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Diversity and community dynamics of protistan microplankton in Sagami Bay revealed by 18S rRNA gene clone analysis

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Abstract: The diversity and short-term changes in the protistan microplankton community from April to June 2006 in Sagami Bay were revealed by 18S rRNA gene clone analysis. A total of 1,076 clones consisted of 68 phylotypes of dinoflagellates, 96 phylotypes of diatoms and 27 phylotypes of other protists affiliated with Ciliophora, Prymnesiomonada, Chlorophyta, Cercozoa, Chytridiomycota, and Heterokonta. Approximately half of all dinoflagellate phylotypes were affiliated with the following genera: *Ceratium*, *Gonyaulax*, *Gymnodinium*, *Gyrodinium*, *Lepidodinium*, *Neoceratium*, *Prorocentrum*, and *Woloszynskia*. The other half was classified into seven uncultured groups. These dinoflagellate clones were mostly detected in May, in contrast to the diatom clones, which were detected frequently throughout the study period. Diatoms were diverse and consisted of 14 genera and three uncultured groups. The genera *Discostella*, *Thalassiosira* and *Skeletonema* were dominant in April, May and June, respectively. Species richness (number of phylotypes) and diversity (Shannon-Weiner) of the whole protistan microplankton community were highest in May. This is the first example of a comprehensive molecular biological analysis of protistan microplankton community structure, and the results clearly showed a dynamic shift in the protistan community in coastal waters from April to June in Sagami Bay. The results of a direct comparison between the clone analysis and microscopic observations indicated that the clone analysis had the great advantage of enabling identification of plankton that were morphologically indistinguishable, and to reveal detailed information on the biodiversity of protistan microplankton. This advancement in molecular biological analysis will assist in our understanding of the biodiversity of protistan microplankton.

Key words: 18S rDNA, biodiversity, clone analysis, protistan microplankton, Sagami Bay

Introduction

Protists play a key role as marine primary producers and consumers since they produce and supply organic matter to marine ecosystems (Smatacek 1999, Falciatore & Bowler 2002, Han et al. 2002). Furthermore, protists constitute an essential component of food webs and play significant roles in the global carbon and mineral cycles, especially in oligotrophic parts of the oceans (Li 1994). Understanding the structure and diversity of protistan communities is of fundamental importance to biological oceanography and is providing information regarding the activities and evolution of life on our planet.

The major justification for documenting protistan diversity has been a desire to answer basic ecological questions. This is because protists are attractive model organisms to study ecological and evolutionary questions since they have relatively short generation times and can be maintained at large population sizes, facilitating an experimental approach for studies of ecology and evolution (Bell 1997, Reynolds 1997, Coats & Park 2002, Bruin et al. 2003). The genetic diversity of a protistan community plays an important role in explaining the interaction of protistan species with the environment, as these interactions will structure the ecosystem (Medlin et al. 2000). The taxonomic identification of protists using molecular

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techniques provides a new type of data that can be used to reconstruct or verify classifications based on morphological and physiological characters (Lundholm et al. 2002). Although, protistan diversity is difficult to grasp (Hulburt 1983, Martin 2002), there have been several pioneering works endeavoring to reveal protistan diversity and community structure using molecular techniques (Rappé et al. 1998, Caron et al. 1999, Savin et al. 2004, Countway et al. 2005, Countway & Caron 2006). These studies showed the presence of highly diverse undescribed protistan species, and this is believed to have important evolutionary and ecological implications. However, the numbers of clones analyzed in these studies were relatively small, and more comprehensive analyses were called for.

Coastal ecosystems deserve particular attention due to influences from the terrestrial and also because of higher trophic levels (Cebriá & Veliela 1999), suggesting that they could harbor particular protist assemblages that are different to those in the open sea. Coastal sites are also prone to larger temporal variability induced by episodic events. Given such coupling, it is important to understand the dynamics of protistan community structure in coastal areas. Sagami Bay is located in the southeastern part of Japan, and is a semi-circular embayment facing the Pacific Ocean, where the Kuroshio Current flows. The warm water from the Kuroshio Current forms the upper layer water mass (0–200 m depth). Variations in surface temperature and salinity are also observed because the oligotrophic Kuroshio waters flowing into the bay get mixed with eutrophic riverine waters (Hogetsu & Taga 1977, Nakata 1985). Moreover, seasonal stratification caused by the synergic effect of rising surface temperatures coupled with increasing precipitation and freshwater discharge from rivers during the spring period (Satoh et al. 2000, Ara & Hiromi 2008, Hashihama et al. 2008) results in a highly diverse assemblage of organisms, including protists. In addition, Sagami Bay is a popular temperate sampling station in Japan due to its relatively high productivity and biodiversity, and much work has been done on seasonal and annual variation in the plankton (Onoue et al. 2004, Miyaguchi et al. 2008, Yoshiki et al. 2008, Shimode et al. 2009). Many studies on the plankton assemblages in the coastal waters of Sagami Bay have been carried out primarily through microscopic observations. It is believed that molecular biological analyses could offer new views on protistan biodiversity and community structure in Sagami Bay. In a previous study, we succeeded in designing universal PCR primers that allowed amplification of 18S rRNA genes (18S rDNAs) of various protistan microplankters. We applied these primers and 18S rDNA clone analysis to describe the biodiversity and short-term changes in community structure of protistan microplankton from April to June 2006 in Sagami Bay.

