0	0	31	0
contig_62	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	AB847419	<i>Chaetoceros debilis</i>	99	0	0	0	278
					<i>Chaetoceros muellerii</i> clone					
contig_17	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	JF790991	1133	99	52	0	159	0
					<i>Chaetoceros muellerii</i> clone					
contig_14	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	JF790992	1134	97	0	69	0	184
contig_81	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	KJ671692	<i>Chaetoceros</i> sp. 19 LG-2014	99	0	0	2	0

contig_82	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	JF794039	<i>Cylindrotheca closterium</i> strain RCC1985	98	533	928	988	1224
contig_66	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	KF899836	<i>Entomoneis</i> sp. CCMP2396	99	0	0	37	0
contig_64	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	KF177774	<i>Navicula pulchripora</i> isolate UTEX 2604	99	0	0	69	0
contig_1	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	KJ866907	<i>Pseudo-nitzschia granii</i> strain UNC1101	99	492	31	13	184
contig_18	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	AJ535168	<i>Skeletonema menzelii</i>	95	3	20	37	576
contig_12	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	AB948147	<i>Skeletonema menzelli</i>	97	270	66	2085	3043
contig_76	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	HM991696	<i>Thalassiosira oceanica</i>	99	0	0	1049	0
contig_78	Stramenopiles:P	Heterokontophyta	Bacillariophyceae	HQ912630	<i>Papiliocellulus simplex</i> strain CS431	99	0	3	4	2008
contig_51	Stramenopiles:P	Heterokontophyta	Dictyochophyceae	JN934682	Pedinellales sp. RCC2301	91	0	0	0	30
contig_46	Stramenopiles:P	Heterokontophyta	Bolidophyceae	JX401229	<i>Biddulphia alternans</i> isolate ECT3886Balt	93	0	0	142	37
contig_80	Stramenopiles:P	Heterokontophyta	Bolidophyceae	HQ912557	<i>Bolidomonas pacifica</i> strain CCMP1866	94	40	0	0	31
contig_79	Stramenopiles:P	Heterokontophyta	Bolidophyceae	KF422625	<i>Bolidomonas</i> sp. 1657	99	0	5	0	0
contig_32	Stramenopiles:P	Heterokontophyta	Pelagophyceae	EF455763	<i>Pelagomonas calceolata</i>	98	2	0	0	0
contig_47	Stramenopiles:P	Heterokontophyta	Chrysophyceae	JN934680	<i>Dinobryon faculiferum</i> strain RCC2293	96	0	0	2	23
contig_27	Stramenopiles:P	Heterokontophyta	Chrysophyceae	AF123296	<i>Phaeoplaca thallosa</i>	92	2354	7	0	0
contig_25	Stramenopiles: NP	Heterokontophyta	Chrysophyceae	EF432523	Chrysophyceae sp. I8 clone A1	91	21	17	0	0

	Stramenopiles:									
contig_31	NP	Heterokontophyta	Chrysophyceae	AF109326	<i>Paraphysomonas butcheri</i>	89	2	0	0	0
	Stramenopiles:									
contig_55	NP	Heterokontophyta	Chrysophyceae	JQ967323	<i>Paraphysomonas</i> sp. 18 JMS-2012	99	0	0	0	30
	Stramenopiles:									
contig_53	NP	Labyrinthulomyco	Labyrinthulomyce	FJ799796	<i>Oblongichytrium</i> sp. PBS02	87	0	0	0	16
	Stramenopiles:									
contig_37	NP	ta	tes	AY872271	<i>Thraustochytriidae</i> sp. SW9	92	4	0	0	0
	Stramenopiles:									
contig_3	NP	ta	tes	GQ354272	<i>Oblongichytrium</i> sp. PBS03	90	0	78	0	0
	Stramenopiles:									
contig_28	NP	Pseudofungi		JF791043	<i>Pirsonia verrucosa</i> clone 7535	96	16	0	0	0
	Stramenopiles:									
contig_4	NP	Incertae sedis	Nanomonadea	JN848814	<i>Incisomonas marina</i> strain BQ	95	0	4	0	0
	Stramenopiles:									
contig_38	NP (MAST)	NA	NA	NA	MAST3	–	76	0	0	0
	Stramenopiles:									
contig_74	NP (MAST)	NA	NA	NA	MAST7	–	0	0	4	0

* Affiliation to supergroups is indicated according to SSU Ref_NR_99 ver. 115, SILVA. P and NP, photosynthetic- and non-photosynthetic, respectively. MAST, uncultured marine stramenopiles. NA, not applicable (because the OTU belongs to MAST for which no cultured strains were reported).

NA indicates "not applied" because the OTU belonged to MAST in which no cultured strain was reported.

Table 4. Cellular characteristics of cultured *S. menzelii*, *K. veneficum*, and *R. duplex* before and after cryopreservation.

Specific name	No. of strain		No cryopreservation			Cryopreservation			Cellular structural change
			Range (μm)	Mean (μm)	No. of cells	Range (μm)	Mean (μm)	No. of cells	
<i>Skeletonema menzelii</i>	NIES-2842	Length	nd	–	–	nd	–	–	Not changed
		Width	nd	–		nd	–		
<i>Karlodinium veneficum</i>	NIES-1966	Length	10.3–17.7	12.3	9	7.0–12.3	10.2	13	Changed
		Width	7.3–12.3	9.5	9	6.7–11.7	8.7	13	
<i>Rhodomonas duplex</i>	NIES-765	Length	12.7–16.7	15.4	8	17.3–13.0	22.2	7	Changed
		Width	6.0–8.0	7.7	8	13.7–25.3	18.7	7	

nd, not determined

n, number of measurement

Fig.1

PMT5

SS

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

PMT5

SS

Fig. 7

Fig. 8