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ORIGINAL ARTICLE

Taming the smallest predators of the oceans

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Protists (unicellular eukaryotes) arguably account for most eukaryotic diversity and are central players of the biosphere. Known protist diversity and biology is largely based on cultured strains. Yet, environmental molecular surveys have unveiled entirely novel lineages that, as their prokaryotic counterparts, are essentially uncultured. Culture bias is an important drawback for any microbe-related science and is particularly severe for heterotrophic protists, which depend on organic food sources for growth. Here, we show how ecologically significant bacterivorous protists have been brought into culture by mimicking *in situ* conditions. Single cells sorted by serial dilution or flow cytometry were inoculated into seawater amended with natural bacterial assemblage at nearly *in situ* abundances. Strains belonging to lineages only known so far from environmental sequencing were isolated. Among them, *Minorisa minuta* gen. nov. sp. nov. forms a novel branch within Rhizaria, holding a key evolutionary position, and with an average size of 1.4 μm represents one of the smallest bacterial grazers known to date. It has a worldwide planktonic distribution and can account for 5% of heterotrophic protists communities in coastal waters. Physiological features of this strain can partly explain its success in the environment. Culturing ecologically relevant but elusive protists provide invaluable material for ecophysiology, genomics, ecosystem modeling and evolutionary issues.

Introduction

Unicellular organisms are major forces driving our planet ecosystems and are an outstanding reservoir of biological diversity (genes, molecules, metabolic pathways and cellular processes) yet to be discovered (Falkowski *et al.*, 2008). They are also main actors in macro- and microevolutionary processes for life on Earth (Cavalier-Smith and Chao, 2006). Nowadays, culture independent approaches are regularly applied to investigate the diversity and function of microbes in the environment. Despite the valuable information provided by 'omics' environmental studies, culture bias definitely remains as one of the most critical challenges faced by scientists aiming to achieve a full understanding of the ecological role of microbes (Giovannoni and Stingl, 2007) and is currently a bottleneck in ecosystem studies (*Giovannoni et al.*, 2007; Raes and Bork, 2008). Environmental DNA surveys demonstrate the extent to which culturing efforts

poorly capture *in situ* microbial diversity (Pedrós-Alió, 2006). Many lineages unveiled in the last few years and holding key phylogenetic positions to understand macroevolutionary patterns among eukaryotes are essentially composed of environmental sequences (Epstein and López-García, 2008). It is estimated that as little as 0.1% to 1% of bacterial and protist cells can be easily cultured (Caron *et al.*, 1989; Amann *et al.*, 1995). Unfortunately, some of the most highly represented taxa in the environment have not been cultured yet and some of those in culture do not appear to be among the most abundant (Massana *et al.*, 2004; Sherr *et al.*, 2007). Consequently, ultrastructural, physiological and genomic information for many ecologically relevant microorganisms is missing.

This culture bias can be overcome by using original culturing strategies (Button *et al.*, 1993), as demonstrated for prokaryotes like *Candidatus* Pelagibacter ubique and *Nitrosopumilus marinus* (Rappé *et al.*, 2002; Könneke *et al.*, 2005), both initially detected through environmental molecular surveys and later identified as ecologically relevant taxa. *Ca.* P. ubique was brought into culture by mimicking oligotrophic conditions and *Nitrosopumilus marinus* were cultured in media amended with ammonia once molecular data revealed they were ammonia oxidizers. Similar culturing efforts

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have seldom been applied to marine protists, even though culture bias is perceived as a major limitation to investigate further the functional role and ecological significance of photosynthetic protists (Vaulot et al., 2008), being even more severe for the heterotrophic ones (Jürgens and Massana, 2008).

