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ORIGINAL ARTICLE

Morphological Identities of Two Different Marine Stramenopile Environmental Sequence Clades: *Bicosoeca kenaiensis* (Hilliard, 1971) and *Cantina marsupialis* (Larsen and Patterson, 1990) gen. nov., comb. nov.

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Keywords
Bicosoecid; biodiversity; culture-independent molecular methods; environmental DNA sequence surveys; MAST; molecular phylogeny; protist; ultrastructure.

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ABSTRACT

Although environmental DNA surveys improve our understanding of biodiversity, interpretation of unidentified lineages is limited by the absence of associated morphological traits and living cultures. Unidentified lineages of marine stramenopiles are called "MAST clades". Twenty-five MAST clades have been recognized: MAST-1 through MAST-25; seven of these have been subsequently discarded because the sequences representing those clades were found to either (1) be chimeric or (2) affiliate within previously described taxonomic groups. Eighteen MAST clades remain without a cellular identity. Moreover, the discarded "MAST-13" has been used in different studies to refer to two different environmental sequence clades. After establishing four cultures representing two different species of heterotrophic stramenopiles and then characterizing their morphology and molecular phylogenetic positions, we determined that the two different species represented the two different MAST-13 clades: (1) a lorica-bearing *Bicosoeca kenaiensis* and (2) a microaerophilic flagellate previously named "*Cafeteria marsupialis*". Both species were previously described with only light microscopy; no cultures, ultrastructural data or DNA sequences were available from these species prior to this study. The molecular phylogenetic position of three different "*C. marsupialis*" isolates was not closely related to the type species of *Cafeteria*; therefore, we established a new genus for these isolates, *Cantina* gen. nov.

ENVIRONMENTAL DNA sequence surveys combined with microscopic observations have greatly improved our knowledge of eukaryotic microbial diversity, especially from uncultured species (Edgcomb and Bernhard 2013; Kim et al. 2011; Kolisko et al. 2010; Takishita et al. 2010; Tarbe et al. 2011; Wylezich and Jürgens 2011). However, most of the sequences recovered from environmental DNA sequence surveys are unidentified at the cellular level and are deposited in GenBank with ambiguous labels, such as "uncultured eukaryotes". There are two types of sequences designated as uncultured eukaryotic lineages: (1) sequences from novel and undescribed organisms and (2) sequences from previously described species that lack any associated DNA sequence information. In the latter case, morphological information obtained before the routine use of molecular methods and more recently acquired DNA sequences from the same species remain unlinked.

Stramenopiles represent a huge group of photosynthetic eukaryotes that play an important ecological role as

primary producers (e.g. kelps and diatoms); the group also comprises a diverse assortment of non-photosynthetic lineages that function as consumers (e.g. bicosoecids and heterotrophic and mixotrophic chrysophytes), decomposers (e.g. water molds and labyrinthulids) and parasites (e.g. *Blastocystis*) (Andersen 2004; Arndt et al. 2000; Patterson 1989; Zubkov et al. 2001). Environmental PCR surveys of small subunit ribosomal RNA (SSU rRNA) gene sequences from diverse environments have demonstrated a large number of marine stramenopile (MAST) clades without clear cellular identities (Massana et al. 2004). These clades represent cosmopolitan species and encompass a great deal of stramenopile diversity (Gómez et al. 2011; Kolodziej and Stoeck 2007; Massana et al. 2004; Rodríguez-Martínez et al. 2012; Takishita et al. 2010; Zuendorf et al. 2006). Because many stramenopiles are very small (< 10 μm) and lack easily recognizable features, identifying, and describing the organisms corresponding to the MAST clades is challenging. Twenty-five MAST clades have been identified and labeled MAST-1 through MAST-25; however, seven of these MAST clades have been discarded because (1) MAST-5 was found to be a chimeric sequences from a labyrinthulid and a dinoflagellate and (2) six of these clades were shown to affiliate with previously described taxonomic groups (e.g. MAST-14 was subsequently identified as members of the Bicosoecida). Therefore, there are currently 18 MAST clades without a clear cellular identity (Massana et al. 2014). Because they represent a diverse collection of small heterotrophic eukaryotes that diverged before the clade of photosynthetic stramenopiles, improved knowledge of these MAST clades is expected to play an important role in our understanding of the early evolutionary history of stramenopiles as a whole. For example, the anaerobic MAST-12 clade has been unexpectedly shown to be the sister clade to parasitic lineages like the human pathogen, *Blastocystis* (Gómez et al. 2011; Massana et al. 2014). The organisms representing MAST-12 are critical for understanding the evolution of parasites from free-living ancestors, the switch from oxic to anoxic lifestyles and the associated transformation of canonical mitochondria to hydrogenosomes. Nonetheless, the cellular identities of only two clades (MAST-3 and MAST-12) have been demonstrated with morphological data (Gómez et al. 2011; Kolodziej and Stoeck 2007), and a representative from only one of these clades (MAST-3) has been successfully brought into culture (Cavalier-Smith and Scoble 2013) since the first report of MAST clades by Massana et al. (2004).

One important point of confusion is that there are two different "MAST-13" clades that have been reported in the literature. One MAST-13 clade branches within the Bicosoecida (Zuendorf et al. 2006). Another "MAST-13" clade was described by Takishita et al. (2007) as a novel environmental sequence clade from an anaerobic environment, namely NAMAKO-31. Subsequent studies have propagated the double meaning for MAST-13. On one hand, Kolodziej and Stoeck (2007), Park and Simpson (2010), Orsi et al. (2011), Cavalier-Smith and Scoble (2013), and Massana et al. (2014) discussed only Zuendorf et al.'s version of MAST-13 and did not consider Takishita et al.'s version of MAST-13; however, Zuendorf et al.'s version of MAST-13 has been subsequently excluded from the MAST clades (Massana et al. 2014) because of its clear affiliation with the Bicosoecida. On the other hand, Takishita et al.'s (2010) analysis of environmental DNA sequences included only their version of MAST-13 (NAMAKO-31), but not Zuendorf et al.'s version of MAST-13 lineages. Only SSU rRNA gene sequences are available from these environmental sequence clades, and these sequences have never been studied together within the same phylogenetic analysis. Zuendorf et al.'s version of MAST-13 has been demonstrated to be a member of Bicosoecida; however, there is no detailed morphological data available from any of these particular organisms in question. In addition, the lack of cellular identities for these two lineages magnifies how poorly we understand the MAST clades in general.