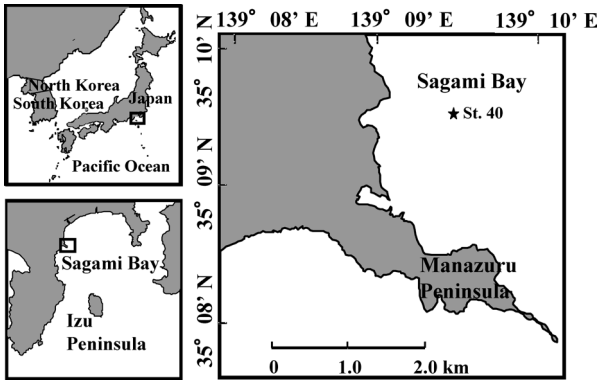

Fig. 1. The location of the sampling site, Station 40, in Sagami Bay, Japan.

Materials and Methods

Sampling and storage of protistan microplankton cells

Sampling was conducted at Station 40 (35°09′30″N, 139°09′25′E, depth 40 m) (Fig. 1), Sagami Bay on 13 April, 18 May, 29 June 2006 using the RV Tachibana (Yokohama National University). Twenty liters of surface seawater were screened through a 180-μm nylon mesh and collected in a tank. The cells for molecular analysis were collected on membrane filters (pore size, 10 μm; Millipore) and fixed with 5% Lugol's solution. Lugol's solution minimizes the impact on downstream analysis without any loss of rDNA sequence information compared to other fixation methods (Galuzzi et al. 2004). Fixed cells were removed from the filters by vibration and collected by centrifugation (Kubota 3700, AF-2724A) for 3 min at 4℃ and 15,000 rpm. This procedure was repeated with additional filtered seawater to ensure that all cells were removed from the membrane filters. The collected cells were stored at −25℃ until further analysis. Cells for microscopic observation, derived from 150 mL seawater, were collected on membrane filters (pore size: 10 μm; Omnipore) by gravity filtration and fixed in 5 mL filtered seawater containing 2.5% glutaraldehyde. The fixed cells were stored at 4℃ until further analysis.

Environmental DNA extraction and nested PCR amplification

The protistan microplankton cells were resuspended in 100 μL of TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0) buffer containing Triton X-100 (0.2%, w/v) and then boiled at 70℃ for 5 min, followed by DNA extraction using a DNA extraction machine (Precision System Science). Extracted DNA was purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare), according to the manufacturer's instructions. The purified DNA was then used for the first PCR amplification of 18S rDNA with the primers PP18S-408F (5′-TACCACATC(T/C)AAGGAAGGCAG, *Alexandrium tamarense* (Lebour) AF022191 position 408–428) and PP18S-1332R (5′-CTCGTTCGTTAACGGAAT-

TAAC, *A. tamarense* AF022191 position 1,313–1,332) using *Ex Taq* DNA polymerase (Takara Bio) with 3 min at 94.0℃, 35 cycles of 30 sec at 94.0℃, 30 sec at 62.0℃, 90 sec at 72.0℃, and a final extension of 5 min at 72.0℃. Subsequently, nested PCR was performed using 1 μL of the first PCR reactant and the primers PP18S-431F (5′-GGCGCG(C/T)AAATTACCCAAT(C/A), *A. tamarense* AF022191 position 431–450) and PP18S-1133R (5′-TCAGCCTTGCGACCATACTC, *A. tamarense* AF022191 position 1,112–1,133) with 3 min at 94.0℃, 30 cycles of 30 sec at 94.0℃, 30 sec at 62.0℃, 1 min at 72.0℃, and a final extension of 5 min at 72.0℃. The amplified DNA was purified using the aforementioned GFX kit.

18S rDNA clone analysis and phylogenetic tree construction

Fragments of 18S rDNA obtained by PCR were cloned into the pT7Blue T-vector (Novagen), and the resulting recombinant plasmids were used for transformation into *Escherichia coli* (Migula) DH5*α*. The transformants were spread on LB plates containing 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-gal, and 0.5 mM IPTG. Blue/white selection was conducted by randomly picking and subculturing individual white colonies in 100 μL of 2×YT medium containing 100 μg mL^{-1} ampicillin in a 96-well plate at 37℃ overnight. The inserted 18S rDNA was amplified by PCR using 1 μL of the culture as the template with the primers PP18S-431F and PP18S-1133R. The PCR procedure was the same as that for nested PCR.

Restriction fragment length polymorphism (RFLP) analysis was conducted to separate clones into related groups. A representative clone from each group was selected for sequencing. RFLP analysis was first conducted with *Afa*I, and clones showing the same RFLP pattern (DNA band pattern on an agarose gel) were grouped together. Additional RFLP analyses were then conducted sequentially with *Hap*II and *Sau*3AI. Clones that were grouped together on the basis of three RFLP analyses were considered to belong to the same operational taxonomic unit (OTU).

The sequences of 18S rDNA of individual OTU representative clones were compared with 18S rDNA sequences published in the National Center for Biotechnology Information DNA database using BLAST (BLASTN; http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990) to identify individual clones. Similarities with known species of more than 98% were considered to indicate the same phylotype, those from 93.0 to 97.9% were considered to indicate the same genus, those from 87.0 to 92.9% were considered to indicate the same family, and less than 86.9% similarity was considered to indicate the same order. Taxonomic classification of Protists was made according to Hausmann et al. (2003).

In order to analyze the phylogenetic relationships among the clones and previously reported protistan 18S rDNA sequences, neighbor-joining trees for dinoflagellates, diatoms, and others were constructed using the CLUSTAL W ver. 1.83 program (Thompson et al. 1994) and GENETYX version 12.1.0 software with the outgroup species *Rhodella violacea* (Kornmann), a member of the Rhodophyta that is situated nearest to the protistan microplankton group in the 18S rDNA phylogenetic tree of Adachi (2000). Bootstrap values were estimated from 1,000 replicates.