Marine heterotrophic flagellates perform key processes in microbial food webs as bacterial grazers, trophic linkers and nutrient remineralizers (Sherr and Sherr, 2002; Pernthaler, 2005). They exhibit a variety of trophic strategies and constitute a diverse assemblage of poorly identified species (Arndt et al., 2000; Vaulot et al., 2002). Up to now, bacterivorous flagellates have been invariably cultured using rich media composed of seawater supplemented with rice grains or yeast extract that promote the growth of very large bacteria at very-high densities. This strategy inexorably retrieves the same species complexes (that is, Cafeteria, Paraphysomonas, or Neobodo spp.) that are known to be rare in marine plankton (Jürgens and Massana, 2008). Abundant taxa identified by molecular surveys still remain uncultured, for example the bacterivorous MAST clades (Massana et al., 2006a).

In order to bring into culture ecologically relevant heterotrophic flagellates, we mimicked oligotrophic marine conditions by amending sterile seawater with a mix of natural bacteria collected from the same sampling site at abundances only slightly higher than in situ. To this media we added a single heterotrophic flagellate cell retrieved by dilution or by flow cytometry. This approach enabled us to obtain several interesting pre-cultures. Some of them were stabilized and maintained for months as stable cultures allowing its phylogenetic, morphological and ecological characterization.

Materials and methods

Isolation by single-cell dilution
Samples were collected at the Blanes Bay Microbial Observatory on the 25th of September 2007. Forty liters were filtered by gravity through 3 μm and incubated at 20 °C in the dark for 2 days. Then, this incubated sample was filtered by 0.45 μm and bacteria in the filtrate were concentrated by tangential flow filtration. Bacterial concentrates were quantified by epifluorescence microscopy using 4',6-diamidino-2-phenylindole (DAPI) staining (Porter et al., 1980) (typically ca 10^8 cells ml^{-1}) and kept frozen. Multi-well plates of 0.1 or 1 ml were filled with aged seawater (3 μm filtered seawater kept in the dark for 2 months, 0.2 μm filtered and autoclaved before use), and an aliquot of the concentrate was added to attain a final concentration of 5×10^6 bacteria ml^{-1}. Heterotrophic flagellates from the same 2-days incubated sample were counted by epifluorescence microscopy after DAPI staining and diluted to add one cell per well. Plates were incubated at 20 °C in the dark and inspected for flagellate growth by inverted microscopy every 3 days during a period of 3 weeks. Wells with observed growth were scaled to 30 ml to establish a pre-culture and later a stable culture.

Isolation by single-cell sorting
Seawater from a second sampling (30th of September 2008) was filtered through 3 μm, and sent to Bigelow Laboratory for Ocean Sciences (Boothbay Harbor, ME, USA) for cell sorting in a MoFlo Flow Cytometer (Dako-Cytomation, Glostrup, Denmark). Digestive vacuoles of heterotrophic protists were stained using the vital stain LysoTracker (Life Technologies, New York, NY, USA) and cells were sorted based on their green fluorescence (Lyso-Tracker fluorescence) and the absence of chlorophyll fluorescence. Details of the staining protocol and flow cytometer setup are described elsewhere (Rose et al., 2004; Heywood et al., 2011). Side scatter was used to select only the smallest protists, approximately < 10 μm in diameter. Individual target cells were deposited into 24-well plates, in which some wells were dedicated for positive controls (10 cells per well) and negative controls (0 cells per well). All wells on the microplates contained 1-ml aged seawater, together with natural bacteria as before. Multi-well plates were sent back to the Institut de Ciències del Mar (Barcelona, Catalonia, Spain) by plane at room temperature (12 h) and treated as above.

Culture maintenance
Cultures were maintained in 50 ml flasks and transferred every 2 to 4 weeks to fresh media (aged seawater with added bacteria as described above) at 1/10 dilution. The full culturing protocol is outlined in Supplementary Figure 1. Physiological parameters were inferred from a batch culture where the abundance of flagellates and bacteria were followed twice a day by epifluorescence microscopy after DAPI staining. Growth rate was calculated from the exponential increase of flagellate cells. Grazing rate was calculated using the exponential decrease of bacterial cells and the average concentration of flagellates and bacteria during the incubation (calculated according to Frost, 1972). Growth efficiency was calculated from the ratio of protist biomass produced as compared with bacterial biomass consumed.