The aims of this study were to (1) isolate cells that represent each of the two MAST-13 clades from environments that are similar to those where the two lineages had been reported, (2) establish the culture conditions necessary to maintain these isolates, (3) determine the molecular phylogenetic positions of these isolates on the basis of SSU rRNA gene sequences, and (4) describe the morphological traits of these isolates using light and transmission electron microscopy. The four isolates we studied here are very similar to two different flagellate species that have been reported from all over the world using light microscopy alone: *Bicosoeca kenaiensis* and "*Cafeteria*" *marsupialis* (Hilliard 1971; Larsen and Patterson 1990); no established cultures, ultrastructural data or DNA sequences were available from these flagellates prior to this study. The SSU rRNA gene sequences generated from our isolates branched separately, each within one of the two different "MAST-13" clades. These data allowed us to demonstrate the cellular identities for both clades and address taxonomic problems associated with the molecular phylogenetic position of "*Cafeteria*" *marsupialis*.

MATERIALS AND METHODS

Establishment, maintenance, and identification of cultures

An isolate with identical morphological traits as *B. kenaiensis* (NY0214) Hilliard 1971; was collected from surface water in the northwestern Pacific Ocean at Station 1 (29°34′38″ N, 144°34′20″E) on February 14, 2011 during a research cruise (MR 11-02) from February 11 to March 9, 2011 on the research vessel Mirai (Japan Agency for Marine-Earth Science and Technology—JAMSTEC). This culture is maintained in seawater with a grain of barley at 16 °C. Three different isolates with identical morphological traits of *Cafeteria marsupialis* Larsen and Patterson 1990 (=*Cantina marsupialis* gen. nov., comb. nov.) were isolated for this study. Isolate NY0206 was collected from soft mud samples in River Port, Nova Scotia, Canada

(44°17′42″N, 64°19′7″W) on May 18, 2011. Isolate YPF1205 was collected from the sediment of the lagoon, Kai-ike (Satsumasendai, Kagoshima, Japan) (31°51′34″N, 129°52′31″E) on June 7, 2012. Isolate BUSSELTON was collected from a sandy beach in Busselton, Western Australia, Australia (33°38′38″S, 115°20′46″E) on April 24, 2010. NY0206 and YPF1205 are maintained in 3.3% Lysogeny Broth (LB) medium in anaerobic seawater at 16 °C, and BUSSELTON was maintained in seawater-based ATCC 1525 medium in microaerophilic conditions at room temperature. A monoeukaryotic culture of *C. marsupialis* (NY0206) has been deposited at the American Type Culture Collection (ATCC), Manasas VA. *Cantina marsupialis* (YPF1205) has been deposited at the National Institute for Environmental Studies (NIES), Tsukuba, Japan (accession number: NIES-3354). BUSSELTON died after 3 yr of continual (weekly) sterile transfers.

Light and transmission electron microscopies

Light micrographs of *B. kenaiensis* (NY0214) and *C. marsupialis* (NY0206) were taken with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a Leica DC500 digital camera (Leica Microsystems Digital Imaging, Cambridge, UK). Light microscopy on *C. marsupialis* (YPF1205) was performed using an Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a digital camera ORCA-ER (Hamamatsu Photo-nics KK, Hamamatsu, Japan). Light-microscopic observations of *C. marsupialis* (BUSSELTON) were performed using an Olympus Microscope BX51TF light microscope equipped with a DP70 color camera (Olympus Corporation, Tokyo, Japan) and Differential Interference Contrast. For transmission microscopy (TEM), cells of *B. kenaiensis* (NY0214) growing in a Lab-Tek II, eight well glass slide chamber (Nunc, Rochester, NY) were mixed with an equal volume of fixative containing 5% glutaraldehyde and 0.2 M sucrose in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature for 1 h. Cells were rinsed with 0.2 M sodium cacodylate buffer (pH 7.2) and then postfixed in 1% osmium tetroxide in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature for 1 h. The cells were then dehydrated through an ethanol series, which was then substituted with acetone. The cells were infiltrated with 1:1 mixture of JEMBED resin diluted with acetone and ultimately embedded in absolute resin.

Cells of *C. marsupialis* (NY0206) were collected in a 15-ml tube by centrifugation at 1,000 *g* for 5 min. The cell pellet was high-pressure frozen using the Leica EM HPM100 High Pressure Freezer. Cells were freeze-substituted in 1% osmium tetroxide and 0.1% uranyl acetate in HPLC grade acetone using the Leica Automatic Freeze Substitution chamber set to: −85 °C for 3 d, warming to −20 °C over 13 h and held at −20 °C for 8 h before warming to 4 °C over 12 h. The cells were washed in acetone, and then increasing concentrations of a 1:1 mixture of JEMBED resin diluted with acetone. The cells were infiltrated with 100% resin at room temperature for 8 h. The infiltrated samples were polymerized overnight at 65 °C.

Ultra-thin sections were cut on a Leica EM UC6 ultramicrotome and double stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963). The ultra-thin sections were observed using a Hitachi H7600 transmission electron microscope (Hitachi High Technologies Corporation, Tokyo, Japan).

