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Morphology, ontogenesis and molecular phylogeny of *Platynematum salinarum* nov. spec., a new scuticociliate (Ciliophora, Scuticociliatia) from a solar saltern

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Abstract

Platynematum salinarum nov. spec. was discovered in a hypersaline (~120‰) solar saltern in Portugal. Its morphology, ontogenesis, and 18S rRNA were studied with routine methods. *Platynematum salinarum* has a size of about 35 μm × 18 μm and differs from other platynematids (= *Platynematum* and *Pseudoplatynematum*) in having an only slightly flattened body without any spines or notches. Both, the oral and somatic infraciliature resemble other platynematids and the tetrahymenid pattern in general. The ontogenesis is scuticobuccokinetal, being unique in generating protomembranelle 1 from kinetids produced by the paroral membrane of the proter and of the scutica. This composite divides transversely: the right half becomes the paroral membrane of the opisthe, the left half transforms to opisthe's adoral membranelle 1. The scutica and the molecular sequence classify *P. salinarum* into the order Scuticociliatida, family Cinetochilidae. The 18S rRNA sequence shows 92.7% similarity to the closest relative deposited in public databases (the scuticociliate *Sathrophilus holtae*), and our study provides the first sequence for the genus *Platynematum*. Experiments at different salinities show growth between 120‰ and 300‰, survival at 100‰, and cell death around 60‰ salinity, characterizing *P. salinarum* as a true halophile.

Keywords: Biodiversity; Cinetochilidae; Extremophile ciliates; Hypersaline ciliates; *Platynematum marinum*; Portugal

Introduction

The Scuticociliatia is a distinct subclass within the class Oligohymenophorea that comprises six subclasses containing the pets of the protistologists, viz. *Tetrahymena* and *Paramecium* as well as the famous *Vorticella*, a member of the subclass Peritrichia (Lynn 2008). The scuticociliates have a clear morphological identity due to the scutica, a postoral field of specialized kinetids contributing to the oral apparatus of the opisthe (Foissner 1996; Lynn 2008; Ma et al. 2005; Small 1967). However, molecular markers show that several families and genera are non-monophyletic and/or show a rather distinct relationship to two other oligohymenophorean subclasses, viz. the parasitic Apostomatia and the mouthless Astomatia, even with multiple molecular markers (Gao et al. 2012; Zhang et al. 2011). Indeed, morphological and molecular classifications are in conflict within and between various scuticociliatid taxa (Gao et al. 2012; Jankowski 2007; Lynn 2008; Ma et al. 2005; Zhang et al. 2011). Possibly, an improved classification needs intensified morphological and molecular research.

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The scuticociliatia is a highly diverse group comprising hundreds of species, many of which have been re-investigated or discovered in the past 20 years (for a review, see Song et al. 2009). Many are small (≤50 μm) which makes morphological work, especially ontogenetic studies difficult and time-consuming. This applies also to the new species described here, *Platynematum salinarum*, which was discovered in a Portuguese solar saltern, i.e., in an extreme habitat with a salinity of 120‰.

The genus *Platynematum* was established as "*Platynema*" by Kahl (1931). However, "*Platynema*" was pre-occupied, and thus Kahl (1935) changed the name to *Platynematum*.

Material and Methods

Material and cultivation

Platynematum salinarum was isolated in June 2013 from a solar saltern pond in the Ria Formosa Natural Park, close to the town of Faro, Portugal, N 37°0′29.4851″, W 7°57′41.0684″. It was cultivated in sterilized Artificial Sea Water (ASW, Instant Ocean, Aquarium Systems, Ohio, USA) adjusted to 120‰ salinity and amended with two to four wheat grains per 25 ml salt medium to support growth of indigenous bacteria.

Morphological methods

All data are from a cultivated clone. Living cells were studied using a high-power oil immersion objective and differential interference contrast microscopy. Protargol and silver nitrate impregnation were performed as described by Vďačný and Foissner (2012), using Stieve's solution to obtain well-fixed cells for protargol impregnation.

Counts and measurements of silvered specimens were performed at a magnification of 1,000×. In vivo measurements were conducted at magnifications of 100–1,000×. Illustrations of live specimens were based on free-hand sketches and micrographs; those of impregnated cells were made with a drawing device. Most of the ontogenetic stages have been observed in at least two specimens. Terminology follows Corliss (1979), Foissner (1996), and Lynn (2008).

