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Distribution and abundance of uncultured heterotrophic flagellates in the world oceans

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Summary

Heterotrophic flagellates play fundamental roles in marine ecosystems as picoplankton grazers. This recognized importance contrasts with our ignorance of the taxonomic composition of this functional group, which remains mostly unidentified by microscopical and culturing approaches. Recent molecular marine surveys based on 18S rDNA genes have retrieved many sequences unrelated to cultured organisms and marine stramenopiles were among the first reported uncultured eukaryotes. However, little is known about the organisms corresponding to these sequences. Here we determine the abundance of several marine stramenopile lineages in surface marine waters using molecular probes and fluorescent *in situ* hybridization. We show that these protists are free-living bacterivorous heterotrophic flagellates. They were widely distributed, occurring in the five world oceans, and accounted for a significant fraction (up to 35%) of heterotrophic flagellates in diverse geographic regions. A single group, MAST-4, represented 9% of cells within this functional assemblage, with the intriguing exception of polar waters where it was absent. MAST-4 cells likely contribute substantially to picoplankton grazing and nutrient re-mineralization in vast areas of the oceans and represent a key eukaryotic group in marine food webs.

Introduction

Marine protists are found throughout the euphotic zone of the world's oceans at concentrations between 10^2 and 10^4 cells per millilitre, forming assemblages numerically dominated by very small cells (3 m or smaller). They are the most abundant eukaryotes on Earth and play significant roles in global carbon and mineral cycles (Sherr and Sherr, 2000). Many of these small protists are phototrophic and contribute significantly to primary production (Li, 1994), whereas others are heterotrophic, mostly naked flagellates, assumed to be grazers of picoplankton (prokaryotes and small phytoplankton) (Fenchel, 1986). Heterotrophic flagellates, together with marine viruses (Suttle, 2005), maintain picoplankton populations at relatively stable concentrations in seawater (Pernthaler, 2005). As a consequence, they are an important trophic link in microbial food webs and are crucial for nutrient remineralization (Caron and Goldman, 1990). However, there is much uncertainty about the identity of the dominant heterotrophic flagellates in the marine plankton (Arndt *et al.*, 2000). These are enumerated by epifluorescence microscopy, but only a few conspicuous forms can be identified. Approaches that yield good taxonomic resolution, such as electron microscopy for ultrastructural features and live observations for characteristic motility, are not quantitative when applied to natural assemblages (Vørs *et al.*, 1995). There are serious doubts that heterotrophic flagellates in culture represent the dominant cells in the environment, as it has been shown that organisms easily enriched are present at low abundance in natural assemblages (Lim *et al.*, 1999). For these reasons, heterotrophic flagellates are usually treated as a black box, obscuring possible significant taxonomic and functional diversity within the assemblage.

In the last few years, environmental molecular surveys of SSU rRNA genes have provided valuable insights into microbial diversity (Pace, 1997), resulting in a more refined picture of the diversity and ecology of marine bacteria and archaea (Giovannoni and Stingl, 2005). This approach has been applied only recently to marine eukaryotic microorganisms, revealing a considerable large diversity and the existence of novel groups of sequences unrelated to cultured organisms (Díez *et al.*, 2001; López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Dawson and Pace, 2002; Edgcomb *et al.*, 2002; Stoeck *et al.*, 2003; Countway *et al.*, 2005). Many of these novel sequences formed distinct phylogenetic lineages within the stramenopiles, one of eight major eukaryotic radiations (Baldauf, 2003), and were provisionally named marine stramenopiles (MAST) (Massana

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et al., 2004). Marine stramenopiles sequences are particularly frequent in clone libraries of surface marine picoplankton, where they typically account for approximately 20% of all clones and up to 50% of sequences from uncultured groups (Massana *et al.*, 2004). In a previous study we showed that two of these lineages (MAST-3 and -4) were bacterivorous heterotrophic flagellates locally abundant in a coastal Mediterranean system (Massana *et al.*, 2002). In this report we develop and apply molecular probes to identify the ecological role of additional MAST lineages and investigate the extent of the distribution and abundance of these uncultured protists in the world's oceans. We found these lineages of MAST were widespread and abundant marine heterotrophic flagellates.