Sequencing and phylogenetic analysis
For molecular analysis, the whole culture was filtered on 0.6 μm polycarbonate filters, DNA was extracted by standard procedures, and 18S rDNA genes were PCR amplified with eukaryote-specific primers (Díez et al., 2001). Complete sequences of 18S rDNA were obtained with five internal primers by the MACROGEN Genomics Sequencing Services (accession numbers JX272635-JX272636). Sequences

were identified and inspected for chimeras by BLAST and KeyDNATools (http://www.keydnatools.com) and were aligned with a subset of relative sequences using MAFFT (Katoh et al., 2002). Alignments were checked with Seaview 3.2 (Galtier et al., 1996) and highly variable regions of the alignment were removed using Gblocks (Castresana, 2000). Maximum likelihood (ML) phylogenetic trees were constructed with the fast ML method RAxML (Stamatakis, 2006) using the evolutionary model GTRMIXI (which conducted inference under GRTCAT and calculated best tree under GTRGAMMA, but with estimate of proportion of invariable sites). Phylogenetic analyses were done in the freely available University of Oslo Bioportal (www.bioportal.uio.no). Repeated runs on distinct starting trees were carried out to select the tree with the best topology (the one having the best Likelihood of 1000 alternative trees). Bootstrap ML analysis was done with 1000 pseudo-replicates and the consensus tree was computed with MrBayes (Huelsenbeck and Ronquist, 2001).

Scanning electron microscopy (SEM)
SEM was performed as in Garcés et al. (2006). Samples were fixed for 2 h in 2% OsO_4 diluted in seawater, or with 2% glutaraldehyde. Cells were subsequently washed with distilled water (2 h) and filtered onto a 0.6 μm pore size Nuclepore filter (Whatman, Maidstone, UK). Samples were dehydrated in an ethanol series (30%, 50%, 70%, 96 and 100%) for 15 min each, followed by an acetone series (25%, 50%, 75% and 100%) for 15 min each. Samples were critical point-dried in liquid CO_2 using a BAL-TEC CPD 030 critical point drying apparatus (Balzers Union, Balzers, Germany). Filters were subsequently glued to SEM-stubs with colloidal silver, sputter coated with gold palladium, and examined with a Hitachi S-3500 N (Nissei Sangyo Co. Ltd., Tokyo, Japan) SEM operating at 5 kV.

Tyramide signal amplification-fluorescent in situ hybridization (TSA-FISH) analysis)
Samples were fixed with formaldehyde (2% final concentration), filtered on 0.6 μm pore size Nucleopore filters and kept frozen. The probe CRN02 (5′-TACTTAGCTCTCAGAACC-3′) was designed with the ARB package (Ludwig et al., 2004) to have no mismatches with the target sequences (coverage in Figure 1a) and at least two mismatches with nontarget sequences. The probe was purchased with a 5′-aminolink (C6) from Thermo Fisher Scientific (Waltham, MA, USA) and was labeled with the enzyme horseradish peroxidase, HRP (Roche Diagnostic Boehringer, Basel, Switzerland) as described elsewhere (Urdea et al., 1988; Amann et al., 1992). The hybridization was carried out on filter pieces using the temperatures and conditions

described in Not et al. (2002). The signal amplification was done using a tyramide (as the substrate of HRP) labeled with Alexa 488 as in Pernthaler et al. (2004). The probe was tested against the positive culture in a gradient of formamide (10% to 70%) in the hybridization buffer. Optimized conditions were 35 °C and 30% formamide in the buffer. The probe gave a negative signal with several nontarget cultures. Filters were counterstained with DAPI, mounted in a slide with an oil mix containing Vecta Shield, and observed by epifluorescence microscopy at 1000 × under ultraviolet (DAPI signal) and blue light excitation (TSA-FISH signal). Two transects (of around 15 mm) were inspected per filter piece. Counts for Blanes samples were done triplicated (s.e. were typically 20% of the mean), whereas no replicates were done for the worldwide and culture samples. The minimal abundance quantified by this counting strategy was around one cell ml^{-1}.