Molecular methods

DNA extraction and PCR amplification of the small subunit rDNA (SSU rDNA) were conducted as described by Foissner and Stoeck (2011). In short, genomic DNA was extracted from ca. 100 specimens of *Platynematum salinarum* using the DNEasy Tissue Kit (Qiagen), and the SSU rDNA was amplified using the universal eukaryotic primers EukA and EukB (Medlin et al. 1988). The PCR amplification protocol employed 5 min denaturation at 95 °C, followed by 30 identical amplification cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2.5 min, and a final extension at 72 °C for 7 min. The purified PCR product (MiniElute kit, Qiagen) was cloned into a vector using the TA-Cloning Kit (Invitrogen, Carlsbad, CA). M13F- and M13R-sequences were obtained with the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) on an ABI 3730 automated sequencer and assembled in CodonCode Aligner (CodonCode Corcporation, MA, USA).

Phylogenetic analyses

Sequences were quality checked, and PHRED and PHRAP analyses were carried out using CodonCode Aligner v.3.0 (CodonCode Corporation, Dedham, MA). Vector and primer nucleotides were trimmed off. Sequences were aligned to the Oligohymenophorea alignment from Dunthorn et al. (2012), using MUSCLE (Edgar 2004) as implemented in Seaview (Gouy et al. 2010), and subjected to Gblocks (Castresana 2000) for refinement. Manual inspection for further fine-tuning of the alignment was conducted in MacClade v4.05 (Maddison and Maddison 2005). The GTR-I-Γ evolutionary model was the best fitted model selected by AIC in jModeltest v0.1.1 (Guindon and Gascuel 2003; Posada 2008).

Maximum likelihood (ML) analyses were carried out in RaxML-HPC v7.2.5 (Stamatakis et al. 2008). Support came from a majority rule consensus tree of 1,000 multiparametric bootstrap replicates. Bayesian Inference (BI) was carried out by MrBayes v3.2.1 (Huelsenbeck and Ronquist 2003). Posterior probability was estimated using four chains running 20 million generations sampling every 1,000 generation; the first 25% of sampled trees were considered burn-in trees and were thus discarded prior to constructing a 50% majority rule consensus tree. Trees were visualized with FigTree v1.3.1 (Rambaut 2006). Table 1 shows GenBank accession numbers of all sequences used for the phylogenetic analyses. The GenBank accession number for *Platynematum salinarum* is KF301567.

Salt tolerance

To assess the salt tolerance of *P. salinarum*, we conducted growth experiments under different salt concentrations. Therefore, salinities of the ASW were adjusted with distilled water or artificial sea salt to the following concentrations: 60‰, 100‰, 140‰, 180‰, 220‰, 260‰, and 300‰. An equal number of wheat grains was added to all experiments as carbon source for indigenous bacteria, serving as a food for the ciliate. All experiments were conducted in triplicate and growth was observed and recorded daily (24 h) under an inverse microscope (Zeiss Axiovert 40C) for 336 h. Cell growth (μ d^{-1}) was calculated according to Guermazi et al. (2008).

Table 1. GenBank accession numbers of taxa used for phylogenetic analyses.

Taxon	GenBank #
Almophrya bivacuolata	HQ446281
Ancistrum crassum	HM236340
Anoplophrya marylandensis	AY547546
Anophyroides haemophila	U51554
Boveria subcylindrica	FJ848878
Cardiostomatella vermiforme	AY881632
Cinetochilum ovale	FJ870103
Cohnilembus verminus	Z22878
Cyclidium glaucoma	EU032356
Dexiotricha sp.	JQ723963
Dexiotrichides pangi	AY212805
Entodiscus borealis	AY541687
Entorhipidium pilatum	AY541689
Eudrilophrya complanata	HQ446280
Eurystomatella sinica	FJ012143
Glauconema trihymene	GQ214552
Gymnodinioides pitelkae	EU503534
Histiobalantium natans viridis	AB450957
Homalogastra setosa	EF158848
Hyalophysa chattoni	EU503536
Mesanophrys carcini	AY103189
Metanophrys sp.	JN885084
Metaracoelophrya intermedia	HQ446278
Miamiensis avidus	AY550080
Njinela prolifera	HQ446276
Paraclausilocola constricta	HQ446275
Paraclausilocola elongata	HQ446274
Paramecium tetraurelia	X03772
Paranophrys magna	JN885089
Paratetrahymena sp.	EU744176
Paratetrahymena parawassi	FJ876969
Parauronema virginianum	AY392128
Philaster digitiformis	FJ648350
Philasterides dicentrachi	GU572375
Plagiopyliella pacifica	AY541685
Platynematum salinarum	KF301567
Pleuronema coronatum	AY103188
Porpostoma notatum	HM236335
Pseudocohnilembus marinus	Z22880
Pseudocohnilembus persalinus	AY551906
Sathrophilus holtae	FJ868188
Sathrophilus planus	FJ868186
Schizocalyptra sinica	FJ156106
Schizocaryum dogieli	AF527756
Thyrophylax vorax	AY541686
Urocentrum turbo	EF114299
Uronema elegans	AY103190
Uronema marinum	GQ465466
Uronemella filificum	EF486866
Vampyrophrya pelagica	EU503539
Wilbertia typica	FJ490551

