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## ORIGINAL ARTICLE

# Diversity and oceanic distribution of the Parmales (Bolidophyceae), a picoplanktonic group closely related to diatoms

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*Bolidomonas* is a genus of picoplanktonic flagellated algae that is closely related to diatoms. *Triparma laevis*, a species belonging to the Parmales, which are small cells with a siliceous covering, has been shown to form a monophyletic group with *Bolidomonas*. We isolated several novel strains of Bolidophyceae that have permitted further exploration of the diversity of this group using nuclear, plastidial and mitochondrial genes. The resulting phylogenetic data led us to formally emend the taxonomy of this group to include the Parmales within the Bolidophyceae, to combine *Bolidomonas* within *Triparma* and to define a novel species, *Triparma eleuthera* sp. nov. The global distribution of Bolidophyceae was then assessed using environmental sequences available in public databases, as well as a large 18S rRNA V9 metabarcode data set from the Tara Oceans expedition. Bolidophyceans appear ubiquitous throughout the sampled oceans but always constitute a minor component of the phytoplankton community, corresponding to at most ~4% of the metabarcodes from photosynthetic groups (excluding dinoflagellates). They are ~10 times more abundant in the small size fraction (0.8–5 µm) than in larger size fractions. *T. eleuthera* sp. nov. constitutes the most abundant and most widespread operational taxonomic unit (OTU) followed by *T. pacifica*, *T. mediterranea* and the *T. laevis* clade. The *T. mediterranea* OTU is characteristic of Mediterranean Sea surface waters and the *T. laevis* clade OTU is most prevalent in colder waters, in particular off Antarctica.

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## Introduction

The heterokont genus *Bolidomonas* (class Bolidophyceae) contains flagellated picophytoplankton cells (Guillou *et al.*, 1999a). Two species, *Bolidomonas pacifica* and *B. mediterranea*, were initially described, differing in the angle of insertion between flagella, in swimming patterns and in 18S rRNA gene sequences (Guillou *et al.*, 1999a). A variety, *B. pacifica* var. *eleuthera*, was later proposed (but not formally described) based on both cultures and environmental sequences (Guillou *et al.*, 1999b). Analyses of photosynthetic pigments as well as nuclear 18S rRNA and plastid RubisCO large subunit (*rbcL*) sequences (Guillou *et al.*, 1999a; Daugbjerg

and Guillou, 2001) demonstrated the sister relationship between *Bolidomonas* and diatoms, even though *Bolidomonas* possesses two flagella and lacks the siliceous frustule characteristic of diatoms. The Bolidophyceae were thus proposed to be an intermediate group between diatoms and all other Heterokontophyta (Guillou *et al.*, 1999a). Bolidophyceae culture and environmental sequences have been predominantly reported from oligotrophic waters in the Pacific Ocean and Mediterranean at low concentrations (Guillou *et al.*, 1999b).

The Parmales is a group of pico-sized marine protists with cells surrounded by silica plates (Booth and Marchant, 1987), first observed by microscopy in oceanic samples (Iwai and Nishida, 1976). Originally, they were thought to be resting stages of silicified loricate choanoflagellates (Silver *et al.*, 1980), but the discovery of a chloroplast in Parmales cells revealed that they are phytoplankton (Marchant and McEldowney, 1986). The taxonomy of the Parmales is based on morphological features that can only be observed with scanning electron microscopy (SEM).

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Three genera have been described: the family Pentalaminae contains one genus, *Pentalamina*, with one species, and the family Triparmae contains two genera, *Tetraparma* (four species) and *Triparma* (five species, four subspecies and four forma). *Pentalamina* has two circular shield plates of equal size, a larger ventral plate and two triradiate girdle plates. *Tetraparma* has three shield plates of equal size, a smaller ventral plate, a triradiate dorsal plate and three girdle plates. *Triparma* has three shield plates of equal size, a larger ventral plate, a triradiate dorsal plate and three girdle plates (Booth and Marchant, 1987, 1988; Kosman et al., 1993; Bravo-Sierra and Hernández-Becerril, 2003; Konno and Jordan, 2007; Konno et al., 2007). However, morphological variations are observed in field samples suggesting phenotypic plasticity in response to environmental conditions (Konno et al., 2007). Although they are most abundant in polar and subarctic waters (Booth et al., 1980; Nishida, 1986; Taniguchi et al., 1995; Komuro et al., 2005; Ichinomiya et al., 2013; Ichinomiya and Kuwata, 2015), parmales have a worldwide distribution including tropical waters (Silver et al., 1980; Kosman et al., 1993; Bravo-Sierra and Hernández-Becerril, 2003).

Typical heterokont features led Booth and Marchant (1987) to establish the Parmales, a new microalgal order tentatively belonging to the class Chrysophyceae. Recently, a culture strain of the Parmales species *Triparma laevis* was isolated from the western North Pacific off Japan (Ichinomiya et al., 2011). Transmission electron microscope observations showed the typical ultrastructure of photosynthetic heterokonts, with two endoplasmic reticulate membranes surrounding the chloroplast, a girdle and two to three thylakoid lamellae, as well as a mitochondrion with tubular cristae. Molecular phylogenetic analyses based on 18S rRNA and *rbcL* gene sequences revealed that this species did not group with the Chrysophyceae, but rather with the Bolidophyceae (Ichinomiya et al., 2011).

The discovery of the close relationship between the Bolidophyceae and the Parmales is highly relevant for elucidating the evolutionary history of diatoms (Guillou, 2011). Diatoms constitute the most ecologically successful phytoplankton group accounting for ~20% of primary production in modern oceans with an estimated diversity of 200 000 species (Armbrust, 2009). To date, however, phylogenetic analyses have relied on a single strain of Parmales and a few strains of *Bolidomonas*, this coverage being insufficient to determine the relationships between species within this key evolutionary lineage. In addition, only limited information is available from SEM surveys of field samples and from environmental clone libraries concerning the oceanic distribution and relative abundance of *Bolidomonas* and Parmales species.

In recent years, the massive sequencing of metabarcodes, that is, small regions of marker genes such

as the V4 or V9 regions of the 18S rRNA gene (Pawlowski et al., 2012; Logares et al., 2014), has provided in-depth information on the structure of eukaryotic microbial communities. Analyses of samples from the *Tara* Oceans expedition (de Vargas et al., 2015) and the Ocean Sampling Day (Kopf et al., 2015) are providing new opportunities to map the distribution of specific taxonomic groups across world oceans.

In this study, we first used nuclear, plastid and mitochondrial gene sequences to analyze the phylogenetic relationships of several new silicified Parmales and flagellated *Bolidomonas* strains isolated from different oceanic regions as well as related environmental sequences. We then assessed the distribution of the major Bolidophyceae taxa across the world oceans using the V9 rRNA metabarcoding data set obtained in the frame of the *Tara* Oceans expedition.

## Materials and methods

### *Culture isolation and maintenance*

Silicified Parmales strains were isolated from seawater samples collected from the Oyashio region of the western North Pacific in July 2009 during a cruise of the *RV Wakatakamaru* of the Tohoku National Fisheries Research Institute. *T. laevis* f. *longispina* and *T. strigata* were isolated from a sample collected at 50 m at St. A3 (42°30'N, 145°00'E) and *T. aff. verrucosa* from a sample collected at 50 m at St. A7 (41°30'N, 145°30'E), the light intensity at these depths corresponding to <0.5% of surface light intensity. Strains were isolated by serial dilution combined with cell labeling by the fluorescent dye PDMPO (2-(4-pyridyl)-5-([4-(2-dimethylaminoethylaminocarbonyl)-methoxy]phenyl)oxazole) (LysoSensor Yellow/Blue DND-160; Molecular Probe, Eugene, OR, USA) as described previously (Ichinomiya et al., 2011). Strains were subsequently maintained in the f/2 medium (Guillard and Ryther, 1962) at 5 °C under 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (14:10 L:D photoperiod). All new silicified strains were deposited to the NIES (National Institute for Environmental Studies) culture collection (<http://mcc.nies.go.jp>; Table 1). Three novel flagellated Bolidophyceae strains were isolated from North Sea, Atlantic Ocean and Mediterranean Sea samples by serial dilution and maintained in the K/2 medium at the temperature specified in Table 1. These strains were deposited in the Roscoff Culture Collection (RCC; <http://www.roscoff-culture-collection.org>; Table 1). Strains previously isolated were obtained either from the NIES or RCC collections (Table 1).

