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A wide diversity of previously undetected free-living relatives of diplomonads isolated from marine/saline habitats

Martin Kolisko,[1] Jeffrey D. Silberman,[2] Ivan Cepicka,[3] Naoji Yubuki,[4†] Kiyotaka Takishita,[5] Akinori Yabuki,[4] Brian S. Leander,[6] Isao Inouye,[4] Yuji Inagaki,[7] Andrew J. Roger[8] and Alastair G. B. Simpson[1*]

Departments of [1]Biology and [8]Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, Canada.

[2]Department of Biological Sciences, University of Arkansas, Fayetteville, AR, USA.

[3]Department of Zoology, Faculty of Science, Charles University in Prague, Prague, Czech Republic.

[4]Institute of Biological Sciences, Graduate School of Life and Environmental Sciences and [7]Center for Computational Sciences and Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

[5]Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Kanagawa, Japan.

[6]Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada.

Summary

Over the last 15 years classical culturing and environmental PCR techniques have revealed a modest number of genuinely new major lineages of protists; however, some new groups have greatly influenced our understanding of eukaryote evolution. We used culturing techniques to examine the diversity of free-living protists that are relatives of diplomonads and retortamonads, a group of evolutionary and parasitological importance. Until recently, a single organism, *Carpediemonas membranifera*, was the only representative of this region of the tree. We report 18 new isolates of *Carpediemonas*-like organisms (CLOs) from anoxic marine sediments. Only one is a previously cultured species. Eleven isolates are conspecific and were classified within a new genus, *Kipferlia* n. gen. The remaining isolates include representatives of three other lineages that likely represent additional undescribed genera (at least). Small-subunit ribosomal RNA gene phylogenies show that CLOs form a cloud of six major clades basal to the diplomonad-retortamonad grouping (i.e. each of the six CLO clades is potentially as phylogenetically distinct as diplomonads and retortamonads). CLOs will be valuable for tracing the evolution of diplomonad cellular features, for example, their extremely reduced mitochondrial organelles. It is striking that the majority of CLO diversity was undetected by previous light microscopy surveys and environmental PCR studies, even though they inhabit a commonly sampled environment. There is no reason to assume this is a unique situation – it is likely that undersampling at the level of major lineages is still widespread for protists.

Received 16 November, 2009; accepted 16 March, 2010. *For correspondence. E-mail asimpso2@dal.ca; Tel. (+1) 902 494-1247; Fax (+1) 902 494-3637. †Present address: Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada.

Introduction

Over the last decade, conventional culturing approaches have led to the discovery of a selection of novel eukaryotic organisms of major evolutionary importance. For example, *Breviata anathema*, a small amoeboid flagellate, was shown to be a deep branch attached to the supergroup Amoebozoa, and important for understanding the unikont/bikont hypothesis and consequently for evaluating hypotheses about the location of the root of the eukaryote tree (Walker *et al*., 2006; Minge *et al*., 2009; Roger and Simpson, 2009). *Capsaspora owczarzaki* is a single-celled organism that is most closely related to choanoflagellates and/or ichthyosporeans and hence is one of the key taxa for understanding the evolution of multicellularity in animals and fungi (Hertel *et al*., 2002; Ruiz-Trillo *et al*., 2004). *Chromera velia* is a photosynthetic relative of the often-plastid-bearing, but non-photosynthetic apicomplexan parasites (Moore *et al*., 2008). In addition, some organisms, such as centrohelids, *Telonema* and *Fonticula*, were known for some time, but have only recently been studied using molecular techniques and have proven to be of particular phylogenetic importance (Cavalier-Smith and Chao, 2003a; Klaveness *et al*., 2005; Sakaguchi *et al*., 2005, 2007;

Shalchian-Tabrizi *et al*., 2006; Brown *et al*., 2009; Burki *et al*., 2009). Over a similar time period environmental PCR approaches have revealed a number of additional and genuinely novel significant lineages (Massana and Pedrós-Alió, 2008). The most important perhaps include the several 'MAST lineages' of uncultured, probably heterotrophic marine stramenopiles (Massana *et al*., 2004; Massana *et al*., 2006) and the mysterious picobiliphytes/biliphytes (Not *et al*., 2007; Cuvelier *et al*., 2008). On the other hand, the last decade has also seen the widespread incorporation of many morphologically distinct eukaryote lineages into existing major groups (e.g. Cercozoa and Bicosoecida – O'Kelly and Nerad, 1998; Cavalier-Smith and Chao, 2003b; 2006; Bass and Cavalier-Smith, 2004), as well as the refutation of several early claims of substantial novelty of major lineages from environmental PCR studies (Berney *et al*., 2004; Cavalier-Smith, 2004). These latter trends tend to suggest that much of the major-lineage-level diversity of eukaryotes is already known. The extent to which this is accurate has important consequences for understanding eukaryote diversity and cell evolution.