DNA extraction, PCR amplification and phylogenetic analyses

Genomic DNA from *B. kenaiensis* (NY0214) and *C. marsupialis* (NY0206) were extracted using the MasterPure Complete DNA and RNA purification Kit (Epicentre, Maddison, WI). Polymerase chain reactions (PCR) were performed using PuRe Taq Ready-To-Go PCR beads kit (GE Healthcare, Buckinghamshire, UK). Nearly the complete SSU rRNA genes from the two strains were amplified using the following primers, PF1: 5′-GCGCTACCTGG TTGATCCTGCCAGT-3′ and R4: 5′-CCGTTAACGAACGA-GACCTC-3′. The PCR conditions were 95 °C for 2 min; 35 cycles involving 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; and 72 °C for 7 min. The amplified fragments were gel-purified using UltraClean 15 DNA Purification Kit (MO Bio, Carlsbad, CA) and sequenced directly. The extraction of DNA and the PCR amplification of an SSU rRNA gene sequence from the isolate YPF1205 followed the procedure described in Yabuki et al. (2010). Genomic DNA of *C. marsupialis* (BUSSELTON) was isolated using the ZR Genomic DNA II Kit™ (Zymo Research, Irvine, CA). Almost complete SSU rRNA gene was amplified using primers A (5′-CTGGTTGATCCTGCCAG-3′) and B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988) with an annealing temperature of 50 °C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, the Netherlands) and sequenced directly. To increase the diversity of stramenopile SSU rRNA gene sequence in the molecular phylogenetic analyses, an SSU rRNA gene sequence of *Pendulomonas adriperis* (Placididea, Stramenopiles) was also obtained using the same methods described above for NY0214. This new monoeukaryotic strain (NY0181) was isolated from sediment sample taken from Sooke, British Columbia, Canada (48°22′16″ N, 123°43′3″W) on April 24, 2008 and identified by light microscopy based on previous report (Al-Quassab et al. 2002; Karpov et al. 2001; Lee 2006; Tong 1997). The sequences of *C. marsupialis* (NY0206, BUSSELTON, YPF1205), *B. kenaiensis* (NY0214) and *P. adriperis* (NY0181) were deposited into GenBank/EMBL/DDBJ (accession numbers: KM816645–KM816649).

The new SSU rRNA gene sequences were added to the alignment used in Yubuki et al. (2010). The alignment consisted of bicosoecids, placidids, slopalinids, *Blastocystis*, labyrinthulids, hyphochytridiomycetes, oomycetes, *Developayella*, ochrophytes, and at least two sequences from each of the 18 MAST clades; seven sequences representing the Alveolata and Rhizaria were used as the outgroup. All ambiguously aligned sites were manually excluded using Mesquite version 2.75 (Maddison and Maddison 2011) and the 128-taxon alignment contained 1,250

unambiguously aligned nucleotide positions that were determined for the phylogenetic analyses. Maximum-likelihood (ML) analyses of the alignment were performed in Garli 2.0 under the general time reversible (GTR) model incorporating among-site rate variation approximated by a gamma distribution with the proportion of invariable sites (GTR + G+ I model) as suggested by the program jModelTest-2.1.6 (Darriba et al. 2012; Guindon and Gascuel 2003). ML bootstrap analyses were carried out for ML with 500 replicates to evaluate statistical reliability. Bayesian analyses were preformed with MrBayes v3.2.2 (Ronquist et al. 2011) using the same alignment. The program was set to operate with the GTR model incorporating among-site rate variation approximated by a gamma distribution and four Monte-Carlo-Markov chains starting from random trees. A total of 10,000,000 generations until the mean standard deviation of split frequencies based on last 75% of generations was lower than 0.01. The trees and log-likelihoods were sampled at 1,000 generation-intervals. The first 25% of the generations were discarded as burn-in using the sumt command. Posterior probabilities (PP) correspond to the frequency at which a given node was found in the postburn-in trees.

RESULTS

Comparative morphology of the new stramenopile isolates: *B. kenaiensis* (NY0214) and *C. marsupialis* gen. nov., comb. nov. (NY0206)

A single cell of *B. kenaiensis* was manually isolated with a micropipette to establish a mono-eukaryotic culture (NY0214) with environmental bacteria as a food source. The cells are ovoid, 2.42–6.21 μm long (average: 3.59 μm, $n = 30$), 2.25–3.67 μm wide (average: 3.00 μm, $n = 30$) and attach to the lorica with a posterior flagellum (Fig. 1). The anterior flagellum is about four times longer than the cell length and extends anteriorly when the cell is sitting in the lorica. The posterior flagellum contracts and the cell withdraws into the lorica when disturbed, and the anterior flagellum rolls up in to tight coil at the anterior end of the cell (Fig. 1C, 2B). The cells have a conspicuous lip for feeding on the anterior side (Fig 1A, B). The unpedicellate lorica is cylindrical, 8.61–9.65 μm long (average: 7.37 μm), and 3.70–4.48 μm wide (average: 3.31 μm) (Fig 1B). The margin of the lorica curves outward; the bottom of the lorica is rounded. The posterior flagellum is positioned within a groove located to the side of the cell (Fig. 1D, 2A). TEM data showed that the posterior flagellum is thickened by a dense rod that is adjacent to the axoneme (Fig. 2A, C, D). The anterior part of the cell contains a vacuole filled with premature mastigonemes and typical mitochondria with tubular cristae (Fig. 2D). Distinct clusters of ribosomes are distributed around the periphery of the cell (Fig. 2A). These observations are consistent with the original description of *B. kenaiensis* by Hilliard (1971). The morphology of our isolate also resembles the species, *Bicosoeca maris* and *B. szabadosi*, although the isolate described here differs from both of these species by the length of the flagella and the size of the lorica (Hamar 1979; Picken 1941). Moreover, unlike our isolate, *B. maris* lacks a dense rod in the posterior flagellum (Moestrup and Thomsen 1976). Although *B. kenaiensis* was originally described from freshwater habitats, our isolate, NY0214, was collected from seawater. Co-occurrence in seawater, brackish water and freshwater is relatively common in the genus *Bicosoeca*; for instance, mostly freshwater species (e.g. *B. kepneri* and *B. pulchra*) have also been recorded in seawater (Lee 2002; Vørs 1993).