Results and discussion

A phylogenetic tree with complete 18S rDNA sequences showed that MAST formed several independent branches placed among heterotrophic stramenopiles including the oomycetes, bicosoecids and labyrinthulids (Fig. 1A). We chose to target three lineages representative of the diversity spectra of MAST (MAST-1, -2 and -4), which together accounted for one-third of MAST clones in open sea and coastal samples from the Atlantic, Pacific and Southern Oceans and the Mediterranean Sea (Massana *et al.*, 2004). Here we add genetic libraries from the Arctic and Indian Oceans and the Norwegian Sea to significantly expand the geographic coverage and the number of MAST sequences considered. Most sites surveyed yielded sequences from MAST-1 (Fig. 1B), MAST-2 (Fig. 1C) and MAST-4 (Fig. 1D), suggesting a global distribution for these uncultured organisms. Their actual abundance in the marine plankton, however, cannot be directly inferred from these polymerase chain reaction (PCR)-based analyses but require the use of other techniques such as fluorescent *in situ* hybridization (FISH) with specific oligonucleotide probes (Amann *et al.*, 1995). Probe NS4 against MAST-4 presented previously (Massana *et al.*, 2002) targeted the 36 additional MAST-4 sequences retrieved after the original publication (Fig. 1D). The second probe in that earlier study, NS3 for MAST-3, missed a significant number of new target

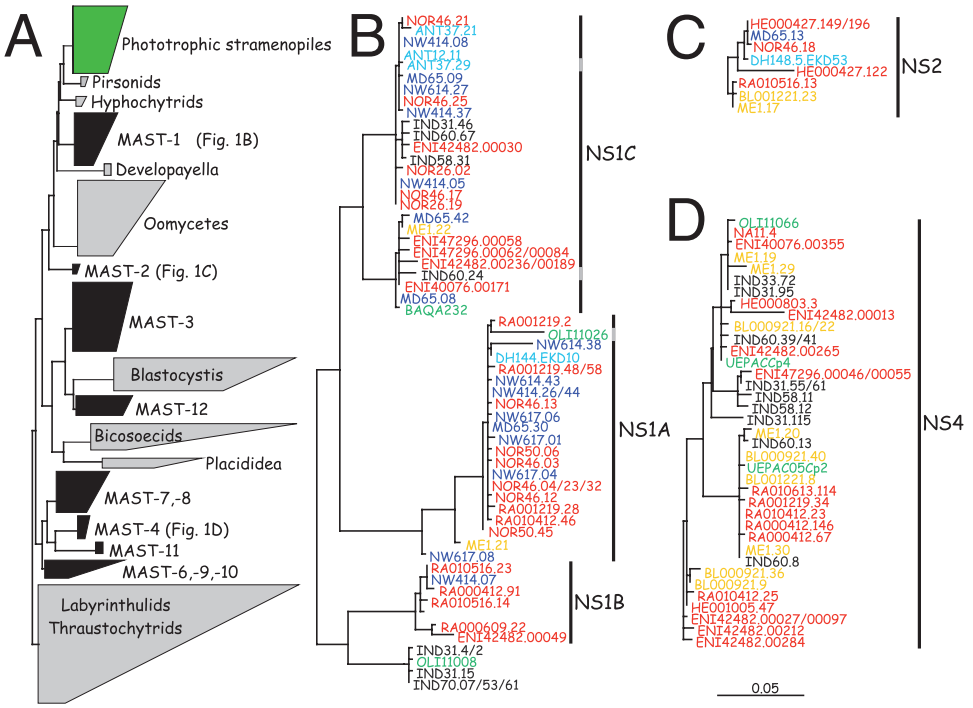

Fig. 1. Phylogeny of marine stramenopiles based on 18S rDNA sequences.
A. Tree with complete sequences [modified from the study by Massana and colleagues (2004)] showing the positions of the different MAST lineages (black boxes) among cultured phototrophic (green box) and heterotrophic (grey boxes) groups.
B. Tree with MAST-1 partial sequences.
C. Tree with MAST-2 partial sequences.
D. Tree with MAST-4 partial sequences.
In B–D, each colour identifies sequences from a different oceanic area (Arctic: dark blue; Antarctica: pale blue; Atlantic: red; Pacific: green; Indian: grey; Mediterranean: orange). Clone numbers separated by slash indicate identical sequences from the same library. Black vertical lines show the coverage of the FISH probes (mismatches are marked by a grey line). The scale bar indicates 0.05 substitutions per position in B–D.