Results

Description of *Platynematum salinarum* (Figs 1–27, Table 2)

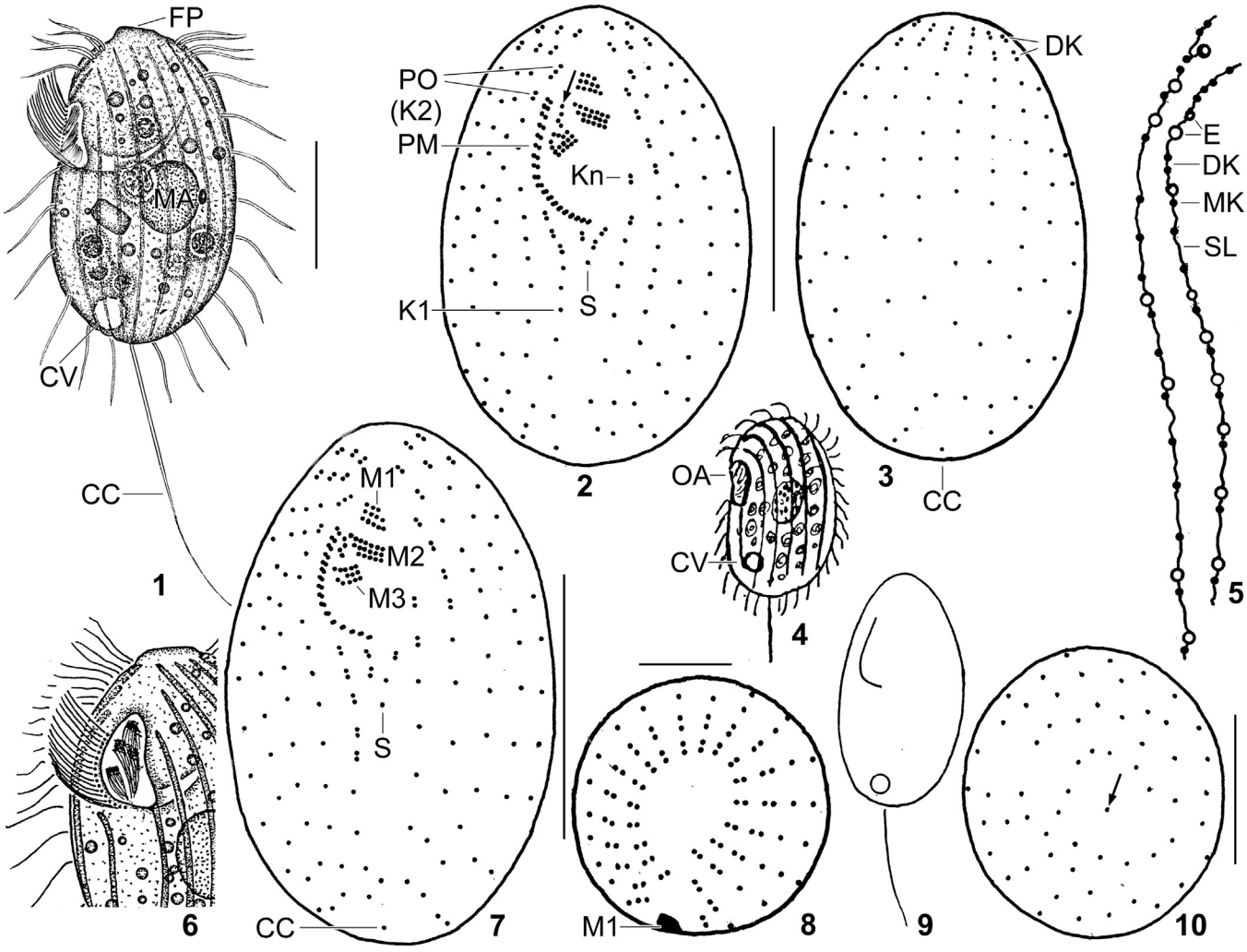
Diagnosis: Size in vivo about 35 μm × 18 μm. Body ellipsoidal, ovate, or bluntly fusiform, slightly flattened laterally, frontal plate obovate to slightly reniform. Nuclear apparatus near cell centre. Contractile vacuole in rear body end. Extrusomes, likely mucocysts, within ciliary rows. Cortex with distinct ridges right of kineties. On average 21 ciliary rows most commencing with two dikinetids; a single caudal cilium about as long as body. Oral apparatus distinctly subapical, tetrahymenid.