Diplomonads, such as the human parasite *Giardia intestinalis*, are among the most interesting and problematic groups of microbial eukaryotes from an evolutionary perspective. Diplomonads are anaerobic or microaerophilic heterotrophic flagellates that live either in anoxic sediments or water bodies, or as parasites or commensals (Kulda and Nohynkova, 1978). They do not possess classical mitochondria and, for a long time, were considered to be ancestrally amitochondriate (Cavalier-Smith, 1983). This, in combination with their tendency to branch at the base of the eukaryotic trees estimated from small-subunit ribosomal RNA (SSU rRNA) and translation elongation factor genes (Sogin *et al*., 1989; Kamaishi *et al*., 1996), led to a widespread view that diplomonads were 'primitive eukaryotes'. However, later studies have shown the presence of genes of mitochondrial origin in diplomonad genomes (Roger *et al*., 1998; Tachezy *et al*., 2001) and tiny mitochondrion-related organelles called mitosomes were subsequently discovered in *G. intestinalis* (Tovar *et al*., 2003). Moreover, the position of diplomonads at the base of the eukaryotic tree is now widely considered to be the result of a long branch attraction artefact stemming from rapid gene sequence evolution in this group (Brinkmann *et al*., 2005; Philippe *et al*., 2005). Thus the true phylogenetic position and evolutionary history of diplomonads remains incompletely understood and there is considerable interest in using comparative genomics and cell biological approaches to better understand diplomonad evolution (Hampl *et al*., 2009).

Until recently, the closest known relatives of diplomonads included retortamonads, which are poorly studied, mostly commensal organisms (Kulda and Nohynkova, 1978), and the more distantly related genus *Carpediemonas*. *Carpediemonas* is a small bacterivorous flagellate found in anoxic marine sediments that was described and characterized relatively recently (Ekebom *et al*., 1996; Simpson and Patterson, 1999; Simpson *et al*., 2002). *Carpediemonas* tends to constitute a shorter branch than diplomonads in molecular phylogenies, and possesses double-membrane-bounded mitochondrion-like organelles that are considerably larger than the mitosomes of *Giardia* (Simpson and Patterson, 1999; Simpson *et al*., 2002, 2006). This makes *Carpediemonas* potentially very important for resolving the phylogenetic position of diplomonads and understanding the reductive evolution of mitochondria-related organelles.

For a long time *Carpediemonas* appeared to be a phylogenetically isolated lineage, although very recently two '*Carpediemonas*-like' organisms (CLOs) have been described, *Dysnectes brevis* (Yubuki *et al*., 2007) and *Hicanonectes teleskopos* (Park *et al*., 2009). In this study we report the isolation and culturing of 18 new isolates of CLOs from oxygen-poor saline and marine habitats around the world. These new isolates are sufficiently distinct in morphology and/or in molecular comparisons to represent several new genus-level groups. We now must envisage CLOs as a phylogenetic cloud of at least six major lineages at the base of the diplomonad-retortamonad-*Carpediemonas* clade (i.e. Fornicata). The existence of such a wide diversity of CLOs was unanticipated, based on both historical microscopy/culturing efforts and recent environmental PCR surveys. This example suggests that a considerable number of evolutionarily important lineages of microbial eukaryotes may still be undiscovered and that culturing approaches remain a valuable avenue for understanding the scope of microbial eukaryotic diversity.

Results

New isolates

We have cultured 18 new isolates of CLOs from marine/saline locations around the world (Table 1). Light microscopy observations of the new isolates show that they usually have a typical excavate morphology, e.g. a visible feeding groove associated with the posterior flagellum (Fig. 1). Most, but not all, broadly resemble *Carpediemonas membranifera* and *D. brevis* in that they are small bean- or crescent-shaped cells that swim relatively slowly with slow rotation or no rotation. One isolate, BICM, is very similar in appearance to the original culture of *C. membranifera* (isolate QB) (Fig. 1A and Table 2). Eleven of the new isolates (isolates GR1, PPP15C, LARNAKA2, NY0173, NY0166, ALLEPEYI, KR3, KR4, KR7, KR8

Table 1. Sampling sites and culture media for all cultured *Carpediemonas*-like organisms.

