Two new species of *Euplotes* with cirrotype-9, *Euplotes foissneri* sp. nov. and *Euplotes warreni* sp. nov. (Ciliophora, Spirotrichea, Euplotida), from the coasts of Patagonia: implications from their distant, early and late branching in the *Euplotes* phylogenetic tree

Alessandro Valbonesi[1], Graziano Di Giuseppe[2], Adriana Vallesi[1,*] and Pierangelo Luporini[1,*]

Abstract

Two new *Euplotes* species have been isolated from cold shallow sandy sediments of the extreme Southern Chilean coasts: *Euplotes foissneri* sp. nov., from a low-salinity site at Puerto Natales on the Pacific coast, and *Euplotes warreni* sp. nov., from a marine site at Punta Arenas on the Atlantic coast. *Euplotes foissneri* has a medium body size (53×36 µm *in vivo*), a dorsal surface marked by six prominent ridges, a double dargyrome, six dorsal and two ventrolateral kineties, a buccal field extending to about 3/4 of the body length, an adoral zone composed of 28–32 membranelles, and nine fronto-ventral, five transverse and two or three caudal cirri. The bulky, hook-, horseshoe- or 3-shaped macronucleus is associated with one sub-spherical micronucleus. The central body region hosts taxonomically unidentified endosymbiotic eubacteria. *Euplotes warreni* has a small body size (39×27 µm *in vivo*), a smooth dorsal surface marked by three deep grooves, a double dargyrome, four dorsal and two ventrolateral kineties, a buccal field extending to about 2/3 of the body length, an adoral zone composed of 23–25 adoral membranelles, and nine fronto-ventral, five transverse and three caudal cirri. The macronucleus is hook- or C-shaped and associated with one spherical micronucleus. Endosymbiotic bacteria belonging to the genus *Francisella* reside preferentially in the anterior cell region. Both species lack the fronto-ventral cirrus numbered 'V/2', whereby their cirrotype-9 conforms to the so-called 'pattern I', which is the basic distinctive trait of the genus *Euplotopsis* Borror and Hill, 1995. Phylogenetic analyses of small subunit rRNA gene sequences, however, classify *E. warreni* into its own early branching clade and *E. foissneri* into a late branching clade. This indicates a polyphyletic nature and taxonomic inconsistency of the genus *Euplotopsis*, which was erected to include *Euplotes* species with cirrotype-9 pattern I.

INTRODuCTION

Euplotes Ehrenberg, 1830 is a very successful colonizer of every aquatic habitat from one to the other pole of the planet [1–6], and one of the most popular genera in experimental ciliatology [7, 8]. Its taxonomy has been traditionally based on the oldest concept of species, that is, the morphospe- cies concept, which separates comparable groups of individuals into two taxa based on relevant differences in their morphology. However, most traditional *Euplotes* diagnostic traits are subject to significant intra-specific as well as intra-clonal variations that blur inter-species boundaries [9, 10]. Two traits are effectively discriminatory because they remain remarkably constant within each species and vary from species to species [1]. One diagnostic trait, known as 'dargyrome' [1], is the geometric pattern (single, double or multiple/complex) of the argentophilic alveolar system that decorates the dorsal cell surface. The second trait is the number of the fronto-ventral cirri, designated as 'cirrotype' [11, 12]. Ten or nine fronto-ventral cirri are present in most species, eight in far fewer species and, exceptionally, 11 in *E. khazarica* Alekperov *et al*., 2006 [13] or seven in some *E. raikovi* Agamaliev, 1966 populations [14, 15]. Different from the fronto-ventral cirri, the two other groups of transverse and caudal/marginal cirri are much less useful for *Euplotes* species determination. There are invariably five transverse cirri arranged in an asymmetric V-shaped pattern in every species, except for *E. strelkovi* Agamaliev, 1967 in which

Author affiliations: [1]Laboratory of Eukaryotic Microbiology and Animal Biology, School of Bioscience and Veterinary Medicine, University of Camerino, Italy; [2]Department of Biology, University of Pisa, Italy.
*Correspondence: Adriana Vallesi, adriana.vallesi@unicam.it; Pierangelo Luporini, piero.luporini@unicam.it
Keywords: Ciliate taxonomy; *Euplotes* phylogeny; cirrotype pattern; *Euplotopsis*; sub-Antarctic biodiversity.
The SSU rRNA gene sequences of *Euplotes foissneri* sp. nov. type strain PN-2A and *Euplotes warreni* sp. nov. type strain Ptg-1, have been deposited in the GenBank database with the accession numbers MT742130 and MT742131, respectively. ZooBank registration number of work: urn:lsid:zoobank.org:pub:28C88B30-52B2-4E8C-BFBD-D74AB6E20D77.

they are six [14]. On the other hand, the number of caudal cirri may vary from two to five even within the same species [10].

The cirrotype and dargyrome patterns were taken by Borror and Hill [16] into primary consideration, along with observations on species ecology and endosymbiotic relationships, to propose a deep taxonomic revision of the historical genus *Euplotes*. The cirrotype-10, pervasive among marine species, was regarded as the ancestral (plesiomorphic) state and all other cirrotypes (the *E. khazarica* cirrotype-11 was unknown at the time of the Borror and Hill's revision) as derived (apomorphic) states. Consistent with this assump- tion, the cirrotype-10 species were divided between *Euplotes* (*sensu stricto*) [type species, *Euplotes charon* (Müller, 1773) Ehrenberg, 1830] and *Moneuplotes* Jankowski, 1979 [type species, *Euplotes vannus* (Müller, 1786) Minkjewicz, 1901]. The former genus is characterized by a double or multiple/ complex dargyrome and a weakly ribbed dorsal surface, while the latter one by a single dargyrome and a smooth dorsal surface.

The other *Euplotes* species were assigned to two new genera, *Euplotoides* Borror and Hill, 1995 [type species, *Euplotoides patella* (Müller, 1773) Ehrenberg, 1838] and *Euplotopsis* Borror and Hill, 1995 [type species, *Euplotopsis affinis* (Dujardin, 1841) Kahl, 1932]. *Euplotoides* includes only lacustrine species carrying a double dargyrome jointly with a cirrotype-9 'pattern II' determined by the loss of the fronto-ventral cirrus numbered V/3. *Euplotopsis* comprises freshwater, euryhaline and marine species possessing a double or multiple/complex dargyrome either in a combination with the cirrotype-9 'pattern I' determined by the loss of the fronto-ventral cirrus V/2 or, more rarely, with the cirrotype-8 distinctive of *E. orientalis* Jiang *et al*., 2009, *E. parkei* Curds, 1974, *E. poljanskyi* Agamaliev, 1966, *E. raikovi* and *E. strelkovi* in which cirrus V/2 is lost together with the second fronto-ventral cirrus of streaks II, III or IV. The fronto-ventral cirral numbers V/2 and V/3 are derived from Wallengren's system of cirrus numbering in hypotrich cili- ates [17]. In *Euplotes*, as illustrated by Curds [1] and shown in Fig. 1g, the longitudinal streaks of fronto-ventral and transverse cirri are numbered I to VI from left to right, and cirri within each streak are numbered 1 to 3 from posterior to anterior.

While this taxonomic subdivision of *Euplotes* is also commonly used in public biodiversity databases, it is increasingly being challenged by molecular phylogenetic approaches [18–23]. With the description of two new Patagonian *Euplotes* species, *E. foissneri* from the Pacific coast and *E. warreni* from the Atlantic coast, we provide additional evidence of the polyphyletic nature and taxonomic inconsistency of the genus *Euplotopsis*. Although both new species share a double dargy- rome and a cirrotype-9 pattern I, which are the diagnostic traits of *Euplotopsis*, they do not cluster together or with other species assigned to *Euplotopsis*. In addition, the finding that
E. warreni represents a new, deep-branching lineage provides insights for updating the *Euplotes* phylogenetic tree.

METhODS

Isolation and cultivation

Samples of sandy sediment–water interface were collected during the second half of January 2008 from shallow pools at intertidal sites in Puerto Natales (Chilean Pacific coast, 51° 43′ 18″ S, 72° 30′ 31″ W) with a water temperature of 5–6 °C and 13–14‰ salinity due to temporary mixing of freshwater flooding with seawater and from Punta Arenas (Chilean Atlantic coast, 53° 08′ 42″ S, 70° 52′ 51″ W) with a water temperature of 4–5 °C and 33‰ salinity. The samples were kept in a refrigerator for 2–3 days prior to inspection under a transportable stereomicroscope for visual recognition and isolation of *Euplotes* specimens. With the aid of a glass micropipette, specimens were transferred into Eppendorf tubes containing fragments of sterile wheat grains (as an initial food source for native bacteria) and natural seawater. Seawater was filtered through Sartorius filters to remove gross debris and micro-fauna, and diluted by the addition of freshwater to roughly half of the native salinity (33‰ salinity) for specimens isolated from Puerto Natales. Stored in a thermos, the Eppendorf tubes were transported to Italy and the surviving *Euplotes* raw cultures were used to raise clonal strains in pasteurized natural seawater with a salinity of 33‰ to which also the strains raised from the Puerto Natales isolates were quickly adapted. Out of the many strains initially grown, only a dozen were able to reproduce. They were maintained in a cold room at 5–6 °C, with 16 h of dark and 8 h of pale light. The green alga *Dunaliella tertiolecta* Butcher, 1959 was used as a standard food source. The description of *E. foissneri* isbased on the type- strain PN- 2A and the description of *E. warreni* on the type- strain Ptg-1.

