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Patchy-distributed ciliate (Protozoa) diversity of eight polar communities as determined by 454 amplicon pyrosequencing

Jae-Ho Jung[a,b,d], Kyung-Min Park[a,b], Eun Jin Yang[c], Hyoung Min Joo[c], Misa Jeon[c], Sung-Ho Kang[c], Han-Gu Choi[b], Mi-Hyun Park[a], Gi-Sik Min[a] and Sanghee Kim[b]*

[a]*Department of Biological Sciences, Inha University, Incheon 402-751, South Korea;* [b]*Division of Life Sciences, Korea Polar Research Institute (KOPRI), 26 Songdomirae-ro, Yeonsu-gu, Incheon 406-840, South Korea;* [c]*Division of Polar Ocean Sciences, Korea Polar Research Institute (KOPRI), 26 Songdomirae-ro, Yeonsu-gu, Incheon 406-840, South Korea;* [d]*Department of Biology, Gangneung-Wonju National University, Gangneung 210-702, South Korea*

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To determine ciliate diversity and community structure in the polar ecosystem efficiently, we applied the pyrosequencing technique to the polar samples. To select the appropriate sequencing depth using a ciliate-specific primer, we evaluated different pyrosequencing depths, ranging 4149–112,306 reads. At a 3% distance cutoff for clustering, 750 operational taxonomic units (OTUs) were identified, and 332 were composed of a single read (singletons). The singletons showed a 1.8-fold increase in OTU richness, although their beta diversity showed no significant changes. The ratio of singletons in each sequencing depth was sharply decreased after reaching the sequencing depth of approximately 10,000 reads, and the singletons did not completely disappear even at 73,435 qualified reads. The data set without singletons showed saturated trends in rarefaction curves. In addition, we built a normalized data set without the singletons (1227 reads × eight samples). Among the samples, one brackish water having a broad range of salinity (3–23 PSU) at the Arctic coast presented the highest OTU richness (103), while a temporal pool at the Antarctic coast with a high salinity of 53.4 PSU, showed a relatively lower OTUs (8). Each normalized sample showed a distinct community structure. Interestingly, a freshwater lake on King George Island shared relatively higher OTUs with salt-water samples (72.1%), suggesting a higher inter-relationship with closely located coastal water environment.

Keywords: community structure; eukaryotic microbial ecology; polar ciliate; pyrosequencing; V4 region

Introduction

High-throughput next-generation sequencing (NGS) technology is a cost-effective method and has replaced clone library method for community analysis (DeLong 2009). Among the various NGS platforms, 454 pyrosequencing is applicable to metagenome, biodiversity, and transcriptome analyses because of the longer read lengths compared to that of other NGS platforms (Glenn 2011). Generally, longer reads contain more reliable information so that each read can be assigned as a species without any assembly. To date, the 454 platform has been the preferred approach for investigations of community structures.

Previous studies have attempted to examine large diverse groups such as meiofauna (Creer et al. 2010), metazoans (Fonseca et al. 2010), and protists/picoeukaryotes (Amaral-Zettler et al. 2009; Stoeck et al. 2009; Cheung et al. 2010; Nolte et al. 2010; Edgcomb et al. 2011; Kilias et al. 2014). To achieve a more thorough understanding of specific ecological groups, we focused on the phylum Ciliophora, a monophyletic unicellular microorganism.

Ciliates are globally distributed in various diverse habitats, ranging from the oceans to terrestrial habitats, and are associated with higher trophic levels. For example, ciliates are consumed by metazoans and play an important role in the ecosystem, as a part of nutrient cycling (Sherr & Sherr 1988). In addition, ciliates can be used as bioindicators for assessing environmental quality by their relatively prompt response to environmental contaminants (Chen et al. 2008; Xu et al. 2013). However, traditional taxonomic approaches to identify ciliates, such as microscopic examination with taxon-specific impregnation methods (Foissner 2014), are often difficult and time-consuming (Dopheide et al. 2008). According to Foissner et al. (2008), approximately 4500 free-living ciliate morphospecies, as valid species, have been described, and it is estimated that approximately 27,000–40,000 ciliates might exist on the earth. Thus, 83–89% of extant ciliates remains and is waiting for our discovery.

The high diversity of ciliates with undiscovered species including endemic ones hampers non-taxonomists in identifying ciliates. However, rapid climate change (e.g. global warming) and human activities have changed the polar ecosystem; thus, the inherent ciliate diversity might have been altered. To assess the ciliate diversity and community

*Corresponding author. Email: sangheekim@kopri.re.kr

structure in the polar ecosystem efficiently, we applied the massive pyrosequencing technique to eight polar samples collected from five coastal, two freshwater, and one pelagic environment. Our aims are as follows: (1) to evaluate the specificity of a ciliate-specific primer in the polar samples, (2) to determine an appropriate pyrosequencing depth that could be utilized for the efficient monitoring of a single community/sample, (3) to evaluate the effect of singletons in data processing, and (4) to examine the relationship among the polar ciliate communities.

Materials and methods

Sample collection

Eight samples were collected from Arctic and Antarctic environments (Figure 1). Detailed information on the samples is presented in Table 1. To compare ciliate diversity in bipolar ecosystems, we collected three salt-water samples and one freshwater sample from each polar environment. Most of the salt-water samples were collected from coastal regions, with the exception of one pelagic sample collected during a research activity on ARAON, a Korean icebreaking research vessel. The bottom of the sampling station was agitated to collect both benthic and planktonic ciliates using a planktonic net (20-μm mesh size). One of the six salt-water samples was collected from the coastal area around King George Island (the Antarctic) using a polyurethane foam unit (PFU). All samples were preserved in ethanol (70% final concentration) and maintained in a freezer at −80°C before the further analysis.