Isolate	Clade	Sampling site	Sampling environment	Media	Taxonomic classification
SIVOTA	CL1	Sivota, Greece (39°23′N, 20°14′E)	Littoral anoxic sediments	802SW	*Dysnectes* sp.
NY0165*	CL1	Kagoshima, Japan (31°20′N, 130°63′E)	Littoral anoxic sediments	NM	*Dysnectes brevis*
PCE	CL2	Prince Cove, USA (41°38′N, 70°24′W)	Littoral anoxic sediments	SW1773	Putative new genus A
NY0171	CL2	Ishigaki Island, Japan (24°48′N, 124°23′E)	Littoral anoxic sediments	NM	Putative new genus A
PCS	CL3	Prince Cove, USA (41°38′N, 70°24′W)	Littoral anoxic sediments	SW1773	Putative new genus B
SB*	CL3	BC, Canada (48°46′N, 123°28′W)	Littoral anoxic sediments	T/S	*Hicanonectes teleskopos*
BICM	CL4	BC, Canada (48°46′N, 123°28′W)	Littoral anoxic sediments	3%LB in 50%SW	*Carpediemonas membranifera*
QB*	CL4	Quibray Bay, Australia (34°02′S, 151°10′E)	Littoral anoxic sediments	3%LB in 50%SW	*Carpediemonas membranifera*
NC	CL5	Nebraska, USA (40°95′N, 96°72′W)	Inland salt marsh sediments	802SW/Horse serum	Putative new genus C
CL	CL5	Mahone Bay, Canada (44°26′N, 64°21′W)	Littoral anoxic sediments	T/S	Putative new genus C
GR1	CL6	Marmara, Greece (38°08′N, 22°21′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
PPP15C	CL6	Halifax, Canada (44°37′N, 63°33′E)	Littoral anoxic sediments	NM	*Kipferlia bialata*, n. gen., n. comb.
LARNAKA2	CL6	Larnaka, Cyprus (34°54′N, 33°38′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
NY0173	CL6	Sagami Bay, Japan (35°0.09′N, 139°13.51′E)	Deep sea anoxic sediments (~1.1 km)	NM	*Kipferlia bialata*, n. gen., n. comb.
NY0166	CL6	Kagoshima, Japan (31°20′N, 130°63′E)	Littoral anoxic sediments	NM	*Kipferlia bialata*, n. gen., n. comb.
ALLEPEYI	CL6	Allapuzha, India (28°07′N, 76°19′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
KR3	CL6	Adelianos Kampos, Greece (35°22′N, 24°32′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
KR4	CL6	Adelianos Kampos, Greece (35°22′N, 24°32′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
KR7	CL6	Adelianos Kampos, Greece (35°22′N, 24°32′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
KR8	CL6	Adelianos Kampos, Greece (35°22′N, 24°32′E)	Littoral anoxic sediments	802SW	*Kipferlia bialata*, n. gen., n. comb.
GSML	CL6	Apalachee bay, USA (~30°04′N, 84°10′W)	Detritus in shipment of sea urchins	802SW	*Kipferlia bialata*, n. gen., n. comb.

Previously described isolates are marked with an asterisk (*). Media formulations are given in *Supporting information* (Table S1).

Fig. 1. Light microscopic photographs of *Carpediemonas* and *Carpediemonas*-like organisms. (A) *Carpediemonas membranifera* QB (source A.G.B. Simpson, unpublished), (B) *C. membranifera* BICM, (C) *Kipferlia bialata* n. gen, n. comb. (source Micro*scope, original micrograph by Won Je Lee), (D) *K. bialata* n. gen, n. comb., isolate KR8, (E) *Dysnectes brevis* NY0165, (F) *Dysnectes* sp., SIVOTA, (G) *Hicanonectes teleskopos* SB, (H) *Carpediemonas*-like organism CL, (I and J) *Carpediemonas*-like organism NC, (K and L) *Carpediemonas*-like organism PCE, (M and N) *Carpediemonas*-like organism NY0171, (O and P) *Carpediemonas*-like organism PCS. Photographs of previously described organisms, *C. membranifera* QB, *K. bialata*, *D. brevis* and *H. teleskopos*, are included for comparative purposes. Scale bar is 5 μm for all figures.

Table 2. Morphological characteristics of *Carpediemonas*-like organisms.

Species/isolate	Clade	Cell shape	No. of flagella	Length of posterior flagellum	Swimming pattern
Carpediemonas membranifera QB	CL4	Bean shaped	2	~3.5× cell length	Slow, with a slow wobbling
Carpediemonas membranifera BICM	CL4	Bean shaped	2	~3.5× cell length	Slow, with a slow wobbling
Dysnectes brevis	CL1	Bean shaped	2	~1× cell length	Very slow, often adheres to surfaces
Hicanonectes teleskopos	CL3	Oval shaped	2	~3× cell length	Relatively fast with rapid rotation
CL	CL5	Bean shaped	2	~2× cell length	Slow with slow rotation/slow wobbling
NC	CL5	Bean shaped	2	~2× cell length	Slow with slow rotation/slow wobbling
PCS	CL3	Spindle shaped	1	~1× cell length	Slow, jerky
NY0171	CL2	Oval shaped	2	~2× cell length	Relatively fast with slow, jerky rotation
PCE	CL2	Oval shaped	2	~2× cell length	Relatively fast, with rapid rotation
SIVOTA	CL1	Bean shaped	2	~1× cell length	Very slow
Kipferlia bialata n. gen n. comb.	CL6	Bean shaped	2	~1.5× cell length	Very slow, often adheres to surfaces, rapid beating of the anterior flagellum

and GSML) are indistinguishable from the previously described morphospecies *Carpediemonas bialata*, which has not been cultured before, and is little-studied (Fig. 1C and D, Table 2). The other isolates all appear to belong to undescribed species as they neither correspond morphologically to previously described species, nor are they very similar at the molecular level (see Table 3). Isolate SIVOTA, which has a short posterior flagellum, resembles *D. brevis*, except the cell shape tends to be more elongated (Fig. 1E and F, Table 2). Isolates CL and NC are bean-shaped cells with a visible groove and a free-trailing posterior flagellum that is approximately twice the length of the cell (Fig. 1H–J, Table 2). Isolates NY0171 and PCE differ substantially from the isolates discussed above – both are oval-shaped cells with a slightly curved feeding groove and they rotate when swimming (Fig. 1K–N, Table 2). Isolate PCS is rod-shaped with a flattened area at the anterior end of the cell (possibly the remnant of the excavate groove), where beats the single visible flagellum. Thus PCS differs substantially from previously described species and from all other new isolates (Fig. 1O and P, Table 2).

