

ORIGINAL ARTICLE

What Can Environmental Sequences Tell Us About the Distribution of Low-Rank Taxa? The Case of *Euplotes* (Ciliophora, Spirotrichea), Including a Description of *Euplotes enigma* sp. nov.Vittorio Boscaro^a , Mitchell J. Syberg-Olsen^a, Nicholas A. T. Irwin^a, Javier del Campo^{a,b} & Patrick J. Keeling^a^a Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, British Columbia, V6T1Z4, Canada^b Department of Marine Biology and Oceanography, Institut de Ciències del Mar – CSIC, Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Catalonia, Spain**Keywords**

18S phylogeny; anoxic environments; ciliates; genus-level analysis; high-throughput sequencing; macronuclear shape; morphological characterization; protist biogeography; SSU rRNA.

Correspondence

V. Boscaro, Department of Botany, University of British Columbia, 3529-6270 University Boulevard, Vancouver, BC, V6T1Z4, Canada

Telephone number: +1 604-822-6089; FAX number: +1 604-822-2845; e-mail: vittorio.boscaro@botany.ubc.ca

Received: 29 May 2018; revised 11 July 2018; accepted July 14, 2018.

Early View publication August 13, 2018

doi:10.1111/jeu.12669

ABSTRACT

Environmental sequences have become a major source of information. High-throughput sequencing (HTS) surveys have been used to infer biogeographic patterns and distribution of broad taxa of protists. This approach is, however, more questionable for addressing low-rank (less inclusive) taxa such as species and genera, because of the increased chance of errors in identification due to blurry taxonomic boundaries, low sequence divergence, or sequencing errors. The specious ciliate genus *Euplotes* partially escapes these limitations. It is a ubiquitous, monophyletic taxon, clearly differentiated from related genera, and with a relatively well-developed internal systematics. It has also been the focus of several ecological studies. We present an update on *Euplotes* biogeography, taking into consideration for the first time environmental sequences, both traditional (Sanger) and HTS. We inferred a comprehensive small subunit rRNA gene phylogeny of the genus including a newly described marine species, *Euplotes enigma*, characterized by a unique question mark-shaped macronucleus. We then added available environmental sequences to the tree, mapping associated metadata. The resulting scenario conflicts on many accounts with previously held views, suggesting, for example, that a large diversity of anaerobic *Euplotes* species exist, and that marine representatives of mainly freshwater lineages (and vice-versa) might be more common than previously thought.

THE phylum Ciliophora (ciliates) is one of the largest and most investigated groups of protists. Ciliates are diverse and morphologically complex unicellular organisms found in almost every environment on Earth (Hausmann and Bradbury 1996; Lynn 2008). While many open questions remain, their phylogeny and large-scale taxonomy have remained largely consistent since the advent of molecular phylogeny (Lynn 2008, 2017), and is currently being refined by phylogenomic analyses (Gentekaki et al. 2014, 2017; Lynn and Kolisko 2017; Lynn et al. 2018). However, among more than 1,500 described ciliate genera, only a handful have been extensively used as model organisms, chiefly *Tetrahymena* (Jiang et al. 2015; Kruger et al. 1982; Vogt and Mochizuki 2013) and *Paramecium* (Aury et al. 2006; Singh

et al. 2014; Sonneborn 1975). In recent years, interest in the genus *Euplotes* (Spirotrichea, Euplotia) has also revitalized, for instance as a model system for the study of symbioses (Boscaro et al. 2013, 2017), mating types (Vallesi et al. 1995), and biogeography (Di Giuseppe et al. 2011).

Euplotes possesses several features that make it a good subject to address many questions. To begin with, it is relatively easy to maintain in standard culture conditions using a single food organism. It is unquestionably monophyletic (Petroni et al. 2002; Yi et al. 2009), and yet molecularly diverse and species-rich (Curds 1975; Syberg-Olsen et al. 2016). With a few important exceptions (see, for example, Dai et al. 2013; Lian et al. 2018; Liu et al. 2015), many of the morphospecies of *Euplotes* are well defined by unique combinations

of discrete and qualitative morphological characters that are supported by molecular data. Intra-genus systematics is being consolidated by small subunit (SSU) rRNA gene analyses (Petroni et al. 2002; Syberg-Olsen et al. 2016) corroborated by additional markers (Zhao et al. 2018). *Euplotes* ecological diversity is rather extensive: *Euplotes* species can be commensals of metazoans (Berger 1965) as well as free-living, and they can harbor obligate symbiotic bacteria (Boscaro et al. 2018; Heckmann et al. 1983; Vannini et al. 2012), algae (Achilles-Day et al. 2008; Lobban et al. 2005), and parasites (Fokin et al. 2008, 2014). Moreover, *Euplotes* has colonized terrestrial, freshwater and brackish habitats in addition to its ancestral marine environment (Curds 1975; Syberg-Olsen et al. 2016).

The habitat can be treated as any other character and mapped on phylogenetic trees to infer evolutionary trends and events, like invasions of freshwater environments by ancestrally marine lineages. These analyses often rely on anecdotal reports, mostly from original species descriptions, and on metadata associated with the sequences used to build phylogenies (e.g. Syberg-Olsen et al. 2016). However, another source of information is becoming increasingly more relevant: environmental sequences, especially those obtained from high-throughput sequencing (HTS) surveys. These sequences are not directly associated with identified organisms, but they are usually accompanied by extensive and more uniformly collected metadata. Processing and classification of environmental sequences can be performed using various pipelines (e.g. Caporaso et al. 2010b; Edgar 2010; Mahé et al. 2014) and reference databases (e.g. del Campo et al. 2018; Guillou et al. 2013; Quast et al. 2013). Inferences on the distribution of high-rank (i.e. more inclusive) taxa are routinely performed and have provided many novel insights in the last decade (e.g. del Campo et al. 2015; Grossmann et al. 2016; Santoferrara et al. 2016). Fewer attempts have targeted low-level taxonomic ranks like genera and species (e.g. del Campo et al. 2017), largely because environmental sequence assignment becomes less precise for these taxa. Nevertheless, well-defined genera such as *Euplotes* provide the perfect opportunity to test the suitability of this approach. Here, we provide a comprehensive *Euplotes* SSU rRNA gene phylogeny, including a novel marine species we describe, *Euplotes enigma*, characterized by the unique shape of its macronucleus. We then use this phylogeny as a reference to examine biogeographic patterns emerging from available environmental Sanger sequences and HTS reads from three major environmental surveys: *Tara* Oceans (de Vargas et al. 2015), VAMPS (Huse et al. 2014), and BioMarkS (Massana et al. 2015).