### *SEM observations*

Silicified cultures were filtered through a polycarbonate membrane filter (0.6  $\mu\text{m}$  pore size;

**Table 1** Origin and culture conditions of Bolidophyceae strains

ID	Species	Previous genus assignment	Strain name	Other name	Ocean origin	Depth of isolation (m)	Growth temperature (°C)	18S rRNA	ITS rRNA	16S rRNA	rbcL	nadH1
NIES-2565	<i>Triparma laevis</i> f. <i>inornata</i>	<i>Triparma</i>	TOY-0807		Western Pacific Ocean	80	5	AB546639	This study	LN735526	AB546640	This study
NIES-3699	<i>Triparma laevis</i> f. <i>longispina</i>		TLLOY-0907		Western Pacific Ocean	50	5	This study	This study	LN735530	This study	This study
NIES-3701	<i>Triparma strigata</i>		TSOY-0907		Western Pacific Ocean	50	5	This study	This study	LN735531	This study	This study
NIES-3700	<i>Triparma</i> aff. <i>verrucosa</i>		TVOY-0907		Western Pacific Ocean	50	5	This study	This study	LN735532	This study	This study
RCC205	<i>Triparma pacifica</i>	<i>Bolidomonas</i>	OLI 31 SE3	CCMP1866, NIES2682	Equatorial Pacific Ocean	15	20	HQ912557, AF123595	This study	LN735325	AF372696	
RCC206	<i>Triparma pacifica</i>	<i>Bolidomonas</i>	OLI 31 SC-A		Equatorial Pacific Ocean	15	20	This study	This study	LN735329	This study	
RCC208	<i>Triparma eleuthera</i>	<i>Bolidomonas</i>	OLI 120 SD-A		Equatorial Pacific Ocean	5	20	AF167154	This study	LN735332	AF333978	This study
RCC210	<i>Triparma eleuthera</i>	<i>Bolidomonas</i>	OLI 46 SE-A		Equatorial Pacific Ocean	15	20	AF167153	This study	LN735333	This study	This study
RCC212	<i>Triparma eleuthera</i>	<i>Bolidomonas</i>	OLI 41 SA-A		Equatorial Pacific Ocean	15	20	AF167155	This study	LN735334	AF333979	This study
RCC216	<i>Triparma pacifica</i>	<i>Bolidomonas</i>	OLI 94 SCH		Equatorial Pacific Ocean	5	20	This study	This study	LN735335	This study	This study
RCC238 <sup>a</sup>	<i>Triparma mediterranea</i>	<i>Bolidomonas</i>	MIN 129–20m Aa	MIN B11E5, CCMP1867, NIES-2681	Mediterranean Sea	20	20	AF123596, HQ710555	This study	AY702144	AF333977	
RCC239	<i>Triparma mediterranea</i>	<i>Bolidomonas</i>	MIN 129–20m Ab	MIN B11E7	Mediterranean Sea	20	20	This study	This study	LN735367	This study	
RCC1657	<i>Triparma</i> sp.		MICROVIR 3K_0	NIES-3369	North Sea	10	15	KF422625	This study	LN735273		This study
RCC1730	<i>Triparma eleuthera</i>		SOLAS F	NIES-3370	Atlantic Ocean	5	20	This study	This study	LN735286	This study	This study
RCC2347	<i>Triparma eleuthera</i>		BOUM117–22	NIES-3371	Mediterranean Sea	25	22	KF422629	This study	LN735356	This study	This study

Abbreviations: ITS, Internal Transcribed Spacer; NIES, National Institute of Environmental Sciences Collection; RCC, Roscoff Culture Collection.

Strains are available from the RCC (<http://www.roscoff-culture-collection.org/>) or the NIES culture collection (<http://mcc.nies.go.jp/>). Lines in bold correspond to novel strains.

<sup>a</sup>This strain has been contaminated and is not available anymore from the RCC collection.

Advantech, Tokyo, Japan) and air-dried at room temperature after rinsing with distilled water to remove salts. The filter was coated with Pt/Pd and examined by SEM (JSM-6390LV; JEOL, Tokyo, Japan).

Flagellated cultures were fixed with a solution of glutaraldehyde at 2.5% containing a 0.1 M sodium cacodylate buffer (pH 7.2) with 0.25 M sucrose. The fixed cells were placed on a polylysine-coated SEM glass plate and kept overnight at 4 °C. The glass plate was rinsed two times with the cacodylate–sucrose buffer, and postfixed with 1%  $\text{OSO}_4$  in cacodylate buffer for 1 h at 4 °C. Another double rinse with cacodylate buffer was carried out before dehydration in a graded ethanol series (30%, 50%, 75%, 90%, 95% and 100%). The 100% ethanol was then substituted two times with 100% of *t*-butyl alcohol. After 1 h at 4 °C, the glass plate was dried in a freeze drying device JEOL JFD-310 (JEOL). Before observation, the glass plate was coated with osmium in a vacuum evaporator coater HPC-1S (Vacuum Device Ltd, Ibaraki, Japan) and then examined by SEM (Hitachi S-4800, Tokyo, Japan).

#### Genomic DNA extraction and PCR amplification

Cells were harvested in exponential growth phase and concentrated by centrifugation. Total nucleic acids were extracted using the Nucleospin Plant II Kit (Macherey-Nagel, Düren, Germany) for RCC strains and the FastDNA SPIN Kit (MP Biomedicals, Solon, OH, USA) for NIES strains. All primer sequences and PCR conditions are listed in Supplementary Table S1. The nearly full-length nuclear 18S rRNA gene was PCR amplified using eukaryotic primers (Lepère *et al.*, 2011). The nuclear region containing the internal transcribed spacer (ITS) 1 and 2, as well as the 5.8S rRNA gene were obtained by PCR amplification using universal primers (White *et al.*, 1990). The plastid-encoded *rbcl* gene was amplified using primers Parma\_rbcLF1 and rbcSR. Parma\_rbcLF1 was designed to amplify the nearly full-length *rbcl* gene (ca. 1700 bp) of Bolidophyceae (Supplementary Table S1). The mitochondrion-encoded NADH dehydrogenase subunit 1 (*nad1*) gene was amplified using nested primer sets designed for Bolidophyceae (but which also amplify a bacterial *nad1*-like gene). All PCR amplifications were conducted using Phusion high-fidelity DNA polymerase (Finnzymes, Thermo Fisher Scientific, Waltham, MA, USA) in a 20  $\mu\text{l}$  reaction volume with 0.25  $\mu\text{M}$  of each primer. The PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sent directly for sequencing either to the Roscoff Genomer service or to the Macrogen Company (Seoul, South Korea). Sequences were deposited to GenBank under the following accession numbers: KR998385–KR998423 and KU160636.

#### Environmental sequences

A reference data set of near-complete sequences of genes coding for nuclear 18S rRNA, plastid 16S rRNA gene, *rbcl*, *nad1* and ITS (ITS1+5.8S rDNA +ITS2) sequences from Bolidophyceae was compiled. In particular, we retrieved all sequences assigned as ‘Bolidophyceae’ in the PR2 database for nuclear 18S rRNA (Guillou *et al.*, 2013) and in the PhytoRef database for plastid 16S rRNA (Decelle *et al.*, 2015). This data set was used to identify similar environmental sequences in the GenBank (release 206.0; February 2015) and CAMERA (<http://camera.calit2.net/>—note that this service no longer exists but the data are available on iMicrobe at <http://data.imicrobe.us/>) databases using MegaBLAST and BLAST (e-value  $e^{-10}$ ). For 18S rRNA, only Genbank sequences longer than 800 bp were kept for analysis (Supplementary Table S2). For CAMERA, only data retrieved from the ‘all 454 reads’ database-matched Bolidophyceae sequences were used.

The nuclear 18S and plastid 16S rRNA environmental sequences were manually checked for chimeras. Two subsequences of 300 bp were extracted from the 5′ and 3′ ends, respectively, and assigned to a taxonomic group based on BLAST searches against GenBank or PhytoRef. Sequences that presented conflicting taxonomic assignment between the two parts were examined in detail to confirm their chimerical nature and not considered further (Supplementary Table S2).

Nuclear 18S rRNA sequences were clustered at 99% similarity with CD-HIT-EST (Li and Godzik, 2006) to obtain OTUs. OTUs with representative sequences on the 18S rRNA phylogenetic tree based on full-length sequences (see below) were annotated following the clades described (Supplementary Table S2).