SSU rRNA gene phylogeny of new isolates

In the phylogenetic analysis of SSU rRNA gene sequences we included our 18 new isolates of CLOs, plus *C. membranifera* QB, *D. brevis* and *H. teleskopos*, 15 environmental SSU rRNA gene sequences similar to those from CLOs as identified by BLAST (Edgcomb *et al*., 2002; Stoeck *et al*., 2007; Takishita *et al*., 2007a), 28 sequences representing diplomonads and retortamonads, and 31 outgroup taxa representing most other major eukaryotic groups. All the CLOs, diplomonads and retortamonads collectively constitute a monophyletic group that we equate with the taxon Fornicata, with high statistical support [97% bootstrap proportion (bp) and a posterior probability (pp) of 1]. Diplomonads and the genus *Retortamonas* form a highly supported clade (100% bp and 1 pp), while the retortamonad *Chilomastix* branches as a sister group to the clade of diplomonads plus *Retortamonas*, but with a very low bootstrap support.

Table 3. Uncorrected genetic distance between and within each CLO clade (SSU rRNA gene).

	CL2	CL3	CL4	CL5	CL6	*Octomitus*	Internal
CL1	0.28	0.3	0.27	0.25	0.27	0.27	0.02
CL2		0.3	0.33	0.3	0.34	0.32	0.01
CL3			0.3	0.29	0.31	0.32	0.22
CL4				0.28	0.31	0.31	0.04
CL5					0.29	0.28	0.1
CL6						0.32	0.01

For context, genetic distances between all the CLO clades and the diplomonad *Octomitus* are also included.

All CLOs branch basally to diplomonads and retortamonads as a non-monophyletic assemblage. The CLOs form six highly distinct and strongly supported clades, here called CL1–CL6 (Fig. 2, Table 1). Clade CL1 contains *D. brevis* and isolate SIVOTA. Clade CL2 is formed by the very similar new isolates NY0171 and PCE. *Hicanonectes teleskopos* (isolate SB), the new isolate PCS and a single environmental sequence, D4P08A09, branch together as clade CL3, although PCS plus D4P08A09 are a group distinct from *H. teleskopos* within this clade. *Carpediemonas membranifera* and new isolate BICM constitute clade CL4, which represents the genus *Carpediemonas* itself. Clade CL5 contains only the new isolates CL and NC. Clade CL6 is a tight cluster containing the rest of the new isolates (GR1, PPP15C, LARNAKA2, NY0173, NY0166, ALLEPEYI, KR3, KR4, KR7, KR8 and GSML) and all environmental sequences except D4P08A09. While clades CL1–CL6 form the basal branches within Fornicata, their interrelationships are essentially unresolved. In the maximum likelihood tree CL1 and CL2 form an unsupported monophyletic group (19 bp; 0.55 pp), and collectively constitute the closest relative of the diplomonads-retortamonads clade, with no