Table 1. Characteristics of five MAST groups.

	MAST-1A	MAST-1B	MAST-1C	MAST-2	MAST-4
Morphology[a]					
Shape	Spherical	Spherical	Spherical	Spherical	Spherical[d]
Mean diameter (m)	7.7 ± 0.3	4.1 ± 0.1	5.6 ± 0.3	6.2 ± 0.6	2.3 ± 0.1
Size versus temperature[b]	NS	NS	–0.092**	–0.266*	NS
Number flagella seen	0	1	1	1	1
Size flagellum (m)	–	8.7 ± 0.6	12.0 ± 1.2	5.7 ± 0.4	3.0 ± 0.1
Trophic mode					
Growth in the dark	Yes	Yes	Yes	Yes	Yes[d]
Bacterivory	Presumed	Observed	Observed	Presumed	Observed[d]
Chloroplasts	Not seen	Not seen	Not seen	Not seen	Not seen
Distribution					
Samples found	17/24	16/24	23/24	11/24	17/24
Oceans found	All	All	All	All but INO/MED	All but polar
Temperature range	–1.6 to 28	–1.6 to 28	–1.6 to 28	–1.6 to 11	5 to 28
Abundance					
Maximal (cells ml^{-1})	86	274	201	10	569
Mean[c] (cells ml^{-1})	11	39	31	4	131
Mean[c] % of HF	1.0	2.8	2.7	0.3	9.2

a. Data from formaldehyde-fixed cells, thus prone to shrinkage or other effects.
b. Slope value and significance (*0.01 > *P* > 0.001; **0.001 > *P*) of the regression between cell diameter and sample temperature. NS, not significant.
c. Mean values calculated from samples with MAST cells. HF, heterotrophic flagellates.
d. Data already published (Massana *et al.*, 2002).
INO, Indian Ocean; MED, Mediterranean Sea.

sequences and was not used further. Here we designed four additional probes, three targeting distinct MAST-1 clades (NS1A, NS1B and NS1C, Fig. 1B) and one targeting MAST-2 sequences (NS2, Fig. 1C). A fourth MAST-1 clade formed by equatorial and Indian sequences remained untargeted (Fig. 1B). Probe sequences are: NS1A (5′-ATTACCTCGATCCGCAAA-3′; *Saccharomyces cerevisiae* positions 857–839), NS1B (5′-AACG CAAGTCTCCCCGCG-3′; 729–710), NS1C (5′-GTGTTC CCTAACCCCGAC-3′; 739–723) and NS2 (5′-CGGGTC CCGAGCACGACA-3′; 738–722). Each probe targets the vast majority of sequences within the group (only three exceptions in the 111 sequences surveyed, Fig. 1B and C), and has two (NS1A) or three (NS1B, NS1C and NS2) internal mismatches with the closest non-target sequence. All probes were experimentally validated with natural samples and non-target cultures. The variability among the sequences covered by each probe was significant, with maximal sequence divergence of 3% in the complete 18S rDNA gene.

These molecular probes were used to visualize and quantify MAST cells in marine samples using FISH. Data on cell morphology, trophic mode, distribution and abundance for the five groups are summarized in Table 1, and micrographs of these cells (MAST-1B and -1C as examples) are shown in Fig. 2. Combined FISH and nucleic acid staining with DAPI showed that MAST cells have the well-defined nucleus typical of protists (Fig. 2A and B). A combination of FISH and protein staining with FITC showed a single flagellum of one to three cell diameters in length (Fig. 2C and D) in all groups except MAST-1A. The second flagellum typical of stramenopiles was likely too short to be observed by epifluorescence as occurs in other stramenopiles (Andersen *et al.*, 1999) or, alternatively, it was lost during sample manipulation. All cells