MATERIALS AND METHODS

Isolation and characterization of *Euplotes enigma* sp. nov

Sampling was performed with a 20- μ m mesh plankton net off a dock close to the Victoria International Airport (Canada, BC) on July 20, 2016 (salinity: 29‰; coordinates:

48°39'11"N, 123°26'51"E). Several *Euplotes* morphotypes were observed in the sample. Individual cells were collected with a glass micropipette, washed three times in sterile isosmotic water, and cultured in autoclaved seawater (salinity 30‰) at 20 °C with a 12:12 h light/dark cycle. *Dunaliella tertiolecta* was added regularly as food. Other monoclonal strains were identified to the species level as *Euplotes crassus* and *Euplotes minuta*, while strain MaS2 represented a novel species.

The morphological characterization of strain MaS2 was carried by differential interference contrast (DIC) microscopy using an Axioplan 2 compound microscope (Zeiss, North York, Canada) and a Zeiss Axiocam 503 color digital camera, by Chatton–Lwoff silver nitrate staining (Gallagher and Kozloff 1971) and by fluorescent microscopy on specimens kept in 8 μ g/ml DAPI-infused water solution (Lesard et al. 1996).

About 100 cells were hand-picked with a glass micropipette from a starving MaS2 subculture, washed several times and fixed in 70% ethanol. Total genomic DNA was extracted from the fixed sample with the MasterPure™ Complete DNA and RNA Purification Kit (Epicenter, Madison, WI), and the SSU rRNA gene was PCR-amplified with primers 18S F9 Euk (5'-CTGGTTGATCCTGCCAG-3') and 18S R1513 Hypo (5'-TGATCCTTCYGCAGGTTTC-3'), then sequenced with three internal primers (Rosati et al. 2004). The 1,818 bp sequence is deposited in the GenBank/EMBL/ENA database (accession number: LT732572).

Phylogenetic analyses

A first phylogenetic inference included SSU rRNA sequences belonging to organisms identified at the genus level (dataset 1). Only one sequence per species was included, except in the cases of *E. euryhalinus*, that is represented by two related but significantly different groups of sequences, and *E. charon*, that is represented by two unrelated sequences, none of which is tied to a morphological characterization. The monophyly of other species represented by more than one sequence was confirmed in a previous study (Syberg-Olsen et al. 2016). In total, 48 *Euplotes* sequences and 14 outgroup sequences (genera *Aspidisca*, *Euplotidium*, *Certesias*, and *Gastrocirrhus*) were aligned with MAFFT (Kato and Standley 2013). The character matrix was inspected with AliView (Larsson 2014) and trimmed at both ends to remove any column with missing data (final size: 2,135 columns). The analysis was performed on RAxML (Stamatakis 2014) (1,000 random starting trees; GTRGAMMA model; 1,000 nonparametric standard bootstraps) and corroborated by IQ-TREE (Nguyen et al. 2015) (GTR+I+G4 model, selected by the best-fitting model search algorithm of the software; 1,000 nonparametric standard bootstraps) and MrBayes (Ronquist et al. 2012) (GTR+I+G4 model; three independent runs, each with one cold and three heated chains; iterated for 1,000,000 generations sampled every 100 generations).

Subsequently, GenBank sequences longer than 1,000 bp and with a similarity of 80% or higher with entries in dataset 1 were collected regardless of

taxonomic identification. Potential chimeras were filtered out following the EukRef pipeline (del Campo et al. 2018) and consulting the list of known deposited ciliate chimeric sequences (Boscaro et al. in press). The 219 remaining sequences, obtained from isolated organisms as well as environmental long-read surveys, were clustered into operational taxonomic units (OTUs) using USEARCH (Edgar 2010) to reduce redundancy in the data and balance errors from clone sequences. A 99% similarity threshold, appropriate for sub-genus taxa (Bachy et al. 2013), was applied, resulting in 58 OTUs. The longest sequence in each OTU was used as representative. A phylogenetic tree including all representative sequences (dataset 2) was inferred on RAxML using the same parameters and outgroups employed for dataset 1.

Analysis of environmental HTS sequences

The 58 *Euplotes* representative sequences from the reference tree were used to retrieve similar sequences using BLAST (Altschul et al. 1997) from three publicly available SSU rRNA datasets: *Tara* Oceans, VAMPS, and BioMarkS (Huse et al. 2014; Massana et al. 2015; de Vargas et al. 2015). The analyzed dataset contains both V4 and V9 region reads. The FASTA file containing all environmental HTS reads was combined with the *Euplotes* reference sequences, sorted by length using vsearch (Rognes et al. 2016) and clustered into OTUs with 99% similarity. OTUs formed by environmental HTS reads not clustered within reference sequences were then aligned with the reference alignment using PyNAST (Caporaso et al. 2010b) embedded in QIIME (Caporaso et al. 2010a) (align_seqs.py). The reference alignment was the same alignment that was used to generate the reference phylogenetic tree (dataset 2). OTUs that the PyNAST algorithm failed to align were discarded. The PyNAST alignment output was merged with the reference alignment and filtered for gap positions using QIIME (filter_alignment.py) with gap filtering threshold set to 0.99 and entropy threshold set to 0.0001. Identification of *Euplotes* reads used a maximum likelihood phylogenetic approach by mapping the OTUs onto our reference tree using the Evolutionary Placement Algorithm (EPA) of RAxML (Berger et al. 2011). OTUs that were not placed within *Euplotes* were removed. Trees using the remaining sequences were built consecutively until no more reads were placed outside our group of interest. OTUs and their clustered sequences were then annotated according to their placement. For novel groups containing only short reads we adopted the same annotation as for the environmental exclusive groups retrieved from GenBank. The annotated OTU table and corresponding metadata were processed for community analysis using QIIME.