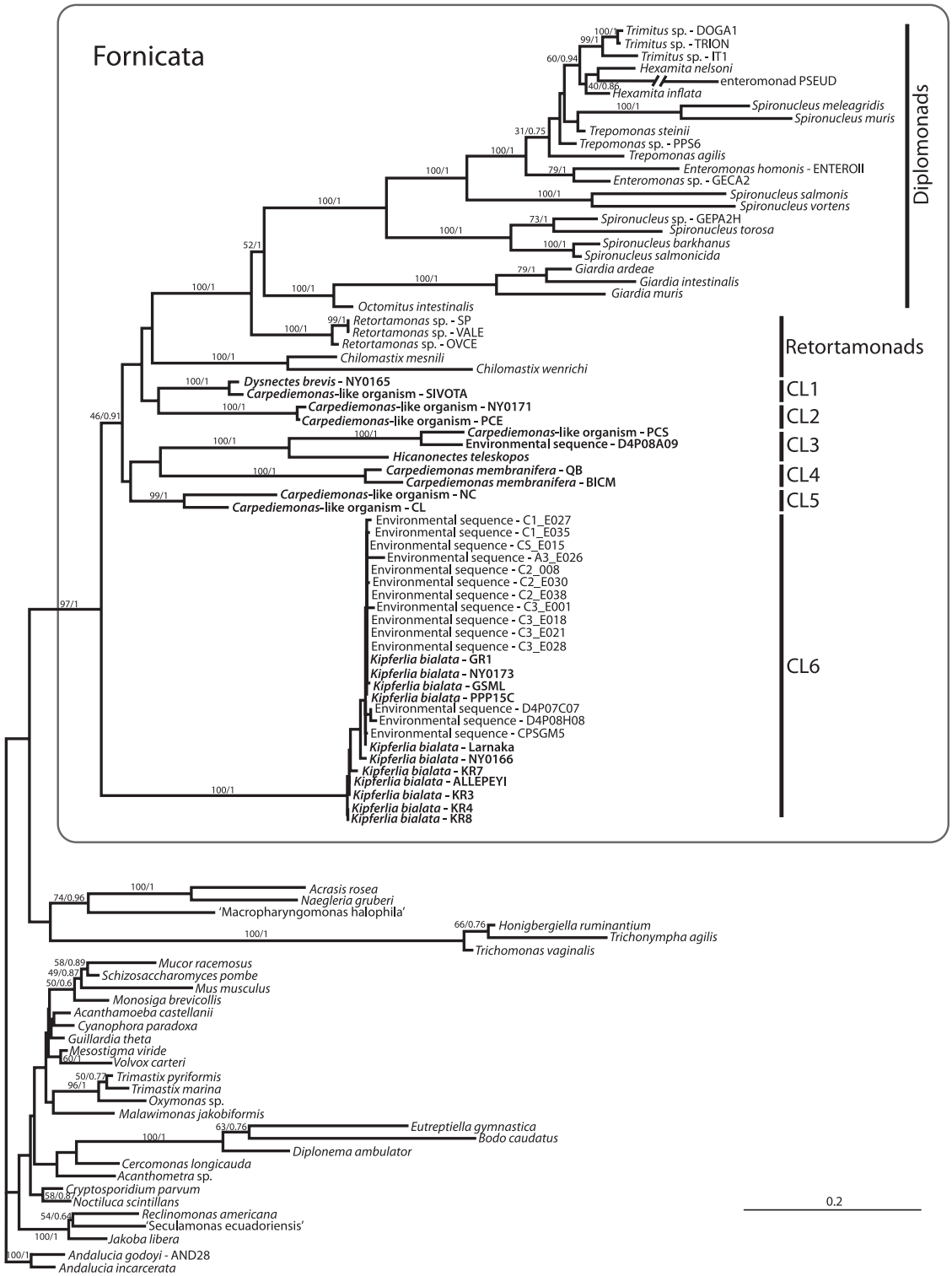

Fig. 2. Maximum likelihood tree based on SSU rRNA genes from Fornicata, including new isolates of *Carpediemonas*-like organisms (CLOs). The tree is rooted using a 31-taxon eukaryotic outgroup. The GTR + I + Γ model of sequence evolution was used. *Carpediemonas* and *Carpediemonas*-like organisms are depicted in bold. Statistical support is as follows: bootstrap proportion, based on 10 000 replicates/MrBayes posterior probabilities. Statistical support is not shown for nodes with support lower than 50 bp and 0.7 pp.

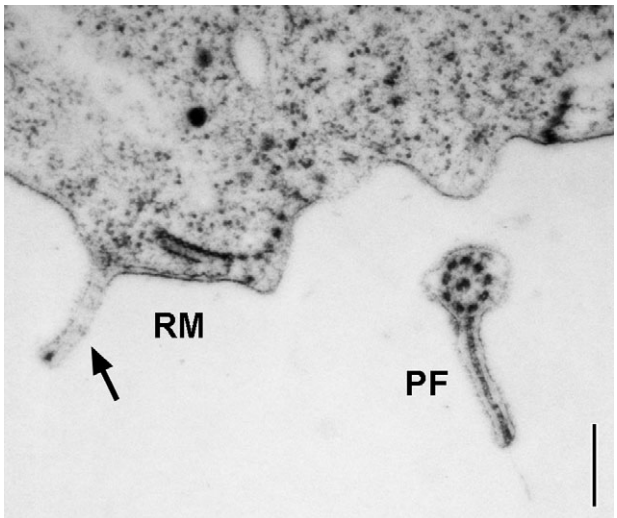

Fig. 3. Transmission electron micrograph of isolate NY0173 of *Kipferlia bialata* n. gen. n. comb. (clade CL6), showing the right portion of the ventral groove in transverse section. The image is shown looking from anterior to posterior, with the ventral side of the cell facing downwards, thus the right side of the cell is towards the left side of the micrograph. The right margin of the groove (RM) is extended by a substantial membrane (arrow). The poster flagellum (PF) bears a single broad vane on its ventral side. Scale bar represents 500 nm.

statistical support. Clades CL3, CL4 and CL5 constitute a separate monophyletic group, but again with no statistical support (11 bp and 0.51 pp). Clade CL6, representing the *C. bialata* morphospecies (here renamed *Kipferlia bialata* n. gen. n. comb., see below), branches independently as the most basal group of Fornicata, with very weak support (46 bp and 0.91 pp). There is no evidence of a specific relationship between CL6 and CL4 (i.e. *Carpediemonas* proper).

Electron microscopy of NY0173

Preliminary transmission electron microscopy of isolate NY0173 from clade CL6 (i.e. *Kipferlia bialata* n. gen. n. comb.) shows that the right margin of the groove is extended substantially into a thin membrane (Fig. 3). The posterior flagellum bears a broad ventral vane, but a dorsal vane is absent or very small (Fig. 3).