The nucleotide sequences of 203 phylotypes have been made available in the DDBJ/EMBL/GenBank databases under accession numbers AB694523–AB694725.

Diversity coverage and index

The diversity coverage (homologous coverage) *Cx* was calculated as follows: $Cx=1-N/n$, where N is the number of phylotypes in the sample, and n is the total number of analyzed clones (Good 1953, Singleton et al. 2001). The Shannon-Weiner diversity index H was calculated as follows: $H=-\Sigma\ (pi)\ (\ln pi)$, where pi is the proportion of the ith phylotype (Margalef 1958). The evenness was calculated by $E=H/\ln S$, where S is the phylotype richness (Pielou 1969).

Microscopic observation

The fixed samples were transferred to a counting chamber (Zählkammern N. fuchs-rosenthal, Hirschmann Laborge). Cell-counting and identification were carried out using a phase contrast microscope (Axioskop 40 Carl Zeiss, Germany) at 400x magnification. This procedure was repeated 10 times on each sample and a total of 4,207 cells were examined.

Results

Community composition and diversity revealed by 18S rDNA clone analysis

Three 18S rDNA clone libraries were constructed independently using the water samples collected on 13 April, 18 May, 29 June 2006 in Sagami Bay, Japan. A total of 1,076 clones consisted of 502 clones of dinoflagellates (dinoflagellata), 538 clones of diatoms (Bacillariophyceae), and 36 clones affiliated with other protists such as Ciliophora, Prymnesiomonada, Chlorophyta, Cercozoa, Chytridiomycota, and Heterokonta (other than diatoms). (Table 1).

The dinoflagellate clones were mostly detected in May with much lower frequencies in April and June. In contrast to the dinoflagellates, diatom clones were detected frequently throughout the study period.

Dinoflagellate (Dinoflagellata) community

The phylogenetic affiliations of 502 dinoflagellate clones are shown in Table 1. These clones were classified into 68 phylotypes consisting of eight genera; *Ceratium*, *Gonyaulax*, *Gymnodinium*, *Gyrodinium*, *Lepidodinium*, *Neoceratium*, *Prorocentrum*, *Woloszynskia*, and seven uncultured groups. The uncultured groups made up 87% of the total number of dinoflagellate clones, and most of them be-

Table 1. Protistan microplankton genera detected by the 18S rDNA clone analysis.

Phyla Subphyla Classes	Genera	Number of phylotypes (Number of clones) April	May	June	Throughout the study period
Alveolata					
Dinoflagellata*	*Ceratium*	1 (1)	1 (2)	–	1 (3)
	Gonyaulax	1 (1)	4 (11)	–	5 (12)
	Gymnodinium	–	1 (3)	–	1 (3)
	Gyrodinium	3 (8)	4 (13)	–	7 (21)
	Lepidodinium	–	1 (1)	–	1 (1)
	Neoceratium	1 (1)	4 (4)	–	5 (5)
	Prorocentrum	–	1 (2)	–	1 (2)
	Woloszynskia	–	5 (16)	1 (1)	6 (17)
	Syndiniales Group I (MALV)	1 (1)	14 (250)	5 (16)	17 (267)
	MALV	–	1 (1)	–	1 (1)
	Uncultured Gymnodiniales I	1 (1)	7 (65)	–	7 (66)
	Uncultured Gymnodiniales II	1 (2)	4 (91)	–	5 (93)
	Uncultured Gymnodiniales III	2 (2)	3 (4)	–	5 (6)
	Other uncultured Gymnodiniales	–	4 (4)	–	4 (4)
	Uncultured Peridiniales	–	–	1 (1)	1 (1)
	(subtotal for Dinoflagellata)	11 (17)	54 (467)	7 (18)	68 (502)
Ciliophora	Uncultured Oligotrichia	–	2 (2)	–	2 (2)
	Uncultured Tintinnida	–	1 (1)	–	1 (1)
Chromista					
Heterokonta					
Bacillariophyceae**	*Arcocellullus*	–	2 (8)	–	2 (8)
	Chaetoceros	3 (5)	1 (1)	4 (4)	8 (10)
	Cyclotella	–	–	4 (15)	4 (15)
	Cylindrotheca	–	1 (1)	–	1 (1)
	Discostella	2 (99)	1 (1)	1 (2)	2 (102)
	Eucampia	1 (4)	–	2 (2)	2 (6)
	Fragilariopsis	–	1 (1)	–	1 (1)
	Gyrosigma	2 (3)	1 (1)	1 (1)	3 (5)
	Leptocylindrus	–	4 (5)	–	4 (5)
	Minisdiscus	–	2 (3)	–	2 (3)
	Pseudo-nitzschia	2 (2)	3 (11)	10 (47)	12 (60)
	Rhizosolenia	2 (2)	–	–	2 (2)
	Skeletonema	–	–	9 (165)	9 (165)
	Thalassiosira	6 (8)	13 (96)	13 (30)	31 (134)
	Uncultured Bacillariales	1 (1)	3 (3)	2 (9)	6 (13)
	Uncultured Cymatosirales	–	5 (6)	–	5 (6)
	Unknown order	–	1 (1)	1 (1)	2 (2)
	(subtotal for Bacillariophyceae)	19 (124)	38 (138)	47 (276)	96 (538)
others	*Ectocarpus*	–	1 (1)	–	1 (1)
	Sargassum	1 (1)	–	–	1 (1)
	Solenicola (MAST-3)	–	1 (1)	–	1 (1)
	MAST-3	–	1 (1)	–	1 (1)
	MAST-12	1 (1)	2 (2)	2 (2)	5 (5)
	Novel Stramenopiles Group X	2 (2)	–	–	2 (2)
Prymnesiomonada	*Emiliania*	–	1 (1)	–	1 (1)
Viridiplantae					
Chlorophyta	*Chlorococcum*	–	1 (1)	–	1 (1)
	Pseudoscourfieldia	–	1 (9)	–	1 (9)
	Tetraselmis	–	–	3 (3)	3 (3)
	Uncultured Pyramimonadales	–	1 (1)	–	1 (1)
Cercozoa	*Cryothecomonas*	–	1 (1)	–	1 (1)
	Uncultured Cryomonadida	–	2 (2)	–	2 (2)
Opisthokonta					
Chytridiomycota	Uncultured Chytridiales	2 (3)	–	–	2 (3)
other protists		–	1 (1)	–	1 (1)
Total		36 (148)	108 (629)	59 (299)	191 (1,076)