Results

Pre-cultures based on serial dilution yielded a 5.2% success rate (growth observed in 25 of the 480 inoculated wells). Out of the 25 pre-cultures, 12 were stable and were scaled to 30 ml volumes. Based on their 18 S rDNA, four pre-cultures were identified as *Paraphysomonas* spp. and the others represented taxa closer to environmental sequences than to any known culture, including a cercozoan, a rhizarian (two pre-cultures), a choanoflagellate, a chrysophyte, two unclassified stramenopiles, and a MAST (Table 1). After a second step of single cell inoculation from the latter eight flasks, some pre-cultures were lost and others were substituted by a different cell type. The two resulting unieukaryote clonal and stable cultures included a rhizaria related to chlorarachniophytes (Figure 1a) and a stramenopile distantly related to *Developayella elegans* (Supplementary Figure 2). Single-cell sorting by flow cytometry was carried out to avoid time-consuming serial dilution steps. From the 400 wells inoculated using single-cell sorting, growth was detected in five wells (1.25% success rate). All of them were identified as the same rhizarian lineage obtained by the dilution method. SEM performed on the two stable cultures revealed extremely small cells with little morphological features (Figure 1b and Supplementary Figure 3). Based on these SEM images we observed that rhizarian cells (40 cells measured) had one flagellum and measured 0.8–2 μm in width (mean of 1.3 μm, s.d. of 0.4) and 1.0–2.1 μm in length (mean of 1.5 μm, s.d. = 0.4), whereas *Developayella*-like cells (13 cells measured) had two flagella (one with hairs) and measured 1.2–3 μm in width (mean of 2.1 μm, s.d. = 0.6) and 1.5–3.0 μm in length (mean of 2.3 μm, s.d. = 0.5).

The rhizarian isolate was distant to any described organism, its 18 S rDNA sequence being only 90.6% similar to *Chlorarachnion reptans*. The 18 S

Table 1 Taxonomic affiliation of the obtained pre-cultures and stable pure cultures based on blast similarity (March 2012)

	Closest cultured match	%	Lineage	Closest match	%
Pre-cultures					
Rhizaria					
IE1.2.D2	*Chlorarachnion reptans*	90.5	Chlorarachniophyte	SSRPE06	99.8
IE1.4.B4	*Chlorarachnion reptans*	90.9	Chlorarachniophyte	SSRPE06	99.9
IE4.4.A1[a]	Cercozoa sp. CC-2009d	94.4	Cercozoan	SA2.4G8	96.8
Opisthokonta					
IE1.4.D1	*Stephanoeca cauliculata*	91.6	Choanoflagellate	Band 4DB38	97.7
Stramenopiles					
IE1.1.A5	*Paraphysomonas foraminifera*	99.4	Chrysophyte		
IE1.3.B3	*Paraphysomonas imperforate*	99.4	Chrysophyte		
IE1.3.B6	*Developayella elegans*	94.4	Stramenopile	RM2-SGM49	96.6
IE1.4.D5[b]	*Pseudobodo tremulans*	95.7	Bicosoecida	Biosope_T123.046	95.7
IE2.4.A6[a]	Chrysophyceae sp. CCMP2296	91.5	Chrysophyte	CD8.S17	98.8
IE3.4.B1	*Bolidomonas pacifica*	88.3	MAST-3	NIF.1E11	97.0
IE4.6.B5	*Paraphysomonas imperforate*	98.7	Chrysophyte		
IE4.8.C6	*Paraphysomonas imperforate*	99.9	Chrysophyte		
Stable cultures					
Rhizaria					
Minorisa minuta[c]	*Chlorarachnion reptans*	99.6	Chlorarachniophyte	SSRPE06	99.6
Stramenopiles					
Developayella-like	*Developayella elegans*	94.4	Stramenopile	RM2-SGM49	96.6