Type locality: Solar saltern pond (salinity 120‰) in the Ria Formosa National Park near to the town of Faro, Portugal (N 37°0′29.4851″, W 7°57′41.0684″).

Type material: One holotype and three paratype slides with protargol-impregnated specimens and four paratype slides with silver nitrate-impregnated (Chatton–Lwoff method) cells have been deposited in the Biologiezentrum of the Oberösterreichische Landesmuseum in Linz (LI), Austria, reg. no. 2013/38-45. Relevant specimens have been marked by black ink circles on the coverslip. GenBank accession number is KF301567.

Etymology: The specific name is a noun in plural genetive and thus does not change the gender when combined with, e.g., a masculine genus; it refers to the habitat, i.e., growing or living in a solar saltern.

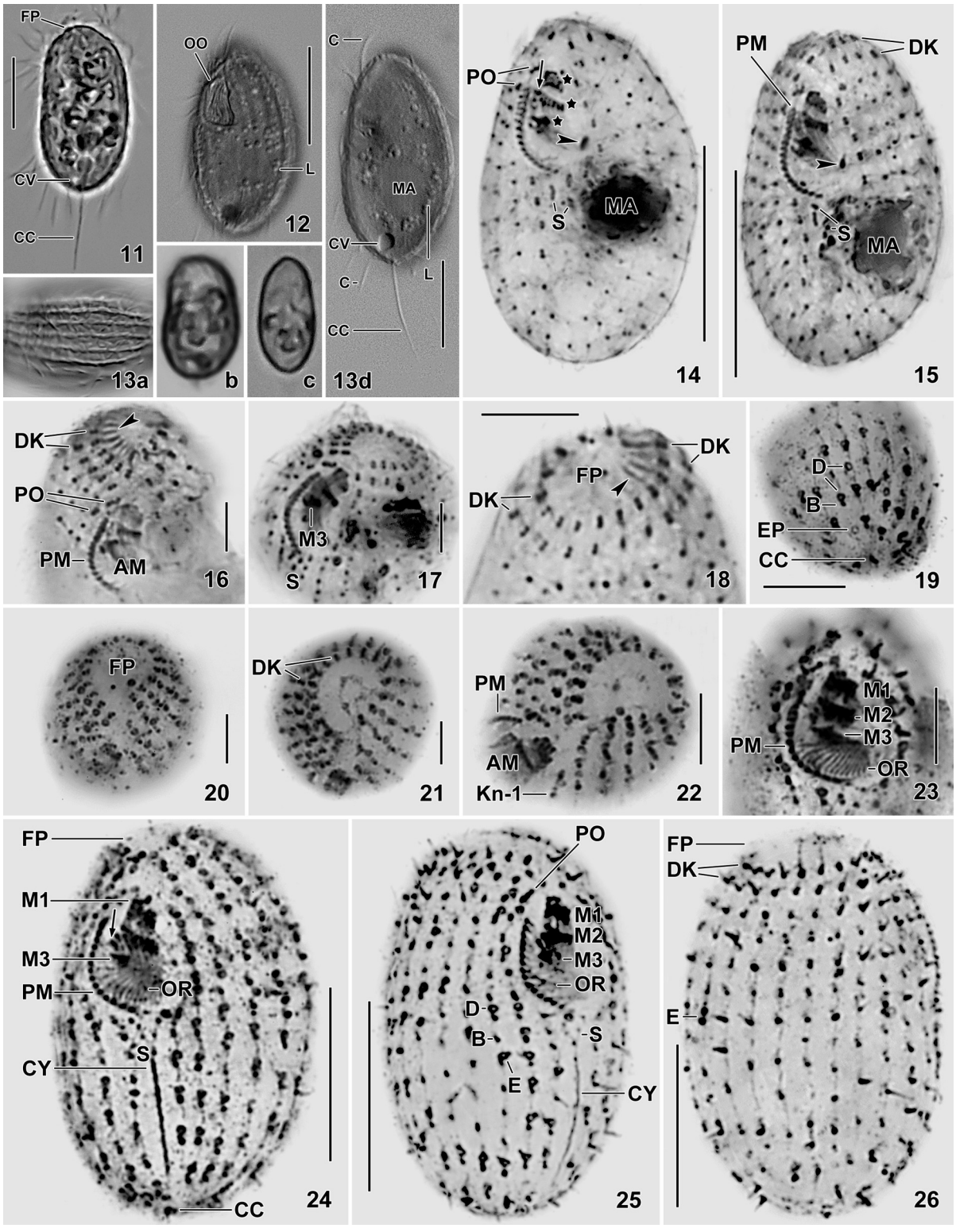
Description: Size in vivo 25–40 μm × 15–20 μm, usually about 35 μm × 18 μm, as calculated from measurements of live and prepared specimens, adding 5% (silver nitrate) or 20% (protargol) for preparation shrinkage (Table 2); silver nitrate-prepared specimens slightly inflated (Figs 24–26), protargol-prepared cells shrunken by about 20%. Shape ellipsoidal, slightly ovate or bluntly fusiform, depending on specimen and culture conditions (Figs 1, 11, 12, 13d). Laterally flattened by 5–25% (Fig. 13b, c); frontal plate easily recognizable in vivo, obovate to slightly reniform because left side ciliary rows slightly longer than right side rows (Figs 1, 6, 8, 20–22); a shallow concavity in mouth area (Figs 1, 13b); spines or distinct notches definitely absent. Salinity changes and culture age did not produce distinctly flattened cells. Nuclear apparatus without peculiarities, in mid-body slightly closer to dorsal side. Macronucleus in vivo finely granulated, about 8 μm across. Micronucleus about 2 μm in size, attached to macronucleus (Figs 1, 13d, 14, 15). Contractile vacuole in rear body end or slightly subterminal, excretory pore in ventral pole area; contraction not observed (Figs 1, 13d, 19). Cytopyge extends between scutica and posterior body end, marked by a long, straight, intensely impregnated line (Figs 24, 25). Most extrusomes within ciliary rows, likely broadly ellipsoidal and of mucocyst type, hardly recognizable in vivo but distinct in silver nitrate preparations, appearing as minute rings when docked and as dark granules when just extruded (Figs 5, 19, 24–26). Cortex about