*This group is referred to as "dinoflagellates" in the text.
**This group is referred to as "diatoms" in the text.

longed to the Marine Alveolates Group (MALV) (Díez et al. 2001, López-Gracía et al. 2001, Moon-van der Staay et al. 2001) or uncultured Gymnodiniales groups (I~III) (Table 1, Fig. 2). Approximately 93% of dinoflagellate clones were detected in May.

The most frequently detected uncultured group was the Syndiniales Group I (Groisillier et al. 2006) of MALV, which shared approximately 53% of the total dinoflagellate clones. Within this group, the phylotype PM63 was dominant and showed 99% sequence similarity with uncultured marine eukaryote clone CD8.17 isolated from seawater incubations (Massana et al. 2006). In the same group, there were two other major phylotypes, PA29 (showed ≥98% similarity with PM1 and PJ16) and PM23 (showed ≥98% similarity with PJ55), that showed 97–99% similarity with environmental clones isolated from the Atlantic Ocean and Mediterranean Sea (Guillou et al. 2008). The phylotypes PM58 and PM90, showed significant similarity with uncultured eukaryotic 18S rDNA clone DSGM27 isolated from a methane cold seep sediment in Sagami Bay (Takishita et al. 2007b). This Syndiniales Group I was detected as only one clone in April and dramatically increased in May.

The uncultured Gymnodiniales groups I, II and III (UGG-I, UGG-2, and UGG-3) were also major uncultured groups and comprised about 32% of the total number of dinoflagellate clones. The most frequent phylotype in the UGG-I, PM44, had a 98% similarity to the "uncultured eukaryotic 18S rDNA clone SCM28C1 isolated from the deep chlorophyll maximum in the Sargasso Sea" (DNA database Acc. No. AY664890). The dominant phylotype, PM65, in the UGG-II had a 98% similarity to a "marine dinoflagellate off the coast of southeastern North Carolina in America" (DNA database Acc. No. FJ914470). The cluster with uncultured Gymnodiniales I and II accounted for the second and third highest number of dinoflagellate clones, respectively. However, these groups disappeared in June. All the phylotypes in UGG-III showed significant similarities with an "Uncultured marine dinoflagellate off the coast of southeastern North Carolina" (DNA database Acc. No. FJ914494).

Diatom (Bacillariophyceae) community

The phylogenetic affiliations of 538 diatom clones (April: 124 clones; May: 138 clones; June: 276 clones) are shown in Table 1. These clones were much more diverse compared to dinoflagellate, and were classified into 96 phylotypes. These consisted of 14 genera; *Arcocellulus*, *Chaetoceros*, *Cyclotella*, *Cylindrotheca, Discostella*, *Eucampia*, *Fragilariopsis*, *Gyrosigma*, *Leptocylindrus*, *Minidiscus*, *Pseudo-nitzschia*, *Rhizosolenia*, *Skeletonema*, *Thalassiosira*, two uncultured groups affiliated with the orders Bacillariale and Cymatosirales, and a group from an unknown Order. Even though the genus *Skeletonema* was only detected in June, it was outstanding in term of clonal frequency. The genus *Thalassiosira* was the second most dominant group within the diatoms and consisted of a high diversity of phylotypes. In April, *Discostella* accounted for 19% of the total number of diatom clones but it almost disappeared in May and June. *Pseudo-nitzschia* was also frequently detected in Sagami Bay. This genus was detected every month, increasing in both clone number and phylotype number over the study period.

Other members of the protistan community

The taxonomically-identified clones other than dinoflagellates or diatoms were affiliated with the Ciliophora (three phylotypes), Prymnesiomonada (one phylotype), Chlorophyta (six phylotypes), Cercozoa (three phylotypes), Chytridiomycota (two phylotypes), or Heterokonta (11 phylotypes other than diatoms). They were mostly detected in May and were relatively diverse, even though these clones comprised only 3% of the total number of clones found in this study (Table 1, Fig. 4).

In the Prymnesiomonada, the phylotype PM34, affiliated with the genus *Emiliania*, was detected in May. Three phylotypes, PM88, PM89, and PM93 in the Cercozoa group, were also detected in May. The phylotype PM93 was identified as a member of the genus *Cryothecomonas*, and another two phylotypes were affiliated with the order Cryomonadida. The Ciliophora group was also detected only in May, and consisted of three uncultured phylotypes affiliated with the subclass Oligotrichia (PM76 and PM96) and the order Tintinnida (PM5). On the other hand, two phylotypes detected in April (PA35 and PA38) were affiliated with the order Chytridiales within the Chytridiomycota.

In the Chlorophyta, six phylotypes were detected from May to June. The phylotype PM68, which was detected in May, was the most frequent clone, and was identified as a member of the genus *Pseudoscourfielda*. One phylotype (PM113) of the genus *Chlorococcum* was also detected in May. Three phylotypes of the genus *Tetraselmis* (PJ60, PJ20, and PJ36) were detected in June. The remaining phylotype, PM95, which was detected in May showed no significant similarity with any known species. However, it had a 99% sequence similarity with the "uncultured eukaryote clone A95F13RJ3A10 isolated from Cariaco Basin, Caribbean" (Edgcomb et al. 2011) and was therefore classified in the order Pyramimonadales.