Discussion

Most of our new isolates apparently represent novel lineages at least at the 'genus level', based mainly on the dissimilarity of their SSU rRNA genes (Table 3) and pattern of phylogenetic relationships (Fig. 2). The only new isolate that is assignable to a previously cultured species is BICM, which is indistinguishable by light microscopy from *C. membranifera* isolate QB (Simpson and Patterson, 1999) and is very closely related in the SSU rRNA gene phylogeny (uncorrected genetic distance is 0.04). The new isolates in clade CL6 are indistinguishable in light microscopic appearance to the previously described, but never cultured or sequenced, *C. bialata* (Lee and Patterson, 2000). The CL6 isolates are also nearly identical to each other in their SSU rRNA gene sequence (the average uncorrected genetic distance within the clade is 0.01). Therefore we consider that they all represent this one species. However, genetic distance between clade CL6 and *C. membranifera*, the type species for the genus *Carpediemonas* (CL4), is considerable (0.31 – the same distance as between *C. membranifera* and the diplomonad genus *Octomitus*, Table 3), and the two groups do not constitute a clade in our SSU rRNA gene tree. There are also substantive ultrastructural differences: The membrane-like extension of the right margin of the groove in CL6 is not seen in *Carpediemonas*, nor in other CLOs examined to date. CL6 lacks the broad dorsal flagellar vane that is characteristic of *Carpediemomas* among CLOs. The organism currently called *Carpediemonas bialata* should therefore be considered a member of a separate genus. We propose the new genus *Kipferlia* n. gen., and transfer *C. bialata* to this new genus as its type species, *Kipferlia bialata* (Ruinen, 1938) n. comb. (see taxonomic summary below).

Clades CL2 (isolates NY0171 and PCE) and CL5 (isolates CL and NC) are markedly dissimilar in SSU rRNA gene sequence from all formally described genera, and neither shows a reliable sistergroup relationship with members of a described genus. Isolate PCS does form a robust phylogenetic relationship with *Hicanonectes* (both are within clade CL3), but the genetic dissimilarity between the two is still substantial (0.22), and they are unalike morphologically. It is very likely that each of these three groups will be also be recognized as a new genus in the future. Isolate SIVOTA is most closely related to *D. brevis* but is molecularly distinguishable (Table 3), and differs slightly in appearance (Fig. 1E and F, Table 2), and might be considered as a separate species. The further characterization of these other new isolates and determination of their possible assignment into new genera will be the subject of future work.

The availability of a wide diversity of basal lineages within Fornicata will be valuable for understanding the evolution of diplomonads and their mitochondrial organelles. For example, it will now be possible to perform comparative analyses of inferred mitochondrial proteins in several different *Carpediemonas*-like lineages, together with diplomonads/retortamonads. An important prerequisite for such comparative analyses is a robust understanding of the actual phylogenetic relationships among the *Carpediemonas*-like lineages, and their relationship to diplomonads and retortamonads. Unfortunately, these deep relationships are poorly and/or inconsistently sup-

ported in SSU rRNA gene phylogenies. For example, in our analysis, clade CL6 is recovered as the most basal clade of Fornicata, consistent with previous analyses in which CL6 is represented by environmental sequences (Takishita *et al*., 2007a; Park *et al*., 2009), but support decreases as taxon sampling increases. Meanwhile, like Park and colleagues (2009) and Cepicka and colleagues (2008) we recover a clade that includes *Dysnectes* as the closest relatives of the diplomonad-retortamonad clade, but this conflicts with the analysis of Yubuki and colleagues (2007). It seems that considerably more sequence data (i.e. additional genes for analysis) will be necessary to reliably resolve the relationships among the major clades of CLOs.

Until very recently (2007), *C. membranifera* was the only species other than diplomonads and retortamonads within the clade Fornicata, and appeared to be a phylogenetically isolated organism. It is now clear that *C. membranifera* is merely one representative of a 'cloud' of free-living CLOs. CLOs were mostly undetected by both historical microscopy-based studies and more recent environmental PCR approaches. The very limited detection of CLO sequences in clone libraries generated from environmental PCR is particularly striking, as the habitats in which these organisms have been isolated – low-oxygen marine/saline sediments – have been frequently sampled (Dawson and Pace, 2002; Edgcomb *et al*., 2002; Stoeck *et al*., 2003, 2007; Takishita *et al*., 2005, 2007a,b; Behnke *et al*., 2006; Epstein and López-García, 2008). Only three of these studies recovered CLO sequences, although only two reported them in their results (Edgcomb *et al*., 2002; Stoeck *et al*., 2007; Takishita *et al*., 2007a), and all but one of the environmental sequences were from just one of the six CLO clades – CL6. Environmental PCR-based studies of low-oxygen marine/saline water column sites have also not recovered CLO sequences (Stoeck *et al*., 2003; Behnke *et al*., 2006).