A single cell of *C. marsupialis* was manually isolated with a micropipette to establish a mono-eukaryotic culture (NY0206) with environmental bacteria as a food source. *C. marsupialis* (NY0206) is a microaerophilic stramenopile with D-shaped cells that are 7.23–12.1 μm long (average: 8.55 μm, $n = 30$) and 5.03–9.13 μm wide (average: 6.24 μm, $n = 30$) (Fig. 3A, B). A lorica is not present. Cells swim with one flagellum oriented forward and one

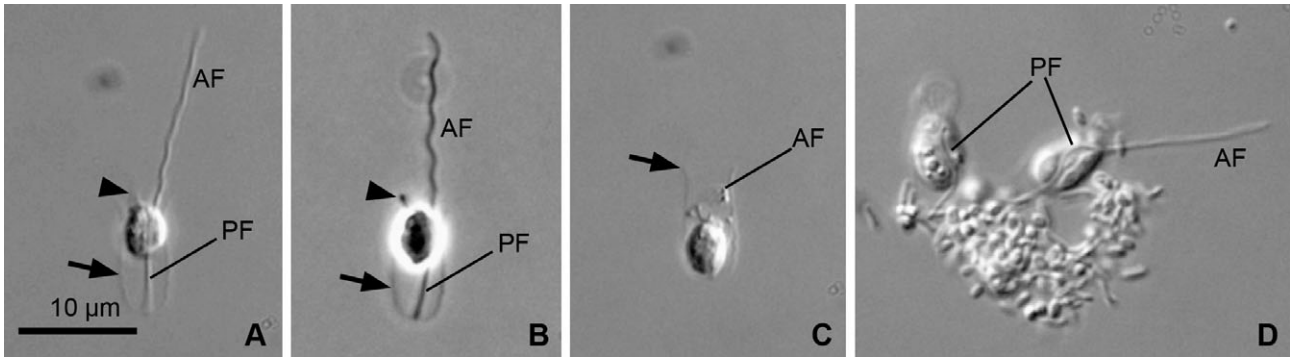

Figure 1 Light micrographs of *Bicosoeca kenaiensis* (NY0214). Arrows and arrowheads indicate the lorica and the feeding lip, respectively. AF = anterior flagellum; PF = posterior flagellum. All images are at the same scale. **A.** Differential interference contrast (DIC) light micrograph showing the lorica, anterior flagellum (AF) and posterior flagellum (PF). **B.** Phase contrast light micrograph showing the unpedicellate lorica. **C.** DIC light micrograph showing the cell withdrawing into the lorica and the anterior flagellum (AF) rolled into a tight coil. **D.** DIC light micrograph showing the posterior flagellum (PF) lying within the groove of the cell.