RESULTS

Morphology of *Euplotes enigma* sp. nov

Living specimens of strain MaS2 measure about $41 \times 25 \mu\text{m}$, exhibiting an average length:width ratio of

1.7. Silver-stained cells do not appreciably shrink, and maintain approximately the same shape (Fig. 1, Tables 1 and 2). The cell outline is oval, with left and right sides roughly equally curved (Fig. 1A, D, E). Both ends are rounded. The ventral side presents deep furrows (Fig. 1A, G), and the dorsal surface is marked by six regularly spaced ridges (Fig. 1H). The peristome is large, long, and deep, extending well beyond half the cell length (Fig. 1A, B). There are 23–26 conspicuous membranelles in the adoral zone (AZM), arranged in a regular and shallow curve that does not become angular in proximity of the cytostome. The AZM and the dorsal argyrome are clearly stained by the Chatton–Lwoff method (Fig. 1B, C), while other components of the silverline system (e.g. cirral basal bodies and the paroral membrane) were sometimes less defined, due in part to inconsistent staining and in part to large colored granules concentrated in the center of cells, present even in starved specimens. The dorsal argyrome is of the double-*eurystomus* type (two approximately equal rows of alveoli between each kinety). There are five to seven (usually six) dorsolateral kineties, with seven to nine dikinetids in the mid-dorsal row. The bases of dorsal bristles are large, sometimes visible in living cells (Fig. 1H). There are 4 thin marginal/caudal cirri, 5 thick transverse cirri, and 10 frontoventral cirri (Fig. 1B)—kinetosomes are not always well-stained in fixed specimens, but the pattern is confirmed by in vivo observations (Fig. 1G). The macronucleus has a unique morphology: the common C-shape of most *Euplotes* species is still discernible in the anterior region, but at around two-third of the length there is a drastic decrease in diameter, resulting in a thin, faintly stained thread connecting the main body to a bulbous posterior appendage (Fig. 1F). This question mark-like shape is consistently observed in all cells, both alive and fixed, stained with DAPI (Fig. 1I), and can be seen even in silver-stained specimens (Fig. 1J). The micronucleus, as is the rule in *Euplotes*, is single, round, fairly large, and close to the macronucleus in the anterior-left area.

Phylogenetic analyses

The phylogenetic analysis results (Fig. 2, 3) were consistent with recently published topologies (Chen et al. 2013; Di Giuseppe et al. 2014; Fotedar et al. 2016; Syberg-Olsen et al. 2016; Zhao et al. 2018). In particular, the 5 major clades and 15 species clusters detected by Syberg-Olsen et al. (2016) were recovered and most were strongly supported by statistical values. Clade A was consistently obtained in all trees, although not always with high support. The clustering of *Euplotes trisulcatus* and *Euplotes* cf. *antarcticus* with *Euplotes euryhalinus* and *Euplotes magnicirratu*s, suggested in the previous study, was confirmed by the analysis on dataset 1 but not the analysis on dataset 2. Since this association seems to be at least partially dependent on sequence sampling, we further split this species cluster. The sequence of *Euplotes* sp. SNK-2011 (accession number: HM140406, unpublished) does not cluster within any major clade and possesses unusual features, such as the absence of insertions in the SSU rRNA gene that are typical of *Euplotes*