Environmental DNA preparation and pyrosequencing

Genomic DNA (gDNA) from the samples was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Co., Carlsbad, CA), because the humic acid in the samples prevents direct amplification of the target gene fragment. A pair of ciliate-specific primers, Pyro-F (5'-AGC AGC CGC GGT AAY HCC-3') and CiliPyro-R (5'-TAS GAC GGT ATC TGA TCG TCT AT-3'), was used to amplify the hypervariable V4 region of the small subunit ribosomal RNA (SSU rRNA) gene from the environmental gDNA. The reverse primer specifically binds to the V4 region of ciliates based on a single-nucleotide polymorphism (SNP)-based phylum-specific polymerase chain reaction (PCR) amplification technique (SPAT) (Jung et al. 2012). PCR was performed in a total volume of 35 μL, which contained 3.5 μL of 10 × AccuPrime PCR Buffer II, 10 pmol of each primer, and 0.5 units (0.1 μL) AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) by using the following cycle conditions: one cycle of 180 s at 92°C (initial denaturation), 30–35 cycles of denaturation, primer annealing, and elongation (96°C for 10 s, 52°C for 20 s, and 68°C for 60 s), and a subsequent 7-min final extension step at 72°C. Twelve PCRs were performed for each gDNA, and the PCR products were pooled together to reduce biased amplification of specific taxa (Lahr & Katz 2009). These PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Co., Hilden, Germany). The purified products were then concentrated up to 100 ng/μL by using a Centricon YM-3 (EMD Millipore, Billerica, MA) and were pooled together. The pooled purified product was sequenced using a 454 FLX Titanium sequencer. The

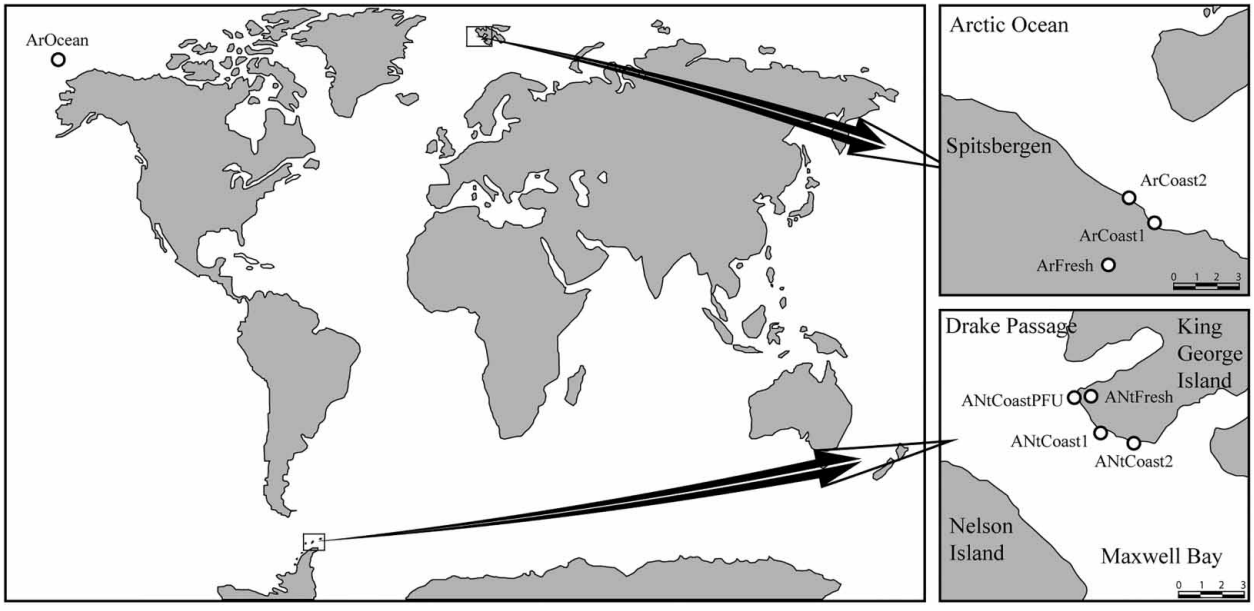

Figure 1. Map of eight sampling stations. Scar bars: km.

Table 1. Summary of sample localities and analyses conducted on samples from the Svalbard (Arctic), Arctic Ocean, and King George Island (Antarctic).

	Antarctic				Arctic			
	Coastal area, King George Island	Coastal area, King George Island	Coastal area, King George Island[a]	Freshwater, King George Island	Coastal area, Svalbard	Coastal area, Svalbard	ARAON, Arctic Ocean	Freshwater, Svalbard
Sample name	ANtCoast1	ANtCoast2	ANtCoastPFU	ANtFresh	ArCoast1	ArCoast2	ArOcean	ArFresh
Location	62°14′S, 58°46′W	62°14′S, 58°46′W	62°13′S, 58°47′W	62°20′S, 58°47′W	78°54′N, 11°57′E	78°55′N, 11°55′E	73°07′N, 168°56′W	78°54′N, 11°52′E
Date	21 January 2010	21 January 2010	20–30[a] January 2010	21 January 2010	18 July 2010	15 July 2010	20 July 2010	14 July 2010
Temperature (°C)	5.6	1.5	0.5–2	1.4	9.5–12.2	8.2	−1.5	11
Salinity (PSU)	53.4	34.3	33–34.5	0.1	3–23	28.4	31	0.1
No. of raw reads	30,159	7502	112,306	4877	4149	8307	5071	6270
No. of high-quality reads	21,393	2367	80,633	2787	3598	7342	4522	5551
No. of high-quality reads in Ciliophora (singleton)	19,690 (1351)	2339 (1112)	73,435 (7058)	2774 (728)	3541 (1026)	7312 (1001)	4481 (936)	5413 (1061)
% of ciliate specificity	92	98.8	91.1	99.5	98.4	99.6	99.1	97.5
No. of ciliate OTUs (3% cutoff)	43	128	301	121	219	69	102	66
No. of ciliate OTUs (3% cutoff without singletons)	17	70	123	66	116	30	78	51
No. of OTUs in normalized data[b] without singleton (Shannon diversity)	8 (1.09)	69 (2.81)	48 (1.75)	61 (2.27)	103 (3.17)	26 (1.95)	67 (2.44)	39 (2.51)