Some very recent studies use 454 sequencing of SSU rDNA environmental PCR samples to examine protist diversity (Stoeck *et al*., 2009; 2010), potentially providing a much deeper coverage of diversity than sequencing of clone libraries. We performed a detailed search for CLOs in two 454 environmental surveys of anaerobic environments (Stoeck *et al*., 2009; 2010; see *Supporting information*). We still identified representatives of just two CLO clades – CL1 and CL6 – all from a single sampling site (Stoeck *et al*., 2009).

It is likely that the true diversity of major lineages of CLOs is still greater than we have described in this study. All but one of our six major clades are represented by only two isolates, leaving the strong possibility that additional readily cultivable lineages have been missed through pure chance. Other lineages could occur in environments other than oxygen-poor saline sediment, or may simply require different culturing approaches. Still others may be difficult to culture and may be detected most effectively through environmental PCR with taxon-specific primers.

By far the most commonly encountered clade is CL6 (*Kipferlia bialata* n. gen., n. comb.), which includes over half (11/18) of our new isolates and all but one of the previously reported environmental sequences. This may suggest that clade CL6 is much more abundant in the environment than the other clades. Alternatively, clade CL6 may be over-represented due to the conditions for culturing and/or environmental PCR. Culturing bias cannot be ruled out as the majority of isolates from clade CL6 were isolated using 802SW media, while other isolates were usually obtained using various other types of media (Table 1). It is possible that 802SW media selects for CL6 over the other clades. In contrast, we found little evidence to suspect a PCR bias towards CL6. SSU rRNA gene sequences from this clade do not constitute better targets for the particular PCR primers used by the environmental studies that yielded CL6 sequences (Edgcomb *et al*., 2002; Takishita *et al*., 2007a). Also we have performed preliminary experiments on mixtures of DNA from different CL clades, and did not find a strong PCR bias towards representatives of clade CL6 (data not shown).

Concluding remarks

The current understanding of the diversity of single-celled eukaryotes is based on microscopy and culturing going back more than 150 years, and more recently on environmental PCR surveys (López-García *et al*., 2001; Bass and Cavalier-Smith, 2004; Groisillier *et al*., 2006; Massana *et al*., 2006; Not *et al*., 2007; Epstein and López-García, 2008). We have explored an important 'region' of the eukaryotic tree that was, until recently, seemingly populated by a single isolated lineage. Our application of straightforward culturing techniques revealed a large number of very distinct lineages in this region of the tree. Moreover, these were isolated from marine anoxic sediments (except isolate GSML), a relatively easily accessible and often-sampled habitat type. The bulk of these lineages had been completely missed both by the historical microscopy/culturing efforts, and by environmental PCR endeavours targeting similar habitats.

The reasons for this limited prior detection of the diversity of CLOs are not clear, but might involve a low abundance of most of these organisms in the environment. Indeed, some of the environmental sequences in clade CL6 (CPSGM5) were detected only after crude enrichment (Takishita *et al*., 2007a). In addition, we cannot

exclude the possibility that a role is played by an experimental bias in environmental PCR studies other than primer–target mismatch.

We see no good reason to assume that the overlooking of major-lineage-level diversity we report is unique to the base of Fornicata. We suggest it is more likely that undersampling at the level of major lineages could still be widespread for microbial eukaryotes. Our understanding of eukaryotic evolution would be greatly advanced if this were overcome. It would be particularly interesting to see whether other phylogenetically isolated but evolutionarily important lineages such as *Chromera* (Moore *et al*., 2008) are in fact the tips of large 'icebergs' of high-level lineage diversity. A combination of raw culturing effort and much deeper environmental PCR sampling, perhaps coupled with the use of enrichments (i.e. 'semi-environmental' samples), and/or taxon-specific primers may help to capture a larger portion of the diversity (Takishita *et al*., 2007a; Lara *et al*., 2009).

Taxonomic summary

The new genus *Kipferlia* is described here in accordance with the International Code of Zoological Nomenclature (ICZN, 1999).

***Kipferlia* n. gen.**

Diagnosis. Free-living, biflagellated and colourless cells with a conspicuous ventral groove. The right margin of the groove is markedly extended by a fine membrane, visible by electron microscopy. The posterior flagellum beats within the groove, and bears a single broad vane, located ventrally. Inhabits low-oxygen marine environments. Similar to *Carpediemonas* and *Dysnectes* in typical habitat and general appearance when viewed by light microscopy, but distinct from both in SSU rRNA gene phylogenies (see Fig. 2).

Type species. *Kipferlia bialata* (Ruinen, 1938) n. comb.

Other species. None described

Etymology. Kipferln (sing. Kipferl; German) are small crescent-shaped cookies from southern Germany and Austria. The name refers to the shape of the type species. The name *Kipferlia* is considered to be of feminine gender, in agreement with the species epithet for the type species.

Taxonomic assignment: Eukaryota; Excavata; Fornicata

***Kipferlia bialata* (Ruinen, 1938) n. comb.**

Basionym. *Cryptobia bialata* Ruinen, 1938

Other synonyms. *Carpediemonas bialata* (Ruinen, 1938) Lee and Patterson 2000.

Comments. Originally described in 1938 as *Cryptobia bialata* (Ruinen, 1938), this organism was next identified as a distinct morphospecies by Lee and Patterson (2000), who renamed it *Carpediemonas bialata*.