In the Heterokonta, 11 phylotypes were detected and occurred throughout the whole spring. In this group, the genera *Ectocarpus*, *Sargassum* and *Solenicola* were detected along with two uncultured groups—the Marine Stramenopiles (MAST, Massana et al. 2002) and the novel Stramenopiles Group X. The phylotype PM31 was affiliated with the MAST-3 group, and five phylotypes were affiliated with the MAST-12 group. Another two phylotypes, PA10 and PA24, showed significant similarities to uncultured marine picoplankton clone He000427_201 isolated from the Central German Bight of the North Sea (DNA database Acc. No. AJ965010) and were classified with the novel Stramenopiles Group X (Medlin et al. 2006). The

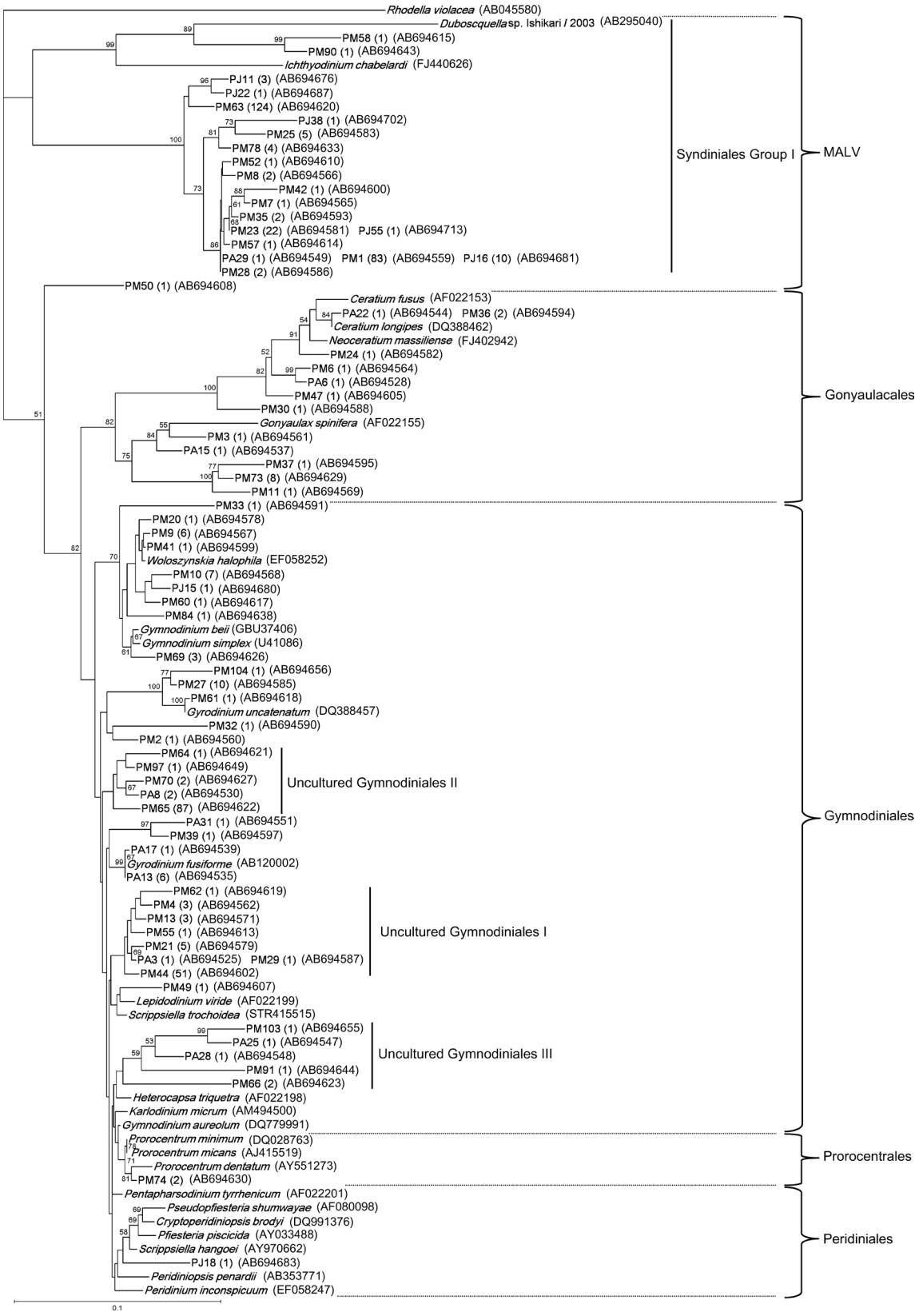

Fig. 2. Neighbor-joining (NJ) tree for Dinoflagellata (dinoflagellate) clones detected from the surface water of Sagami Bay. The sequences are indicated by "PA, PM, PJ and numbers". The number of clones of each phylotype is indicated in the first parentheses followed by the accession number in the second parentheses. Bootstrap values derived from 1,000 replicates are given at respective nodes as percentages (values less than 50% are not shown).