[a]Polyurethane foam unit (PFU) sample soaked in the coastal water for ten days
[b]Normalized data set composed of 1227 high-quality ciliate reads per a sample

amplicons from each environmental sample contained its own multiplex identifier (MID) sequence at the 5′ end of both forward and reverse primers.

Operational taxonomic unit determination and taxonomic assignment

To isolate high-quality reads, we preliminarily filtered the raw reads with the following criteria: complete MID sequences at both ends, minimum sequence length of 300 bp (including PCR primers), no ambiguous nucleotide (N), ≥25 average quality score, no chimera, and no poor quality of alignments with the SILVA eukaryotes, using Mothur software (Schloss et al. 2009). Both ends of the filtered reads were trimmed to extract the hypervariable V4 region. Finally, the V4 region was examined to define operational taxonomic units (OTUs) based on *p*-distance by using Mothur version 1.33.3 and JAGUC version 2.1 (Nebel et al. 2011). USEARCH version 7.0.1090 (Edgar 2010) was implemented to assign the V4 region to SILVA SSU rRNA database release 119 (Pruesse et al. 2007). According to the assigned results, these reads were classified at higher taxonomic levels (kingdom to order level). The output data of the USEARCH were transferred to MEGAN version 5.5.3, and all communities were combined and visualized together (Huson et al. 2009). A Perl script was used to transform these data to the preferred formats of the programs mentioned above and was applied for simple repetitive procedures. Additionally, we generated several data sets to identify the influences of the singletons.

Here, we applied two similarity thresholds (97% and 99%). Reads showing ≥99% similarity with the reference were classified as species level (i.e. taxonomic assignment), and the species list was used to interpret the environmental features based on taxonomic literatures (e.g. occurrence, ecology). Based on the OTU (3% distance cutoff), we calculated the Bray–Curtis similarity, and these values were used to construct dendrograms and to implement non-metric multidimensional scaling (NMDS) in Primer 6.1.16 (Clarke & Gorley 2006) to analyze relationships among polar ciliate communities.

Results

Ciliate specificity of SPAT primers

A total of 178,641 raw reads were composed of 4149–112,306 reads from each of the eight samples. From the raw data set of 178,641 reads, 128,193 reads satisfied our criteria for high quality. Using USEARCH (Edgar 2010), we assigned the high-quality reads to SILVA SSU rRNA database release 119 (Pruesse et al. 2007). A total of 118,985 reads from the high-quality reads were identified as ciliate. The ciliate-specific primer presented high specificity for ciliates, ranging from 91.1% to 99.6% (92.8% on average) (Table 1).

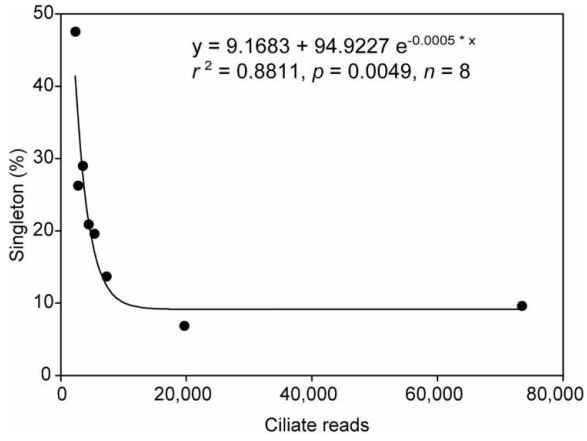

Figure 2. Percentage of singletons in reads with exponential decay fit.

Singletons

The ratio of singletons varied among the samples and showed a range of 6.9–47.5%. ANtCoast2 consisted of 2339 qualified-ciliate reads and showed the highest singleton ratio of 47.5%. The singleton ratio was negatively exponentially correlated with the number of reads (Figure 2). ANtCoastPFU, the biggest data set, was composed of 73,435 qualified-ciliate reads with a singleton ratio of 9.6%. The lowest singleton ratio was 6.9% from 19,690 qualified-ciliate reads of ANtCoast1.

Richness and diversity of operational taxonomic units

The number of raw reads in each sample ranged from 4149 to 112,306, and qualified-ciliate reads showed 43–301 OTUs at a 3% cutoff (750 OTUs in this study). The singletons accounted for 11.8% of the total ciliate reads covering 44.3% of the OTUs (332 out of 750). OTU richness was highly influenced by two factors: non-ciliate sequences and singletons (Figure 3). In particular, singletons highly affected and increased the number of OTUs (1.8-fold). Elimination of both non-ciliate reads and singletons displayed a saturated trend in the rarefaction curves. Therefore, we excluded the non-ciliate reads and singletons for reliable data analysis of ciliate community. From the qualified reads, we built normalized data sets. Each sample of the normalized data set was composed of 1227 ciliate reads without a singleton. OTU richness ranged from 8 to 103 OTUs and Shannon diversity was in the range of 1.09–3.17 (Figure 4, Table 1). ArCoast1 showed the highest diversity among the eight samples.