Morphological analysis

Morphological observations were carried out on morphostatic cells arrested in the G_1/G_0 stage of the reproductive cycle by food deprivation and re-suspension in fresh seawater for no fewer than 2 days. Measurements were done on living cells immobilized by means of a mechanical micro-compressor device [24], with the aid of a calibrated ocular micro-metre mounted on a Nomarski differential interference contrast (DIC) microscope. Counts of diagnostic traits were conducted on cells stained with the Chatton–Lwoff silver nitrate method following Foissner [25]. The nuclear apparatus was visualized with Feulgen and DAPI staining performed with routine procedures [26].

For scanning electron microscopy (SEM), cells were fixed for 30 min at 4 °C in a modified Parducz solution made by mixing six parts of 2% OsO_4 (w/v) in seawater (instead of distilled water) and one part of saturated aqueous solu- tion of $HgCl_2$. Fixed cells were then (1) washed with 0.1 M Na–cacodylate buffer, (2) mounted on cover-slip fragment previously treated with aqueous solution of 0.1% (w/v) l-polylisine, (3) dehydrated in a graded ethanol series, (4) quickly submerged in freon 113, (5) critical point-dried in a Leica EM CPD 300 apparatus, and (6) coated with gold in an Edwards S150B sputter coater. Observations

were performed with a JEOL/JSM-5410 scanning electron microscope.

Fluorescent *in situ* hybridization (FISH)

FISH was performed according to a standard protocol [27] using the eubacterial fluorescein-labelled probe EUB338 (5′-GCTGCCTCCCGTAGGAT-3′) and the *Francisella*-specific Cy3-labelled probe Bwall1448 (5′-CAACCATTCGC- CGGGCCT-3′). After having been re-suspended every day for 1 week in fresh seawater with no food added (to avoid undigested material in the *Euplotes* cytoplasm), cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature, transferred onto glass slides, permeabilized by addition of ethanol 70% (v/v) in water and dehydrated in absolute ethanol for 10 min. Hybridization was carried out in a humid chamber at 46 °C for 3 h, using the fluorescent probes (20 µl aliquots) in hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl pH 7.0, 15% formamide, 0.1% SDS). Slides were flushed with washing buffer (318 mM NaCl, 20 mM Tris–HCl, pH 7.0, 0.1% SDS) and distilled water at 48 °C, air-dried, embedded in antifading mounting medium and examined with a Nikon confocal microscope.

DNA extraction and small subunit (SSu) rRNA gene amplification and sequencing

DNA was extracted from cell cultures previously resuspended for 2 days in fresh seawater using the QIAamp DNA Micro Kit (Qiagen) and following the manufacturer's instructions. The full-length small subunit (SSU) rRNA coding gene was amplified via PCR with the universal forward F9 primer (5′-CTGGTTGATCCTGCCAG-3′) and the 18S Hypo reverse R1513 primer (5′-TGATCCTTCYGCAGGTTC-3′) [28, 29].

Amplifications were performed by adding DNA aliquots (100 ng) to a 50 µl reaction mixture containing 2 mM $MgCl_2$, 250 mM dNTP, one unit of Taq DNA polymerase (Polymed), and 0.2 mM of each primer. PCRs were run in a GenAmp PCR system 2400 (Applied Biosystems), following a standard programme [4, 5]. Amplified products were purified on Quantum Prep PCR Kleen Spin columns (Bio-Rad) and sequenced in both directions with an ABI Prism 310 auto-
mated DNA sequencer (Applied Biosystems). To minimize amplification errors, the sequences of products from two distinct amplifications were compared.

Phylogenetic analysis

Euplotes SSU rRNA gene sequences were retrieved from the GenBank/EMBL database, narrowing the search for sequences longer than 1500 nucleotides and including only one sequence for each species, except for *E. charon*, which is represented by two unrelated sequences in the database and neither has associated morphological information. The sequence alignment was prepared in ClustalX 1.81 [30] with default parameter settings and edited with BioEdit 7.0.0 [31]. The resulting alignment had 2109 nucleotide positions.

Maximum-likelihood (ML), maximum-parsimony (MP) and Bayesian inference (BI) analyses were performed with the Tree-Puzzle 5.0 [32], PAUP 4.b10 [33] and MrBayes 3.1 [34] programs, respectively, using the GTR+G+I model selected under the Akaike information criterion by ModelTest 3.7 [35]. In the Bayesian analysis, two sets of Markov chain Monte Carlo (MCMC) simulations were run and each set had four chains. The number of generations in the MCMC simulations was 1110000, with trees sampled every 100th generation. The first 250000 generations were discarded as burn-in. The reliability of internal branches in the ML and MP phylogenetic trees was evaluated with the bootstrap method and 1000 replicates [36], while that of the BI tree by calculating posterior probabilities.

RESULTS

Euplotes foissneri sp. nov.

Morphology (Fig. 1a–m, Table 1)

Cells *in vivo* range from 46 to 58 µm long and from 32 to 40 µm wide, and have a length/width ratio of about 1.5 (Table 1). The body is oval in shape, with the frontal region gently convex and the rear region slightly pointed and asymmetric (Fig. 1a). Starved cells, with more transparent cytoplasm, are filled with many 2–4 µm brownish vesicles, (lipid?) droplets and fine refractile grains, and do not show any contractile vacuole.

The dorsal surface (Fig. 1b–e) is markedly pleated and decorated with six longitudinal ridges the crests of whichslope slightly towards the left side. It bears six kineties, each positioned midway on the left slope of each ridge and formed by 12–16 dikinetids. The equatorial and subequatorial dikinetids appear to be not ciliferous. Two other kineties run laterally along the body sides. The kinety on the left side extends only sub-equatorially and includes seven or eight non-ciliferous dikinetids, while the kinety on the right-side body margin is only a little shorter than the dorsal kineties and includes 10–12 dikinetids. The dargyrome is of the double type and formed by rectangular cortical alveoli, 14–16 of which form the two most central rows running between kineties 4 and 5. In silver-stained specimens, alveoli on the left side of each kinety appear slightly wider than those on the kinety right. However, as documented in *E. bisulcatus* Kahl, 1932 [37], this difference in the alveolar width very likely reflects an optical effect caused by the presence of the prominent dorsal ridges.

The ventral surface (Fig. 1f–i) invariably bears a cirrotype-9 pattern I, which is characterized by very closely apposed cirri IV/2 and IV/3. The transverse cirri are five in number and do not invariably take the typical V-shaped arrangement. In at least 5% of cells, the left transverse cirrus (II/1) lies in line with the other four (Fig. 1i). There are usually three, rarely two (in about 20% of cells) caudal cirri. The left caudal/marginal cirrus is inserted 5 µm behind the rear end of the adoral zone and is thicker than the two other caudal cirri lying very close to each other in the middle of the cell rear. The buccal field extends along 70–75% of the cell length and is paved with variously shaped cortical alveoli. The outer border is delimited with 28–32 adoral membranelles of which 11 or 12 (collar) are inserted frontally and the others (lapel) extend down to the bottom of the buccal field to join the paroral membrane. As revealed by the SEM (Fig. 1j), the insertion sites of adoral