[a]Sequences with ambiguities.
[b]Lost after 1 year of being stable.
[c]Culture isolated by Flow Cytometry.
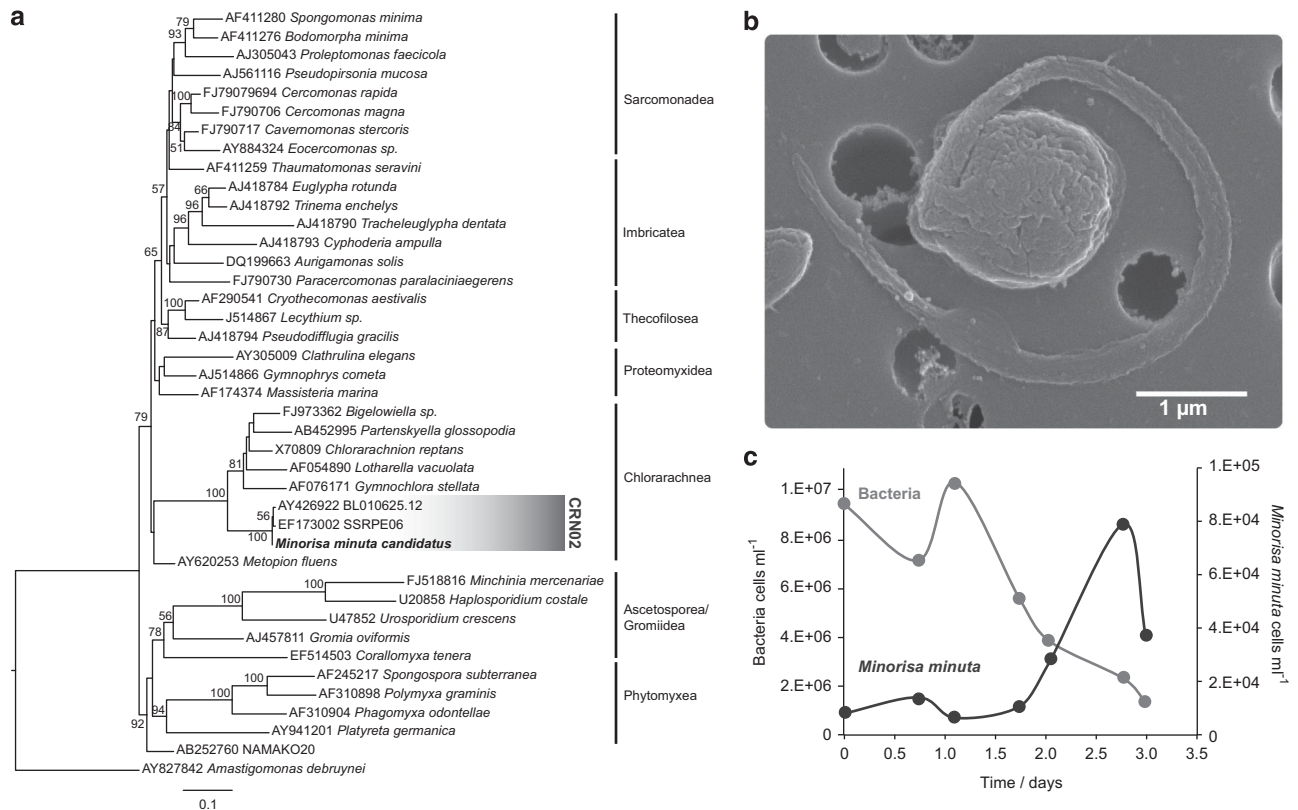
Figure 1 (**a**) ML phylogenetic tree with complete 18S rDNA sequences showing the position of *Minorisa minuta* within the Cercozoa. The scale bar indicates 0.1 substitutions per position. The coverage of the specific probe CRN02 is shown in grey. (**b**) SEM image of a *Minorisa minuta* cell of 1.6 μm in size and possessing a single flagellum. (**c**) Growth of *Minorisa minuta* with natural bacteria as prey in a batch culture.
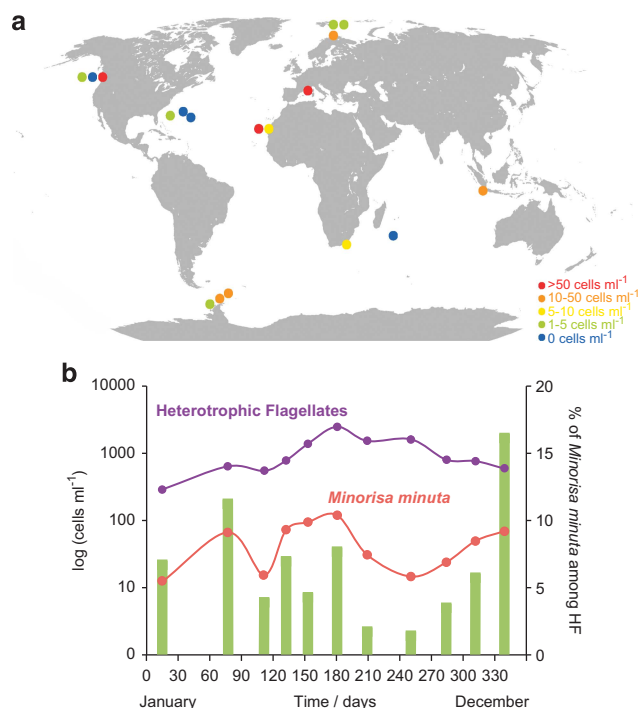
Figure 2 (**a**) Abundances of *Minorisa minuta* cells at various sampling sites as estimated by TSA-FISH counts. (**b**) Abundances of *Minorisa minuta* cells during a temporal study at Blanes Bay (year 2007), together with the abundance of heterotrophic flagellates, estimated by epifluorescence (log scale). Bars represent the contribution (in %) of *Minorisa minuta* cells to heterotrophic flagellates.