Community structure

In the normalized data sets, a total of 258 OTUs was identified, and Antarctic and Arctic samples were composed of

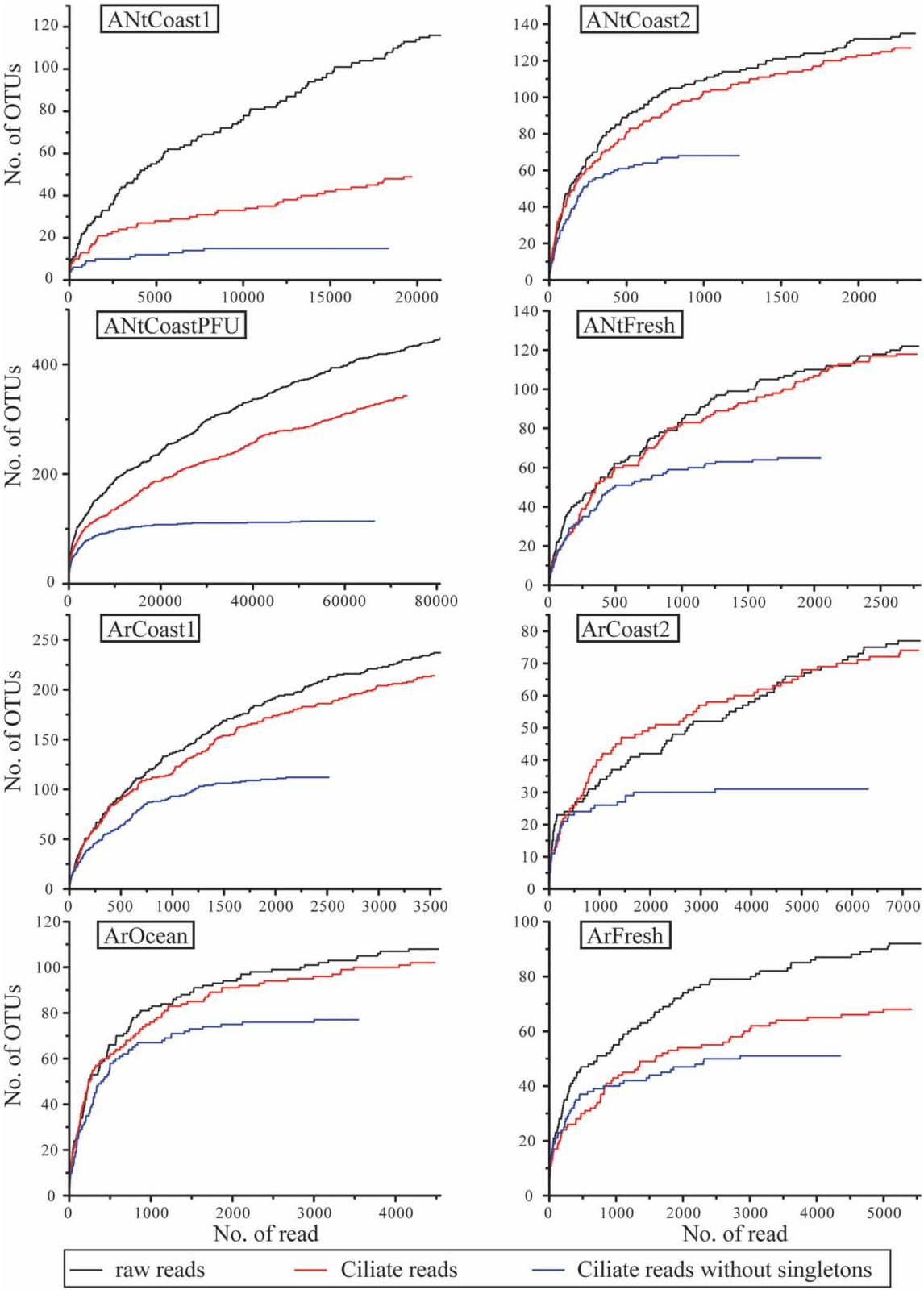

Figure 3. Rarefaction curves of three different data sets from eight samples. The raw reads are high-quality reads that satisfied the filter criteria using Mothur (Schloss et al. 2009). Based on USEARCH results (Edgar 2010), the ciliate reads were separated from the raw reads. Additionally, we constructed one additional data set that excluded the singletons from the ciliate reads, and these three data sets were analyzed for their rarefaction curves by using JAGUC (Nebel et al. 2011). The number of OTUs differed slightly from that generated using Mothur (Schloss et al. 2009), which may be attributable to variations in algorithms in each software.

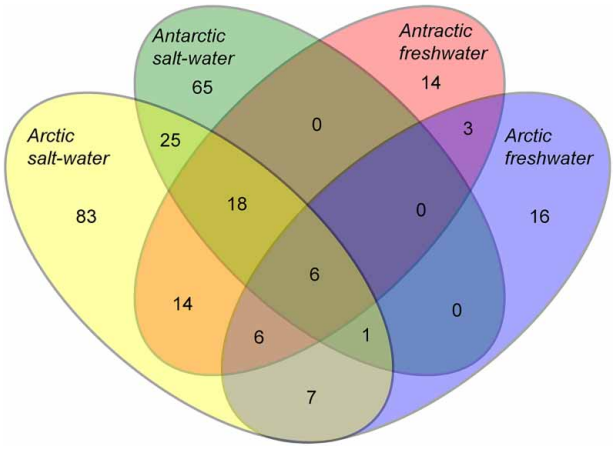

Figure 4. Venn diagram showing co-occurring OTUs using a normalized data set without singletons.