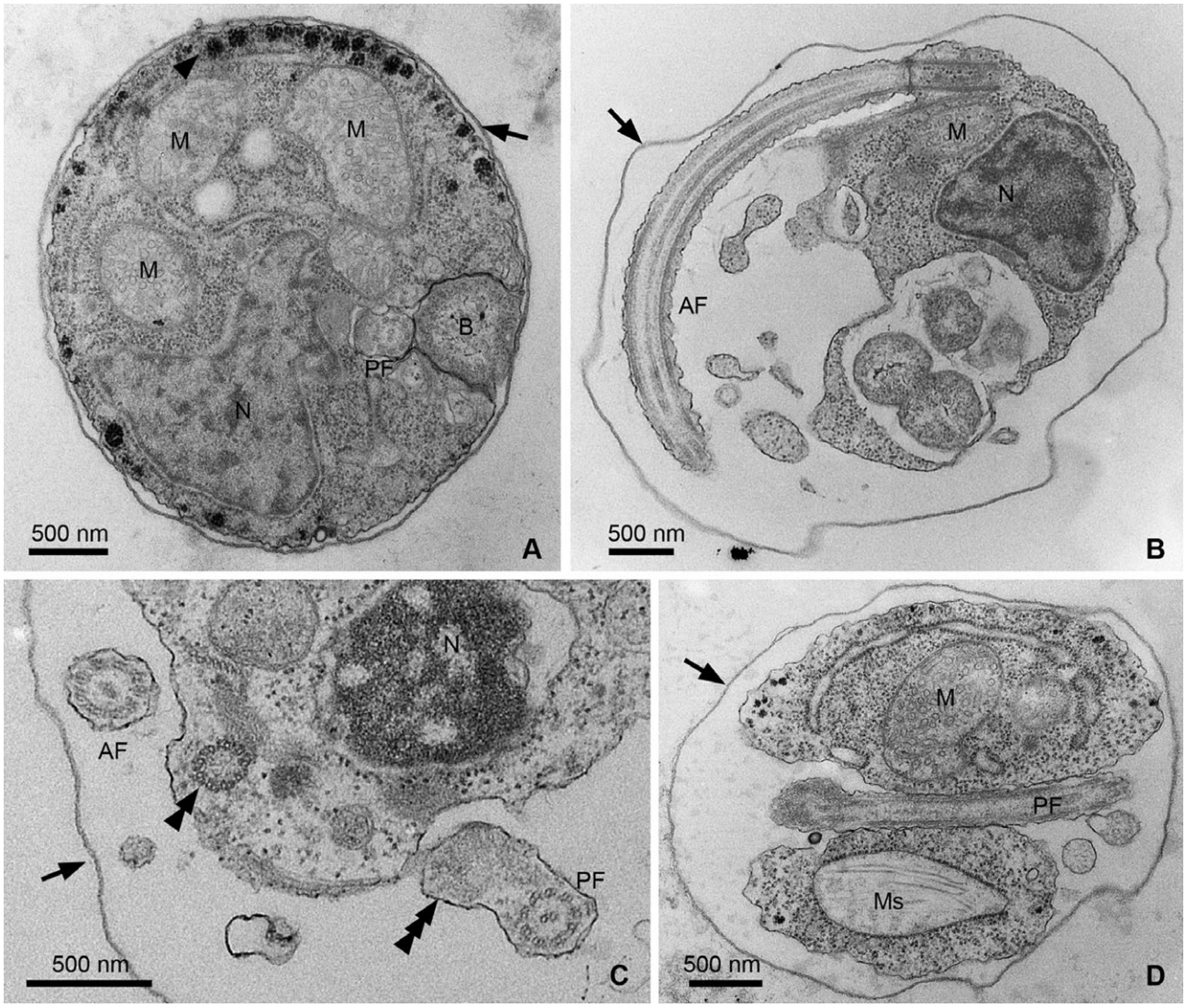

Figure 2 Transmission electron micrographs (TEM) of *Bicosoeca kenaiensis* (NY0214). Arrows indicate the lorica; AF = anterior flagellum; B = bacteria; M = mitochondria; N = nucleus; PF = posterior flagellum. **A.** Cross section of the cell showing the lorica, the PF and clusters of ribosomes (arrowhead) positioned in the periphery of the cell. **B.** Longitudinal section through the anterior flagellum (AF). **C.** Cross section showing a dense rod (triple arrowhead) adjacent to the axoneme of the posterior flagellum (PF) and the basal body (double arrowhead). **D.** TEM showing that the posterior flagellum (PF) sits within the feeding groove. Premature mastigonemes (Ms) are observed in the vacuole.

flagellum trailing behind. When attaching to the substrate with the posterior flagellum, the cells beat the anterior flagellum laterally. The anterior flagellum is about 1.5 times longer than the cell length; the posterior flagellum is about 1.2–1.5 times longer than the cell length. Bacteria are ingested within a deep ventral groove. The food vacuoles are located on the middle to posterior part of the cell (Fig. 3). Morphology of the two other isolates of *C. marsupialis* (YPF1205, BUSSELTON) is entirely consistent with that of the isolate NY0206 (Fig. 3C, D). These observations also agree with the original description of *C. marsupialis* by Larsen and Patterson (1990) and subsequent reports of this species (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee 2006; Lee et al. 2003; Tong 1997). TEM data demonstrated that the cells have mitochondria (M) with a few tubular cristae (Fig. 4A, B). A striated rhizoplast is situated between the Golgi body and the nucleus and originated from the posterior side of the posterior basal body (Fig. 4C).

Phylogenetic positions of the new isolates as inferred from SSU rRNA gene sequences

We determined almost entire SSU rRNA gene sequences from *B. kenaiensis* (NY0214) and the three isolates of *C. marsupialis* (NY0206, YPF1205, BUSSELTON). The molecular phylogenetic tree inferred from an analysis of the 128-taxon alignment of previously described heterotro-

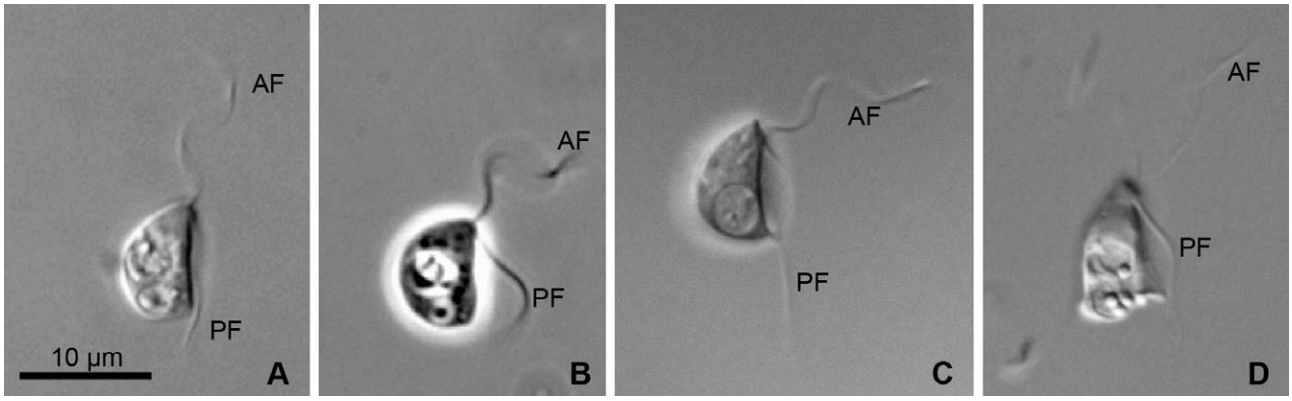

Figure 3 Light micrographs of three different strains of *Cantina* (formerly in *Cafeteria*) *marsupialis* gen. nov., comb. nov. AF = anterior flagellum; PF = posterior flagellum. All images are at the same scale. **A.** Differential interference contrast (DIC) light micrograph of *C. marsupialis* strain NY0206. **B.** Phase contrast light micrograph of *C. marsupialis* strain NY0206. **C.** DIC light micrograph of *C. marsupialis* strain YPF1205. **D.** DIC light micrograph of *C. marsupialis* strain BUSSELTON.

phic stramenopiles and representatives from all 18 MAST clades, is shown in Fig. 5. The monophyly of each MAST lineage is supported with moderate to high bootstrap values (BS) and Bayesian PP as demonstrated previously (Massana et al. 2004, 2014). Our isolate, *B. kenaiensis* (NY0206) nested within *Bicosoeca* (BS = 68, PP = 1.00). Moreover, it was more closely related to the aerobic marine environmental sequences associated with Zuendorf et al.'s (2006) version of MAST-13 (BS = 88, PP = 1.00) than *B. vacillans* and *B. petiolata*. The sequences from the three strains of *C. marsupialis* (NY0206, YPF1205, BUSSELTON) formed a monophyletic group with an environmental sequence NAMAKO-31 with high statistical support (BS = 100, PP = 1.00), which represents Takishita et al.'s (2007) version of MAST-13 from anaerobic environments. Our *Cantina marsupialis* (previously *Cafeteria marsupialis*) and MANAKO-31 do not group with the type species of *Cafeteria* (*C. roenbergensis*), Bicosoecida nor any stramenopile lineages with robust statistical support (Fig. 5).