Figs 1–10. *Platynematum salinarum* (1–3, 5–10) and *P. marinum* (4) from life (1, 4, 6, 9), after protargol impregnation (2, 3, 7, 8, 10), and in a silver nitrate preparation (5). **1:** Left lateral view of a representative specimen, length 35 μm. **2, 3:** Infraciliature of ventral and dorsal side. The arrow marks isolated basal bodies between paroral membrane and the second adoral membranelle. **4:** *Platynematum marinum* from Kahl (1935), length 30–35 μm. **5:** Two ciliary rows after silver nitrate impregnation. **6:** Ventral view of oral apparatus, showing the reniform oral opening. **7:** Ventral view of holotype specimen, length 29 μm. **9:** Left lateral outline of a slender specimen. **8, 10:** Anterior and posterior polar view. The arrow marks the basal body of the caudal cilium. CC – caudal cilium, CV – contractile vacuole, DK – dikinetids, E – docked extrusomes, FP – frontal plate, Kn – kinety n, K1, 2 – somatic kineties 1 and 2, M1–3 – adoral membranelles, MA – macronucleus, MK – somatic monokinetid, OA – oral apparatus, PM – paroral membrane, PO – polymerization of kinetids in anterior region of somatic kinety 2, S – scutica, SL – silverline. Scale bars 5 μm (8), 10 μm (2, 3, 10), and 15 μm (1, 7).