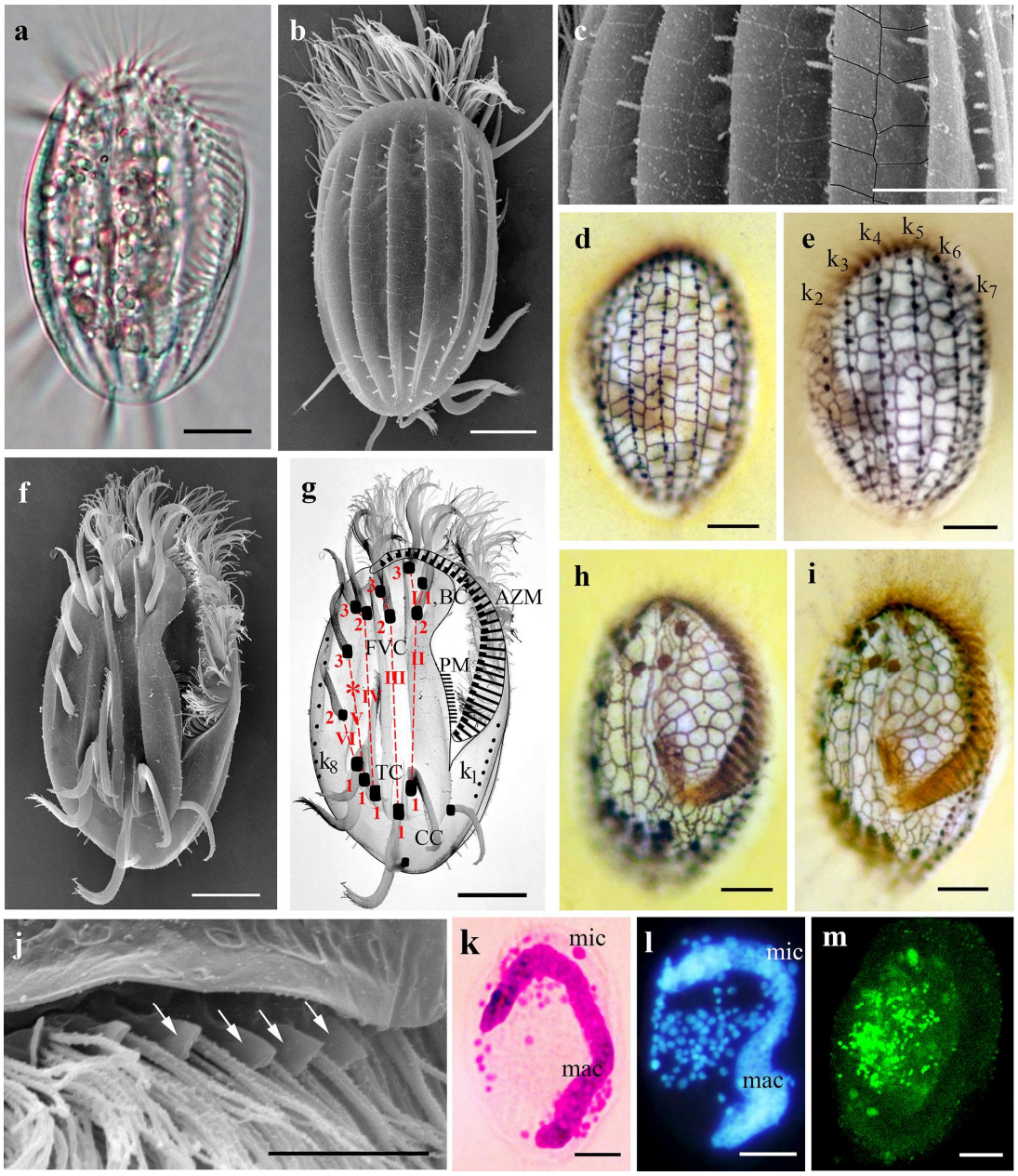


Fig. 1. *Euplotes foissneri* sp. nov. (a) Living specimen in ventral view. (b) SEM image of the dorsal surface carrying six ridges each ornamented with a kinety. (c) Magnified section of the previous SEM image highlighting the kinety location on the left slope of each ridge and the even outline (highlighted locally in black) of cortical alveoli. (d, e) Dorsal view of silver nitrate-stained specimens, showing the dargyrome of double type, with some alveoli irregularly shaped. (f) SEM image of the ventral surface, showing the cirrotype-9 pattern I. (g) Previous SEM image processed to highlight the insertion sites of the fronto-ventral, transverse and caudal cirri (FVC, TC and CC, respectively), and the disposition of adoral membranelles (AZM), paroral membrane (PM), and the leftmost (k_1) and rightmost (k_8) lateral kineties. Numbering of cirri (in red) follows the Wallengren's system as applied to *Euplotes* by Curds [1], who labels I/1 the buccal cirrus (BC). Fronto-ventral and transverse cirri originating from the same streak are connected by dashed lines. The asterisk marks the putative position of the missing cirrus 'V/2' that determines the cirrotype-9 pattern I. (h) Ventral view of the silver nitrate-stained holotype specimen, showing the argyrome composed of irregular alveoli and the arrangement of the cirri. (i) Occasionally, the transverse cirri appear arranged in a line. (j) SEM image of the cell anterior left border, showing the foliaceous crests (arrows) intercalated between the adoral membranelles. (k, l) Feulgen (k) and DAPI (l) stained specimens, showing the bulky macronucleus (mac) associated with one micronucleus (mic) and surrounded with roundish coloured bodies of unknown origin. (m) Cell, showing a dense population of cytoplasmic bacteria after hybridization with the green fluorescein-labelled universal eubacteria probe EUB338. Scale bars: 5 µm (c, j) and 10 µm (a, b, d–i, k–m).

Table 1. Morphometric data on *Euplotes foissneri* sp. nov.

SD, standard deviation; CV, coefficient of variation in %; n, number of specimens investigated.

Characteristic	Min–Max	Mean	SD	CV	n
Body, length (μm)[a]	46–58	53.6	4.61	8.6	30
Body, width (μm)[a]	32–40	36.2	3.26	9.0	30
Buccal field, length (μm)[a]	34–42	38.8	2.25	5.8	23
Adoral membranelles[b]	28–32	28.5	0.78	2.7	20
Fronto-ventral cirri[b]	9	9	0	0	24
Transverse cirri[b]	5	5	0	0	24
Caudal cirri[b]	2–3	2.8	0.39	13.6	18
Dorsolateral kinety[b]	8	8	0	0	28
Dikinetids in mid-dorsal kineties[b]	12–16	14.6	1.07	7.3	28
Dorsal grooves[c]	6	6	0	0	15

Data based on: *a,* immobilized living cells; *b,* silver-stained cells; *c,* SEM-processed cells.

membranelles are interspaced from one another by leaf-like cortical crests. These were originally described in *E. crassus* (Dujardin, 1841) Kahl, 1932 as sites of membrane morphogenesis in mating cells [38]. The inner border of the buccal field has a sinuous, question mark-like profile. It broadens anteriorly into a sickle-shaped lip that rims the insertion of the frontal membranelles and is the site of insertion of the buccal cirrus, labelled I/1 according to Curds [1], and cirri II/2 and II/3. Posteriorly, it protects the well-developed, 8–9 μm long paroral membrane and extends sub-equatorially in the form of a sharp ridge that separates the left caudal/marginal cirrus from all the other cirri. Two other prominent longitudinal ridges run on the right half of the ventral surface. One ridge extends medially over nearly the entire body length, tracing a boundary between cirri of streaks III and IV; the second and shorter one extends between the insertions of cirri VI/1 and VI/2.

The macronucleus is quite bulky and varies markedly from a more common hook-like shape to a horseshoe- or 3-like shape. Its posterior arm is quite extended and folded back on itself. There is invariably one sub-spherical micronucleus (3 μm in diameter), which is associated with the anterior external side of the macronucleus and surrounded by a plethora of small (1.5–2 μm in diameter) DAPI- and Feulgen-stained bodies of unknown origin (Fig. 1k and l).

Endosymbionts, eco-ethological and biological remarks

Severely starved cells, hybridized *in situ* with the fluorescein- labelled universal eubacteria probe EUB338 (Fig. 1m), revealed the pervasive presence of subspherical (0.4–0.8 μm in diameter) symbiotic bacteria of a yet-to-be determined taxon. Bacteria were densely concentrated in the central body region.

Resting stages (cysts) were not observed in response to severe starvation (2–3 weeks), or in response to variations in salinity from 10 to 45‰, temperature from 0 to 18 °C, and population cell density from 10 to approximately 10^3 cells ml^{-1}. On the substratum, cells alternate between periods of motionless and fast crawling along essentially rectilinear trajectories; they tend not to swim much. At 5–6 °C (the standard cultivation temperature) and in the continuous presence of food, the reproduction rate was assessed to be roughly one cell fission every 4–5 days. It increases to one division every 3–4 days at 9–10 °C and decreases to less than one division every week at 1–2 °C. A temperature of 20 °C is not tolerated for more than 2–3 days. No information was obtained on sex manifested as conjugation, because no strain combination was found to regularly raise mating pairs in a sufficient number for analysis.

Euplotes warreni sp. nov.

Morphology (Fig. 2a–m, Table 2)

Cells *in vivo* range from 34 to 45 μm long and from 25 to 30 μm wide, and have a length/width ratio of about 1.4 (Table 2). The body shape is roughly oval and slightly asymmetrical, with a more curved left side and a more rectilinear right side (Fig. 2a). Starved cells show cytoplasm rich in tiny concretions and (lipid?) droplets mostly concentrated in the central body region. A large contractile vacuole (5 μm in diameter) is situated in a median position close to the body posterior end.