152 and 179 OTUs, respectively. Seventy-three OTUs (28.3%) co-occurred in both polar regions. The normalized eight samples were categorized as salt-water or freshwater, and each category of Antarctic and Arctic samples was composed of 3681 (1227 reads × 3 salt-waters) and 1227 reads (1227 reads × 1 freshwater), respectively (Figure 4). The number of co-occurring OTUs between the Antarctic and Arctic regions was 50 OTUs (22.2% in salt-water category) and 15 OTUs (17.6% in freshwater category), respectively. Six OTUs co-occurred in all categories from bipolar regions, and their top hit taxa were as follows: OTU050—Hypotrichia, with a similarity of 85.8–91.6%; OTU054—Hypotrichia, with a similarity of 98.2–100%; OTU067—Oligotrichia, with a similarity of 86.1–89.2%; OTU105—*Tintinnopsis* sp., with a similarity of 99.1–100%; OTU155—*Euplotes* sp., with a similarity of 99.6–100%; and OTU197—*Euplotes balteatus,* with a similarity of 94.9–96%.

The most abundant classes in the normalized data set were Spirotrichea (43.6%), Litostomatea (33.1%), and Oligohymenophorea (10.6%) (Figure 5). However, the ratios of the abundant groups varied among the samples. For example, the most abundant class Spirotrichea showed a minimum of 5.3% (ANtCoast1) and a maximum of 86% (ANtFresh) (Figure 5). The most abundant orders were Pleurostomatida (Litostomatea, 19.6%), Tintinnida (Spirotrichea, 10.4%), and Haptorida (Litostomatea, 10%). Spirotrichea showed more diverse orders and greater evenness than did the other orders. For example, the class Litostomatea was mainly composed of three orders, Pleurostomatida, Haptorida, and Cyclotrichida, while Spirotrichea was composed of nine orders.

The normalized data set was used to analyze beta diversity among the eight samples (Figures 6 and 7). Each community showed a distinct community structure and low similarity between the samples (Bray–Curtis similarity with read abundance, 0–24.3%; Bray–Curtis similarity without read abundance, 0–38.3%; Figure 7). One additional normalized data set was constructed to evaluate the effect of singletons on beta diversity. The community similarity values were not significantly affected by the singletons (Figure 6). The normalized data set was employed for taxonomic assignments using USEARCH (Edgar 2010) and reads showing ≥99% sequence similarity with the reference SILVA were assigned to species level. Each normalized data set was composed of 1227 reads and the number of assigned reads was in the range of 8–819 (0.7–66.7%, Table 2). The ratio of reads showing ≥99% similarity was loosely positively correlated with number of OTUs ($y = 32.1065 + 0.9364x$, $r^2 = 0.5190$, $p < 0.05$, $n = 8$). As anticipated, the assigned taxa usually showed its occurrence at appropriate environments, such as terrestrial, freshwater, and salt-water. However, ANtFresh contained many marine species such as *Apokeronopsis bergeri*, *Cyclotrichium cyclokaryon*, *Euplotes nobilii*, *E. sinicus*, *Loxophyllum rostratum*, *Psammomitra retractilis*, *Pseudoamphisiella alveolata*, *Spathidiopsis buddenbrocki*, *Tintinnopsis rapa*, and *T.* spp.

Discussion

Appropriate sequencing depth with SPAT primer

We analyzed the ciliate community structure of eight polar environments using the ciliate-specific primer we previously designed, and the SPAT primer had showed high specificities of 94.5% for cloning and 99.2% for the pyrosequencing method (Jung et al. 2012). Although several primers targeting partial ciliate groups have been reported (Costas et al. 2007; Bachy et al. 2012a; Liu & Gong 2012), the SPAT primer encompasses the majority of the phylum Ciliophora both in silico and experimentally, thus indicating its applicability to mass pyrosequencing of diverse polar ciliate communities.

The singleton was mainly responsible for increasing the number of OTUs. Even though we increased the sequencing depth up to a maximum of 112,306 reads to recover rare species, singletons were not completely removed, and the rarefaction curve with singletons showed a non-saturated trend (Figure 3). If we exclude singletons from the data set, the curves showed more saturated trends at a range of 1000–20,000 reads. The OTUs showing low abundance (or consisting of a single read) could be rare species (Sogin et al. 2006), while Behnke et al. (2011) reported that singletons should be eliminated for reliable data interpretation. Thus, removal of singletons from data sets could be an appropriate way to avoid overestimation of ciliate diversity in despite of loss of few rare species. Even though the singletons were not completely disappeared with the increased reads, the singleton ratio sharply decreased at approximately 10,000 reads (Figure 2).