#### Phylogenetic analysis

All culture and environmental sequences were aligned with MAFFT using the E-INS-i algorithm (Katoh *et al.*, 2002). Gaps were treated as missing characters. CAMERA reads were excluded from phylogenetic analysis as they represented different regions of a given gene. Only 18S rRNA sequences longer than 1500 bp were used for phylogenetic reconstruction. Phylogenetic analyses were performed with three different methods: maximum-likelihood (ML), distance (neighbor-joining) and Bayesian analyses. The TrN+I+G, K2+G, GTR+G, T92+G and TN93+G+I models were selected for the 18S rDNA, 16S rDNA, *rbcl*, ITS and *nad1* sequence data, respectively, using Akaike information criterion in Modeltest 2.1.4 (Posada, 2008). Neighbor-joining and ML analyses were performed using MEGA 6.0 (Tamura *et al.*, 2013) and PhyML 3.0 (Guindon *et al.*, 2010) with Subtree Pruning and Regrafting tree topology search operations and approximate likelihood ratio test with

Shimodaira–Hasegawa-like procedure (Guindon *et al.*, 2010), respectively. Markov chain Monte Carlo iterations were conducted for 1 000 000 generations sampling every 100 generations with burning length 100 000 using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). MAFFT and MrBayes programs were run within Geneious 7.1.7 (Kearse *et al.*, 2012). For the definition of environmental clades, we followed the criteria of Guillou *et al.* (2008): a clade has to contain two or more environmental sequences obtained from different locations and/or samples in a given location and has to have strong phylogenetic support.

#### *Tara Oceans 18S rDNA metabarcodes*

The *Tara Oceans* project sampled oceanic waters over the course of a two and a half year expedition (Pesant *et al.*, 2015). Here, we used the primary eukaryotic metabarcoding data set, consisting of >530 million quality-checked eukaryotic V9 rDNA reads obtained from 293 size-fractionated plankton communities sampled at 47 stations (Supplementary Figure S1), two depths (surface and deep chlorophyll maximum (DCM)) and four size fractions (0.8–5, 5–20, 20–180 and 180–2000 µm). V9 rDNA metabarcodes (129 bp on average) were sequenced with Illumina HiSeq (Genoscope, Evry, France) at a typical depth of 2 million reads per sample (Supplementary Table S3). Detailed information on sampling and analysis is provided in Pesant *et al.* (2015) and de Vargas *et al.* (2015), respectively

Metabarcodes sequences for each data set were clustered into OTUs using the SWARM software (Mahé *et al.*, 2014). Each OTU corresponds to a set of sequences differing by one base at most. For the present analysis, we only considered OTUs that contained at least 10 reads and that had a BLAST similarity higher than 80% to an 18S rRNA sequence from GenBank. OTUs were assigned to a taxonomic group based on ggsearch against an annotated eukaryotic V9 database (de Vargas *et al.*, 2015) derived from the PR<sup>2</sup> database (Guillou *et al.*, 2013), which provides an eight-level taxonomic hierarchy (kingdom, superdivision, division, class, order, family, genus, species). Taxa were regrouped at the class level and we extracted the 36 OTUs that were classified as Bolidophyceae-and-relatives (Supplementary Table S4). We compared the contribution of Bolidophyceae metabarcodes to the total number of ‘photosynthetic’ metabarcodes. Groups considered as photosynthetic included Chlorophyta, Rhodophyta, Cryptophyta, Haptophyta, Ochrophyta (i.e., photosynthetic heterokonts such as diatoms), but not dinoflagellates for which the differentiation of photosynthetic vs heterotrophic species is highly complex. *Tara Oceans* data have been deposited to the International Nucleotide Sequence Database Collaboration and are accessible at <http://doi.pangaea.de/10.1594/PANGAEA.843017>

and 843022. Analyses, statistics and graphics of the *Tara Oceans* data were performed with the R software using the following libraries: ggplot2, mapproj, maps, mapdata, reshape2, scales and gridExtra (R Core Team, 2015).

## Results

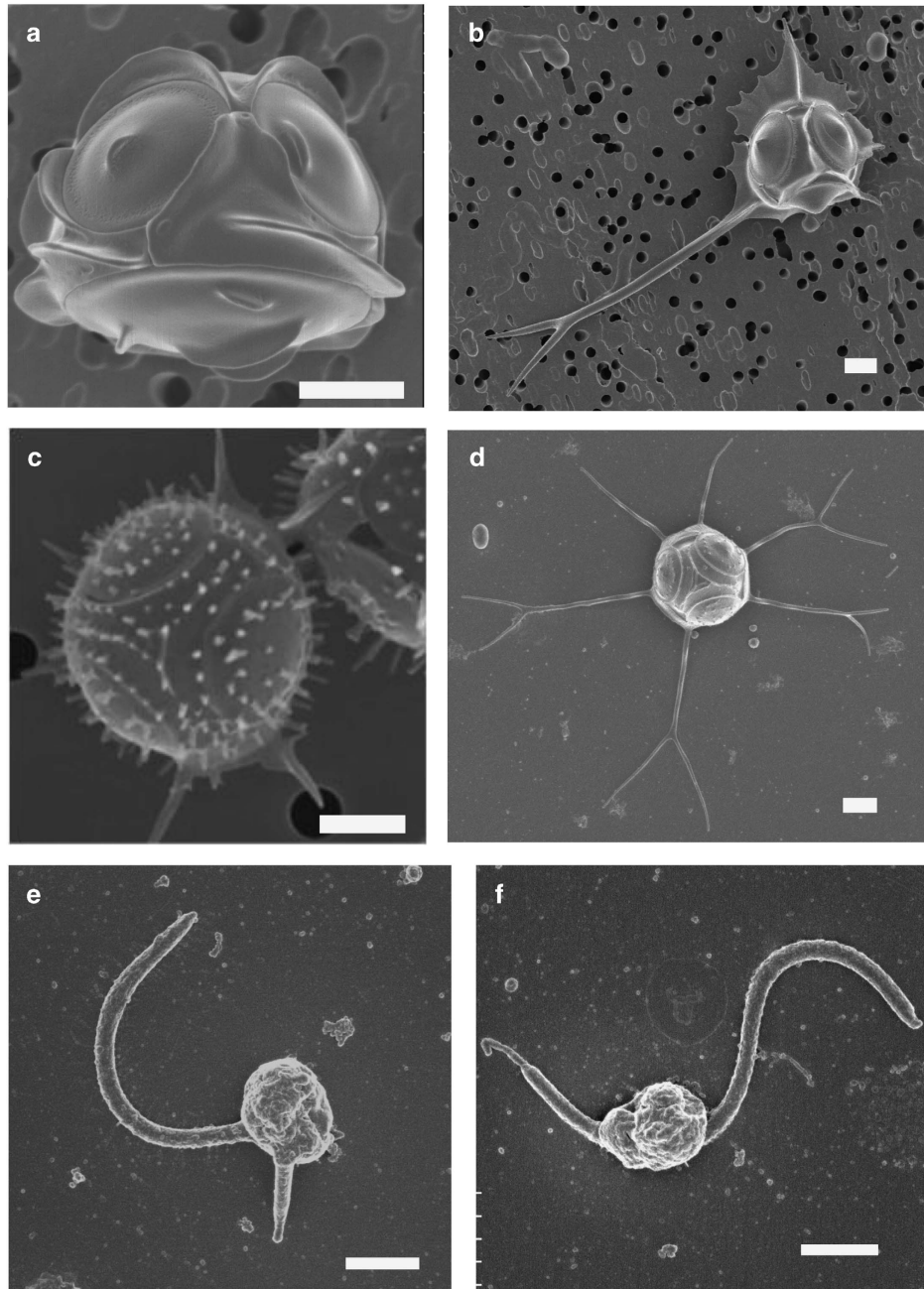
### *Characterization of novel strains*

The silicified Parmales strain initially isolated (NIES-2565) and the three new strains (NIES-3699, NIES-3700 and NIES-3701) have cells with a diameter in the range 2.4–3.6 µm (Figures 1a–d), surrounded by three shield plates, one dorsal plate, one ventral plate and three girdle plates, demonstrating that they all belong to the genus *Triparma* (Booth and Marchant, 1987). Strains NIES-2565 and NIES-3699 possess three girdle plates with a single wing without any surface ornamentation (Figures 1a and b), corresponding to the description of *T. laevis* (Ichinomiya *et al.*, 2011). More precisely, in NIES-2565 each girdle plate has a rounded wing at the margin without spines (Figure 1a), which is characteristic of the *inornata* form of *T. laevis* (Konno *et al.*, 2007). In contrast, for NIES-3699, each wing on the girdle plate is irregular at the margin and has a buttressed spine (Figure 1b). One of the spines is very long (up to 14 µm) and bifurcated at the end, thus being typical of the *longispina* form of *T. laevis* (Konno *et al.*, 2007). All plates in cells of NIES-3701 are covered by straight or forked tubular processes (Figure 1c). Girdle plates do not have a wing, but have two straight spines. These features are typical of the species *T. strigata* (Booth and Marchant, 1987; Kosman *et al.*, 1993). The morphological features of the fourth strain, NIES-3700 (Figure 1d), have never been reported before. This strain shares features with both *T. strigata* and *T. verrucosa*. It lacks central area structures on shield and ventral plates like *T. strigata*, but no triradiate keel is found. It possesses two long girdle-plate spines like *T. verrucosa*, but lacks dense radiating papillae characteristic of this latter species (Kosman *et al.*, 1993). For these reasons, this strain is hereafter identified as *T. aff. verrucosa*.