The dorsal surface is deeply ploughed over the entire length by three unevenly spaced large grooves (Fig. 2b–e). Two grooves extend close to each other over the left body half, while the third groove runs over the right body half separated by a smooth, 14–15 μm wide central plateau. Four kineties run over the dorsal surface and each consists of 9–11 dikinetids that are ciliferous only at the kinety extremities. Three of them lie on the left slope of the three grooves, while the most central one lies medially on the smooth central plateau. Two other kineties run along the lateral cell margins. The kinety on the left side extends only sub-equatorially and is formed by only four or five not ciliferous dikinetids, while the kinety on the right side matches the length of the dorsal kineties and includes eight or nine dikinetids. The dargyrome is of the double type and formed by large, often irregularly shaped quadrangular cortical alveoli (4–5 μm), 8–10 of which form two rows running alongside kinety 4.

The cell ventral surface bears a cirrotype-9 pattern I, which is characterized by a close insertion of cirri IV/2 and IV/3, one immediately behind the other (Fig. 2f–h). Few specimens have been observed to bear a cirrotype-8, which is determined by the lack of cirrus III/2 (Fig. 2g). Transverse cirri are constantly five in number and they are markedly thicker and longer (20 μm) than all the other cirri. Caudal cirri are three in number, and the left caudal/marginal cirrus is inserted 5–6 μm behind the proximal end of the buccal field and is more conspicuous than the two other cirri, which lie in a median position and are inserted just on the cell rear margin. The buccal field extends 64–67% (approximately 2/3) of the cell length and is paved with a few large, variously shaped

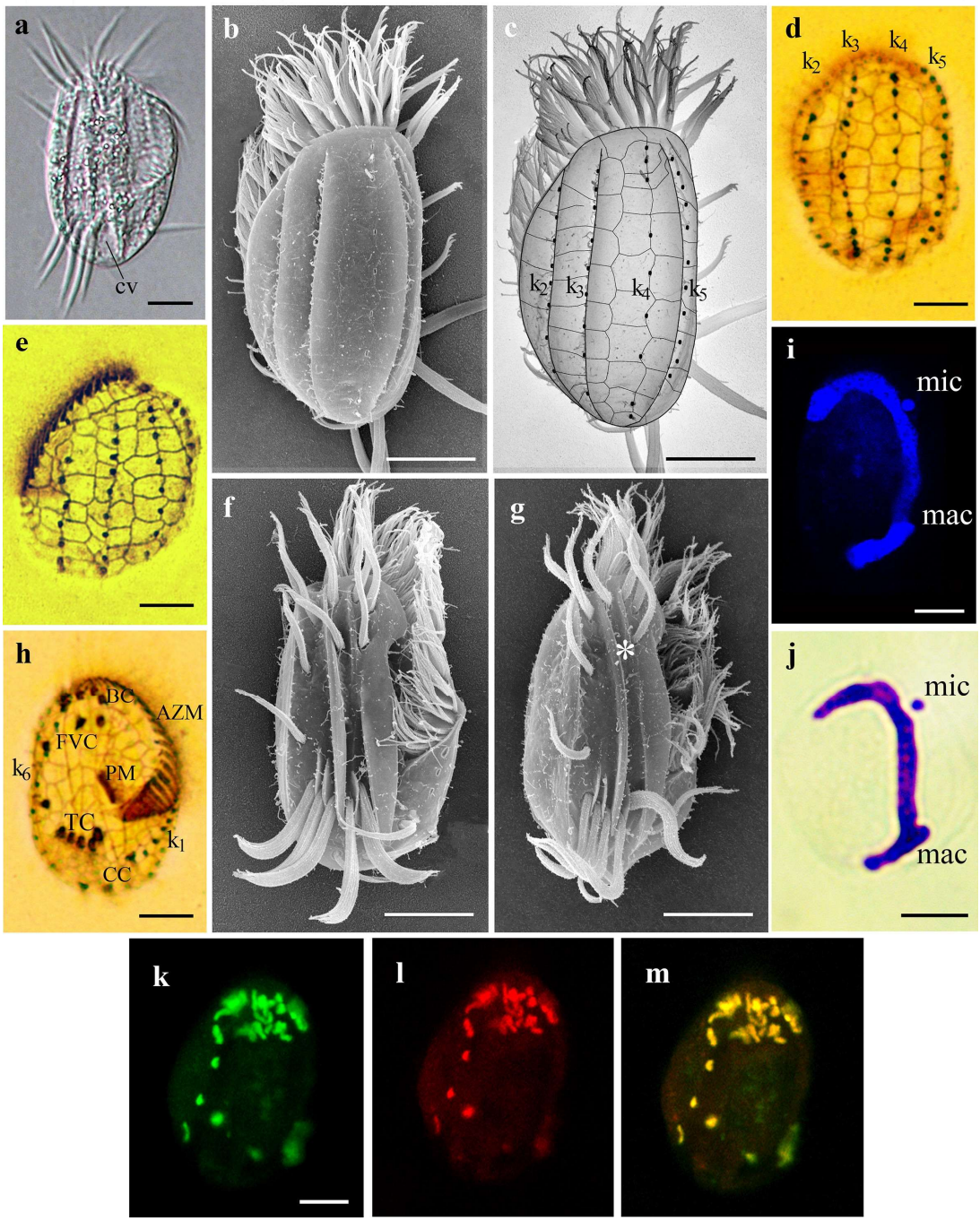


Fig. 2. *Euplotes warreni* sp. nov. (a) Living specimen in ventral view. cv, Contractile vacuole. (b) SEM image of the cell dorsal surface with three unevenly spaced grooves. (c) Previous SEM image processed to highlight the dargyrome pattern and the four dorsal kineties (k_2 to k_5). (d) Dorsal view of a silver-nitrate stained specimen, showing the dargyrome of double type formed by unusually large alveoli and the four dorsal kineties. (e) Dorso-frontal view of a silver-nitrate stained specimen, showing the arrangement of the more frontal (collar) adoral membranelles. (f) SEM image of the cell ventral surface, showing the standard cirrotype-9 pattern I. (g) SEM image of the ventral surface of an exceptional cell with cirrotype-8 due to the loss of cirrus III/2 (whose putative position is indicated by an asterisk). (h) Ventral view of the silver-stained holotype specimen, showing the argyrome composed of irregular alveoli and the arrangement of fronto-ventral, transverse and caudal cirri (FVC, TC and CC, respectively), adoral membranelles (AZM), paroral membrane (PM) and lateral kineties (k_1 and k_6). (i, j) DAPI (i) and Feulgen (j) stained specimens, showing the C- or hook-shaped macronucleus (mac) associated with one spherical micronucleus (mic). (k–m) Cell showing cytoplasmic *Francisella* bacteria identified after hybridization with the green fluorescein-labelled universal eubacteria probe EUB338 (k), the red Cy3-labelled *Francisella*-specific probe Bwall1448 (l) and merging (yellow) of the two probes (m). Scale bars, 10 µm.

Table 2. Morphometric data on *Euplotes warreni* sp. nov.

SD, standard deviation; CV, coefficient of variation in %; n, number of specimens investigated.

Characteristic	Min–Max	Mean	SD	CV	n
Body, length (μm)[a]	34–45	39.5	3.38	8.6	30
Body, width (μm)[a]	25–30	27.5	2.83	10.3	30
Buccal field, length (μm)[a]	23–30	26.5	1.78	6.1	25
Adoral membranelles[b]	23–25	24.1	0.78	3.2	22
Fronto-ventral cirri[b]	8–9	8.9	0.32	3.6	28
Transverse cirri[b]	5	5	0	0	28
Caudal cirri[b]	3	3	0	0	24
Dorsolateral kinety[b]	6	6	0	0	30
Dikinetids in mid-dorsal kineties[b]	9–11	9.7	0.83	8.6	30
Dorsal grooves[c]	3	3	0	0	15

Data based on: *a*, immobilized living cells; *b*, silver-stained cells; *c*, SEM-processed cells.

cortical alveoli (Fig. 2h). Its outer border bears 23–25 quite long (15 μm) adoral membranelles, 9–10 of which (collar) lie frontally and the others (lapel) extend down along the ventrolateral margin. The inner border shows a distinct question mark-like shape. Anteriorly, it widens to form a pronounced lip that accommodates the insertion of buccal cirrus and cirri II/2 and II/3, and extends frontally to rim the insertion of collar adoral membranelles. Posteriorly, it covers and protects the 5–6 μm long paroral membrane. A prominent rib runs centrally along the right half of the ventral surface, over nearly the entire length and traces a sharp boundary between cirri of streaks III and IV.

The nuclear apparatus (Fig. 2i, j) consists of a hook- or C-shaped macronucleus, with an extended posterior arm folded back on itself and one spherical micronucleus (2–3 μm in diameter) usually associated with a shallow depression of the macronuclear antero-lateral surface.