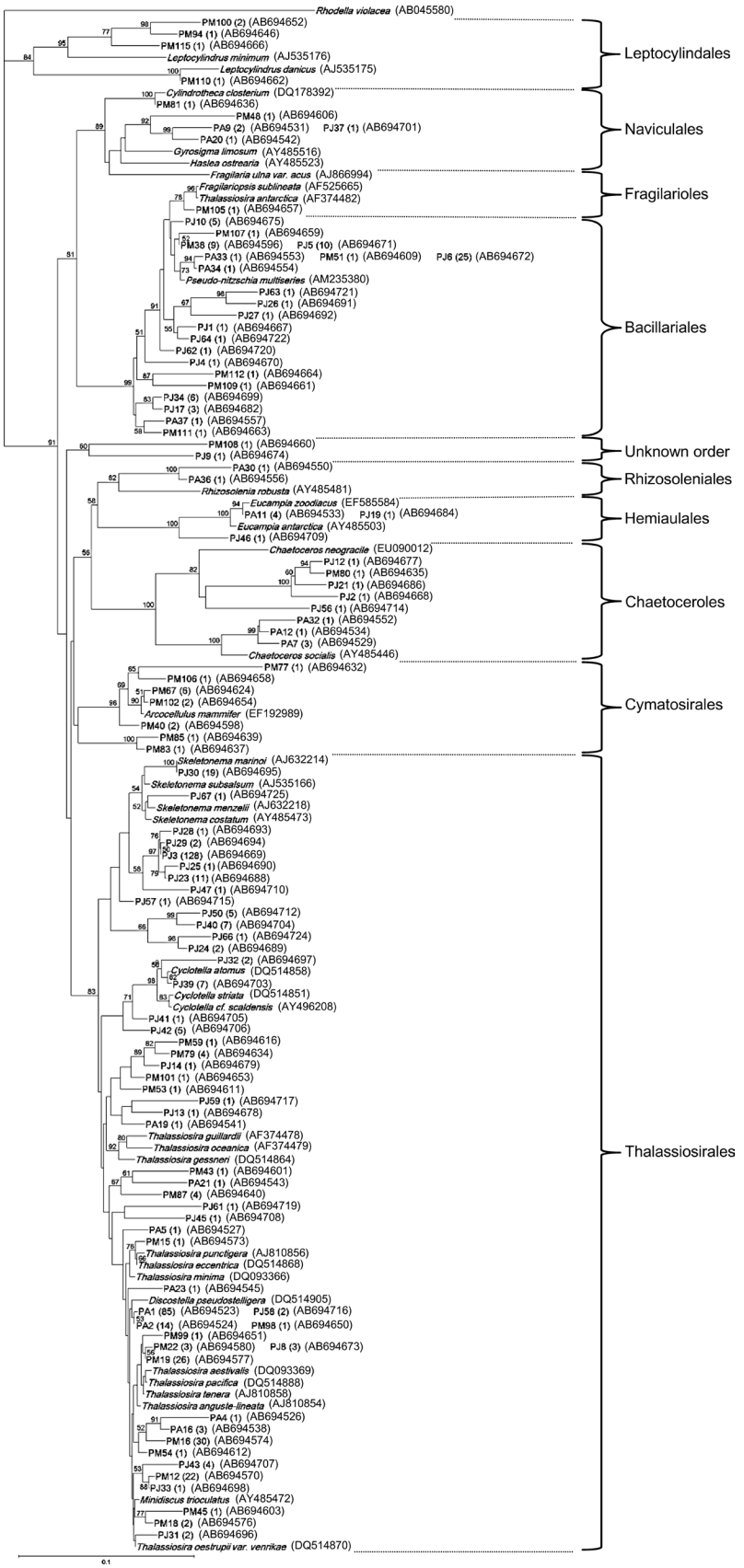

Fig. 3. Neighbor-joining (NJ) tree for Bacillariophyceae (diatom) clones detected from the surface water of Sagami Bay. See Fig. 2 for further explanation.