In contrast to the significant morphological variation observed between silicified strains, all new flagellated strains isolated from the Atlantic Ocean, North Sea and Mediterranean Sea (Table 1) possess the typical minimalist morphology of the genus *Bolidomonas* with very small cells possessing two flagella of unequal length and morphology (Figures 1e and f).

### *Phylogenetic analysis*

Full or partial DNA sequences were obtained for *Triparma* and *Bolidomonas* strains for the genes coding for nuclear 18S rRNA and ITS, plastid 16S rRNA and *rbcl* and mitochondrial *nad1* (Table 1).

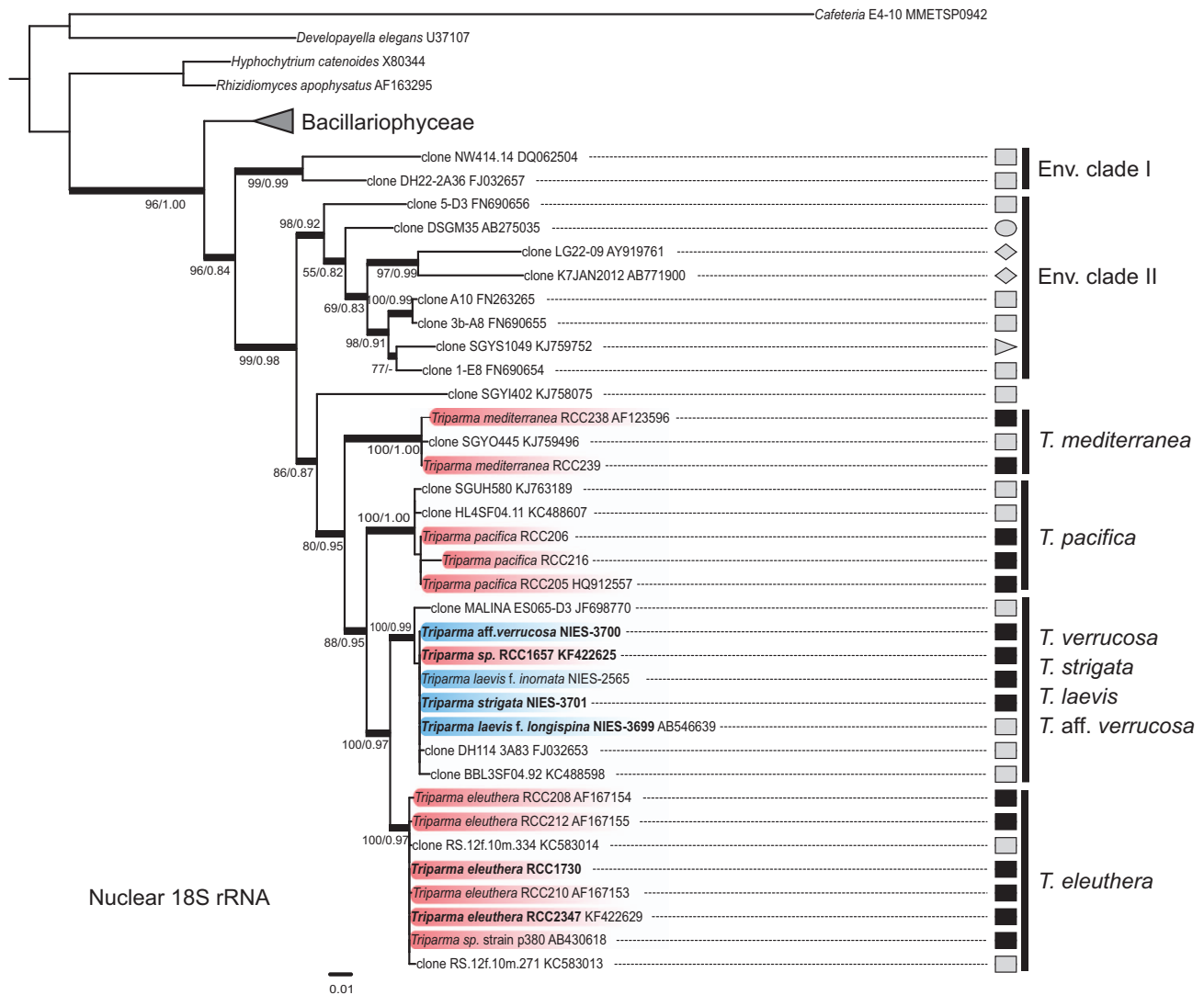


**Figure 1** SEM of the novel strains used in this study. (a) *T. laevis* f. *inornata* NIES-2565, (b) *T. laevis* f. *longispina* NIES-3699, (c) *T. strigata* NIES-3701, (d) *T. aff. verrucosa* NIES-3700, (e) *T. eleuthera* sp. nov. RCC2347 and (f) *Triparma* sp. RCC1657. Scale bar = 1 µm.

We also included environmental sequences related to the Bolidophyceae lineage (Supplementary Table S2) in all phylogenetic analyses.

In agreement with the work of Ichinomiya *et al.* (2011), the phylogeny based on full-length nuclear 18S rRNA gene sequences clearly demonstrates that the genus *Bolidomonas* (Guillou *et al.*, 1999a) is polyphyletic and that the order Parmales falls within the class Bolidophyceae (Figure 2). This prompted us to emend several taxonomic levels (see Taxonomic Appendix) and in particular to combine the genus *Bolidomonas* into *Triparma* following the anteriority

rule of the International Code of Botanical Nomenclature. Remarkably, the full-length nuclear 18S rRNA gene sequences from the strains of *T. laevis* f. *inornata*, *T. laevis* f. *longispina*, *T. strigata*, *T. aff. verrucosa* and from the flagellated strain RCC1657 were very similar, sharing between 99.9% and 100% identity (Figure 2) despite obvious morphological differences (Figure 1). These sequences, together with the environmental sequence MALINA ES065-D3 from the Arctic, formed a well-supported clade (Figure 2) that we hereafter call for convenience the '*T. laevis* clade'. The *T. laevis* clade was sister to the



**Figure 2** ML tree inferred from nuclear 18S rRNA sequences belonging to the Bolidophyceae lineage. Only bootstrap and SH-like support values higher than 50 and 0.7, respectively, are shown. Nodes supported by Bayesian posterior probabilities over 0.95 are shown by thick branches. Sequences in bold correspond to novel strains. Sequences in blue and red correspond to non-motile silicified and motile strains, respectively. Symbols represent the origin of the sequences: environmental samples (gray), cultures (black), euphotic zone (square), deep-sea (right-pointing triangle), fish microbiome (left-pointing triangle), sediments (circle) and freshwater (diamond).