DISCUSSION

Bicosoecids are abundant heterotrophic flagellates with a cosmopolitan distribution in freshwater, marine, brackish, and hypersaline environments (Arndt et al. 2000; Kim et al. 2010; Lee 2006; Lin et al. 2012; Park et al. 2006). Both *B. kenaiensis* and *C. marsupialis* gen. nov., comb. nov. (=*Cafeteria marsupialis*) have been previously described as bicosoecids. Molecular phylogenetic analyses in our present study demonstrated that these two species are members of different environmental sequence clades; however, both of them have been labeled "MAST-13" by different authors (Takishita et al. 2007; Zuendorf et al. 2006). Zuendorf et al.'s version of MAST-13 includes our isolate of *B. kenaiensis*, which is nested within the more inclusive *Bicosoeca* (Fig. 5). The sequences from our three microaerophilic strains of *C. marsupialis* formed a robust monophyletic group that branched closely with the environmental sequence "NAMAKO-31" (Takishita et al.'s MAST-13) which was also collected from a low-oxygen seawater environment (Takishita et al. 2007). Based on small divergence of the SSU rRNA gene sequence among NAMAKO-31 and our strains of *C. marsupialis*, it is reasonable to assume that NAMAKO-31 represents *C. marsupialis*. The environments from which the sequences in both environmental sequence clades were collected correspond to the environments where we collected our isolates of *B. kenaiensis* and *C. marsupialis*, respectively.

Park and Simpson (2010), Cavalier-Smith and Scoble (2013), and Massana et al. (2014) suggested that MAST-13 represents *Bicosoeca* based on molecular phylogenetic evidence, which is consistent with our results (Fig. 5). Small subunit rRNA gene sequences from only two other described species of *Bicosoeca* (*B. vacillans* and *B. petiolata*) have been reported prior to our study. No detailed morphological information other than from light micrographs and line drawings has been reported from *B. vacillans* and *B. petiolata* so far. Our strain of *B. kenaiensis* (NY0214) improved our understanding of morphological traits in the environmental sequence clade previously recognized as MAST-13. Of the 40 accepted species of *Bicosoeca* (Preisig et al. 1991), only several species (e.g. *B. lacustris*, *B. kepneri*, *B. maris*, *B. planctonica*) have been studied at the ultrastructural level prior our study (Belcher 1975; Mignot 1974; Moestrup and Thomsen 1976); the descriptions of most species are based only on light microscopy and line drawings. The morphological characters in some bicosoecid species descriptions, especially those from older literature, are difficult to interpret, so some of these descriptions could represent the same species with different names (synonyms). Indeed, some authors have already pointed out the need to revise the taxonomy of *Bicosoeca* (Al-Quassab et al. 2002; Aydin and Lee 2012; Lee 2007; Tong 1997; Tong et al. 1998). The current work offers a good example for how to improve the systematics of this genus by using a combination of detailed morphological traits and molecular phylogenetic