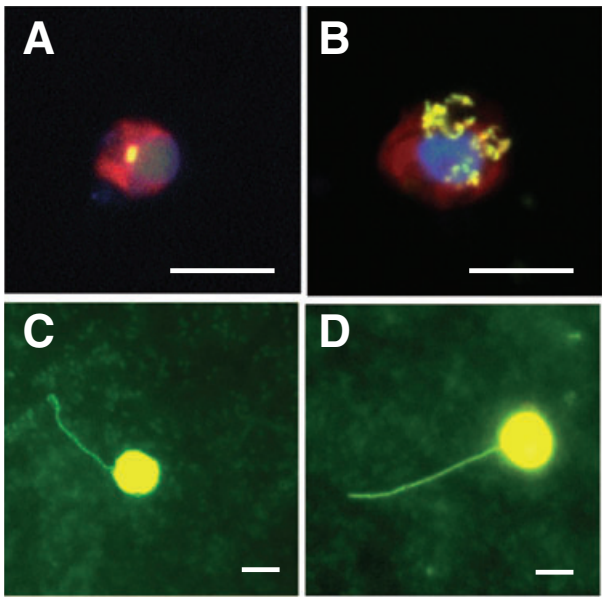

Fig. 2. Epifluorescence micrographs of MAST-1B (left panels) and MAST-1C (right panels) cells.
A and B. Picture overlays of the same cell observed under UV radiation (showing blue nucleus after DAPI staining), green light (red cytoplasm after FISH) and blue light excitation (yellow fluorescent labelled bacteria).
C and D. Flagella of MAST cells observed under blue light excitation after FITC staining. Scale bar is always 5 m.

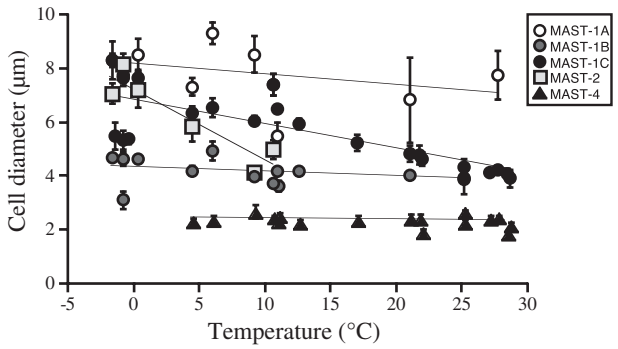

Fig. 3. Relationships of MAST cell size measured by epifluorescence after FISH (mean diameter and standard error) and seawater temperature in each sample of the marine survey. A linear regression was fitted for each of the five groups (see Table 1 for slopes and significance).

were spherical or quasi-spherical, but the cell size varied (Fig. 3): some groups showed a consistent size in all samples analysed (MAST-4 around 2 m, MAST-1B around 4 m and MAST-1A around 8 m), whereas other groups (MAST-1C and -2) followed the 'temperature-size rule' of decreasing body size with increasing temperature (Atkinson *et al.*, 2003).

The trophic mode of these uncultured MAST organisms was studied in unamended dark incubations of 3 m-filtered surface seawater. We previously reported the rapid growth of MAST-4 cells in such incubations using a coastal sample from the Mediterranean Sea (Massana *et al.*, 2002). We observed the growth of the other MAST cells (MAST-1A, -1B, -1C and -2) in similar unamended dark incubations with coastal and offshore Norwegian Sea samples (R. Massana, R. Terrado, I. Forn and C. Pedrós-Alió, submitted). During these incubations, vacuoles containing bacteria were often seen inside MAST cells. Experiments to test for bacterivory using fluorescent labelled bacteria (FLB) at the peak of MAST cells were positive (Fig. 2A and B), showing their capacity to ingest bacteria. In addition, we found no evidence of chloroplasts within MAST cells. Firstly, we did not detect the autofluorescence of chloroplasts, still visible in blue light in FISH samples. Secondly, a double FISH hybridization with a MAST probe and a bacterial probe that targets chloroplasts of many phytoplankton cells was always negative in MAST cells. We concluded that cells from these five MAST groups are bacterivorous, free-living heterotrophic flagellates.