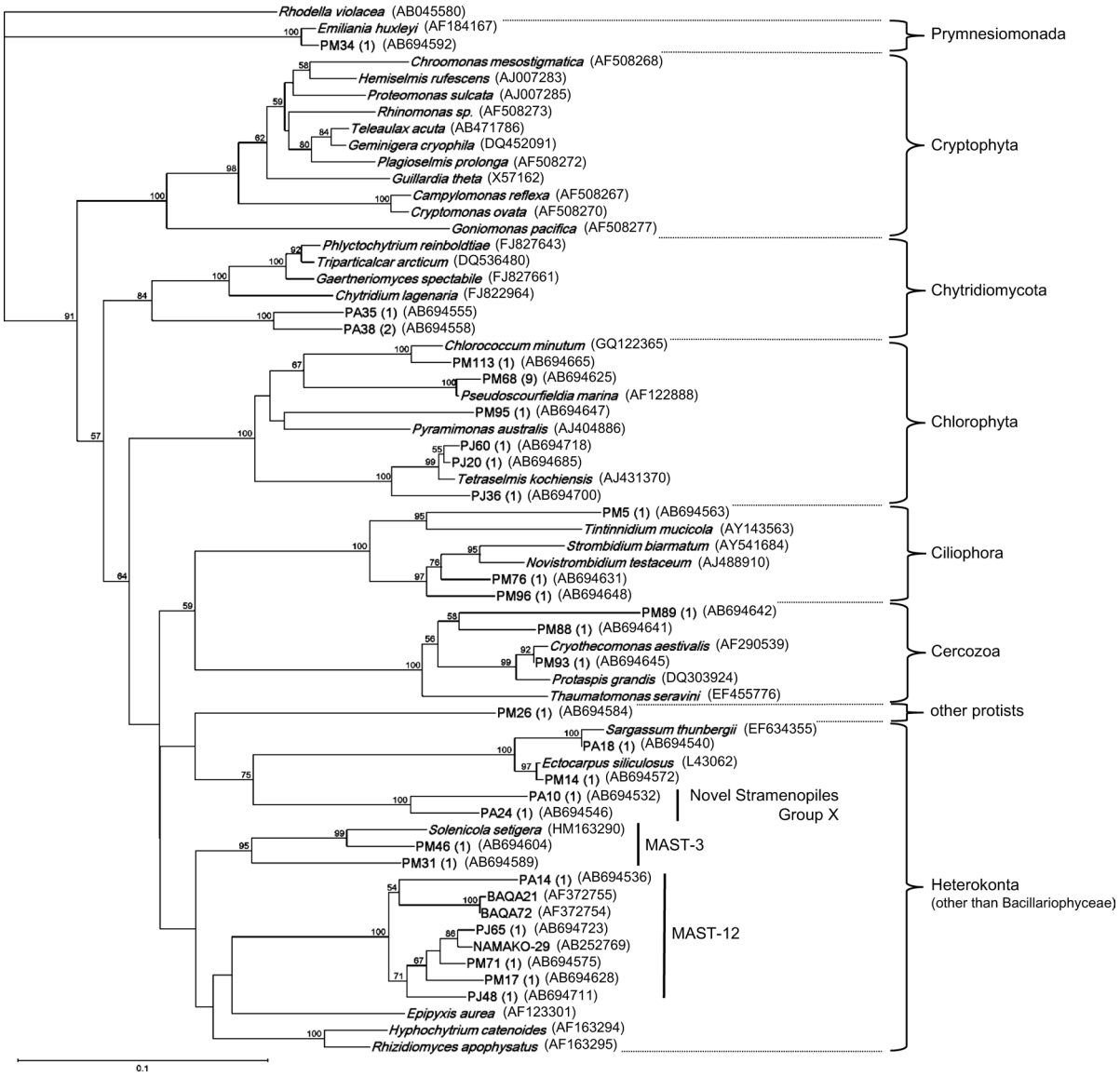

Fig. 4. Neighbor-joining (NJ) tree for clones other than Dinoflagellata and Bacillariophyceae detected from the surface water of Sagami Bay. See Fig. 2 for further explanation.

phylotype PM26 did not exhibit a significant phylogenetic relationship with any known species of protistan microplankton.

Protistan microplankton richness and distribution

The number of phylotypes (richness) and the frequency distribution of the phylotypes (evenness) in each clone library were evaluated using a variety of standard diversity indices (Table 2). According to the richness values (*S*) obtained with the 18S rDNA clone libraries, April had a relatively low phylotype richness (36 phylotypes), followed by June (56 phylotypes) and the highest richness was in May (108 phylotypes). This order was similar to the results of Shannon-Weiner index (*H*) analysis, where the April library had the lowest diversity (*H*=2.66), followed by June (*H*=5.56) and May recorded the highest diversity with a value of 9.55. The clone analyses from April, May and June had homologous coverage of 0.76, 0.83, and 0.80, respectively. In order to understand the equitability of phylotype distributions in each library, evenness in each library was measured and the values were 0.74, 2.00, and 1.36, respectively.

Community composition and diversity analyzed by morphological identification

A total of 20 genera of protistan microplankton were identified by microscopic observation (Table 3). Total abundance of microplankton was highest in April, decreased in May, and increased again in June. Diatoms were most frequently observed and were diverse in all the samples. *Chaetoceros Eucampia*, *Guinardia*, *Leptocylindrus*, *Pseudo-nitzschia*, *Rhizosolenia*, and *Thalassiosira* were

observed in all three months; with *Pseudo-nitzschia* recorded the highest cell numbers in April (123 cells mL^{-1}), followed by *Leptocylindrus* in May (38 cells mL^{-1}) and *Chaetoceros* in June (89 cells mL^{-1}). *Lauderia* were observed in April and June. *Skeletonema* was observed in May and June. *Diatoma* and *Fragilaria* were only observed in April. *Ditylum* and *Cylindrotheca* were only observed in May and June, respectively. In the dinoflagellate group, *Ceratium* and *Prorocentrum* were observed in April and May, followed by *Gonyaulax*, *Peridinium* and *Scripsiella* in June. Chryosomonadea, genus *Distephanus* and *Dictyocha*, were only observed in May.

Table 2. Statistic analysis of the protistan microplankton community.

Index	Sagami Bay community		
	April	May	June
Phylotype richness *S*	36	108	59
Shannon-Weiner diversity index *H*	2.66	9.55	5.56
Homologous coverage *C*	0.76	0.83	0.80
Evenness *E*	0.74	2.00	1.36

Discussion

Protistan microplankton community structure and diversity in Sagami Bay revealed through clone analysis

The biodiversity and short-term changes in the protistan microplankton community in Sagami Bay were revealed by 18S rDNA clone analysis. We detected a total of 191 protistan phylotypes based on 1,076 clones derived from surface seawater in April to June 2006. The community consisted of eight genera of dinoflagellates, 14 genera of diatoms, eight genera of other protists, and many uncultured groups, including parasitic endosymbionts. The values of homologous coverage suggested that approximately a further 20% of protistan phylotypes are still unrevealed in the surface waters of Sagami Bay. This study is much more comprehensive than previous molecular biological analyses of protistan diversity (Savin et al. 2004, Countway et al. 2005), and succeeded in detecting a broad range

Table 3. Protistan microplankton analyzed by microscopic observations.