Table 2 Counts of *Minorisa minuta* (cells ml^{-1}) in a set of samples from diverse oceans and comparison with the counts of heterotrophic flagellates (cells ml^{-1}). Data of heterotrophic flagellates and codes of samples are from Massana *et al.* (2006a)

System	Date	*Minorisa minuta*	*Heterotrophic flagellates*	*%-Minorisa minuta*
Atlantic ocean				
ATL1	24-Aug-02	4	652	0.7
ATL2	27-Aug-02	9	1003	0.9
ATL3	29-Aug-02	9	584	1.6
ATL4	23-Sep-02	54	1302	4.1
ATL5	30-Sep-02	13	814	1.6
ATL6	12-Jul-04	9	214	4.1
ATL7	15-Jul-04	0	443	0.0
ATL8	30-Jul-04	0	593	0.0
Pacific ocean				
PAC1	09-May-02	12	–	–
PAC2	15-May-02	0	–	–
PAC3	18-May-02	60	–	–
Indian ocean				
INO1	16-May-03	33	687	4.9
INO2	23-May-03	0	626	0.0
INO3	06-Jun-03	5	719	0.7
Southern ocean				
ANT1	03-Dec-02	7	1562	0.4
ANT2	05-Dec-02	4	1668	0.3
ANT3	11-Dec-02	2	2367	0.1
Mediterranean sea				
BL07	2007[a]	52	1039	5.0

[a]Average of a whole-year sampling.

rDNA sequence of our isolate was highly similar to environmental sequences retrieved from the Mediterranean Sea (BL000921.31 and BL010625.12; Massana *et al.*, 2004), the Sargasso Sea (SSRPE06; Not *et al.*, 2007), and the English Channel (RA070625T.047; Marie *et al.*, 2010). Therefore, we decided to focus in the rhizarian isolate for the rest of our study, given its taxonomic novelty and its similarity with environmental marine sequences. This small heterotrophic flagellate has been named *Minorisa minuta* gen. nov. sp. nov. (see formal description at the end of this section).

Following *Minorisa minuta* abundance and that of the bacterial prey for several days in a batch culture has assessed its physiological properties. This flagellate grew relatively fast on natural bacteria (doubling time of 10.6 h) and reduced bacterial abundances from 10^7 cells ml^{-1} to around 10^6 cells ml^{-1} (Figure 1c). The estimated growth efficiency (bacterial biomass converted to protist biomass) of this protist in this batch culture was 30%, and its grazing rate was seven bacteria flagellate^{-1} h^{-1}.