We estimated MAST cell abundance by FISH in surface waters from different world regions (Table 2), including the five major oceans and the Mediterranean Sea. Summarized results are shown in Table 1. MAST-1 cells were detected in all samples (Fig. 4A), with a mean abundance of 63 cells ml^{-1} (range between 2 and 548 cells ml^{-1}). Within this group, MAST-1C (found in all but one sample) and MAST-1B cells dominated over MAST-1A. Cells from these three groups were particularly abundant in Antarctic samples. MAST-4 cells were present in all samples except those from Arctic and Antarctic Oceans (Fig. 4B). In non-polar samples, MAST-4 averaged 131 cells ml^{-1} (range between 8 and 569 cells ml^{-1}) and were always more abundant than MAST-1. The last group, MAST-2 (not shown in Fig. 4), had a limited distribution and abundance (maximal abundance of 10 cells ml^{-1}) but still was found in many distant samples. The origin of MAST sequences in clone libraries (Fig. 1 and Fig. 4) agreed with the patterns revealed by FISH counts: MAST-4 was found in most temperate samples and never in polar sites, MAST-1 was also widely distributed and seemed more prominent in polar sites and MAST-2 was the least represented group.

The ecological relevance of MAST becomes clear when comparing their abundances with those of heterotrophic flagellates in the same sample. These five MAST groups accounted for 10–20% of heterotrophic flagellates <5 m in most samples (13 ± 2% on average), with a maximal representation of 35% in one Antarctic sample. A single group (MAST-4) accounted for almost 10% of heterotrophic flagellates in non-polar systems. These values are significant, given the assumption of high *in situ* diversity of heterotrophic protists (Vaulot *et al.*, 2002) and the large number and disparate phylogenetic affiliations of

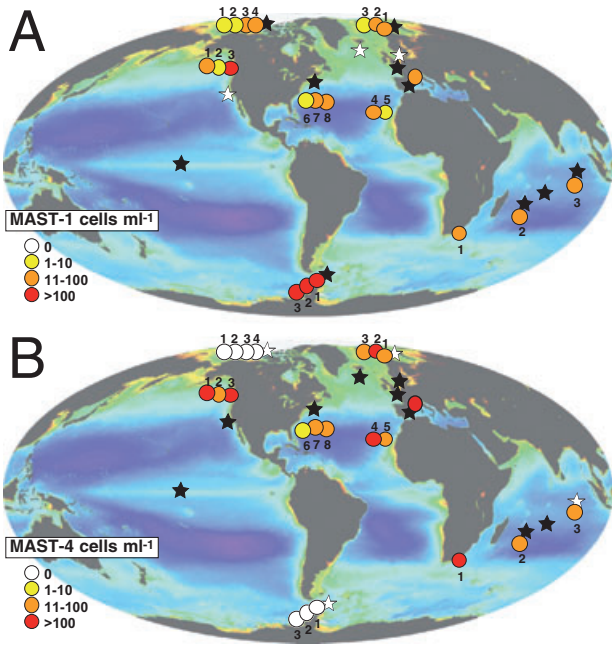

Fig. 4. Global distribution and abundance in the world oceans of MAST-1 (A) and MAST-4 cells (B). Stars indicate sites where 18S rDNA clone libraries have been constructed, black if the library contains the MAST sequence and white if it does not. Dots indicate sites where FISH counts have been performed, in different colour depending on the recorded cell abundance. Sampling details are given in Table 2.

Table 2. Station locations (dots in Fig. 4), surface seawater parameters and cell counts (cells ml^{-1}).