Phyla / Subphyla / Classes	Genera	Abundance (cells mL^{-1})		
		April	May	June
Alveolata				
Dinoflagellata*	*Ceratium*	<1	<1	–
	Gonyaulax	–	<1	1
	Peridinium	–	–	2
	Prorocentrum	1	2	–
	Scrippsiella	–	–	<1
	others	5	6	3
	(subtotal for Dinoflagellata)	6	8	6
Chromista				
Heterokonta				
Bacillariophyceae**	*Chaetoceros*	3	16	89
	Cylindrotheca	–	–	<1
	Diatoma	<1	–	–
	Ditylum	–	<1	–
	Eucampia	16	1	2
	Fragilaria	<1	–	–
	Guinardia	1	1	<1
	Lauderia	<1	–	<1
	Leptocylindrus	3	38	15
	Pseudo-nitzschia	123	14	34
	Rhizosolenia	1	4	6
	Skeletonema	–	1	5
	Thalassiosira	3	7	1
	Others	9	<1	3
	(subtotal for Bacillariophyceae)	159	82	155
Chryosomonadea	*Distephanus*	–	<1	–
	Dictyocha	–	<1	–
other protists		12	5	6
Total		177	95	167

*This group is referred to as "dinoflagellates" in the text.
**This group is referred to as "diatoms" in the text.

of protists.

In the dinoflagellate community, we identified one phylotype of the genus *Ceratium* which has been reported to be a dominant red tide genus in Sagami Bay (Baek et al. 2007). We also identified five phylotypes affiliated with the genus *Neoceratium*, which is a relatively newly-established genus of dinoflagellate reported by Gómez et al. (2010). In addition, we detected another red tide causing phytoplankter–the genus *Gonyaulax*. Also, the genus *Woloszynskia*, which consists of relatively small-sized species, was detected. *Neoceratium* and *Woloszynskia* were not detected by the detailed microscopic analyses conducted by Shimode et al. (2009), Ara et al. (2011) or the present study, indicating the superior sensitivity of molecular surveys for detecting rare taxa.

On the other hand, clones affiliated with uncultured dinoflagellate groups were much more abundant than cultured species. In particular, clones affiliated with the parasitic endosymbiont Syndiniales Group I comprised 53% of dinoflagellate clones. This uncultured group was most diverse and abundant in May, and was detected for the first time in Sagami Bay. Syndiniales is known to parasitize dinoflagellates and ciliates (Coats & Park 2002, Chambouvet et al. 2008, Guillou et al. 2008). The frequent clonal detection of this dinoflagellate likely was correlated to a high abundance of their host cells in Sagami Bay. Other than Syndiniales Group I, the marine Stramenopiles group (MAST-3 and MAST-12) and novel Stramenopiles Group X were also detected for the first time in Sagami Bay. MASTs and novel Stramenopiles Group are novel uncultured sequences reported from diverse marine environments (Massana et al. 2002, Massana et al. 2004, Takishita et al. 2007a).

Sagami Bay is seasonally characterized by a diatom spring bloom, dominated by *Chaetoceros*, *Leptocylindrus*, *Eucampia, Pseudo-nitzschia*, and *Skeletonema* according to microscopic analyses (Shimode et al. 2009). Our clone analysis results revealed a similar diatom community structure, except that *Discostella* comprised a high percentage of the clones in April, *Thalassiosira* in May, and *Skeletonema* in June. We also identified other diatom genera that have only rarely been reported in Sagami Bay, such as *Arcocellulus*, *Cyclotella*, and *Discostella*.

Diatoms have been reported to be predominant in Sagami Bay all year round except for the blooming season of dinoflagellates during summer (Ara & Hiromi 2008, Ara et al. 2011). In our clone analysis results, however, the protistan microplankton community changed abruptly in May, and members of the dinoflagellate, ciliate and MALV groups made up about 40% of the clones in the library. The high percentage of Syndiniales clones in May suggested that parasitic activity by this group occurred during this period. In June, the diatom community dominated again to comprise 92% of the total number of clones. It has been reported that seasonal variation in diatom size is related strongly to the physical structure of the ambient aquatic environment, and that small diatoms such as *Skeletonema*, *Chaetoceros*, and *Pseudo-nitzschia* dominate the final stages of the spring bloom in temperate waters (Nishikawa et al. 2007, Ara et al. 2011). Similarly, the highest percentage of clones of the genera *Skeletonema* and *Pseudo-nitzschia* were also detected in June in our study, marking the final stage of the spring bloom in Sagami Bay.

Advantages and limitations of clone analysis and microscopic observation

In the aquatic environment, there are many protistan microplankton species that do not have morphological features distinct enough from each other to tell them apart easily. Also, some species of dinoflagellate are known to be endosymbionts or parasites of larger plankton. These protistan plankton might be difficult to identify or to count under an optical microscope. In this study, the number of detected phylotypes of protistan microplankton according to the clone analysis was remarkably higher than the number found by microscopic observations, even though the number of cells examined by microscope was much higher than the number of analyzed clones. This result indicates that the clone analysis allows identification of plankton that were morphologically indistinguishable, and helps to reveal detailed information on the biodiversity of protistan microplankton. On the other hand, the diatoms *Chaetoceros* and *Pseudo-nitzschia* were less frequently detected by the clone analysis compared to the microscopic observations. One possible reason of this observation could be a lower DNA extraction yield from some phytoplankton species. Dorigo et al. (2002) and Jasti et al. (2005) reported that the different morphological characteristics and cell wall structures affect DNA extraction yield of phytoplankton cells. Also, we should consider the possibility of a different copy number of 18S rDNA among species, which might give biases to the percentage composition of clones. However, despite the current obstacles, an advancement in molecular biological analysis could help our understanding of the biodiversity of protistan microplankton.

Conclusion

This is the first example of a comprehensive molecular biological analysis of protistan microplankton community structure. The results clearly showed a shift in biodiversity and community structure of protistan microplankton in coastal waters. Advancements in molecular biological analysis will enrich the genetic database of protistan microplankton and help our understanding of aquatic ecosystems.

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