Minorisa minuta was a significant component of marine heterotrophic flagellates on a global scale, being both widely distributed and abundant (Figure 2a). TSA-FISH counts using a newly designed specific probe revealed abundances up to 60 cells ml^{-1} (17 cells ml^{-1} on average) in the Atlantic, Pacific, Indian and Southern Oceans and the Mediterranean Sea. It accounted for 1.8% of heterotrophic flagellates in these samples, a value that increased to 5% when considering coastal sites only (Table 2). *Minorisa minuta* was detected all year long in a coastal oligotrophic station in the NW Mediterranean Sea (Figure 2b), ranging from 12 to 120 cells ml^{-1} (52 cells ml^{-1} on average) and accounting for 1–12% of heterotrophic flagellates (5% on average). In this site, its abundance was well correlated with bacterial cells numbers (Figure 3a). Sizing *Minorisa minuta* cells in natural marine assemblages using TSA-FISH observations confirmed its picoeukaryotic character with cell size varying from 1 to 3 μm (Figure 3b).

Minorisa minuta J. del Campo gen. nov. sp. nov.
Naked and spherical or ovoid cells: 0.8–2 μm (mean = 1.3) in width and 1.0–2.1 μm (mean = 1.5) in length. They possess a single flagella that could be as long as four times the cell length. They are swimmers and active bacterial grazers.

Holotype: Figure 1b from culture BMO.6.C1, isolated from Blanes Bay Microbial Observatory (NW Mediterranean Sea) and maintained at the Institut de Ciències del Mar (Barcelona). Scanning microscopy samples and FISH samples are also preserved there. 18S rDNA sequence deposited in GenBank under accession number: JX272635.

Etymology: Latin Minorisa, Manresa, author′s born town. Latin minuta, tiny, refers to the small size of the organisms.
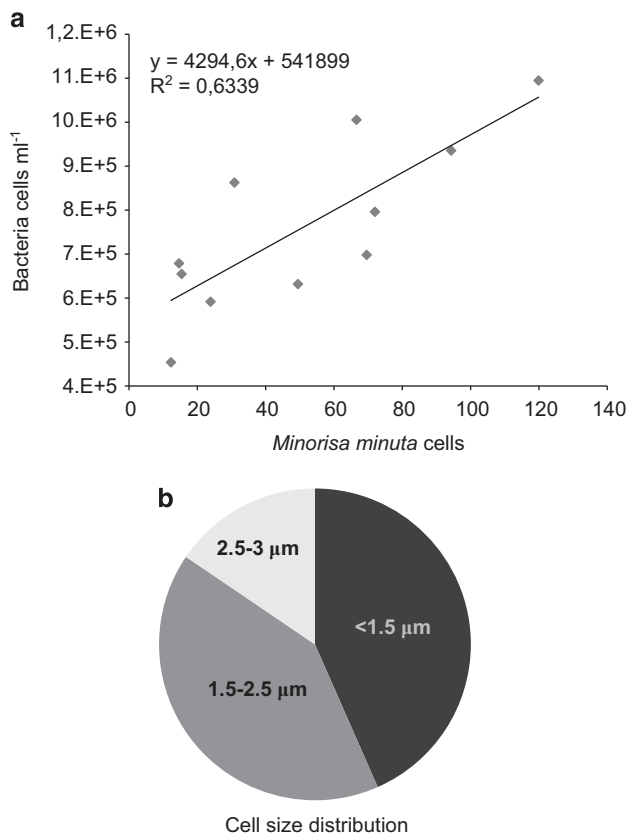
Type locality: Blanes Bay (Catalonia, Spain).

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a

$y = 4294{,}6x + 541899$
$R^2 = 0{,}6339$

b

Cell size distribution

Figure 3 (**a**) Correlation between the abundances of *Minorisa minuta* and natural bacteria during the seasonal study (year 2007) at Blanes Bay. (**b**) Cell size distribution of *Minorisa minuta* in these samples (1148 cells measured by TSA-FISH).