Endosymbionts, eco-ethological and biological remarks

Like *E. foissneri*, severely starved *E. warreni* specimens contain cytoplasmic symbiotic bacteria (Fig. 2k), as observed after *in situ* hybridization with the fluorescein-labelled universal eubacteria probe EUB338. However, these bacteria markedly differ from those of *E. foissneri* in that they were of larger dimensions (1.0–1.5 μm long) and preferred a peripheral localization in the cell anterior body region, similarly to *Fran- cisella* bacteria previously isolated from an Antarctic marine species, *E. petzi* Wilbert and Song, 2008 [27]. This supposi- tion was supported by positive results from the second *in situ* hybridization carried out with the Cy3-labelled *Francisella*- specific probe Bwall1448 (Fig. 2l–m).

As in *E. foissneri*, resting stages were not observed in response to variations in salinity, temperature, cell density and food deprivation. Likewise, it was not possible to obtain information on sex manifested as conjugation. On the substratum, cells stop motionless for several seconds after fast and short left-turning crawling, while they swim slowly in monotonous counter-clockwise spirals in the water column. The reproduction rate of one division every 3–4 days at 5–6 °C was observed to increase to one division every 2–3 days at 9–10 °C, and decrease to one division every 6–7 days at 1–2 °C. Exposition for more than 2–3 days at 20 °C was lethal.

Phylogenetic relationships of *E. foissneri* sp. nov. and *E. warreni* sp. nov.

The SSU rRNA gene sequence of the *E. foissneri* type-strain PN-2A is 1838 nt long with a G+C content of 43.5mol%, and the sequence of the *E. warreni* type-strain Ptg-1 is 1767 nt long with a G+C content of 45.5mol%. The difference in length between the two gene sequences is due to multiple insertions/deletions of single nucleotides or segments of 2–21 nucleotides.

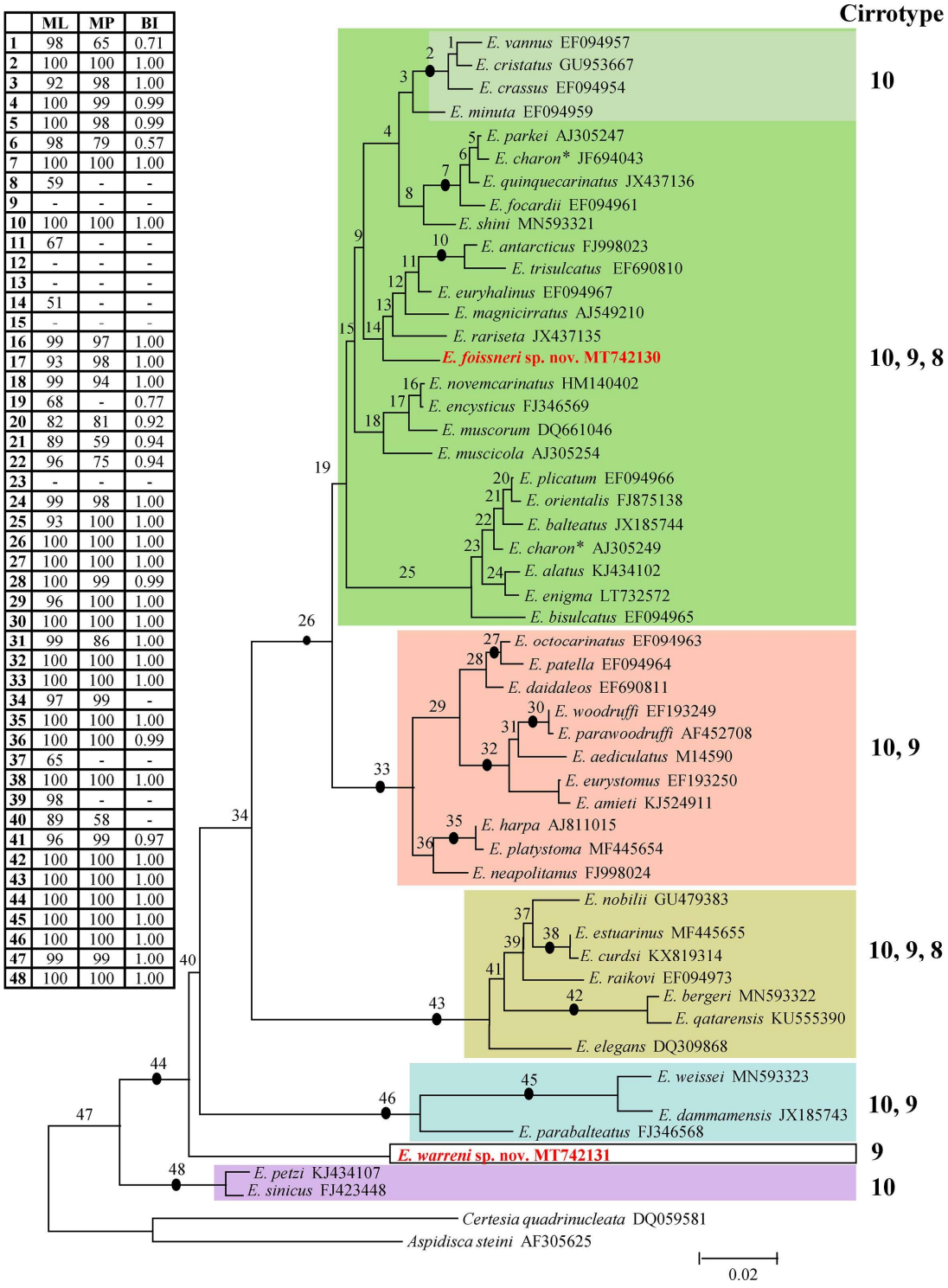
In the blast analysis, the *E. foissneri* sequence shows the closest relatedness (96.4% identity) to a sequence (accession number JX437135) from strain Ubt22 of *E. rariseta* Curds *et al*., 1974. The *E. warreni* sequence shows maximal relatedness (89.4% identity) to a sequence (accession number HM140406) from an unidentified *Euplotes* sp. SNK-2011 isolate.

In the *Euplotes* SSU rRNA gene sequence-based phylogenetic tree, *E. foissneri* and *E. warreni* are distinctly separated from each other (Fig. 3). *Euplotes foissneri* clusters within the largest and least resolved clade that includes the vast majority of marine species with cirrotype-10, along with *E. bisulcatus* with cirrotype-9 and *E. orientalis* with cirrotype-8. Within this clade, *E. foissneri* groups with *E. antarcticus* Fenchel and Lee, 1972, *E. euryhalinus* Valbonesi and Luporini, 1990,
E. magnicirratus Carter, 1972, *E. rariseta* and *E. trisulcatus* Kahl, 1932. However, statistical support is very weak. On the other hand, *E. warreni* does not cluster with any of the major clades—either with the six clades numbered I to VI from bottom to top [39, 40] or the five clades labelled A to E from top to bottom [18, 19]—that are generally recognized in the *Euplotes* phylogenetic tree. It represents a new, independent lineage that branches off immediately after the clade formed by *E. petzi* and *E. sinicus* Jiang *et al*., 2010. The base of the tree is well resolved and statistically strongly supported.

DISCUSSION

Morphological comparison of *E. foissneri* sp. nov. and *E. warreni* sp. nov. with congeners (Table 3)

Euplotes foissneri may be considered a brackish species in relation to the low-salinity site from where it was isolated, or a marine species in light of its immediate adaptation to cultivation in natural seawater. In the former case, the comparison narrows to *E. elegans* Kahl, 1932 which, according to the accu- rate re-description by Schwarz *et al.* [41], has a dargyrome



	ML	MP	BI
1	98	65	0.71
2	100	100	1.00
3	92	98	1.00
4	100	99	0.99
5	100	98	0.99
6	98	79	0.57
7	100	100	1.00
8	59	-	-
9	-	-	-
10	100	100	1.00
11	67	-	-
12	-	-	-
13	-	-	-
14	51	-	-
15	-	-	-
16	99	97	1.00
17	93	98	1.00
18	99	94	1.00
19	68	-	0.77
20	82	81	0.92
21	89	59	0.94
22	96	75	0.94
23	-	-	-
24	99	98	1.00
25	93	100	1.00
26	100	100	1.00
27	100	100	1.00
28	100	99	0.99
29	96	100	1.00
30	100	100	1.00
31	99	86	1.00
32	100	100	1.00
33	100	100	1.00
34	97	99	-
35	100	100	1.00
36	100	100	0.99
37	65	-	-
38	100	100	1.00
39	98	-	-
40	89	58	-
41	96	99	0.97
42	100	100	1.00
43	100	100	1.00
44	100	100	1.00
45	100	100	1.00
46	100	100	1.00
47	99	99	1.00
48	100	100	1.00

Fig. 3. Positions of *E. foissneri* and *E. warreni* in the *Euplotes* SSU rRNA gene phylogenetic tree. Nodes are numbered progressively. Bootstrap values (≥50%) and posterior probability values (≥0.50) are provided for each node. ML, Maximum-likelihood support; MP, maximum-parsimony support; BI, Bayesian inference support. Statistically fully supported branches are marked with solid circles. The scale bar corresponds to two substitutions per 100 nucleotide positions. Following Jiang *et al*. [39, 54] and Chen *et al*. [55], *Certesia quadrinucleata* and *Aspidisca steini* were used as outgroups. Asterisk marks the two unrelated sequences of *E. charon*. No gene sequence is available from *E. affinis*, which represents the type species of *Euplotopsis*. The five or six clades generally recognized in phylogenetic analyses are highlighted with different colours. The new clade represented by *E. warreni* is boxed. The species cirrotype is indicated alongside clades.