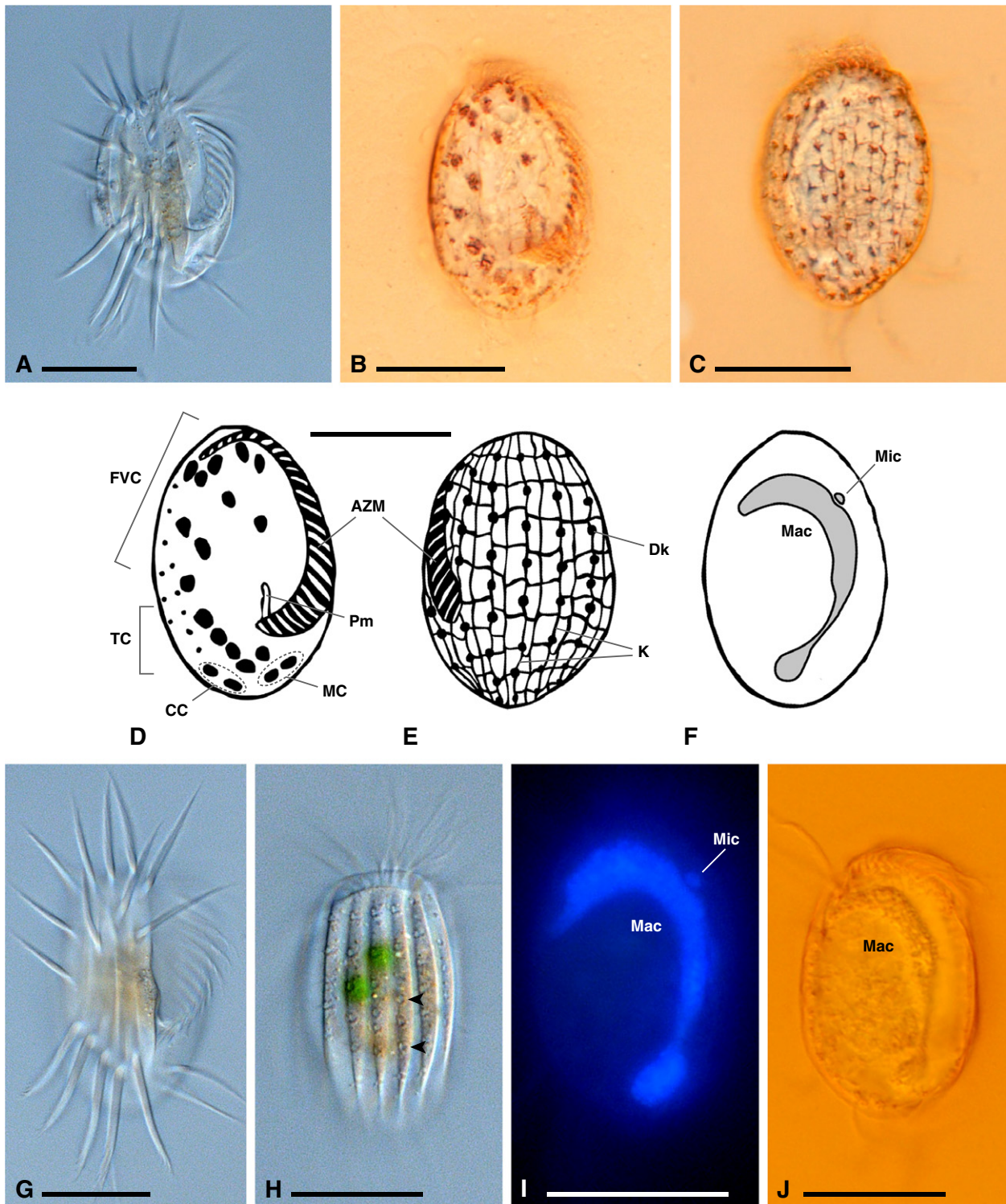


Figure 1 Morphological features of *Euplotes enigma* sp. nov. (type strain MaS2). **(A)** Ventral view of a live specimen; the ventral surface and the base of most cirri and oral membranelles are in the plane of focus. Ventral **(B)** and dorsal **(C)** argyrome features after silver staining. Schematic drawings of ventral **(D)** and dorsal **(E)** argyrome features and of the nuclear apparatus **(F)**, based on multiple cells. **(G)** Ventral view of a cell with the 10 frontoventral cirri in the plane of focus. **(H)** Dorsal view of a cell showing the prominent dorsal ridges and the location of bristle kinetosomes (arrowheads). Nuclear apparatus as visible in a DAPI-stained living cell **(I)** and a silver nitrate-impregnated specimen **(J)**. AZM, adoral zone membranelles; CC, caudal cirri; Dk, dkinetids; FVC, frontoventral cirri; K, kineties; Mac, macronucleus; MC, marginal cirri; Mic, micronucleus; TC, transverse cirri. All scale bars represent 20 μm.

(Petroni et al. 2002). To our knowledge, there is no available description of this strain. It is here treated as *Euplotes* sp. in its own species cluster.

The novel strain MaS2 is most closely related to *Euplotes alatus* (Fig. 2), and belongs to a species cluster that shares many of its morphological features, in particular the double-*eurystomus* dargyrome type and the 10 frontoventral cirri pattern (modified in the most derived representatives, *E. orientalis* and *E. bisulcatus*). All these species were originally described from marine environments, although the sequenced strain of *E. alatus* was collected in a freshwater pond (Di Giuseppe et al. 2014). The SSU rRNA gene sequences of MaS2 and *E. alatus* strain EHHS (accession number: KJ434102) differ at 16 nucleotide positions (0.88%) according to BLAST.

With the exception of already mentioned minor differences, the two datasets produced very similar results, especially concerning supported nodes. Clades A–E are always monophyletic and their relationships consistent. The topology of species clusters within clades is instead mostly unresolved. At least seven lineages including only environmental sequences can be identified (Fig. 3). These lineages are mostly associated with species-poor clades D and E.

HTS OTUs and ecological metadata

The 227 OTUs collected from major HTS surveys were distributed across all but three species clusters (the *fo-cardii*, *rariseta*, and *platystoma* clusters). They were

Table 1. Morphometric measurements performed on MaS2, type strain of *Euplotes enigma* sp. nov.

	Mean	SD	Min.–Max.	<i>N</i>
Length (in vivo)	41.1 µm	3.3 µm	35.9–46.9 µm	23
Width (in vivo)	24.7 µm	3.1 µm	19.1–30.9 µm	23
Length:width ratio (in vivo)	1.68	0.13	1.44–1.99	23
Length (Chatton–Lwoff staining)	39.9 µm	3.5 µm	32.6–45.7 µm	26
Width (Chatton–Lwoff staining)	26.5 µm	2.2 µm	20.5–31.5 µm	26
Length:width ratio (Chatton–Lwoff staining)	1.51	0.11	1.34–1.72	26
Peristome:cell length ratio (in vivo)	72%	5%	65–80%	16
Peristome:cell length ratio (Chatton–Lwoff staining)	73%	4%	67–82%	17
Adoral zone membranelles	24	1	23–26	17

Max., maximum value; Min., minimum value; *N*, number of observed cells; SD, standard deviation.