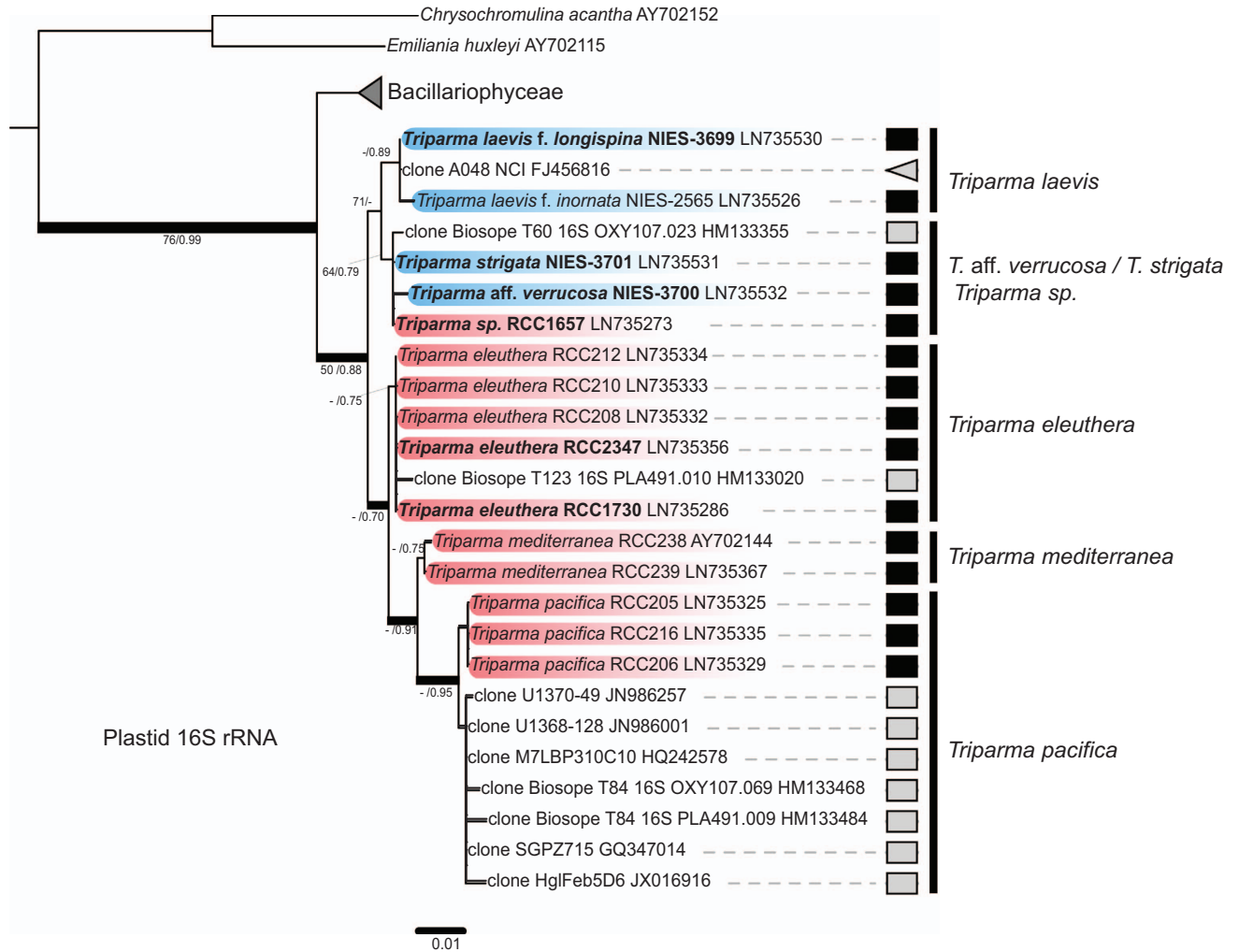
clade of *T. eleuthera*, a novel species defined here for a previously invalid taxon, *B. pacifica* var. *eleuthera* (Guillou *et al.*, 1999b), which contains RCC strains and two environmental sequences from the Red Sea. These two clades clustered with the *T. pacifica* clade, which included two environmental sequences from the Atlantic and Pacific Oceans. Finally, *T. mediterranea* formed the most basal group together with one environmental sequence from the Gulf Stream.

A number of environmental 18S rRNA gene sequences retrieved from PR<sup>2</sup>/GenBank according to their affiliation with Bolidophyceae did not group with *Triparma* sequences and formed two distinct clades (env. I and II) basal to the Bolidophyceae. These sequences originated from a range of environments including freshwater and marine sediments

(Figure 2). The monophyly of these clades with the Bolidophyceae was supported by neighbor-joining bootstrap, ML and Bayesian analyses.

In contrast to the situation for the 18S rRNA gene, all other molecular markers (plastid 16S rRNA (Figure 3), plastid *rbcL* (Supplementary Figure S4), ITS rRNA (Supplementary Figure S5), mitochondrial *nad1* (Supplementary Figure S6)) revealed the presence of two distinct subclades within the silicified *Triparma* species. One subclade contained the two forms of *T. laevis* (f. *inornata*, and *longispina*) and the other included *T. strigata*, *T. aff. verrucosa* and the flagellated strain RCC1657. The subclades were well supported in the *rbcL* and ITS trees but not in the plastid 16S rRNA tree (Figure 3). The close relationship between silicified *Triparma* and flagellated *T. eleuthera* was observed in all of





**Figure 3** ML tree inferred from plastid 16S rRNA gene nucleotide sequences belonging to the Bolidophyceae lineage. Legend as in Figure 2.

our ML and Bayesian analyses, except for the plastid 16S rRNA gene for which the former group was allied to a clade formed by the three flagellated species *T. eleuthera*, *T. mediterranea* and *T. pacifica* (Figure 3).

#### Oceanic distribution from sequences available in public databases

To determine the oceanic distribution of Bolidophyceae, we first searched for similar sequences in GenBank and in PR<sup>2</sup> (Guillou *et al.*, 2013) as well as PhytoRef (Decelle *et al.*, 2015), which contain annotated eukaryotic nuclear 18S and plastid 16S rRNA sequences, respectively, as well as CAMERA, which contains large-scale environmental metagenomic data sets (Supplementary Table S2). Only environmental sequences from nuclear 18S and plastid 16S rRNA genes were retrieved from these databases. The absence of *rbcL*, ITS or *nad1* sequences in GenBank is explained by the fact that these genes are typically not used as molecular

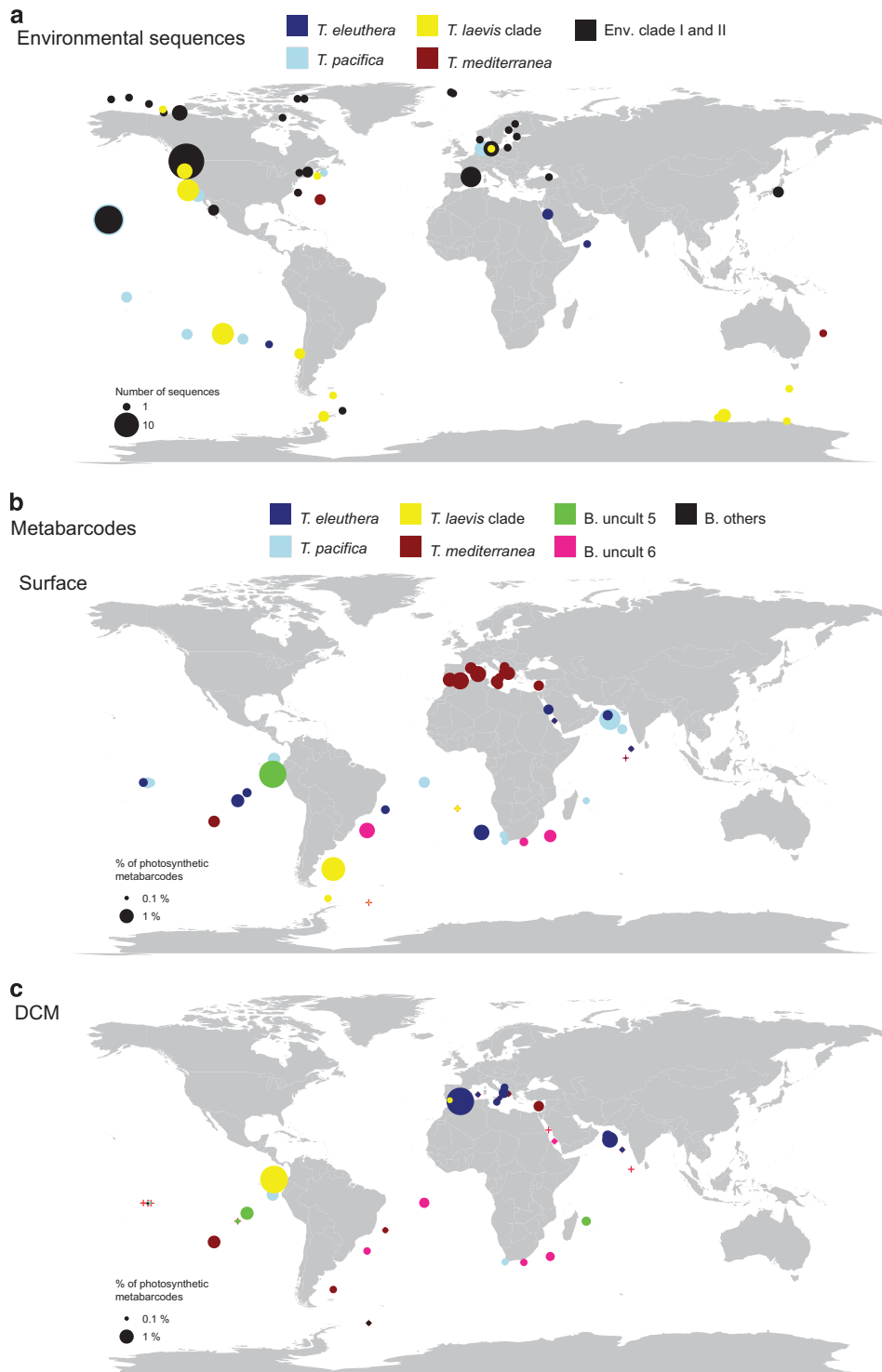
markers in environmental diversity studies. The data retrieved from CAMERA consisted of 135 reads that were reduced to 32 reads closely related to Bolidophyceae after careful checking by alignment with the full sequences used for the phylogenetic analysis and exclusion of reads without information on sample location.