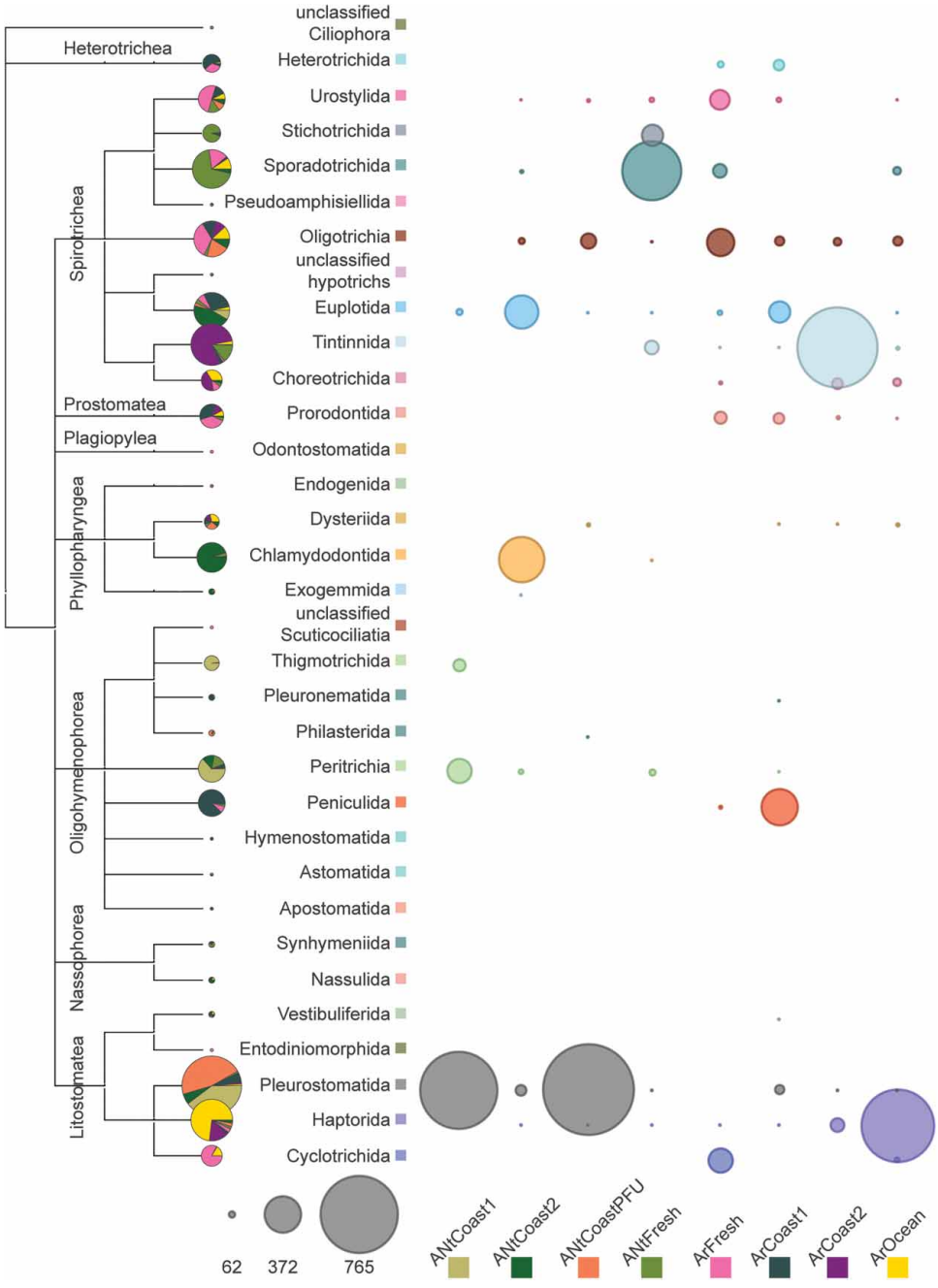

Figure 5. Megan tree showing assigned reads at higher taxonomic level with their abundances (circles).

Based on these results, we propose that the appropriate sequencing depth using the SPAT primer could be 10,000 reads per sample, because this number of reads showed a very low singleton ratio and could recover most of the species in a sample. Similarly, Lundin et al. (2012) proposed that 5000 denoised reads are effective in describing trends for bacterial communities. Because ciliate biodiversity is usually lower than bacterial biodiversity, a lower number of reads than the 10,000 reads would be adequate for ciliate monitoring to capture existing trends of ciliate diversity.

The number of OTUs significantly varied depending on the data processing criteria, cutoff threshold, and target region (Schloss 2010; Behnke et al. 2011; Bachy et al. 2013). Recently, Meng et al. (2012) investigated ciliate diversity by broad sampling of offshore sediments from the Yellow Sea with silver impregnation, and the number of morphospecies (ranging from eight to 65 in each station; a total of 198 morphospecies) was higher than that from the intertidal sediments, as reported by Hamels et al. (2005). Meng et al. (2012) reported that the observed higher morphospecies richness might have been attributable

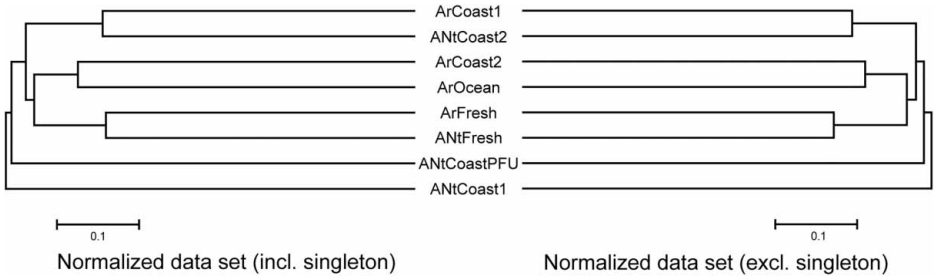

Figure 6. Dendrogram of two normalized data sets based on the Bray–Curtis similarity at 3% cutoff. These dendrograms consist of 18,712 reads and 9816 reads, respectively. The topology of these dendrograms is nearly identical whether the singletons are included or excluded.