Table 2. Morphological comparisons between *Euplotes enigma* sp. nov. and six similar species with 10 frontoventral cirri and a double-*eurystomus* dargyrome type

	<i>E. enigma</i> sp. nov.	<i>E. parabalteatus</i>	<i>E. alatus</i>	<i>E. curdsi</i>	<i>E. balteatus</i>	<i>E. trisulcatus</i>	<i>E. octocirratius</i>
Size	36–47 µm	30–35 µm	36–43 µm	45–65 µm	30–150 µm	35–50 µm	55–60 µm
Shape	Oval; ends rounded	Oval	Oval	Oval; ends rounded	Oval	Elongated; pointed posterior end	Elongated oval
Peristome	~70% of the body; wide and deep	~67–75% of the body; prominent	~50% of the body	~70% of the body; narrow	~67–75% of the body; wide	~66% of the body; narrow	~66% of the body; wide
Adoral zone membranelles	23–26	19–23	~26	25–34	25–80	25–36	~30
Dorsal ridges	6, prominent	Inconspicuous	Inconspicuous	5–6, inconspicuous	3–5 , prominent	Prominent	N/A
Dorsolateral kineties	5–7	6–7	8	6–7	7–8	7	7
Dikinetids in mid-dorsal row	7–9	8–11	10–12	10–12	10–16	Up to 11	Up to 14
Macronucleus	Question mark-shaped	C-shaped	C-shaped	C-shaped	C-shaped	C-shaped	C-shaped
References	This paper	Jiang et al. (2010a,b)	Borror (1968) and Curds (1975)	Syberg-Olsen et al. (2016)	Chen et al. (2013) and Curds (1975)	Curds (1975) and Tuffrau (1960)	Agamaliyev (1967) and Curds (1975)

Unambiguously different characters are in bold.

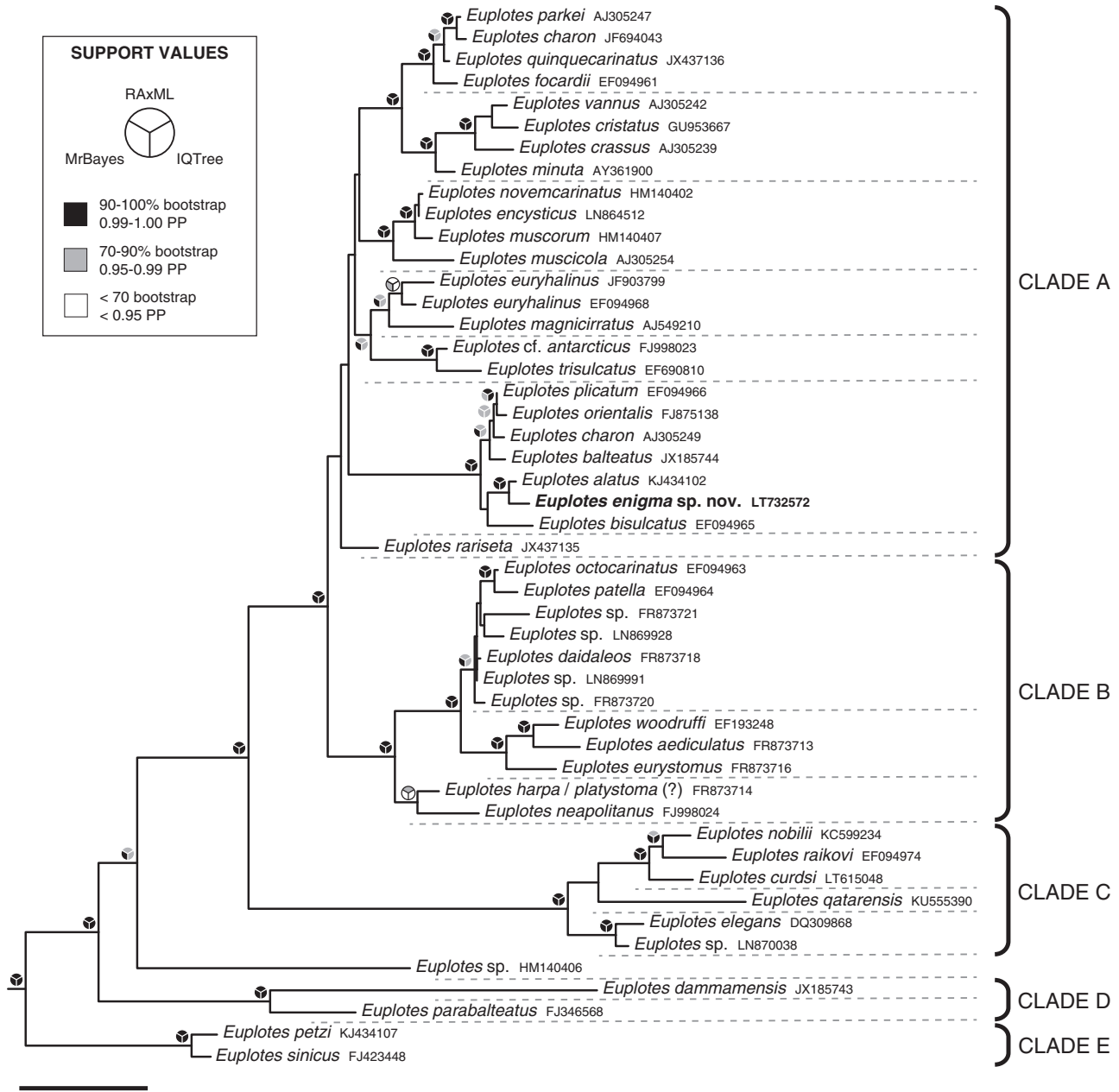


Figure 2 Maximum Likelihood phylogenetic tree of *Euplotes* species based on SSU rRNA gene sequences. Pie charts associated with nodes summarize statistical support values for RAxML, IQ-TREE and MrBayes analyses (see insert). The RAxML tree is shown. The nomenclature of clades follows Syberg-Olsen et al. (2016). The question mark after *Euplotes harpa* is due to recent reports suggesting that strains previously classified as *E. harpa* may actually belong to *Euplotes platystoma* (Lian et al. 2018; Yan et al. 2018). Dotted lines separate species clusters used in biogeographic analyses (Fig. 3). The bar represents an estimated divergence of 0.5.

additionally found within three of the seven environmental-only long-read sequence clusters: ENV1, ENV5, and ENV6 (no HTS read clustered instead within ENV2-4 and ENV7). In addition, 79 environmental HTS OTUs could not be reliably assigned to any cluster, and were divided into 15 HTS short-read-only clades (SRC, Fig. 3). Unlike long-read environmental sequence clusters, short-read OTUs seem to be more evenly spread across the tree.