After clustering, 50% of the environmental 18S rRNA gene sequences could not be assigned to a specific clade (Supplementary Table S2). Sequences related to *T. pacifica* were the most abundant followed by those from the *T. laevis* clade (Supplementary Figure S3). *T. pacifica* sequences originated mostly from the Pacific Ocean and *T. eleuthera* from the Red Sea and Pacific Ocean, whereas the *T. laevis* clade was characteristic of the Southern Ocean and was also found near the Pacific coast (Figure 4a). A large number of sequences from Arctic or sub-Arctic locations, as well as from the Baltic Sea, were short and difficult to align and could not be affiliated to the four major Bolidophyceae clades.

Oceanic distribution based on *Tara Oceans* 18S rRNA V9 metabarcodes

Thirty-six *Tara Oceans* 18S V9 rDNA OTUs were classified as Bolidophyceae, representing a total

of 105 113 reads (Supplementary Table S4). Their contribution to metabarcodes affiliated to photosynthetic groups (excluding dinoflagellates) reached a maximum of 3.8% and was slightly higher,



**Figure 4** Oceanic distribution of Bolidophyceae. (a) Based on available environmental sequences from GenBank and CAMERA databases (see Supplementary Table S2). (b) Based on *Tara Oceans* V9 metabarcodes from the 0.8 to 5  $\mu$ m plankton size fraction from surface waters. (c) Idem but at the DCM. Color indicates for subpanel a, the taxonomic affiliation of the sequences (Supplementary Table S2) and for subpanels b and c, the dominant OTU at each station (OTUs 7–36 have been regrouped in the category ‘B. others’). Surface of the circle is proportional to the number of sequences (a) or to the total contribution of Bolidophyceae OTUs to sequences from photosynthetic groups (b and c). Red crosses correspond to the location of the *Tara Oceans* stations where no Bolidophyceae metabarcodes have been detected.

on average, in surface waters compared with at the DCM (Table 2). Bolidophyceae were present in the 0.8–5 µm plankton size fraction at virtually all stations. In contrast, they were absent at more than a third of the stations in the larger size fractions and their mean contribution decreased ~5-fold compared with the 0.8–5 µm fraction (Table 2), confirming that this group is essentially pico-/nanoplanktonic in size.

In surface waters, Bolidophyceae in the 0.8–5 µm size fraction displayed the highest contribution to photosynthetic groups in the Mediterranean Sea and in regions relatively close to the coast (but not strictly coastal since *Tara* Oceans only sampled offshore pelagic waters) of the Indian, Atlantic and Pacific Oceans. In contrast, at the DCM their contribution appeared significant at only two stations, one near Gibraltar and the second in the equatorial Pacific Ocean (Figure 4c).

The six most abundant OTUs contributed 86% of total Bolidophyceae sequences in the four size fractions considered (Supplementary Table S5). Among these, the four most abundant OTUs shared 100% identity with the sequences of, respectively, *T. eleuthera*, *T. pacifica*, *T. laevis* and *T. mediterranea*. Very clear signatures for these four clades are present in the V9 region of the 18S rRNA gene, which would therefore be suitable for the design of fluorescence *in situ* hybridization or quantitative real-time PCR probes (Supplementary Figure S2). A single other OTU (no. 11) with 647 reads matched an environmental sequence (KJ758075) from the Ross Sea (Antarctica) (Supplementary Table S4). In contrast, all other OTUs did not match any known sequences, not even those from the two environmental clades defined on the basis of the full 18S rRNA gene sequences (Figure 2 and Supplementary Table S4).

We estimated the contribution of the six most abundant OTUs (regrouping the other OTUs into a single ‘other’ category) in the 0.8–5 µm fraction in

surface and DCM waters. *T. pacifica* and *T. eleuthera* were the most abundant and ubiquitous clades, occurring at more than 79% of the stations (Supplementary Table S5). *T. eleuthera* was more widespread in surface waters than at the DCM. In surface waters (Figures 4b and 5a), *T. pacifica* seemed to be absent in the Mediterranean Sea (Stns TARA\_007 to TARA\_030), whereas both species were absent in the Southern Ocean (Stns TARA\_082 to TARA\_085). In contrast, surface waters of the Indian Ocean and Pacific Ocean were completely dominated by these two OTUs (Figures 4b and 5a). At the DCM (Figures 4c and 5b), the situation was roughly similar. The two OTUs were absent from cold waters below 15 °C, but present up to 30 °C (Supplementary Figure S7B).

*T. mediterranea* was much less ubiquitous, occurring at about 50% of stations (Supplementary Table S5). It was more characteristic of surface waters but was also found very deep, beyond 150 m (Supplementary Figure S7A). It dominated Mediterranean Sea surface waters (Figures 4b and 5a) and was also present in subtropical South Atlantic and South Pacific waters. It was much less abundant at the DCM (Figures 4c and 5b), although it dominated at some stations, again in the South Atlantic and South Pacific. In contrast to *T. eleuthera* and *T. pacifica*, *T. mediterranea* appears to have a narrower temperature range (Supplementary Figure S7B).

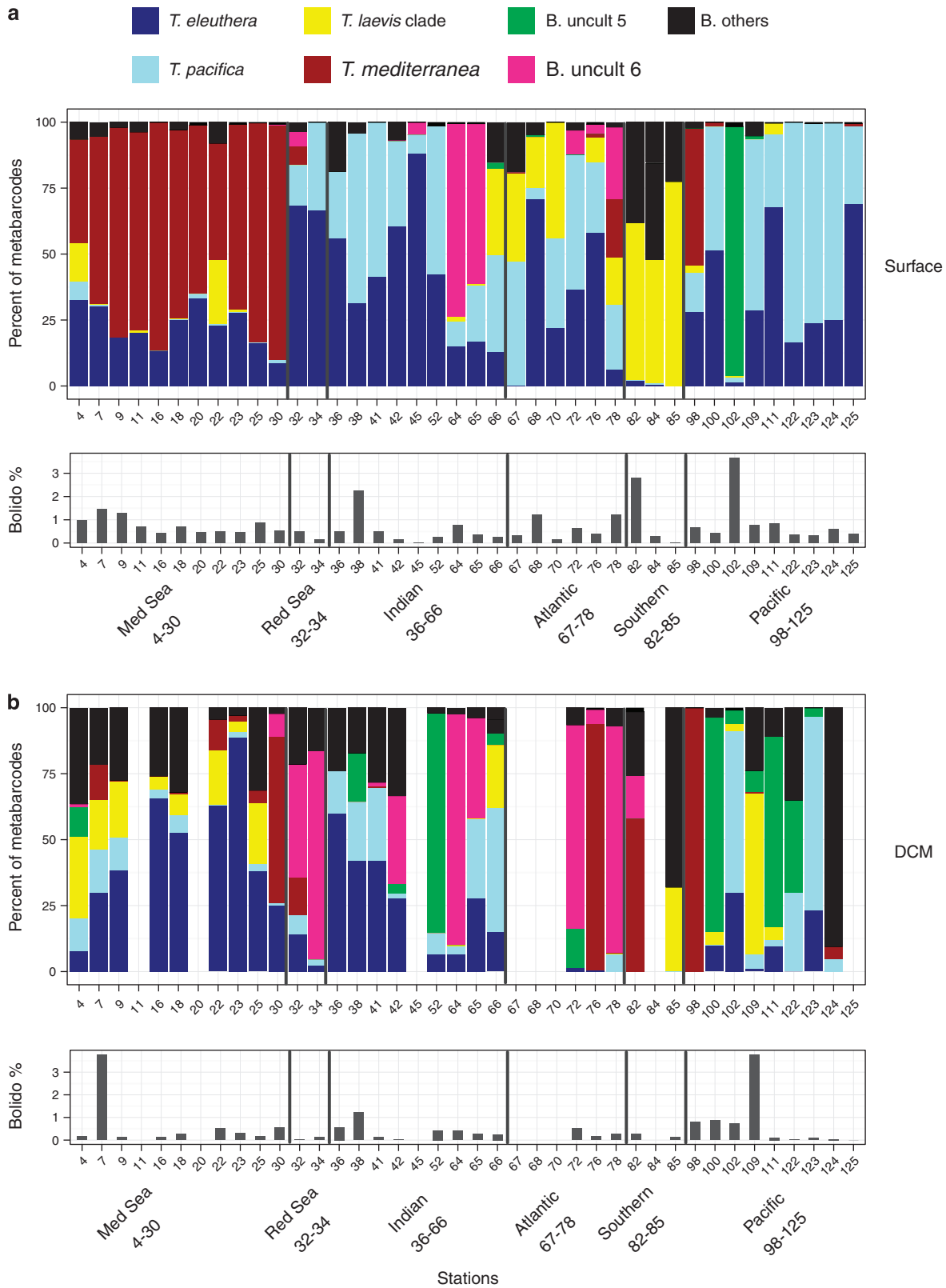
The *T. laevis* clade was also present in ~50% of stations and was more characteristic of surface waters (Figure 5 and Supplementary Table S5). Its distribution contrasted to that of *T. mediterranea*. Its highest contributions in surface waters were off South Africa and in the Southern Ocean (Figure 4b and Figure 5a). At the DCM, the *T. laevis* clade provided significant contributions in the Mediterranean Sea and in the Pacific offshore Costa Rica (Figure 4c and Figure 5b). In agreement with its geographical localization, it extended to very cold water (Supplementary Figure S7B).