Table 3. Morphological comparison of *Euplotes foissneri* sp. nov. and *Euplotes warreni* sp. nov. with marine (m) and brackish (b) congeners with double dargyrome and cirrotype-9, pattern I and II. nd, Not Determined; *n*, number.

Species	Body shape	Average body size (μm)	Buccal field/ body length ratio	Cirrotype-9 pattern	Adoral membranelles (*n*)	Kineties (*n*)	Dikinetids in mid-dorsal kineties (*n*)	Caudal cirri (*n*)	Macronucleus	Dorsal surface
E. apsheronicus (m)[y]	Ellipsoid	55×35	1/2	I	30–35	9	15	4	C-shaped	nd
E. bisulcatus (m)[z]	Oval	40×30	2/3	I	~17	7[d]	9–10[d]	3	C-shaped	Two shallow grooves[d]
E. dogieli (m)[a]	Ellipsoid	60×30	2/3	II	35–38	7	13	3	C-shaped	nd
E. elegans (b)[b]	Elongated oval	118×65	2/3	II	47–64	9–10	18	4	C-shaped	Seven, eight ridges
E. latus (m)[y]	Roughly oval	70×46	2/3	II	35–40	6	15	4	open angular C-shaped	nd
E. weissei (m)[c]	Roughly trianglular	37.5×25	2/3	I	24	6–7	9	3	C-shaped	Two deep grooves
E. zenkewitchi (m)[a]	Elongated	80×50	1/2	I	50–55	10	18	3–4	C-shaped, with pointed tail	nd (smooth?)
E. foissneri sp. nov. (m/b)	Oval	53×36	3/4	I	28–32	8	12–16	2–3	Hook-, horseshoe- or 3-shaped Hook-, open C-shaped	Six prominent ridges
E. warreni sp. nov.	Roughly	39×27	2/3	I	23–25	6	9–11	3		Three deep

a, After Curds [1]; *b*, after Schwartz *et al.* [41]; *c*, after Lian *et al.* [19]; *d*, after Valbonesi and Luporini [37].

of the double, not complex/multiple type as earlier reported by Tuffrau [42]. This species clearly differs from *E. foissneri* in the cirrotype-9 pattern (II vs I), body size (118×65 μm vs 53×36 μm) as well as in the number of adoral membranelles (47–64 vs 28–32), kineties (9–10 vs 8), dikinetids in mid-dorsal kineties (18 vs 12–16), and ridges on the dorsal surface (7–8 vs 6).

On the other hand, if *E. foissneri* is regarded as a marine species, the comparison comes to include many more species, i.e. *E. apsheronicus* Agamaliev, 1966, *E. bisulcatus*, *E. dogieli,* Agamaliev, 1967, *E. latus* Agamaliev, 1967, *E. weissei* Lian *et al*., 2020, and *E. zenkewitchi* Burkovsky, 1970. *Euplotes dogieli* and *E. latus* differ from *E. foissneri* in the cirrotype-9 pattern (II vs I), the number of adoral membranelles (35–38 and 35–40, respectively, vs 28–32), the number of kineties (7 and 6, respectively, vs 8) as well as, limited to *E. latus*, in the body size (70×46 μm vs 53×36 μm). Among the other comparable congeners which have a cirrotype-9 pattern I like *E. foissneri*,
E. zenkewitchi has a larger body size (80×50 μm vs 53×36 μm), a relatively wider buccal field, nearly double the number of adoral membranelles (50–55 vs 28–32), more kineties (10 vs 8) and dikenitids in mid-dorsal kineties (18 vs 12–16). *Euplotes weissei* and *E. bisulcatus* have a smaller body size than *E. foissneri* (37.5×25 μm and 40×30 μm, respectively, vs 53×36 μm) as well as fewer adoral membranelles (24 and 17, respectively, vs 28–32) and dikinetids in the mid-dorsal kinety (9 and 5–7, respectively, vs 12–16). In addition, *E. weissei* has an eccentric 'triangle-shaped' body and a dargyrome of a rather spurious double-type, which is likely generated by the two grooves that deeply furrow the cell dorsal surface [21]. Lastly, *E. apsheronicus*, which practically equals in body size to *E. foissneri*, is unique in having an indentation midway down the cell anterior margin that forms a distinct collar region. In addition, it differs in the length of the buccal field (extending over half vs 3/4 of the body length) and in the number of kineties (9 vs 8) and caudal cirri (4 vs 2–3).

The morphological comparison of *E. warreni* with congeners remains practically restricted to *E. bisulcatus* and *E. weissei*. They are the only ones, among the *Euplotes* marine species with double dargyrome and cirrotype-9 pattern I or II, that share a small body size and a furrowed dorsal surface with *E. warreni*. However, *E. warreni* has three and unevenly spaced grooves on its dorsal surface, whereas *E. bisulcatus* and *E. weissei* are characterized by two and symmetrically spaced grooves. In addition, *E. weissei* appears to be unique in having a roughly triangular body shape and a 'mixed' single-double dargyrome pattern [20]. *Euplotes bisulcatus* further differs from *E. warreni* in the number of adoral membranelles (17 vs 23–25) and kineties (7 vs 6).

CONCLUSIONS

The remarkable species richness and the cosmopolitan distribution of *Euplotes* are supported by unceasing descriptions of species new to science. As many as eight novel taxa, *E. bergeri* Lian *et al*., 2020 [20], *E. curdsi* Syberg-Olsen *et al*., 2016 [23],

E. domenicanus Živaljić *et al*., 2020 [18], *E. enigma* Boscaro

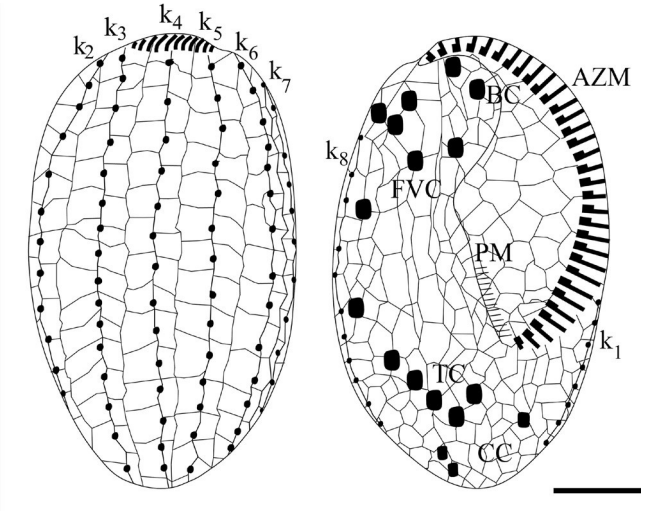


Fig. 4. Diagrammatic presentation of *E. foissneri*, showing dorsolateral kineties (k_1 to k_8), adoral zone of membranelles (AZM), paroral membrane (PM), and position of fronto-ventral, transverse and caudal cirri (FVC, TC and CC, respectively). Scale bar, 10 µm.

et al., 2019 [19], *E. estuarinus* Yan *et al*., 2018 [43], *E. shini* Lian *et al*., 2020 [20], *E. weissei* [20] and *E. wuhanensis* Lian *et al*., 2019 [21], have been recognized in the past 3 or 4 years, and *E. foissneri* and *E. warreni* can now be added on this list. However, most old *Euplotes* descriptions remain merely typo- logical and based on counts and measurements of individuals from temporary cultures raised from only few natural isolates. In general, they lack information on morphological details that can be clearly visualized only at the ultrastructural level.