The HTS sequences from the *Tara* Oceans, VAMPS, and BioMarKs surveys are associated with a variety of metadata, some of which are shown in Fig. 3. Information on the Sanger sequences environment, extracted from the EukRef-Ciliophora database (Boscaro et al. in press), is also shown for comparison. Neither set of data can be considered exhaustive by any means, because classical reports on *Euplotes* species are still far more numerous

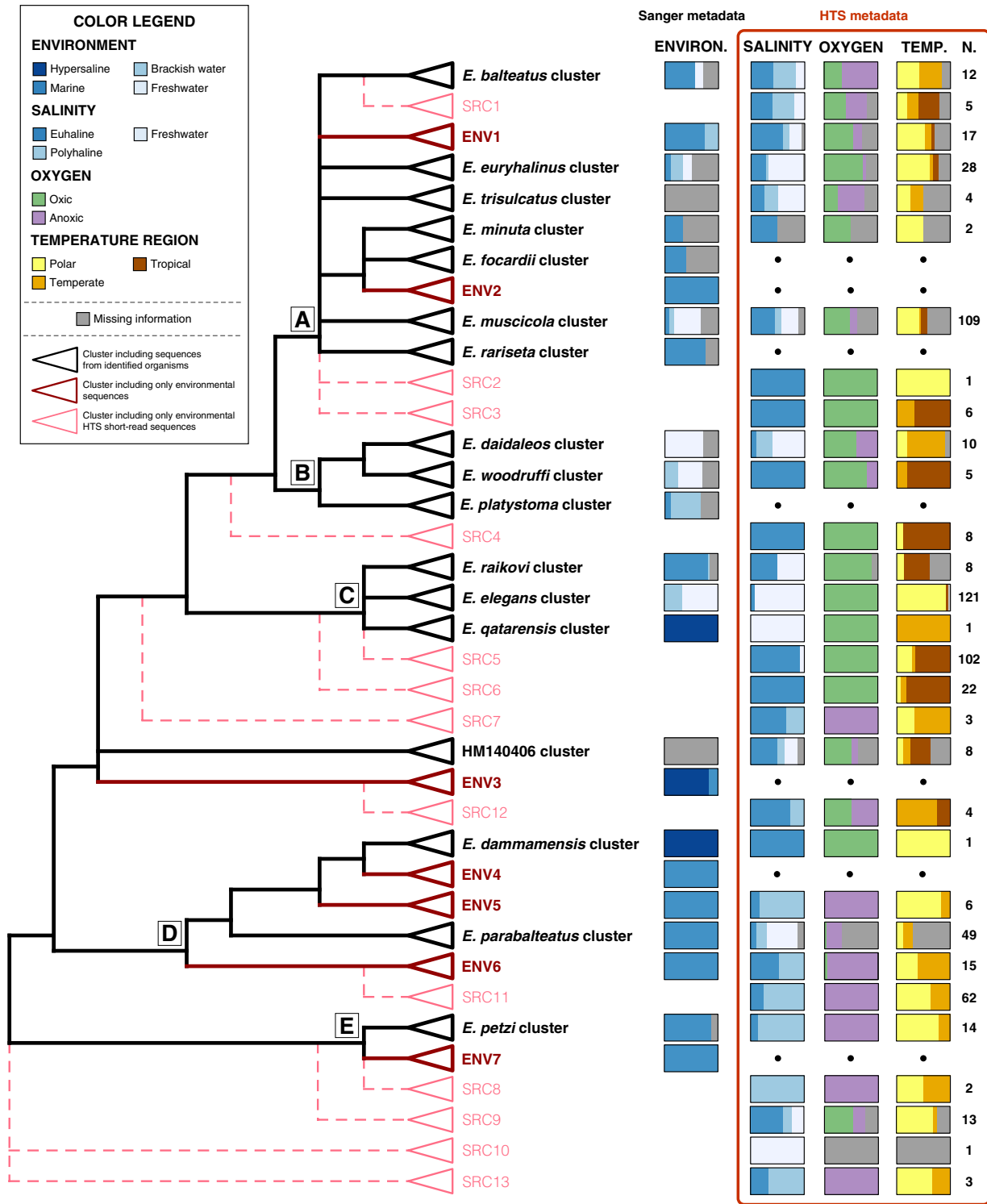


Figure 3 The *Euplotes* EPA tree, with HTS OTUs mapped on a scaffold Maximum Likelihood tree of Sanger-obtained SSU rRNA sequences. Closely related lineages are grouped into clusters. Seventeen clusters (in black) include representatives of described species, seven clusters (in dark red) include environmental “long” (Sanger) sequences but no isolated organism. Short HTS reads are scattered across most of these clusters and thirteen addition ones (in pink). The main *Euplotes* clades (A–E) are labeled. Metadata are mapped on the right. Environmental profiles for Sanger sequences are reported in the first column, HTS read metadata (salinity according to the Venice system, oxygen level, and temperature region) in the remaining columns (within the red rectangle). Within each bar, relative abundances of sequences belonging to each category (detailed in the insert) are depicted. N. stands for “number of HTS operational taxonomic units”.

than those in the molecular literature, and molecular surveys in general are biased toward certain environments (e.g. marine rather than freshwater). However, important differences are already apparent. Sanger sequences (both environmental and from isolated organisms) and reported morphospecies distributions depict a fairly consistent pattern, with most lineages still inhabiting the ancestral saline (marine or brackish) habitats. With a few other exceptions, invasions of freshwater seem to have happened mainly in clade B and in the *muscicola* cluster within clade A (Syberg-Olsen et al. 2016). On the contrary, freshwater HTS sequences are spread through most lineages in all clades, except possibly clade E. Similarly, marine HTS sequences are detected from supposedly freshwater lineages, such as the *woodruffi* and *daidaleos* clusters. An even more striking discrepancy is the widespread presence of HTS reads from anoxic environments in most lineages from all clades except C. All known *Euplotes* species are obligate aerobes, although a few *Euplotes* Sanger environmental sequences from anoxic environments are reported in the EukRef database (Boscaro et al. in press), corroborating the HTS results.