Experimental procedures

Culture isolation and light microscopy

All isolates except isolate GSML were cultured from anoxic sediments; the locations of the sampling sites, as well as media used for cultivation, are listed in Table 1 (media formulations are given in *Supporting information*, Table S1). Isolate GSML was cultured from detritus accompanying a shipment of sea urchins collected in an estuarine bay (Gulf Specimen Marine Lab cat# E-1610) and received at the University of Arkansas. Monoeukaryotic cultures were usually established via transferring the cultures at the point where CLOs were the most common eukaryotes, which slowly diluted out other eukaryotes. Isolates CL and BICM were purified away from ciliates by filtering the culture through 3 μm filters. A single cell of each of four isolates: NY0165 (CL1), NY0166 (CL6), NY0171 (CL2) and NY0173 (CL6) was isolated by micropipeting from the enrichment culture and inoculated into the medium, which was prepared as a low-oxygen environment beforehand. All cultures were xenic and grown in nutrient-rich media (see *Supporting information*). The low-oxygen environment was maintained by high bacterial growth and by the large volume of media relative to the size of the culturing tubes (i.e. a small headspace). The actual oxygen levels were not monitored. Light microscopy observations utilized a Zeiss Axiovert 200M microscope equipped with an Axiocam HR digital camera (Zeiss, Germany), a Leica DMR light microscope (Leica, Germany) equipped with a Keyence VB6010 digital chilled CCD camera (Keyence, Osaka, Japan), a Zeiss Axioskop 2 equipped with a JVC KY-F75U colour digital camera using Automontage (Syncroscoy, Frederick, MD) or an Olympus BX51 microscope BX51 and Olympus DP70 camera (Olympus America). For transmission electron microscopy (Fig. 3), cells were high-pressure frozen using a Leica HPM100. The procedure for the high-pressure freezing fixation, dehydration and embedding was the same as that described by Yubuki and colleagues (2010). Ultra-thin sections were cut on a Leica EM UC6 ultra-microtome, double stained with 2% (w/v) uranyl acetate and lead citrate and observed using a Hitachi H7600 electron microscope.

DNA isolation and sequencing

The DNA was isolated from the cultures using the CTAB (cetyltrimethylammonium bromide) protocol of Clark (1992), a modified CTAB protocol (Ishida *et al*., 1999), a simple phenol/chloroform protocol (Garriga *et al*., 1984), a High Pure PCR template kit (Roche Applied Science, UK), or a Gentra PureGene DNA isolation kit (Qiagen, USA). Universal eukaryotic primers 5′ primer A, 3′ primer B (Medlin *et al*., 1988), or 18S Fw (5′-aacctggttgatcttgccag-3′) and 18S Re (5′-cygcaggttcacctacggaa-3′) were used to amplify almost-complete SSU rRNA genes of all but one isolate (PCS). The SSU rRNA gene of isolate PCS was amplified using 5′ primer A and PCS_1600R (5′-ccatgtccaaacaacttgcc-3′). Fragments of expected size were purified from agarose gels using the Qiagen Gel extraction kit (Qiagen, USA) or GeneElute Gel extraction kit (Sigma-Aldrich, USA) and either directly sequenced or cloned using the TOPO-TA cloning kit (Invitrogen, USA) or Promega T-easy Vector

system (Promega, USA). In the latter cases, several clones were partially sequenced and their identity was checked using BLAST (Altschul *et al*., 1990) before 1–10 pooled clones were fully bidirectionally sequenced by an oligonucleotide primer-walking approach. All 18 new sequences are deposited in GenBank database under Accession No. GU827588–GU827605.

Phylogenetic analyses

A eukaryotic secondary structure-based alignment was downloaded from the European SSU rRNA gene database (http://bioinformatics.psb.ugent.be/webtools/rRNA/). Missing and new taxa were realigned to the downloaded alignment with the program CLUSTALX (Thompson *et al*., 1997). The final dataset contained 63 Fornicata sequences and 31 sequences from other eukaryotes. The resulting alignment was edited by eye and ambiguously aligned regions were discarded, leaving 914 nt positions. The relatively low number of truly unambiguously aligned positions was due to the divergent nature of diplomonad SSU rRNA genes.

The phylogenetic trees were constructed using maximum likelihood and Bayesian methods. The GTR + I + Γ model of sequence evolution was selected by the Akaike information criterion implemented in the program Modeltest7.0 (Posada and Crandall, 1998). The maximum likelihood tree was constructed using PAUP*4b10 (Swofford, 2002) with 20 random sequence stepwise addition replicates and tree-bisection-reconnection rearrangements. Bootstrap support was estimated from 10 000 bootstrap replicates using RAxML 7.0 (GTR + I + Γ) (Stamakis, 2006). The Bayesian analyses was performed with MrBayes 3.1 (Huelsenbeck, 2000) using a GTR + I + Γ model and was run for 20 million generations (stationarity was reached after 500 000 and burnin was set to 500 000 generations, while other parameters were left at their default values).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Media formulations used to cultivate *Carpediemonas*-like organisms.

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