					DAPI counts		FISH counts						
Station	Location	Date	Temp. (C)	Chl *a* (g l^{-1})	HF	PF	MAST-1A	MAST-1B	MAST-1C	MAST-2	MAST-4	Euks	% HF
Arctic Ocean													
ARC1	74 29′N-158 01′W	4/9/02	–0.8	0.056	–	–	1	4	2	1	0	2 073	–
ARC2	76 47′N-148 58′W	27/8/02	–1.4	0.070	–	–	1	0	1	1	0	1 613	–
ARC3	70 09′N-133 31′W	3/10/02	–0.4	0.210	–	–	2	0	9	1	0	5 019	–
ARC4	70 03′N-126 17′W	6/8/04	2.8	0.147	–	–	0	12	6	0	0	1 253	–
Atlantic Ocean													
ATL1	70 30′N-20 04′E	24/8/02	12.6	0.936	652	5 346	1	4	7	2	94	21 321	16.6
ATL2	76 19′N-23 45′E	27/8/02	4.5	2.815	1 003	4 421	34	12	29	7	101	4 905	18.2
ATL3	76 20′N-03 59′E	29/8/02	6.0	0.606	584	6 658	2	1	7	2	48	5 508	10.3
ATL4	20 56′N-25 59′W	23/9/02	25.2	0.158	1 302	692	0	6	19	0	160	3 202	14.2
ATL5	21 00′N-17 50′W	30/9/02	21.8	1.441	814	676	0	4	4	0	37	2 599	5.5
ATL6	28 56′N-75 36′W	12/7/04	28.7	–	214	236	0	1	1	0	8	1 524	4.7
ATL7	30 40′N-65 15′W	15/7/04	27.1	0.041	443	240	1	0	14	0	51	1 809	14.9
ATL8	29 03′N-63 37′W	30/7/04	27.7	0.035	593	403	3	0	15	0	22	1 244	6.7
Pacific Ocean													
PAC1	44 39′N-127 06′W	9/5/02	9.2	0.414	–	–	9	24	46	10	569	20 733	–
PAC2	44 39′N-124 53′W	15/5/02	11.1	0.346	–	–	0	9	0	0	30	4 572	–
PAC3	44 39′N-124 25′W	18/5/02	10.6	2.930	–	–	3	121	24	5	496	9 478	–
Indian Ocean													
INO1	35 03′S-23 44′E	16/5/03	21.0	1.300	687	13 205	2	8	15	0	101	17 775	18.3
INO2	31 50′S-52 37′E	23/5/03	22.0	0.147	626	1 289	1	0	16	0	66	2 536	13.3
INO3	12 13′S-96 47′E	6/6/03	28.5	0.122	719	1 357	0	0	11	0	58	2 990	9.6
Southern Ocean													
ANT1	62 45′S-59 11′W	3/12/02	–0.8	1.170	1 562	2 008	86	274	188	2	0	6 183	35.2
ANT2	64 23′S-62 04′W	5/12/02	0.4	1.240	1 668	4 362	16	57	39	4	0	5 074	7.0
ANT3	66 13′S-68 18′W	11/12/02	–1.6	0.660	2 367	2 433	23	45	201	6	0	3 083	11.6
Mediterranean Sea													
MED1a	41 40′N-02 48′E	4/3/03	11.0	1.350	1 222	20 886	2	35	4	0	75	20 532	9.5
MED1b	41 40′N-02 48′E	13/5/03	17.0	0.520	1 460	6 545	1	0	44	0	102	4 910	10.1
MED1c	41 40′N-02 48′E	4/8/03	25.2	0.250	1 524	2 400	0	0	10	0	215	5 131	14.8

Heterotrophic (HF) and phototrophic (PF) flagellates (< 5 m) were counted in DAPI-stained preparations. FISH counts were performed with the MAST probes and the eukaryotic probe Euk502 (Lim *et al.*, 1999). The last column shows the percentage of HF cells represented by the five MAST groups.

cultured marine strains (Cavalier-Smith, 2002). Our study shows that MAST-1 and -4 cells represent important members of heterotrophic flagellate assemblages, but still give plenty of room for the presence of other organisms in the functional group. Previously recorded species from cultures and enrichments (Fenchel, 1986; Vørs *et al*., 1995), other MAST lineages (Massana *et al*., 2004), and perhaps novel alveolates also detected in molecular surveys (Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001) should account for the remaining heterotrophic flagellates. It is likely that MAST are yet-to-be cultured organisms, as so far they have not been reported in culture collections and do not appear in current culturing efforts that characterize the 18S rDNA of the isolates (Scheckenbach *et al*., 2005). Given their ecological relevance a major effort is required to culture MAST organisms, and their growth in unamended incubations seems promising as first stages in an isolation strategy targeting directly MAST cells using the FISH probes.