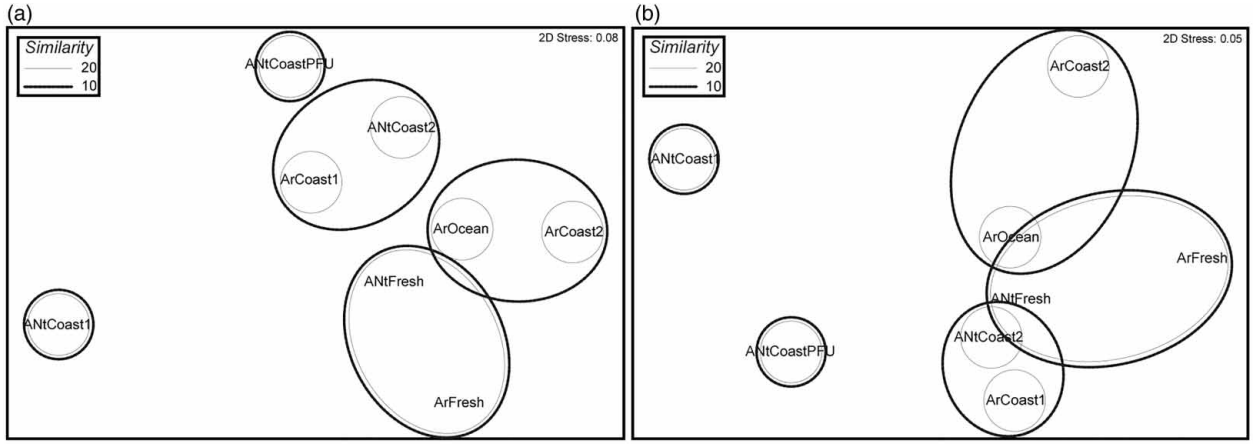

Figure 7. Multidimensional scaling with cluster analysis of Bray–Curtis similarity matrix by using abundance (a) and presence/absence (b) data.

to their broad sampling approach. In terms of species richness, the polar ciliate diversity does not deviate from the microscopic approach and slightly higher. However, some closely related ciliate species could have identical 18S sequences (some congeners in *Tetrahymena*; see Lynn & Strüder-Kypke 2006). Here we should note that the number of OTUs could be underestimated because of the inherent nature of the 18S rRNA gene.

Community structure of bipolar regions

Each sample showed a distinct community structure (Bray–Curtis similarity with abundance data, 0–24.3%), and the number of co-occurring OTUs between the eight polar samples was 0–33 OTUs (Bray–Curtis similarity of presence/absence data, 0–38.3%). The singletons increased the number of OTUs while they did not significantly affect the beta diversity among the eight samples because of their low abundance. The distinct structure of each sample made it difficult to understand their relationships (e.g. indigenous species). Previously, Foissner (1996) reported a patchy distribution of soil ciliates from Antarctica and proposed the "moderate endemicity model" (Foissner 1999, 2004; Foissner et al. 2008). Petz also reported that some ciliates show a restricted geographic distribution (Petz 2003; Petz et al. 2007). Based on the molecular approach of terminal restriction fragment length polymorphism (T-RFLP) (Engel et al. 2012), the intertidal ciliate community, collected from the German island of Sylt in the Wadden Sea showed a highly patchy structure. Our results show trends similar to those of these previous studies and emphasize the need for a broader sampling to understand polar environments better. In addition, we should be aware that cell size (Zhu et al. 2005) and 18S rDNA copy number (Gong et al. 2013) could affect the read abundance in an OTU. With the distinctness of the structures, we found some interesting results from the polar samples.

The two freshwater samples ANtFresh and ArFresh contained and shared some OTUs with salt-water samples, and ANtFresh shared more OTUs than did ArFresh. Assigned taxa in Table 2 indicate that the shared OTUs were typically marine ciliates, and ANtFresh, in particular, showed 12 marine species (vs. 4 in ArFresh). The sampling station of ANtFresh is located about 50 m away from the coastline and salt-water can easily penetrate

Table 2. Ciliate species showing high similarity (≥99%) with pyrosequencing data, compiled with SILVA release 119.