In addition, in *Euplotes* species and spirotrichs in general, it is difficult to integrate the morphological criteria with breeding analyses demanded for applying the species biological concept. *Euplotes* species regulate the sexual phenomenon

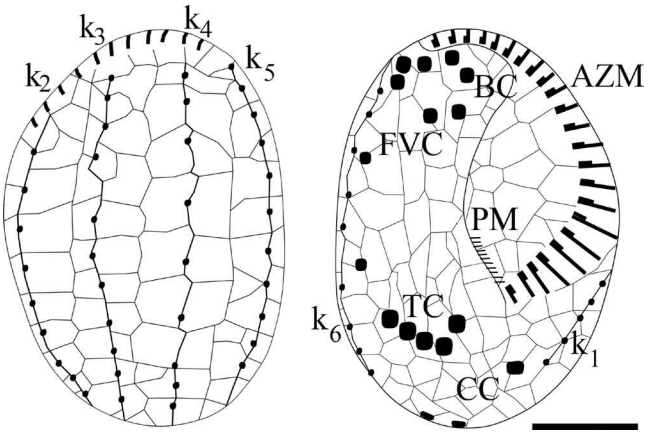


Fig. 5. Diagrammatic presentation of *E. warreni*, showing dorsolateral kineties (k_1 to k_6), adoral zone of membranelles (AZM), paroral membrane (PM), and position of fronto-ventral, transverse and caudal cirri (FVC, TC and CC, respectively). Scale bar, 10 µm.

of conjugation and, hence, their inter-population gene flow through mating systems of the high-multiple, virtually 'open' type. This necessarily limits comparative breeding analyses to strain collections that are very likely far from reflecting the actual dimensions of the species genetic variety in the wild [44–46]. Much different is the case of *Paramecium* and *Tetrahymena* species in which 'closed' binary and low-multiple mating systems greatly facilitate breeding analyses on strain collections that are comprehensive of all naturally occurring cell types [47–50]. In addition, the high-multiple mating systems strongly favour cross-mating phenomena that tend to relax the genetic boundaries between species living in sympatry, such as *E. crassus*, *E. vannus* and *E. minuta* Yocum, 1930 [46] or *E. octocarinatus* Carter, 1972 and *E. patella* [50]. Also, they may include populations in which genetic separation is favoured by the self-fertilization phenomena consequent to autogamic sex [51] and pheromone-induced conjugation between genetically identical cells [52, 53].

The division of *Euplotes* into *Euplotes* (*sensu stricto*), *Euplo- toides*, *Euplotopsis* and *Moneuplotes* [15] has further compli- cated the systematics of this taxon, because phylogenetic analyses recognize only *Moneuplotes* as a monophyletic taxon [40]. The other three genera are instead found as polyphyletic, separated into distinct clades in which marine species with double or multiple/complex dargyrome and cirrotype-10 cluster along with freshwater species with cirrotype-9 pattern II, or with marine or freshwater species with double or multiple/complex dargyrome and cirrotype-9 pattern I or cirrotype-8.

Although *E. foissneri* and *E. warreni* share the cirrotype-9 pattern I and the double dargyrome, which would justify their taxonomic assignment to *Euplotopsis*, they are quite far apart on the *Euplotes* phylogenetic tree. While *E. foissneri* joins marine species with cirrotype-10 within a statistically not well-supported clade, *E. warreni* forms an independent, deep-branching lineage that branches off immediately after the basal clade of *E. petzi* and *E. sinicus*, which are marine species with double dargyrome and cirrotype-10. This early branching of *E. warreni* thus suggests that the fronto-ventral cirrus V/2 was lost in *Euplotes* species most likely before the loss of any other fronto-ventral cirrus and this loss (distinctive of the cirrotype-9 pattern I) might have preceded the transition of *Euplotes* species from marine to freshwater habitats.

Taxonomic summary

Euplotes foissneri sp. nov.

ZooBank registration number: urn:lsid:zoobank.org:act:CA3E0555-58F8-4C5A-A016-209E9C15B46A.

Diagnosis (Fig. 4)

Brackish/marine, medium-sized *Euplotes* species (on average 53×36 µm), with cirrotype-9 pattern I, five transverse cirri and three, rarely two caudal cirri. Buccal field extending about 3/4 of body length, 28–32 adoral membranelles. Dorsal surface with six prominent ridges, double dargyrome; eight dorsolateral kineties. Macronucleus bulky, variously shaped

with a curled posterior arm; one sub-spherical micronucleus. Endosymbiotic eubacteria present.

Type locality
Sandy beach in Puerto Natales, Chile (51° 43′ 18″ S, 72° 30′ 31″ W).

Type material
A holotype slide (reg. no. MNS-3584) and a paratype slide (reg. no. MNS-3585) containing silver nitrate-impregnated specimens of the type strain PN-2A have been deposited at the Museo di Storia Naturale of the University of Pisa, located in Calci (PI). Living cell and DNA samples of the type-strain PN-2A are available upon request from the authors' laboratories.

Etymology
The species is named after Prof. Wilhelm Foissner (1948–2020; Paris Lodron University of Salzburg, Salzburg, Austria), in recognition of his milestone contribution to the study of ciliate taxonomy and ecology.

Euplotes warreni sp. nov.

ZooBank registration number: urn:lsid:zoobank.org:act:2FC12015-EB6B-4D96-AA33-3348967E2817.

Diagnosis (Fig. 5)
Marine, small *Euplotes* species (on average 39×27 μm) with cirrotype-9 pattern I (rarely cirrotype-8 due to loss of cirrus III/2), five transverse and three caudal cirri. Buccal field extending nearly 2/3 of body length, 23–25 adoral membranelles. Dorsal surface with three deep, unevenly spaced grooves, double dargyrome; six dorsolateral kineties. Macronucleus hook- or C-shaped with a curled posterior arm; one spherical micronucleus. Cytoplasm hosting symbiotic proteobacteria of the genus *Francisella*.

Type locality
Sandy beach in Punta Arenas, Chile (53° 08′ 42″ S, 70° 52′ 51″ W).

Type material
A holotype slide (reg. no. MSN-3586) and a paratype slide (reg. no. MSN-3587) containing silver nitrate-impregnated specimens of the type strain Ptg-1 have been deposited in the Museo di Storia Naturale of the University of Pisa, located in Calci (PI). Living cell and DNA samples of the type strain Ptg-1 are available upon request from the authors' laboratories.

Etymology
The species is named after Dr. Alan Warren (Natural History Museum, London, UK) in recognition of his milestone contribution to the study of ciliate taxonomy and ecology.

Funding information
This work was financially supported by the PNRA (Programma Nazionale di Ricerca in Antartide) and the Italian Ministry of University and Research (MIUR).

Acknowledgements
The authors greatly appreciate Dr. Simone Gabrielli for skilful assistance in processing specimens for SEM, Prof. Weibo Song for providing the *Euplotes bergeri* SSU-rRNA gene sequence before being published, and Dr. Sheila Beatty for language editing in the manuscript. The authors also are particularly grateful to two anonymous reviewers and the editor, Dr. Peter Vdacny, for valuable suggestions to improve the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References
1. Curds CR. A guide to the species of the genus *Euplotes* (Hypotrichida, Ciliatea). *Bull Br Mus (Nat Hist) Zool* 1975;28:1–61.
2. Berger H. *Bibliography of Hypotrichs and Euplotids (Ciliophora)*. *Verlag*. Salzburg, Austria: Helmut Berger; 2006.
3. Di Giuseppe G, Barbieri M, Vallesi A, Luporini P, Dini F. Phylogeographical pattern of *Euplotes nobilii*, a protist ciliate with a bipolar biogeographical distribution. *Mol Ecol* 2013;22:4029–4037.
4. Di Giuseppe G, Erra F, Frontini F, Dini F, Vallesi A *et al*. Improved description of the bipolar ciliate, *Euplotes petzi*, and definition of its basal position in the *Euplotes* phylogenetic tree. *Eur J Protistol* 2014;50:402–411.
5. Di Giuseppe G, Erra F, Dini F, Alimenti C, Vallesi A *et al*. Antarctic and Arctic populations of the ciliate *Euplotes nobilii* show common pheromone-mediated cell-cell signaling and cross-mating. *Proc Natl Acad Sci USA* 2011;108:3181–3186.
6. Di Giuseppe G, Dini F, Vallesi A, Luporini P. Genetic relationships in bipolar species of the protist ciliate, *Euplotes*. *Hydrobiologia* 2015;761:71–83.
7. Di Giseppe G, Dini F, Vallesi SA, Luporini P. *Euplotes (Dorsoventrally Flattened Ciliates)*. John Wiley & Sons, Ltd; 2015.
8. Nanney DL. *Experimental Ciliatology*. New York: Wiley and Sons; 1980.
9. Gates MA. Morphometric variation in the hypotrich ciliate genus *Euplotes*. *J Protozool* 1978;25:338–350.
10. Hufnagel LA, Torch R. Intraclonal dimorphism of caudal cirri in *Euplotes vannus*: cortical determination. *J Protozool* 1967;14:429–439.
11. Gates MA. Analysis of positional information applied to cirral patterns of the ciliate *Euplotes*. *Nature* 1977;268:362–364.
12. Gates MA. Pattern analysis in biology: a simple method for Morphogenetically constrained systems. *Am Nat* 1979;114:344–349.
13. Alekperov I, Buskey E, Snegovaya N. New and little-known free-living ciliates from the plankton of the Caspian sea. *Protistology* 2006;4:195–201.
14. Agamaliev FG. Faune des ciliés mésopsammiques de la Côte ouest de la Mer Caspienne. *Cah Biol* 1967;8:359–402.
15. Washburn ES, Borror AC. Euplotes raikovi Agamaliev, 1966 (Ciliophora, Hypotrichida) from New Hampshire: description and morphogenesis. *J Protozool* 1972;19:604–608.
16. Borror AC, Hill BF. The order Euplotida (Ciliophora): taxonomy, with division of *Euplotes* into several genera. *J Eukaryot Microbiol* 1995;42:457–466.
17. Wallengren H. Zur Kenntnis Der vergleichenden Morphologie Der Hypotrichen. *Bih K Svenska Vetensk-Akad Handl* 1900;26:1–31.
18. Živaljić S, Scherwass A, Schoenle A, Hohlfeld M, Quintela-Alonso P *et al*. A barotolerant ciliate isolated from the abyssal deep sea of the North Atlantic: *Euplotes dominicanus* sp. n. *Eur J Protistol* 2020;73:125664.
19. Boscaro V, Syberg-Olsen MJ, Irwin NAT, Del Campo J, Keeling PJ. 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. *J Eukaryot Microbiol* 2019;66:281–293.