Habitat and ecology: In Blanes Bay, the organism was detected in coastal plankton all year long. It has been also detected in different world oceans, but the maximum abundances have always been observed in coastal areas.

Discussion

Using the culturing approach developed here, we isolated several small heterotrophic flagellates belonging to previously uncultured taxa and from distant lineages within the eukaryotic tree of life (Table 1). When applied at different temporal and spatial scales, this strategy will potentially give access to a wealth of novel heterotrophic protists in culture. This is important because the presence of heterotrophic flagellates in culture collections is scarce (Gachon *et al.*, 2007), and among the ones present in culture collections there are few with a significant representation in the environment (Lim *et al.*, 1999).

The tiny uniflagellated *Minorisa minuta* stands up as one of the smallest bacterivore known so far, together with the minuscule phagotrophic bicosoecid *Symbiomonas scintillans* isolated from open oligotrophic waters (Guillou *et al.*, 1999). Moreover, it represents the only heterotrophic represen-

tative within the chlorarachniophytes, the single photosynthetic lineage within rhizaria. Chlorarach-nyophytes are marine amoeboflagellated cercozoans (Cavalier-Smith and Chao, 2003) that together with the Euglenophytes represent the two unrelated eukaryotic groups that acquired a chloroplast by secondary endosymbiosis with a green alga (Delwiche, 1999). The discovery of this unpigmen-ted protist could be another evidence indicating that the acquisition of the green chloroplast took place independently in both lineages (Rogers *et al.*, 2007). Nevertheless, this is not a definitive clue as the absence of chloroplast could also derive from a posterior loss of it. Therefore, the genome analysis of *Minorisa minuta* is of primary interest to study the transition to secondary plastid endosymbiosis and, as for its photosynthetic counterparts in the oceans, is expected to reveal unprecedented cellular, bio-chemical and evolutionary pathways (Worden *et al.*, 2009).

We have shown here that *Minorisa minuta* has a worldwide marine distribution and is a significant member of heterotrophic flagellate assemblages, particularly in coastal waters. In the same set of samples considered for the present study, *Minorisa minuta* followed in abundance the uncultured MAST-4, MAST-1C and MAST-1B groups (Massana *et al.*, 2006a), which is remarkable given that its probe is clearly more specific than MAST probes (roughly species versus family level). Our data suggest that the importance of this organism in coastal waters is comparable to the importance of MAST in open waters, considered as abundant bacterivores in the sea. *Minorisa minuta* represents one of the main players in the eukaryotic picoplank-ton of coastal ecosystems, possibly having a relevant role in the carbon fluxes and in controlling bacterial populations. Nevertheless, many more samples need to be processed in order to verify the patterns of its abundance, distribution and coastal prefer-ence. Its growth rate is double of that observed in MAST in unamended incubations (Massana *et al.*, 2006b), and similar to or lower than maximal growth rates observed for other cultured heterotrophic flagellates (Eccleston-Parry and Leadbeater, 1994, Boenigk *et al.*, 2007). The estimated growth effi-ciency and its grazing rate are again within the range of known cultured strains. The functional res-ponse of *Minuta minuta* exhibits a half-saturation constant much lower than that of other cultured flagellates, below 10^6 cells ml^{-1} (Rodríguez-Martí-nez in preparation). This suggests that this grazer is adapted to live at the usual bacterioplankton concentrations in marine waters. The physiological properties of *Minorisa minuta* can explain its ecological success and set this species as a good example of dominant marine heterotrophic flagel-lates (Montagnes *et al.*, 2012), whose parameters could be used to improve ecological models (Caron *et al.*, 2009). New experiments must be performed in order to determine its functional behavior and the

degree of its influence over the coastal microbial community.

This is the first time that a widely distributed and abundant heterotrophic flagellate has been obtained in culture. Getting the environmentally relevant bacteria *Ca.* P. ubique in culture led to a leap forward toward a better understating of microbes′ function in the oceans and opened up several research directions (Tripp *et al.*, 2008). Taming small marine predators with ecological relevance holds promise for similar future discoveries.

Acknowledgements

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