Species (≥99% with Ref seq)	Ant Coast1	Ant Coast2	Ant CoastPFU	Ant Fresh	Ar Coast1	Ar Coast2	Ar Ocean	Ar Fresh	Habitat
Amphisiella annulata GU170843	0	4	0	0	0	0	0	0	S/B
Apokeronopsis bergeri DQ777742	0	0	0	18	3	0	13	0	S/B
Arcuseries scutellum FJ156105	0	0	0	0	1	0	0	0	S/B
Aspidisca steini AF305625	0	0	0	0	0	0	1	0	S/B
Cyclotrichium cyclokaryon FJ876971	0	0	0	8	0	87	127	0	S/P
Diophrys scutum DQ353851	0	0	0	0	3	0	0	0	S/B
Diophrys sp. HE664174	8	0	0	0	0	0	0	0	S/B
Euplotes focardii EF094960	0	0	2	0	0	0	0	0	S/B
Euplotes nobilii GU479383	0	0	0	2	4	0	0	0	S/B
Euplotes nobilii GU479391	0	3	0	2	1	0	0	0	S/B
Euplotes rariseta JX437134	0	3	0	0	1	0	0	0	S/B
Euplotes sinicus FJ423448	0	8	0	2	4	0	0	2	S/B
Euplotes sp. EF193242	0	12	0	0	0	0	0	0	S/B
Gastrostyla steinii AF508758	0	0	0	0	0	0	0	1	F/B
Halteria grandinella AF508759	0	2	0	151	0	0	0	0	F/P
Laboea strobila AF399154	0	0	0	0	0	3	0	0	S/P
Loxophyllum rostratum DQ190465	0	3	5	8	5	0	1	0	S/B
Metaurostylopsis antarctica JF906730	0	0	0	0	4	0	0	0	S/B
Metaurostylopsis cheni GU170204	0	2	0	0	0	0	4	0	S/B
Orthoamphisiella sp. JQ723974	0	0	0	204	0	0	0	0	F?/B
Oxytricha trifallax AMCR01020474	0	0	0	0	0	0	0	29	F/B
Parauronema longum AY212807	0	0	16	0	0	0	0	0	S/B
Pleuronema setigerum JX310015	0	6	0	0	0	0	3	0	S/B
Psammomitra retractilis EF486865	0	0	1	3	2	0	0	0	S/B
Pseudoamphisiella alveolata DQ503583	0	0	0	6	2	0	0	0	S/B
Pseudotontonia sp. JX178817	0	0	0	0	0	0	1	0	NA/P
Spathidiopsis buddenbrocki HM051056	0	2	0	4	2	0	0	0	S/P
Stentor multiformis FN659821	0	0	0	0	0	0	1	62	F/B
Strombidinopsis sp. JQ028734	0	2	0	0	0	0	0	0	S/P
Strombidinopsis sp. AM412524	0	0	0	0	0	0	1	0	S/P
Strombidium biarmatum AY541684	0	0	0	0	3	0	5	0	S/P
Strombidium rassoulzadegani AY257125	0	0	0	0	47	0	0	0	S/P
Strombidium sp. AY143564	0	17	10	0	2	0	0	0	S/P
Tintinnopsis cylindrica JN831811	0	0	0	0	0	2	18	0	S/P
Tintinnopsis rapa JN831833	0	0	0	23	7	164	5	1	S/P
Tintinnopsis sp. JX178854	0	0	0	0	0	2	0	0	S/P
Tintinnopsis sp. JX178862	0	0	0	0	0	3	0	0	S/P
Tintinnopsis sp. JX178881	0	0	0	2	0	1	0	0	S/P
Tintinnopsis sp. JX178900	0	3	0	90	0	557	0	1	S/P
Uroleptus gallina AF508779	0	0	0	0	0	0	6	185	F/B
Uroleptus willii EU399543	0	0	0	0	0	0	0	15	F/P
Uronema marinum GQ465466	0	0	3	0	0	0	3	0	S/B
Uronychia setigera HQ380023	0	22	0	0	1	0	3	0	S/B
Uronychia setigera JF694042	0	3	0	0	83	0	0	0	S/B
Vorticella microstoma DQ868347	0	6	0	0	0	0	0	0	F/B
Zoothamnium sp. JX178767	0	0	0	0	0	0	0	3	NA/B
Total	8	98	37	523	175	819	192	299	

Notes: NA, data not available; B, benthic; F, freshwater; P, planktonic; S, salt-water. Soil environment was classified as freshwater in this study.

the island and reach to the freshwater lake. However, most OTUs were not assigned to the species level because of low similarity with SILVA DB. Because of this limitation of the reference sequence, we could not ascertain whether the other co-occurring OTU is a marine or freshwater species. However, we can assume that these samples shared some OTUs and they were likely euryhaline species or were in their resting stage (cyst) or were cell debris. All sampling stations of each polar region were closely situated along the coastline, except for one

pelagic sample. Thus, these environments might easily influence each other by physical forces such as wind and waves. Additionally, we need to consider a dispersal by resting cysts of terrestrial ciliates (including freshwater species) to coastal waters because live cells are very fragile for the dispersal as an active form (Foissner 2011). Moreover, the two freshwater samples share only 15 OTUs (17.6% of total freshwater OTUs) that might be related with the limited distribution of limnetic ciliates as reported by Petz et al. (2007).

Among the eight polar samples of the normalized data set, ArCoast1 showed the highest number of OTUs (103). The sample ArCoast1 includes ciliate species collected from a sandy sediment. According to Hamel et al. (2005), the grain size of bottom environments is positively related with species richness because small size of grains such as silt may prevent their movement and colonization. In addition, ArCoast1 was collected from a coastal area and showed a broad range of salinity (3–23 PSU). This broad salinity range probably increased the OTU richness, while ANtCoast1 showed the lowest number of OTUs (8). The sample ANtCoast1 was collected from a temporal pool, which was approximately $50 \times 50 \times 15$ cm (length × width × depth) in size, with high salinity (53.4 PSU), and was situated on a rock approximately 80 m from the coastline, isolated from other surrounding coastal environments. This slightly extreme environmental condition likely affected the community structure and decreased the number of OTUs, and its community structure has a distinct pattern from the others (Figure 7). Some ciliates are also found in such a high salinity environments. Filker et al. (2015) reported a negative relationship between salinity (40–380 PSU) and OTU richness that appears to be related with a lower OTU richness in ANtCoast1.

The artificial substrate, PFU, could host diverse protozoan communities including planktonic, periphytic, and benthic species (Xu et al. 2002). The PFU of ANtCoastPFU was soaked in costal water for 10 days. Contrary to our expectation, the OTU richness of ANtCoastPFU was not much greater than the others and its community structure showed a distinct pattern from other polar stations with the exception of the extreme environment of ANtCoast1 (Figure 7). The origin of introduced species in PFU should be from the surrounding environment of costal water but its community structure showed a lower OTU richness than ANtCoast2 and was not clustered with ANtCoast2. Further study on introduced species in PFU and its driving force for structuring is needed to evaluate the representativeness of natural community structures using the PFU system.

In conclusion, it is prerequisite to reduce potential technical bias of 454 amplicon pyrosequencing as to more reliably reveal eukaryotic community structures; these include (i) the discrepancy between cell and read numbers (Stoeck et al. 2014), (ii) the deficiency in reference gene sequences for taxonomic assignment (Pawlowski et al. 2012), and (iii) the low resolution of 18S rRNA for closely related species (Lynn & Strüder-Kypke 2006).

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ORCID

Jae-Ho Jung http://orcid.org/0000-0001-5497-8678

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