DISCUSSION

Comparison of *E. enigma* sp. nov with similar species

The “question mark”-shaped macronucleus observed in strain MaS2 is unique within *Euplotes*. The vast majority of congeneric species have a C-shaped macronucleus or a slight variation in this conserved structure (a more or less prominent curvature in the middle in species with a “3-shaped” macronucleus, or particularly elongated branches in species with a “horseshoe-shaped” macronucleus) (Curds 1975). The variability in length, curvature, and degree of association with the micronucleus are usually considered of low systematic importance due to the considerable plasticity observed within strains. The only other species that is unambiguously identified by its unique (T-shaped) macronucleus is *Euplotes woodruffi* (Borror 1963; Gaw 1939). The macronuclei of both *E. woodruffi* and *E. enigma* are clearly derived from the ancestral (and most common) C-shape, but are different enough to constitute unambiguous diagnostic characters.

Regardless of nuclear features, only a handful of species possess a set of morphological characteristics comparable to those of *E. enigma* (Table 2). *Euplotes parabalteatus* is possibly the most similar taxon, but it is slightly smaller (30–35 μm) and displays no prominent dorsal ridge (Jiang et al. 2010a); the SSU rRNA gene sequence of the type strain confirms that the two species are only distantly related (Fig. 2). *Euplotes alatus* is the closest relative and shares the same size as *E. enigma* (Borror 1968; Kahl 1932). But while *E. alatus* is identifiable by one of the smaller peristome to cell length ratio in the genus (about 50%), *E. enigma* displays one of the larger; additionally, *E. alatus* possesses eight dorsolateral kineties (vs. no more than seven in *E. enigma*). Other species whose subtle somatic differences from *E. enigma* are

confirmed by considerable genetic distances are *Euplotes curdsi* (larger size: 46–68 μm , inconspicuous dorsal ridges, and 10–12 dikinetids in mid-dorsal row vs. 7–9 in *E. enigma*) (Syberg-Olsen et al. 2016) and *Euplotes balteatus* (five or less dorsal ridges vs. six in *E. enigma*, 10–16 dikinetids in mid-dorsal row) (Chen et al. 2013; Kahl 1932; Tuffrau 1964). The only available sequence of *Euplotes trisulcatus* (accession number: EF690810) is not confirmed by a published morphological characterization, but the diagnostic characters of this species include a pointy posterior end, a narrow peristome, and 25–36 AZM (Kahl 1932; Tuffrau 1960). Finally, *Euplotes octocirratu*s has not been yet molecularly barcoded, but can be distinguished by *E. enigma* for its slightly bigger size (55–60 μm), up to 14 dikinetids in the mid-dorsal kinety, and the presence of only three caudal/marginal cirri (Agamaliev 1967). All the mentioned species possess a C-shaped macronucleus.

Considerations on the systematics of *Euplotes*

This study, as others before (Syberg-Olsen et al. 2016; Zhao et al. 2018), confirmed that species taxonomy in the genus *Euplotes* is overall robust. The high number of described taxa is reflected by molecular diversity. Even subtle morphological differences correspond to different SSU rRNA sequences, while morphologically indistinguishable strains share identical or extremely similar barcodes, even when collected in distant areas (e.g. Dai et al. 2013; Lian et al. 2018; Syberg-Olsen et al. 2016). This relatively rare situation among protists is due to the high number of quantitative and qualitative characters that can be observed using standardized techniques, careful guidelines devised in the past (Curds 1975; Tuffrau 1960) and general “ease” of handling of *Euplotes* populations.

However, some cautionary reminders are in order. First, while many recent papers do provide SSU rRNA barcode sequences together with the description of new species (e.g. Chen et al. 2013; Fotedar et al. 2016; Jiang et al. 2010a,b; Syberg-Olsen et al. 2016) or even redescription of old ones (e.g. Chen et al. 2013; Lian et al. 2018), most of the molecular articles published between 2002 and 2012 released sequences assigned to various species without a morphological context. While there is no particular reason to mistrust these identifications, it is important that eventually at least one sequence per species is tied to a complete morphological characterization. Many investigators now rely on barcodes alone for species identification, making potential errors in the database more and more difficult to correct as time goes by. Hence, confirmatory descriptions of sequenced strains belonging to old species should be welcomed. Second, and complementary, while it is safe to trust barcodes confirmed by morphology, cursory morphological observations are emphatically insufficient for *Euplotes* species identification. Given the elevated number of taxa, often differing only slightly to each other, morphological identification can stand on their own only when thorough (i.e. including morphometry, silver staining, and DNA staining on a significant number of specimens). Relying on a few characters

or antiquated guides often leads to incorrect conclusions and creates biases toward the more “famous” members of a genus (Boscaro et al. 2014). Whenever possible, we encourage researchers to obtain SSU rRNA sequences to confirm identifications, especially if a complete morphological investigation is not feasible, or to use noncommittal labeling (“*Euplotes* sp.”) whenever there is a margin of doubt, in order to avoid the propagation of mistakes.