0.8 μm thick, with distinct ridges right of ciliary rows; ridges about 1 μm wide and 0.6 μm high, hardly notching body ends but producing a distinct striation (Figs 1, 6, 12, 13a). Cytoplasm colourless, finely granulated, contains few to many lipid droplets 0.5–2 μm across and food vacuoles 4–6 μm in diameter. Feeds on about 3 μm long, very thin bacteria (Fig. 1). Swims rapidly when disturbed mechanically or by strong light; undisturbed cells stand still on bottom of culture dish or at margin of organic debris spreading cilia more or less distinctly (Figs 11, 13d).

scutica (S), and the macronucleus (Figs 13, 14). The arrow in Figs 14, 24 marks isolated basal bodies right of adoral membranelle 2. The arrowhead in Figs 14, 15 denotes the anteriormost dikinetid of kinety n. **16–18:** Ventral (16, 17) and dorsal (18) views of oral (anterior) body region. The arrowheads mark fibres originating from the anteriormost dikinetids and extending into the frontal plate. **19:** Oblique posterior polar view. **20–22:** Anterior polar views, showing the obovate to slightly reniform frontal plate surrounded by two circles of dikinetids. AM – adoral membranelles, B – basal bodies, C – ordinary somatic cilia, CC – caudal cilium, CV – contractile vacuole, CY – cytopyge, D – docked extrusomes, DK – dikinetids, E – extruded extrusomes, EP – excretory pore of contractile vacuole, FP – frontal plate, Kn−1 – penultimate somatic kinety, L – lipid droplets, M1–3 – adoral membranelles, MA – macronucleus, OO – oral opening, OR – oral ribs, PM – paroral membrane, PO – polymerization of kintetids in anterior region of kinety 2, S – scutica. Scale bars 5 μm (16–23) and 15 μm (11–15, 24–26).



Figs 11–26. *Platynematum salinarum* from life (11–13d), after protargol impregnation (14–18), and in silver nitrate preparations (19–26). **11:** Lateral view of a representative specimen. **12:** Ventral view of an ovate specimen, showing the reniform oral opening. **13a:** The cortex has distinct ridges (anterior end of cell is right). **13b, c:** Lateral and ventral view of a large (38 μm), blunt specimen, showing 23% body flattening. **13d:** A bluntly fusiform specimen, showing the proximal half of the caudal cilium. **14, 15, 23–26:** Infraciliature of ventral (14, 15, 23, 24) right (25), and dorsal (26) side, showing the general organization of *P. salinarum*, i.e., the meridional ciliary rows with various specializations, the oral apparatus composed of a paroral membrane and three short, thick adoral membranelles (Fig. 14, asterisks; 23), the

Table 2. Morphometric data on *Platynematum salinarum*.

Characteristics[a]	$\bar{x}$	*M*	SD	SE	CV	Min	Max	*n*
Body, length (in vivo), µm	34.8	35.0	3.1	1.0	8.9	28.0	38.0	9
Body, width (in vivo), µm	17.6	17.0	2.3	0.8	13.1	15.0	21.0	9
Body length:width, ratio (in vivo)	2.0	1.9	0.2	0.1	10.1	1.8	2.4	9
Body, length (protargol), µm	26.4	26.0	2.0	0.4	7.6	23.0	30.0	21
Body, width (protargol), µm	17.1	17.0	2.0	0.4	11.4	13.0	20.0	21
Body length:width, ratio (protargol)	1.6	1.5	0.1	0.1	8.1	1.4	1.9	21
Body, length (silver nitrate), µm	31.2	31.0	2.2	0.5	7.2	28.0	37.0	21
Body, width (silver nitrate), µm	21.1	21.0	1.9	0.4	9.1	18.0	25.0	21
Body length:width, ratio (silver nitrate)	1.5	1.5	0.7	0.1	4.7	1.4	1.7	21
Posterior end of paroral to posterior end of body, µm	14.6	15.0	2.1	0.5	14.4	10.0	18.0	21
Anterior body end to adoral membranelle 1, distance, µm	4.4	4.5	1.1	0.3	25.8	2.0	7.0	21
Macronucleus, length, µm	5.9	6.0	0.7	0.2	11.4	5.0	7.0	21
Macronucleus, width, µm	4.8	4.5	0.9	0.2	18.2	4.0	6.0	21
Micronucleus, length, µm	1.4	1.5	0.3	0.1	18.0	1.0	2.0	15
Micronucleus, width, µm	1.1	1.0	0.2	0.1	18.8	1.0	1.5	15
Ciliary rows, number	21.1	21.0	–	–	–	21.0	22.0	21
Kinetids in a dorsal ciliary row, number[b]	12.1	12.0	0.8	0.2	6.7	10.0	13.0	21
Somatic kinety 1, number of kinetids in anterior polymerization	3.2	3.0	–	–	–	3.0	4.0	21
Somatic kinety n−1, number of dikinetids in anterior region	4.1	4.0	–	–	–	4.0	5.0	21
Somatic kinety *n*, number of dikinetids in anterior region	1.9	2.0	–	–	–	1.0	2.0	21
Postoral kinety, number of dikinetids in anterior region	3.8	4.0	0.6	0.1	15.8	3.0	5.0	21
Scutica, number of basal bodies[c]	8.2	8.0	1.0	0.2	12.7	7.0	11.0	21
Isolated basal bodies right of adoral membranelles, number	3.4	3.0	–	–	–	3.0	4.0	21