Despite the lack of some groups in a few samples, which could be because numbers were below the detection limit, our FISH counts showed that the three MAST-1 clades and MAST-4 were widely distributed in surface marine waters. This global distribution agrees with the view, based on the morphological species concept, that microbial eukaryotes have high dispersal rates and are cosmopolitan (Finlay, 2002). In fact, the cell size constancy (or significant decrease with temperature) of MAST groups in our marine survey (Fig. 3) was surprising given the large variety of systems analysed and the potential for different physiological demands of protists within each assemblage (Fenchel, 1986). This indicates that indeed each MAST group is a coherent assemblage of cells. However, there was significant genetic variability within each MAST group (*c*. 3% of the 18S rDNA), sufficient for physiological differences that may represent ecotypes with different optima for environmental factors, as reported for other picoeukaryotes (Rodríguez *et al*., 2005). In the latter study, *Ostreococcus* strains differing 2% in their 18S rDNA represented ecotypes adapted to the light regimes at different water depths, just as described for *Prochlorococcus* (Moore *et al*., 1998). Marine stramenopiles ecotypes, if they exist, could be adapted to different temperatures. Therefore, we argue for a global distribution of MAST-1 and MAST-4 as a group, the later excluded from very cold environments, and a significant genetic diversity within each group, perhaps including different ecotypes. The real ecological significance of this intra-group diversity and whether or not genetically identical organisms have cosmopolitan distributions are open issues for future research.

We have demonstrated that some novel sequences recovered from recent molecular surveys correspond to abundant and globally distributed marine heterotrophic flagellates. These uncultured protists could be more abundant than some typical marine organisms such as the choanoflagellates (Vørs *et al*., 1995). Current views of marine food webs assume that heterotrophic flagellates, together with viruses, keep prokaryotic assemblages relatively stable by cropping prokaryotic production (Pernthaler, 2005), and it is surprising how little we know about the phylogenetic and functional diversity of these grazers. Each MAST group represents a different cell type with idiosyncratic patterns of distribution and activity and will probably collaborate in the fine-tuning of the complex interactions within the microbial food web. The identification of MAST-4 parallels the discovery of previously unrecognized and abundant marine prokaryotes such as the SAR11 clade (Morris *et al*., 2002) or the marine crenarchaeota (Karner *et al*., 2001), and is the first report for an equivalently significant picoeukaryotic group. Considering its relative abundance, MAST-4 cells alone could be responsible for 10% of bacterivory and nutrient re-mineralization in vast areas of the upper ocean.

Experimental procedures

Phylogenetic analyses

18S rDNA sequences of MAST derive from previous articles (Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001; Dawson and Pace, 2002; Massana *et al*., 2004; Countway *et al*., 2005), from GenBank (UEPAC clones) and from 12 additional genetic libraries presented here. Arctic libraries were from samples collected at station ARC2 (Table 2) at 50 m (NW414), station ARC3 at surface (MD65) and station 75 59′N, 156 53′W at surface (NW617) and 50 m (NW614). Norwegian Sea libraries were from samples collected at station ATL2 at surface (NOR26) and station ATL3 at surface (NOR46) and 60 m (NOR50). Indian libraries were from samples collected at station INO2 at surface (IND31) and 74 m (IND33), station INO3 at surface (IND70) and station 17 11′S, 83 41′E at surface (IND58) and 85 m (IND60). Picoplanktonic biomass (between 0.2 and 3 m) was collected on filters and community DNA was extracted for all these samples. Complete 18S rDNA genes were PCR-amplified with the eukaryotic-specific primers EukA and EukB and the PCR products were cloned. Between 50 and 100 clones were partially sequenced with primer 528f in each library. Details of filtering set-up, DNA extraction protocol, primers used and PCR and cloning conditions are described elsewhere (Díez *et al*., 2001; Massana *et al*., 2004).