20. Lian C, Wang Y, Li L, AL-Rasheid KAS, Jiang J *et al*. Taxonomy and SSU rDNA-based phylogeny of three new *Euplotes* species (Protozoa, Ciliophora) from China seas. *J King Saud Univ Sci* 2020;32:1286–1292.
21. Lian C, Zhang T, Al-Rasheid KAS, Yu Y, Jiang J *et al*. Morphology and SSU rDNA-based phylogeny of two *Euplotes* species from China: *E. wuhanensis* sp. n. and *E. muscicola* Kahl, 1932 (Ciliophora, Euplotida). *Eur J Protistol* 2019;67:1–14.
22. Liu M, Fan Y, Miao M, Hu X, Al-Rasheid KAS *et al*. Morphological and morphogenetic redescriptions and SSU rRNA gene-based phylogeny of the poorly-known species *Euplotes amieti* Dragesco, 1970 (Ciliophora, Euplotida). *Acta Protozool* 2015;54:171–182.
23. Syberg-Olsen MJ, Irwin NAT, Vannini C, Erra F, Di Giuseppe G *et al*. Biogeography and character evolution of the ciliate genus *Euplotes* (Spirotrichea, Euplotia), with description of *Euplotes curdsi* sp. nov. *PLoS One* 2016;11:e0165442.
24. Yan Y, Jiang L, Aufderheide KJ, Wright GA, Terekhov A *et al*. A Microfluidic-enabled mechanical microcompressor for the immobilization of live single- and multi-cellular specimens. *Microsc Microanal* 2013;20:1–11.
25. Foissner W. An update of 'basic light and scanning electron microscopic methods for taxonomic studies of ciliated protozoa'. *Int J Syst Evol Microbiol* 2014;64:271–292.
26. Abraham JS, Sripoorna S, Maurya S, Makhija S, Gupta R *et al*. Techniques and tools for species identification in ciliates: a review. *Int J Syst Evol Microbiol* 2019;69:877–894.
27. Vallesi A, Sjödin A, Petrelli D, Luporini P, Taddei AR *et al*. A new species of the γ-Proteobacterium *Francisella*, *F. adeliensis* sp. nov., endocytobiont in an Antarctic marine ciliate and poten- tial evolutionary forerunner of pathogenic species. *Microb Ecol* 2019;77:587–596.
28. Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene* 1988;71:491–499.
29. Petroni G, Dini F, Verni F, Rosati G. A molecular approach to the tangled intrageneric relationships underlying phylogeny in *Euplotes* (Ciliophora, Spirotrichea). *Mol Phylogenet Evol* 2002;22:118–130.
30. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 1997;4:4876–4882.
31. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp* 1999;41:95–98.
32. Schmidt HA, Strimmer K, Vingron M, von Haeseler A. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* 2002;18:502–504.
33. Swofford DL. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sunderland, Massachusetts: Sinauer Associates; 2003.
34. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003;19:1572–1574.
35. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 1998;14:817–818.
36. Felsenstein J. Phylogenies from molecular sequences: inference and reliability. *Annu Rev Genet* 1988;22:521–565.
37. Valbonesi A, Luporini P. *Euplotes bisulcatus*, a key species for a better understanding of the organization and evolution of *Euplotes* argyrome patterns. *Eur J Protistol* 1995;31:32–37.
38. Dallai R, Luporini P. Ultrastructural aspects of the development of the cytoplasmic connection between mating cells of the ciliate *Euplotes crassus*. *Eur J Protistol* 1989;24:125–132.
39. Jiang J, Zhang Q, Warren A, Al-Rasheid KAS, Song W. Morphology and SSU rRNA gene-based phylogeny of two marine *Euplotes* species, *E. orientalis* spec. nov. and *E. raikovi* (Ciliophora, Euplotida). *Eur J Protistol* 2010;46:121–132.
40. Zhao Y, Yi Z, Warren A, Song WB. Species delimitation for the molecular taxonomy and ecology of the widely distributed microbial eukaryote genus *Euplotes* (Alveolata, Ciliophora). *Proc R Soc. B* 2018;285:20172159.
41. Julian Schwarz MV, Zuendorf A, Stoeck T, Schwarz MVJ. Morphology, ultrastructure, molecular phylogeny, and autecology of *Euplotes elegans* Kahl, 1932 (Hypotrichida; Euplotidae) isolated from the anoxic Mariager fjord, Denmark. *J Eukaryot Microbiol* 2007;54:125–136.
42. Tuffrau M. Révision du genre *Euplotes*, fondée sur la comparaison des structures superficielles. *Hydrobiologia* 1960;15:1–77.
43. Yan Y, Fan Y, Luo X, El-Serehy HA, Bourland W *et al*. New contribution to the species-rich genus *Euplotes*: Morphology, ontogeny and systematic position of two species (Ciliophora; Euplotia). *Eur J Protistol* 2018;64:20–39.
44. Valbonesi A, Ortenzi C, Luporini P. The species problem in a ciliate with a high multiple mating type system, *Euplotes crassus*. *J Protozool* 1992;39:45–54.
45. Gianni A, Piras L. Species structure in *Euplotes crassus* (Ciliophora, Hypotrichida). *J Eukaryot Microbiol* 1993;40:155–161.
46. Nobili R, Luporini P, Dini F. Breeding systems, species relationships and evolutionary trends in some marine species of Euplotidae (Hypotrichida, Ciliata). In: Battaglia B, Beardmore J (editors). *Marine Organisms, Genetics, Ecology and Evolution. Plenum Press*. New York; 1978. pp. 591–616.
47. Sonneborn TM. The *Paramecium aurelia* complex of fourteen sibling species. *Trans Am Microsc Soc* 1975;94:155–178.
48. Przybos E, Fokin SI. Data on the occurrence of species of the *Paramecium aurelia* complex world-wide. *Protistology* 2000;1:179–184.
49. Simon EM, Nanney DL, Doerder FP. The "*Tetrahymena pyriformis*" complex of cryptic species. *Biodivers Conserv* 2008;17:365–380.
50. Kuhlmann HW, Sato K. Interspecific mating reactions between *Euplotes octocarinatus* and *Euplotes patella* syngen 2. *Eur J Protistol* 1993;29:24–31.
51. Dini F, Nyberg D. Sex in ciliates. In: Jones JG (editor). *Advances in Microbial Ecology*, 13. Boston, MA: Springer; 1993.
52. Luporini P, Miceli C, Ortenzi C. Evidence that the ciliate *Euplotes raikovi* releases mating inducing factors (gamones). *J. Exp. Zool.* 1983;226:1–9.
53. Luporini P, Pedrini B, Alimenti C, Vallesi A. Revisiting fifty years of research on pheromone signaling in ciliates. *Eur J Protistol* 2016;55:26–38.
54. Jiang J, Zhang Q, Hu X, Shao C, Al-Rasheid KAS *et al*. Two new marine ciliates, *Euplotes sinicus* sp. nov. and *Euplotes parabalteatus* sp. nov., and a new small subunit rRNA gene sequence of *Euplotes rariseta* (Ciliophora, Spirotrichea, Euplotida). *Int J Syst Evol Microbiol* 2010;60:1241–1251.
55. Chen X, Zhao Y, Al-Farraj SA, Al-Quraishy SA, El-Serehy HA *et al*. Taxonomic descriptions of two marine ciliates, *Euplotes dammamensis* n. sp. and *Euplotes balteatus* (Dujardin, 1841) Kahl, 1932 (Ciliophora, Spirotrichea, Euplotida), collected from the Arabian Gulf, Saudi Arabia. *Acta Protozool* 2013;52:73–89.