[a] Data based, if not stated otherwise, on mounted, protargol-impregnated, and randomly selected specimens from pure cultures. CV, coefficient of variation in %; *M*, median; Max, maximum; Min, minimum; *n*, number of specimens investigated; SD, standard deviation; SE, standard error of arithmetic mean; $\bar{x}$, arithmetic mean.

[b] Dikinetids counted as one kinetid.

[c] Dikinetids counted as two basal bodies.

Somatic cilia in vivo about 7 µm long. Caudal cilium in centre of pole, about as long as body, proximal half as thick as somatic cilia, distal half very fine; very flexible becoming distinctly curved when cell changes swimming direction (Figs 1, 9–11, 13d). On average 21 meridional, equidistant, basically monokinetidal ciliary rows most commencing posterior of frontal plate and ending in posterior pole area (Table 2; Figs 1–3, 7, 8, 10, 14–26). Kinety 1 commences with three to five dikinetids posterior of anterior half of paroral membrane. Kinety 2 slightly shortened anteriorly, extends right of paroral membrane, commences with three or four narrowly spaced monokinetids followed by a single dikinetid. Kineties 3–19 commence posterior of frontal plate with two dikinetids, anterior dikinetid associated with a distinct fibre extending to centre of frontal plate. Kinety 20 commences at level of adoral membranelle 1 with three to five dikinetids, associated with the caudal cilium. Kinety 21 (Kn) commences at level of mid-buccal cavity with one or two dikinetids. Scutica at margin of buccal vertex, composed of 7–11 basal bodies, most as dikinetids (Table 2; Figs 2, 3, 7, 8, 10, 14–26).

Oral apparatus in a shallow, subapical concavity about 10 µm long and thus occupying 38% of body length in protargol preparations; structure and shape tetrahymenid (Table 2; Figs 1, 2, 6, 7, 12, 13b, 14–17, 23–25). Oral opening usually slightly reniform because left margin slightly convex; rarely triangular (Figs 1, 6, 12). Paroral membrane slightly to distinctly curved, composed of 14–18 narrowly spaced dikinetids; proximal half associated with distinct oral ribs. Adoral membranelles short and thick, each likely composed of three rows of basal bodies, details slightly variable and difficult to recognize; three or four scattered basal bodies right of membranelles 2 and 3 (Figs 1, 2, 7, 14–17, 23–25).

Silverline pattern simple because intermeridional connectives lacking or not impregnated. Silverlines extend meridionally containing basal bodies of cilia and extrusomes; buccal cavity with an irregular, widely meshed silverline net connecting adoral membranelles among each other and with paroral membrane and somatic kineties (Figs 5, 23–25).

Ontogenesis of *Platynematum salinarum* (Figs 28–39)

Details of the ontogenesis of *P. salinarum* are difficult to study because of its small size. Thus, the figures must be interpreted with care. Nuclear and cell division occur in the ordinary way (Foissner 1996; Lynn 2008) and are thus not described. Likewise, the somatic infraciliature reproduces as usual.

As typical for scuticociliates, the ontogenesis commences with the scutica (Figs 2, 7) whose dikinetids migrate apart (Fig. 28), each single kinetid then producing a new dikinetid,