Partial sequences from MAST-1, MAST-2 and MAST-4 were independently aligned using CLUSTALW 1.82 (Thompson *et al*., 1994) using a close relative as outgroup (later removed from the final tree). Highly variable regions of the alignment were removed using Gblocks (Castresana, 2000) leaving more than 500 bp informative positions. Maximum likelihood analysis was carried out with PAUP 4.0b10 (Swofford, 2002), with the general time-reversible model assuming a discrete gamma distribution with six rate categories and a proportion of invariable sites. Parameters were estimated from an initial neighbour-joining tree. Sequence data have been

submitted to the GenBank database under accession numbers DQ029011–DQ029023, DQ062469–DQ062486, DQ121419–DQ121431, DQ234593–DQ234596 and DQ337349–DQ337361.

Probe design and FISH

Probes were designed with the Probe_Design option of the ARB program package (http://www.arb-home.de). They were very robust *in silico*, matching almost all target sequences and having at least two to three internal mismatches with all non-target sequences. Probes were supplied labelled with a CY3 fluorophore at the 5′ end and were applied for FISH as explained before (Massana *et al*., 2002). They were tested against seawater incubations of heterotrophic flagellates from the Norwegian Sea (R. Massana, R. Terrado, I. Forn and C. Pedrós-Alió, submitted) in a gradient of formamide (0–50%) in the hybridization buffer and constant temperature (46 C). There were no differences in the hybridization signal and 30% formamide was used for routine counting. Probes were tested against non-target cultures of a prasinophyte, a prymnesiophyte, a cryptophyte, an eustigmatophyte, a chrysophyte and a bicosoecid [as listed in the study by Massana and colleagues (2002)], and against typical heterotrophic flagellates affiliating within the stramenopiles: the bicosoecid *Cafeteria roenbergensis* (two strains) and the chrysophytes *Paraphysomonas* (one strain) and *Spumella* (three strains). Cells from all these non-target cultures did not hybridize with our probes.

Marine survey

Surface samples were taken during several cruises between 2002 and 2004 in different oceanographic regions (Table 2). In general, seawater was collected with Niskin bottles and 100–200 ml was fixed with formaldehyde (3.7% final concentration). Fixed samples were kept at 4 C for 1–24 h, filtered on 0.6 m polycarbonate filters, and stored at −80 C until processed. Filter-immobilized protists were hybridized with CY3-probes, counter-stained with DAPI, and observed by epifluorescence microscopy under UV (DAPI staining) and green light excitation (FISH signal). Duplicate FISH counts were made on separate portions of the filters and mean values were calculated. A minimum of 6 mm^2 of the filter was inspected for each count. Additional variables for each sample included temperature, chlorophyll *a* concentration and epifluorescence counts of DAPI-stained heterotrophic and phototrophic flagellates.

Trophic mode determinations

The growth dynamics of MAST cells was studied in unamended incubations that promoted the development of heterotrophic flagellates. These were prepared by gently filtering seawater through a 3 m pore size polycarbonate filter and incubating the filtrate at *in situ* temperature in the dark (Massana *et al*., 2002). Incubations performed in the Norwegian Sea (R. Massana, R. Terrado, I. Forn and C. Pedrós-Alió, submitted) showed that MAST cells from the five groups analysed here grew in the dark paralleling the dynamics of typical heterotrophic flagellates. To study the bacterivory of MAST cells, FLB uptake experiments (Sherr *et al*., 1987) were performed using heat-killed, DTAF-stained *Brevundimonas diminuta* (syn. *Pseudomonas diminuta*) cells (biovolume 0.065 m^3). Fluorescent labelled bacteria were added at concentrations typical of bacteria (5×10^5 FLB ml^{-1}) to an heterotrophic flagellate seawater incubation from Blanes Bay and after 40 min a subsample was processed for FISH. The presence of FLB inside MAST cells (identified under green light excitation) was observed under blue light excitation. Assays to hybridize simultaneously MAST cells and chloroplasts were performed by mixing a MAST probe and a bacterial specific probe with a horseradish peroxidase (EUB338-HRP). After the hybridization, the bacterial signal was amplified with tyramide labelled with $Alexa_{488}$ following the CARD-FISH protocol (Pernthaler *et al*., 2002). Chloroplasts from diatoms, cryptophytes, pelagophytes and prasinophytes in culture were clearly seen, but not all phototrophic cells in environmental samples gave a clear chloroplast signal.

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