The first of the two other abundant OTUs (no. 5 named ‘B. uncult 5’) that did not match any cultured Bolidophyceae sequence was absent in surface waters at most stations except one offshore Costa Rica (Stn TARA\_102) where it was dominant (Figures 4b and 5a). At the DCM, it was only present at a few stations, but at these stations it was the dominant metabarcodes (Figures 4c and 5b). The last abundant OTU (no. 6 or ‘B. uncult 6’) was present at a few stations in the Indian Ocean near South Africa and in the Atlantic Ocean off Brazil where it dominated (Figures 4b and 5a). It was much more prevalent at the DCM and was dominant at the same stations where it dominated in surface waters (Figures 4c and 5b). These two OTUs appeared to have a narrower temperature ranges than the four dominant OTUs (Supplementary Figure S7). Finally, OTU no. 11 matching an Antarctica GenBank sequence, represented in Figure 2 by the

**Table 2** Bolidophyceae sequences as a function of size fraction and depth in the *Tara* Oceans V9 data set

Fraction (µm)	Depth	No. of samples	% of samples with Bolido sequences	Bolido/photo (%)	
				Mean	Max
0.8–5	SUR	40	100	0.73	3.68
0.8–5	DCM	33	97	0.52	3.78
5–20	SUR	41	68	0.15	0.88
5–20	DCM	33	58	0.10	0.89
20–180	SUR	42	45	0.08	1.00
20–180	DCM	28	43	0.04	0.47
180–2000	SUR	45	47	0.23	3.34
180–2000	DCM	31	55	0.10	1.09

Abbreviations: DCM, deep chlorophyll maximum; SUR, surface. Number of samples analyzed, percentage of samples with Bolidophyceae sequences, mean and maximum contribution of Bolidophyceae to photosynthetic taxa.



**Figure 5** Relative contribution of the major groups of Bolidophyceae OTUs in the 0.8–5  $\mu$ m plankton size fraction at *Tara* Oceans stations sampled in surface waters (a) and at the DCM (b). OTUs 7–36 (Supplementary Table S4) have been regrouped in the category ‘B. others’.

environmental sequence clone SGYI402 (KJ758075), was most abundant at Stn TARA\_085, which was the southern-most station sampled by the *Tara* Oceans expedition.

## Discussion

Multigene phylogenetic analyses including nuclear, plastidial and mitochondrial markers revealed that all silicified *Triparma* species isolated so far form a monophyletic lineage within the Bolidophyceae, strongly supporting the previous hypothesis that the Parmales and Bolidophyceae belong to the same lineage, sister to the diatoms (Ichinomiya *et al.*, 2011).

Each of the three flagellated *Triparma* species that were previously classified in the genus *Bolidomonas* form monophyletic groups according to all gene markers studied. *T. pacifica* and *T. eleuthera* were originally thought to belong to the same species based on the absence of clear morphological differences (Guillou *et al.*, 1999b). In the present study, they were never monophyletic for any of the genes analyzed, leading to the description of a new species *T. eleuthera*. The branching order of the different cultivated Bolidophyceae clades was not consistent across all genes, making it difficult to draw any firm conclusions about their genetic relationships. However, the hypothesis that silicified *Triparma* species are most closely related to *T. eleuthera* was supported by all gene phylogenies except for that of the plastid 16S rRNA gene.

We observed two distinct environmental clades (env. I and II; Figure 2). The monophyly of these clades with the Bolidophyceae group is well supported by neighbor-joining bootstrap, ML and Bayesian analyses. As these clades do not contain any cultured representatives, it is difficult to say whether these groups represent other Bolidophyceae species or distinct heterokont forms. These sequences were recovered from similar locations (Figure 4) to where silicified Parmales have been observed by microscopy, in particular polar regions (Silver *et al.*, 1980; Marchant and McEldowney, 1986; Nishida, 1986; Booth and Marchant, 1987; Kosman *et al.*, 1993), supporting the idea that they could correspond to species from this group. In contrast, no environmental clade related to the Bolidophyceae was observed with the plastid 16S rRNA gene, which is probably explained by the lower number of eukaryotic surveys using this gene.

All genes analyzed, with the exception of the 18S rRNA, allowed discrimination of two subclades within silicified *Triparma* species, one corresponding to the two forms of *T. laevis* and the other to *T. strigata* and *T. aff. verrucosa*. *Triparma* sp. RCC1657, although being a flagellated organism with no evidence of possessing a silica covering (Figure 1e), was always affiliated to silicified strains in all analyses, grouping with *T. strigata* and *T. aff.*

*verrucosa* for the plastid 16S rRNA and *rbcl*, the nuclear ITS rRNA and the mitochondrial *nad1* genes. Interestingly, RCC1657 is the flagellated strain that has been isolated from the highest latitude so far (North Sea), in waters whose temperature range is similar to that where silicified forms are observed (Ichinomiya and Kuwata, 2015). Flagellated cells have occasionally been observed in cultures of silicified *T. laevis* (MHN, unpublished observations). *Triparma* could therefore have a life cycle that switches between diploid silicified non-flagellated and haploid naked flagellated stages. This hypothesis is supported by the fact that the flagellated *T. pacifica* (RCC205) has been hypothesized to be haploid based on the absence of heterozygous alleles in its transcriptome (Kessenich *et al.*, 2014). Life-cycle switching between non-flagellated and flagellated forms is known in some algal classes. The coccolithophore *Emiliania huxleyi* can exist in a diploid non-motile coccolith-bearing form and in a haploid flagellated non-calcifying form, both stages capable of growing vegetatively (Rokitta *et al.*, 2011), whereas *Calyptroshaera sphaeroidea* exhibits flagellated holococcolith-bearing and non-flagellated heterococcolith-bearing forms (Noël *et al.*, 2004). The 18S rRNA gene sequence of the small coccoid prasinophyte *Pycnococcus provasolii* is known to be almost 100% identical to that of the small planktonic flagellate *Pseudoscourfieldia marina*, leading to speculation that these two forms could be alternate life-cycle stages of the same species (Fawley *et al.*, 1999; Guillou *et al.*, 2004). Extant centric diatoms, which have a diploid vegetative stage, produce naked flagellated haploid male gametes for sexual reproduction (Drebes, 1977). Mann and Marchant (1989) proposed that the ancestral diatom could have been a haploid flagellate that formed a diploid silicified zygote. The mitotic division of the zygote might have taken place preferentially to give rise to the centric diatoms, which are the most ancient diatom group (Kooistra *et al.*, 2007). The conditions that trigger the alternation are often not clear and specific strains may have lost the genetic ability to convert from one form to the other. This is the case of *E. huxleyi* for which isolates from the oligotrophic ocean have lost the genes necessary to form flagella (von Dassow *et al.*, 2015). Future culture studies of the life cycle of the Bolidophyceae, perhaps modulating important environmental factors such as temperature, light or nutrients to attempt to provoke life-cycle phase changes, will shed light on the relationships between motile and non-motile silicified forms and thus on the early evolutionary history of diatoms.

The *Tara* Oceans V9 metabarcoding data set confirms that the Bolidophyceae are mainly limited to the smallest size fraction below 5 µm, which matches the size range of all silicified and flagellated Bolidophyceae strains isolated in culture to date. Their presence in the larger size fractions, in particular the 180–2000 µm fraction where

their contribution increases in comparison with the 5–20 and 20–180 µm fractions (Table 2), could be explained by their consumption by predators and subsequent inclusion into large sinking particles. This hypothesis is reinforced by the fact that parmalian siliceous plates have been observed in the fecal pellets and gut contents of zooplankton (Booth *et al.*, 1980; Marchant and Nash, 1986; Urban *et al.*, 1992; Konno and Jordan, 2012) as well as in marine snow (Marchant *et al.*, 1996). Sequences have also been found in deep-sea sediments and fish microbiota (Supplementary Table S2).