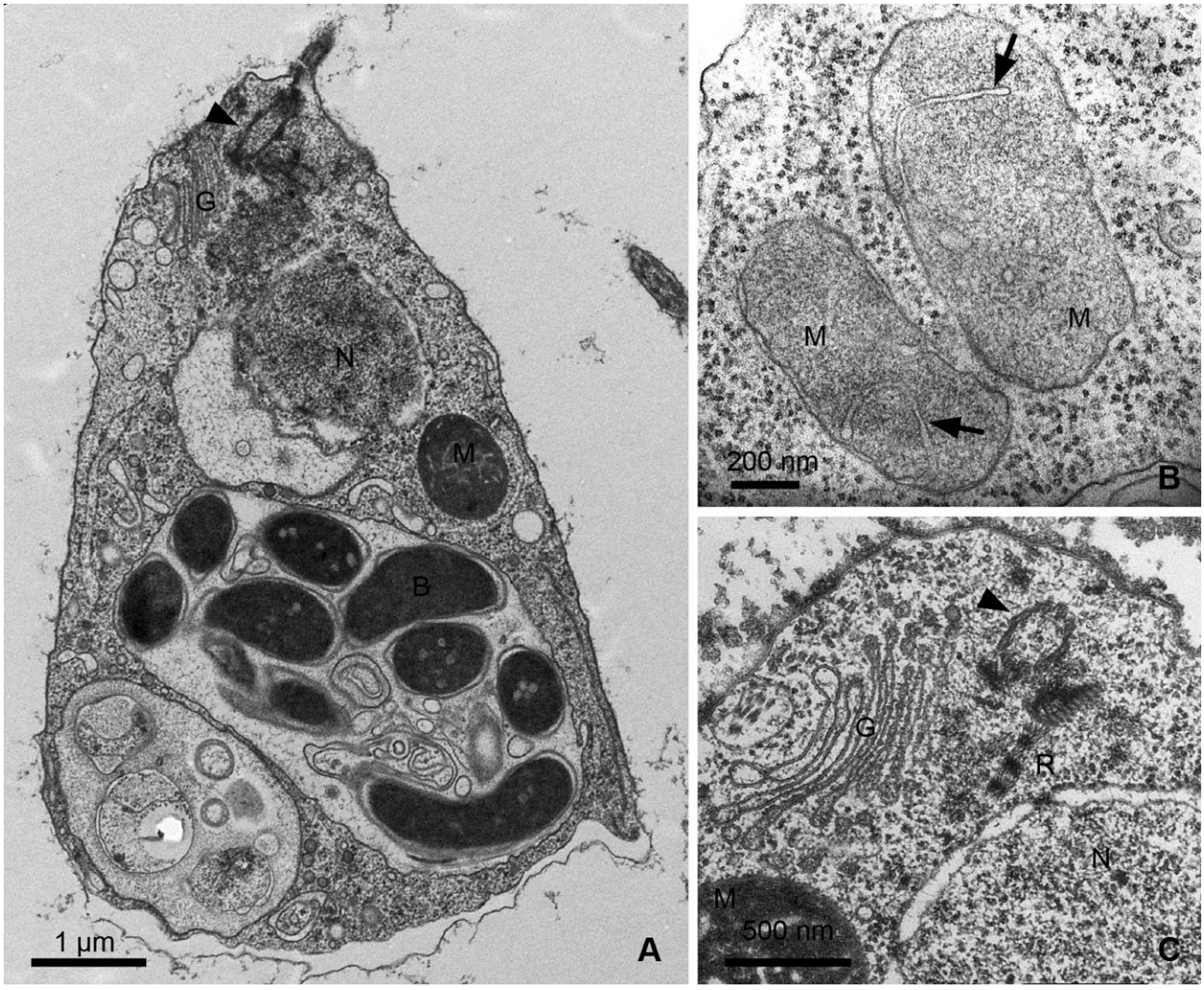

Figure 4 Transmission electron micrographs (TEM) of *Cantina* (formerly in *Cafeteria*) *marsupialis* gen. nov., comb. nov. (NY0206). B = bacteria; G = Golgi body; M = mitochondrion; N = nucleus. Arrowheads indicate the basal body. **A.** Longitudinal section of the cell. **B.** High magnification micrograph showing the small number of tubular mitochondrial cristae (arrows). **C**. High magnification micrograph showing a Golgi body and a rhizoplast (R) connecting a basal body and a microtubular root to the nucleus (N).

data. Nonetheless, *B. kenaiensis* (NY0214) represents the first species of *Bicosoeca* with enough associated data to infer the cellular identity of a previously unclear environmental sequence clade.

Although no DNA sequences were available prior to this study, data from light microscopy suggested that *Cantina marsupialis* (as *Cafeteria marsupialis*) occupies anaerobic sediments all over the world, such as Australia, Brazil, the United Kingdom, Japan and Canada (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee and Patterson 1998, 2000; Lee 2006; Lee et al. 2003; Tong 1997; and this study). Our study demonstrated that an SSU rRNA sequence from *C. marsupialis* was previously reported as an environmental sequence (NAMAKO-31) from an anoxic environment (Takishita et al. 2007) and a few cristae in the mitochondria of *C. marsupialis* observed here reflect this organism's adaptations to low oxygen environments; for instance, *C. marsupialis* may retain a part of the mitochondrial electron transport chain as demonstrated in *Blastocystis* (Stechmann et al. 2008). Environmental DNA surveys are an efficient tool for elucidating broad patterns of species diversity in natural environments, but this approach has several limitations. For instance, some sequences from scarce organisms and divergent sequences from abundant organisms could fail to be recovered altogether. This issue has become evident in free-living fornicate clades (Excavata) from environmental PCR surveys. SSU rRNA gene sequences of one species, namely *Kipferlia bialata*, from these environmental PCR surveys are deposited in GenBank providing a misleading picture of the overall diversity of the Fornicata; culture-dependent methods have subsequently discovered five