Relevance of environmental studies for the diversity and ecology of low-rank taxa

High-throughput sequencing surveys are quickly becoming the most common approach to investigate the microbial diversity of virtually any environment (e.g. Sogin et al. 2006; de Vargas et al. 2015). Ciliates have been extensively targeted by such analyses (e.g. Forster et al. 2015; Santoferrara et al. 2016; Stoeck et al. 2014). Even before the current surge of HTS studies, environmental sequencing had an impact not only on ciliate ecology but also on ciliate systematics, with the establishment of the environmental-only class Ciliacotrichea, including SSU rRNA sequences from deep-sea anaerobic regions (Orsi et al. 2012). The main limitation of environmental sequencing in general, and HTS in particular, is that the output data are bare sequences not directly linked to any observed organism. The identification of environmental sequences and hence, largely, their interpretation, require reliable and extensive references based on multidisciplinary works, particularly correctly annotated reference sequences (del Campo et al. 2018). Issues are compounded for low-rank (less inclusive) taxa, which are often not well defined in protists and, at the same time, too difficult to differentiate using only short sequence fragments. For these reasons, HTS environmental analyses of low-rank taxa such as genera are rare.

Euplotes possesses some characteristics that make it suited as a target for this approach: it is monophyletic and well separated from related genera; species-rich and molecularly diverse; and its internal phylogeny and ecology are reasonably well known. Even so, some issues are still evident. SSU rRNA differences between morphospecies may be subtle, and short fragments cannot be unambiguously assigned to the species level. In this paper, HTS reads were assigned to strongly supported clusters of sequences, often including several closely related species. The second issue is that *Euplotes*, while certainly widespread and common in distribution, is probably never abundant in the environment, and it might fall below the detection threshold of broad surveys. This could explain why *Euplotes*-associated HTS reads were missing from many examined samples even in areas where the genus has been traditionally studied (e.g. the Mediterranean), and were in general not numerous.

Despite all these limitations, several novel insights could be obtained simply by including environmental sequences in the phylogenetic inference. To begin with, the existence of unaffiliated environmental lineages suggests that a good portion of *Euplotes* diversity remains undiscovered. Several known morphospecies have not been molecularly

characterized yet, so it is possible that some of the unassigned sequences correspond to known but understudied taxa. It is, however, likely that most belong to undescribed species, especially considering that some of the habitats these sequences were collected from, such as the deep sea Atalante Basin (Alexander et al. 2009) and Guaymas Basin (Coyne et al. 2013), have never been screened for *Euplotes*. Similarly, the consistent detection of *Euplotes*-affiliated environmental sequences in anoxic samples strongly suggests that there are completely unknown niches occupied by representatives of this genus. As a whole, environmental data support the view that *Euplotes* species are even more widespread and variable than previously realized, despite never contributing substantially to the total biomass.

It is more difficult to interpret the discrepancy in salinity profiles of certain clusters as depicted by long Sanger sequences and HTS reads. For example, all known strains in the *woodruffi* and *daidaleos* clusters inhabit freshwater or very low-salinity brackish systems, and depend on essential bacteria of the genus *Polynucleobacter*, recruited from freshwater free-living populations (Boscaro et al. 2017; Vannini et al. 2012). And yet, several marine HTS sequences that fall into these clusters imply that representatives of the same species or closely related species can be found in the sea (although it will be difficult to estimate their abundance and distribution until we obtain more extensive HTS coverage). It is unlikely that these marine forms have ever been observed, since the morphological synapomorphies of the species clusters are rather clear (Curds 1975; Syberg-Olsen et al. 2016). Finding these potential habitat outliers and anaerobic species may be a noteworthy goal for future exploration of marine *Euplotes* diversity. As mentioned previously, conclusions based only on sequencing have in general to be corroborated by actual observation before being accepted. Including environmental sequences in biogeography analyses, however, can provide useful guidelines about where to look for new insights and intriguing working hypotheses.

This case study on *Euplotes* suggests that interesting information and a few surprises can be gained from environmental sequences even for low-rank taxa, provided that their systematics is sound. The same methodology could be applied to several other protist genera, as a more general test to assert how many traditional biogeography views need to be challenged.

TAXONOMIC SUMMARY

Phylum Ciliophora Doflein, 1901
 Class Spirotrichea Bütschli, 1889
 Subclass Euplotia Jankowski, 1979
 Order Euplotida Small and Lynn, 1985
 Family Euplotidae Ehrenberg, 1838
 Genus *Euplotes* Ehrenberg, 1830

Euplotes enigma sp. nov

Zoobank LSID: urn:lsid:zoobank.org:act:AB875460-A2FA-47AE-A337-B643785BF00F

Diagnosis. Free-living marine *Euplotes*, about 41 × 25 μm in vivo. Shape oval, with both ends rounded. Deep furrows on ventral side, six pronounced and regular ridges on dorsal side. Large, long, and deep peristome (65–80% of body length). Twenty-three to 26 adoral zone membranelles. Dorsal argyrome of the double-*eurystomus* type, with five to seven (most commonly six) kineties and seven to nine dikinetids in the mid-dorsal row. Ten frontoventral, five transverse, and four marginal/caudal cirri. “Question-marked” shape macronucleus. Single round or ellipsoid micronucleus.

Type locality. Surface water around a dock in Victoria (British Columbia, Canada). 48°39'11"N, 123°26'51"E.

Type material. One slide with silver nitrate-stained cells of the type strain MaS2 is deposited at the Beaty Biodiversity Museum (University of British Columbia, Vancouver, Canada; accession number: [V247293]). The holotype and two paratypes are marked following Foissner et al. (2002). The SSU rRNA gene sequence is deposited in GenBank (accession number: LT732572).

Etymology. The specific epithet *enigma* (Latin for “riddle”) refers to the unique question-marked shape of the macronucleus.

ACKNOWLEDGMENTS

The authors thank Greg Gavelis for providing the original marine sample from which strain MaS2 was isolated. This work was supported by a grant (RGPIN-2014-03994) from the Natural Sciences and Engineering Research Council of Canada to P. J. K.

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