For all size fractions, the contribution of Bolidophyceae to phytoplankton remains in general limited (below 3%), matching previous estimates for Bolidophyceae using 18S rRNA probes (<1%; Guillou *et al.*, 1999b; Not *et al.*, 2005) and the low cell concentration (10–100 cell ml<sup>-1</sup>) usually recorded for silicified Parmales in oceanic waters (Ichinomiya and Kuwata, 2015). However, this group is very widely distributed throughout the ocean, being present at over 90% of the *Tara* Oceans stations (Figure 4 and Supplementary Table S5). The global distribution of this group is further stressed by a number of recent metabarcoding studies that reported their presence in the English Channel, the Arctic and the Antarctic Oceans (Kilias *et al.*, 2014; Luria *et al.*, 2014; Taylor and Cunliffe, 2014).

The *Tara* Oceans metabarcoding data set also allows assessment of the distribution of the four phylogenetic clades that can be distinguished based on very clear 18S rRNA signatures. Two OTUs are clearly ubiquitous, *T. eleuthera* and *T. pacifica*. The former has been isolated several times from a wide range of oceanic locations (Pacific and Atlantic Oceans, Mediterranean Sea; Table 1), while in contrast *T. pacifica* has been isolated from a single cruise in the Pacific Ocean (OLIPAC in 1994; Table 1). *T. mediterranea* is particularly well named as this species dominates in surface waters of the Mediterranean Sea (Figures 4 and 5), the only region from which it has been isolated into culture, during a single cruise and at a single station. The *T. laevis* clade appears dominant in cold waters similar to those from which it was first described (Booth and Marchant, 1987) and subsequently isolated (Ichinomiya *et al.*, 2011). In the western North Pacific, the silicified forms only appear to grow actively in low temperature waters that occur during winter–spring and in culture *T. laevis* and *T. strigata* are unable to grow above 10 °C (Ichinomiya *et al.*, 2013; Ichinomiya and Kuwata, 2015). However, reads from the *T. laevis* clade were also present and abundant in the tropical Pacific near the Costa Rica dome at 30 m (Figure 4c) where the recorded water temperature was 25 °C (Supplementary Figure S7B) and environmental sequences have been found in the South East Pacific Ocean (Supplementary Table S2). Silicified Parmales have also been reported by microscopy in upwelling areas

of the tropical Pacific Ocean. Therefore, sequences found in tropical waters could correspond to *Triparma* species for which we have no isolates such as *T. laevis* subsp. *mexicana* or *T. retinervis* (Silver *et al.*, 1980; Kosman *et al.*, 1993; Bravo-Sierra and Hernández-Becerril, 2003).

The genetic diversity observed for the metabarcodes with no matching GenBank sequences could correspond to uncultured taxa, either among the silicified forms for which many morphotypes, in particular corresponding to the genera *Tetraparma* and *Pentalamina*, have not yet been cultured. These unknown metabarcodes could also correspond to flagellated forms previously classified as *Bolidomonas*, which are also quite difficult to bring into culture as demonstrated by the fact that *T. mediterranea* has only been isolated once, almost 20 years ago. Some of these metabarcodes could be more abundant in areas under-represented in this data set such as polar regions, as suggested by OTU no. 11, which could be typical of the Southern Ocean as it matches sequences recovered in the Ross Sea (KJ758075).

The data presented in this paper shed some light on the enigmatic Bolidophyceae group, which is of particular interest given it is phylogenetically close to the diatoms, which constitute the most successful extant phytoplankton group. For the first time, we establish the possibility that some species of Bolidophyceae may have a life cycle with motile flagellated and non-motile silicified stages. However, the *Tara* Oceans metabarcoding data clearly reveal distinct niches between the silicified *T. laevis* clade and the other flagellated species described so far, the former being mostly a cold-water group while the latter have temperate and tropical distributions. One species, *T. mediterranea*, seems to be very characteristic of the Mediterranean Sea, a region known to harbor endemic species (Gómez, 2006). This work demonstrates the power of mixing classical culture and phylogenetic studies with novel massive metabarcoding approaches.

#### Taxonomic Appendix

The multigene approach applied to the new strains of *Bolidomonas* and *Triparma* (Figures 2, 3 and Supplementary Figures S4–S6) confirmed that the genus *Bolidomonas* (Guillou *et al.*, 1999a) is no longer monophyletic and that the class Bolidophyceae must include the order Parmales and the family Triparmaceae (Konno *et al.*, 2007) that were originally classified within the class Chrysophyceae. Therefore, the following emendations to the original definitions of the class, order, family, genus and description of one new species are required.

Class **Bolidophyceae** Guillou et Chrétiennot-Dinet 1999

emend. Ichinomiya et Lopes dos Santos.

*Diagnosis*: This class includes motile naked and non-motile silicified cells. Motile cells with two

unequal flagella, ventrally inserted. Long flagellum directed forward, with tubular flagellar hairs. Short flagellum naked and acronemated. Basal apparatus reduced to basal bodies. Transitional helix absent. No eyespot. Non-motile cells with silicified cell wall composed of five or eight plates, all fitting edge to edge. All cell walls composed of shield plates, girdle plates and a ventral plate. In addition, a dorsal plate is usually present. For both motile and non-motile cells, one chloroplast with a girdle lamella, lamellae with two to three appressed thylakoids. Mitochondria with tubular cristae. Pigment composition includes chlorophyll *a*, *c*<sub>1</sub>+*c*<sub>2</sub> and *c*<sub>3</sub> and fucoxanthin as a major carotenoid. 18S rRNA gene sequences place this class as a sister group to the diatoms.

Type genus: *Triparma* Booth et Marchant

Order **Parmales** Booth et Marchant 1987 emend. Konno et Jordan 2007.

emend. Ichinomiya et Lopes dos Santos.

Diagnosis as for the class.

Family **Triparmaceae** Booth et Marchant 1987 emend. Konno et Jordan 2007 emend. Ichinomiya et Lopes dos Santos.

*Diagnosis*: Motile cells 1–2 µm in diameter, spherical or heart shaped. Two unequal flagella, ventrally inserted. Long flagellum 4–7 µm directed forward, with laterally inserted tubular flagellar hairs. Short flagellum 0.9–2.2 µm naked with a marked acronema. Basal apparatus reduced to basal bodies. Dorsal chloroplast occupies about half the cell. Non-motile cells with silicified cell wall. Cell wall composed of three shield plates, three oblong girdle plates, a triradiate dorsal plate with rounded ends and a large ventral plate. For both motile and non-motile cells, one chloroplast with a girdle lamella, lamellae with two to three appressed thylakoids. Mitochondria with tubular cristae. Pigment composition includes chlorophyll *a*, *c*<sub>1</sub>+*c*<sub>2</sub> and *c*<sub>3</sub> and fucoxanthin as a major carotenoid.

Type genus: *Triparma* Booth et Marchant

Genus ***Triparma*** Booth et Marchant 1987 emend. Konno et Jordan 2007

emend. Ichinomiya et Lopes dos Santos.

Diagnosis as for the family.

Type species: *Triparma columacea* Booth et Marchant.

Synonym: *Bolidomonas* Guillou et Chrétiennot-Dinet in Guillou et al., 1999a

*Triparma eleuthera* Ichinomiya et Lopes dos Santos sp. nov.

*Diagnosis*: Characters of the motile type of the genus. Cells 1.3–2 µm in size, swimming speed variable, with the long flagellum pulling the cell and changes in direction. Two flagella, inserted at 109°. Short flagellum 2–3 µm. Long flagellum 5–7 µm. The combined nucleotide sequences of the nuclear 18S rRNA (KF422629), rRNA ITS (KU160636), plastid 16S rRNA (LN735356) *rbcL* (KR998422) and mitochondrial *nad1* genes (KR998413) are characteristic of this species.

*Etymology*: The specific epithet ‘eleuthera’ is derived from the feminine form of the Greek adjective *eleutheros*, meaning freedom and refers to the frequent changes in swimming direction.

*Authentic strain*: RCC2347 collected by L Garczarek during the BOUM cruise (July 2008), in the Mediterranean Sea at 35°40'N 14°06'E, deposited in the Roscoff Culture Collection, Roscoff, France.

*Holotype*: Figure 1e in this publication.

*Triparma pacifica* (Guillou et Chrétiennot-Dinet) Ichinomiya et Lopes dos Santos. *comb. nov.* Basionym: *Bolidomonas pacifica* Guillou et Chrétiennot-Dinet (in Guillou et al., 1999a, p 371)

*Triparma mediterranea* (Guillou et Chrétiennot-Dinet) Ichinomiya et Lopes dos Santos. *comb. nov.* Basionym: *Bolidomonas mediterranea* Guillou et Chrétiennot-Dinet (in Guillou et al., 1999a, p 371)

## Conflict of Interest

The authors declare no conflict of interest.

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