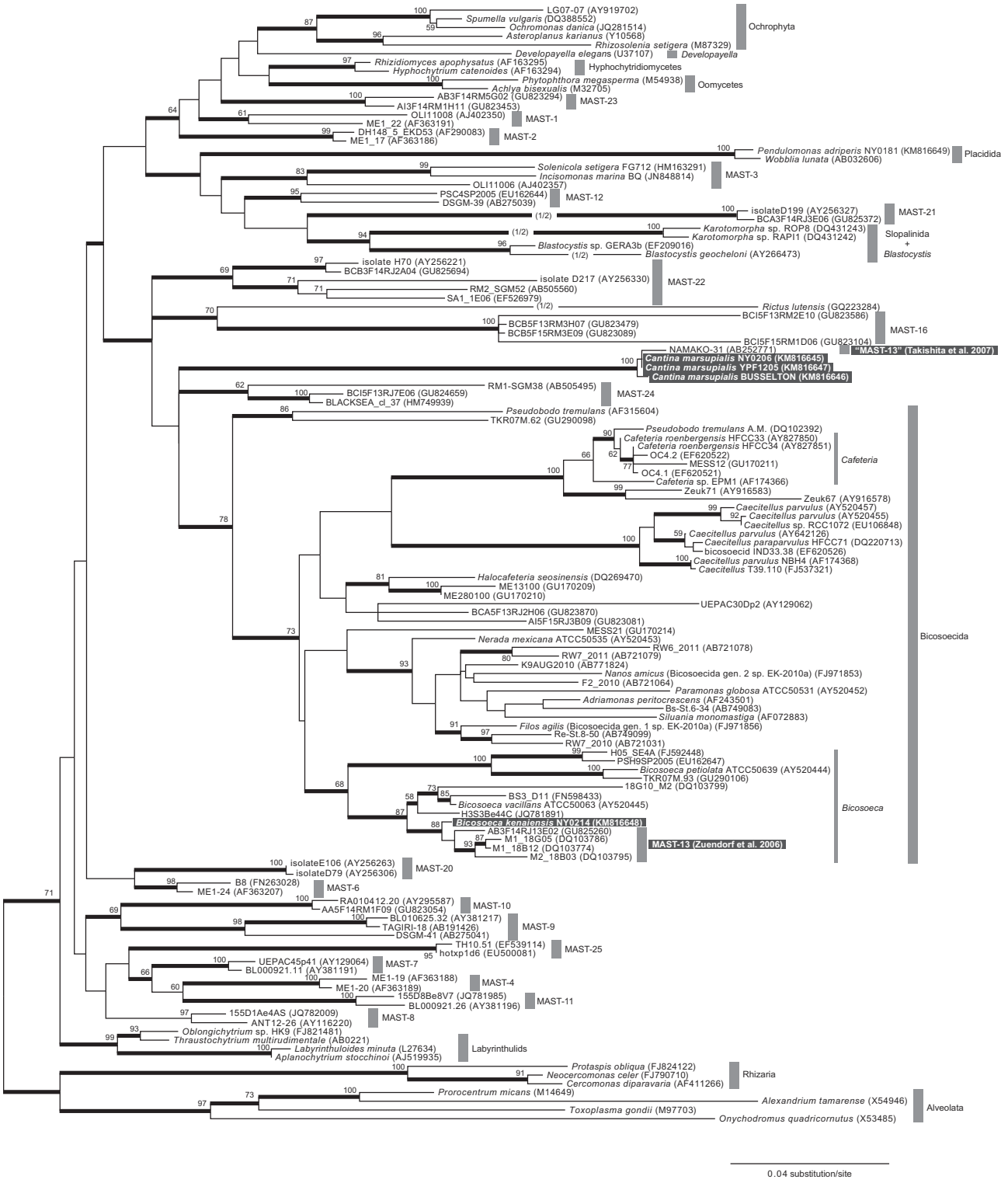

Figure 5 Phylogenetic tree inferred from a 128-taxon alignment of SSU rRNA gene sequences. The tree was inferred with Maximum Likelihood (ML) analyses using a GTR + G + I model. ML bootstrap values less than 50% are not shown. Nodes supported by Bayesian posterior probabilities ≥ 0.95 are shown by thick lines.

other free-living lineages of fornicates providing more comprehensive knowledge of the group (Kolisko et al. 2010; Takishita et al. 2012). The NAMAKO-31 lineage has only been reported once before in environmental DNA surveys, which implies that the distribution of *C. marsupialis* is restricted in nature. As Heger et al. (2014) pointed out,

morphological data and molecular data are insufficient by themselves to understand the diversity, interactions and geographic distributions of different eukaryotic lineages. The integration of both kinds of information as studied in this work leads to much better understanding of these patterns.

Our molecular phylogenetic analyses showed that the anaerobic *Cantina* clade, including NAMAKO-31 in stramenopiles, is not resolved as was also reported in previous studies (Takishita et al. 2007, 2010). The phylogenetic affiliation of the *Cantina* clade with other anaerobic/microaerophilic lineages, such as *Rictus lutensis*, *Blastocystis*, slopalinids, MAST-9, MAST-12, MAST-16, and MAST-20 through MAST-24 is not confirmed in our analysis either, supporting the possibility that the anaerobic lineages within stramenopiles are either a paraphyletic or polyphyletic group (Massana et al. 2014). This would stand in contrast to the pattern observed in other major groups of eukaryotes where anaerobic lineages form only one or two clades, such as in euglenozoans, archamoebae, metamonads, and heteroloboseans (Hampl et al. 2005; Hampl et al. 2009; Pánek et al. 2012, 2014; Yubuki et al. 2013). Additional environmental sequences combined with culture-dependent studies of anaerobic stramenopiles are expected to resolve the phylogenetic position of *Cantina* and the overall phylogenetic pattern of anaerobic lineages within stramenopiles.

So far, marine environmental PCR surveys of SSU rRNA gene sequences have demonstrated that stramenopiles comprise a substantial portion of the total number of eukaryotic sequences recovered. For example, they represent over 30% of the total environmental eukaryotic lineages present in 13 different environmental gene libraries (Richards and Bass 2005) and 42% of total OTUs detected from the suboxic zone of the Black Sea (Wylezich and Jürgens 2011). Sequences from heterotrophic stramenopiles are also one of the most dominant lineages in RNA-derived clone library from an anoxic Danish fjord (Stoeck et al. 2007). Massana et al. (2006) demonstrated that marine stramenopiles occupied up to 35% of the abundance of heterotrophic flagellates in five different oceans, and MAST clades represent a significant fraction of the total number of cells present (e.g. MAST-4 alone represents 9%). Studies of community compositions in both marine and freshwater environments have demonstrated that stramenopile, mainly bicosoecids and chrysomonads, constitute 20–50% of the heterotrophic nanoflagellate biomass (Boenigk and Arndt 2002). Therefore, bicosoecids, including *B. kenaiensis*, contribute significantly to marine food webs, and our culturing-dependent and microscopical approaches help demonstrate the morphological identities of environmental sequences clades and improve our overall understanding of these ecologically important lineages.

TAXONOMIC SUMMARY

The morphological traits present in our three isolates of *C. marsupialis* gen. nov., comb. nov. are consistent with the original description of *C. marsupialis* (Larsen and Patterson 1990) and subsequent reports based on light microscopy (Ekebom et al. 1995/1996; Larsen and Patterson 1990; Lee 2006; Lee et al. 2003; Tong 1997). However, the molecular phylogenetic positions of our isolates were not closely affiliated with the type species of the genus, namely *Cafeteria roenbergensis*, or any other lineage of stramenopiles for that matter (Fig. 5). Therefore, we propose a new genus name and combination for this species based on molecular and ultrastructural data, namely *C. marsupialis* gen. nov., comb. nov.

Cantina gen. nov

Diagnosis. Stramenopiles with D-shaped cells possessing a large groove on the ventral side and two flagella; one oriented anteriorly and one oriented posteriorly. Mitochondria with a few tubular cristae. Microaerophilic, living in marine sediments.

Type species. *Cafeteria marsupialis* Larsen and Patterson 1990 (=*Cantina marsupialis* comb. nov.).

Etymology. "*Cantina*" refers to the English word "canteen", which has a similar meaning to the previous genus name *Cafeteria*. Gender feminine.

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