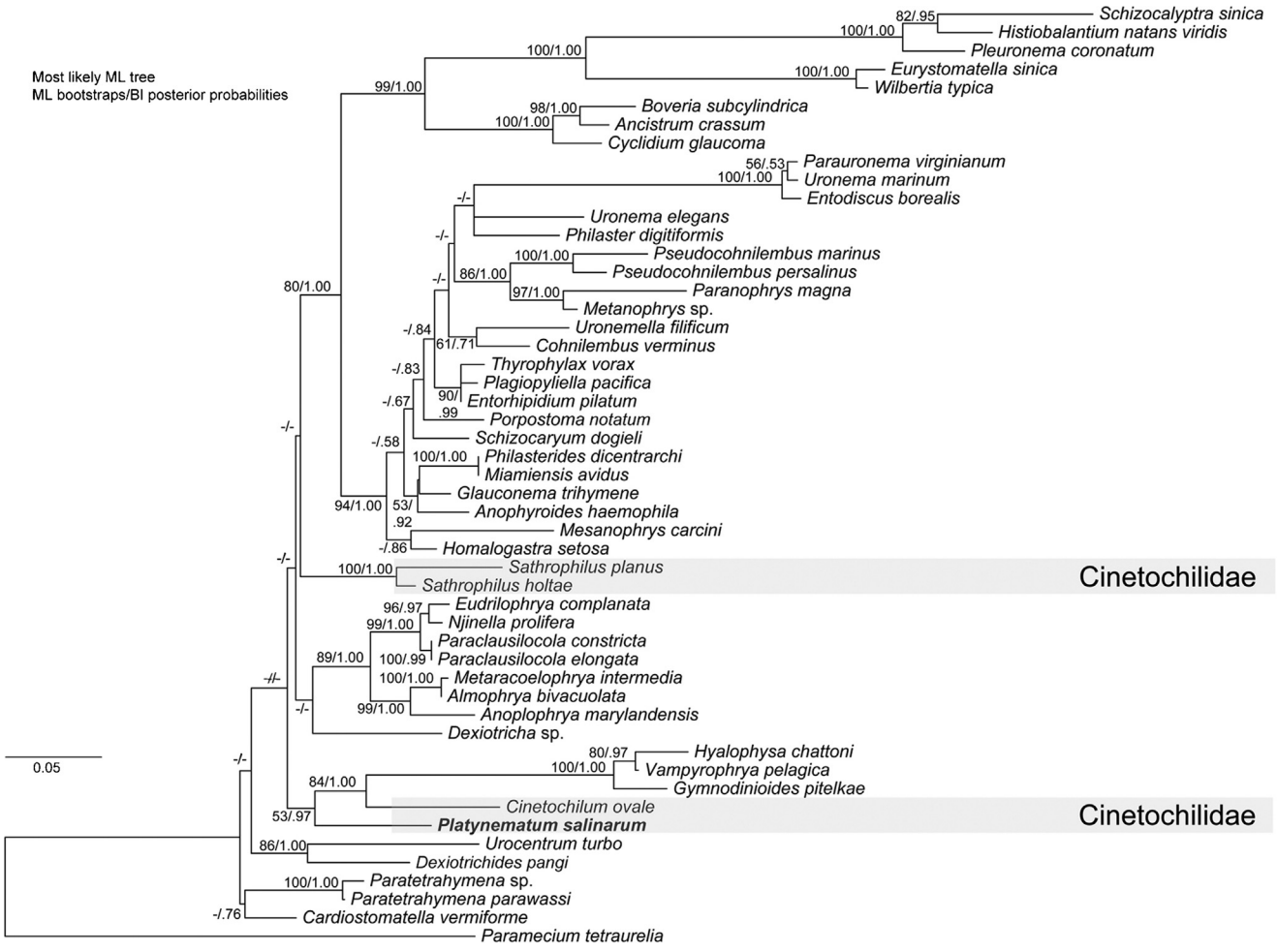


Fig. 27. Maximum likelihood tree of 18S rDNA sequences, showing the phylogenetic placement of *Platynematum salinarum*. Bootstrap values above 50 are given at the individual nodes for the maximum-likelihood (ML, 100 replicates) and Bayesian posterior probabilities. The edited sequence alignment included 1503 characters. *Paramecium* was chosen as outgroup. "–" and "–/–" denote support values <50% (ML bootstrap) and <50%/0.5 (ML bootstrap/Bayesian posterior probabilities, respectively. For details, see Material and Methods section.

forming an anarchic field of dikinetids, i.e., the opisthe oral anlage, together with the anterior dikinetidal portion of kinety 1 (Figs 29, 30). Next, kinety n joins the oral anlage and the parental (proter) paroral splits longitudinally (Fig. 31), producing a long, sigmoidal dikinetidal protomembranelle 1 together with dikinetids from the oral anlage; protomembranelles 2 and 3 are generated by the remaining dikinetids of the anlage (Figs 32, 33).

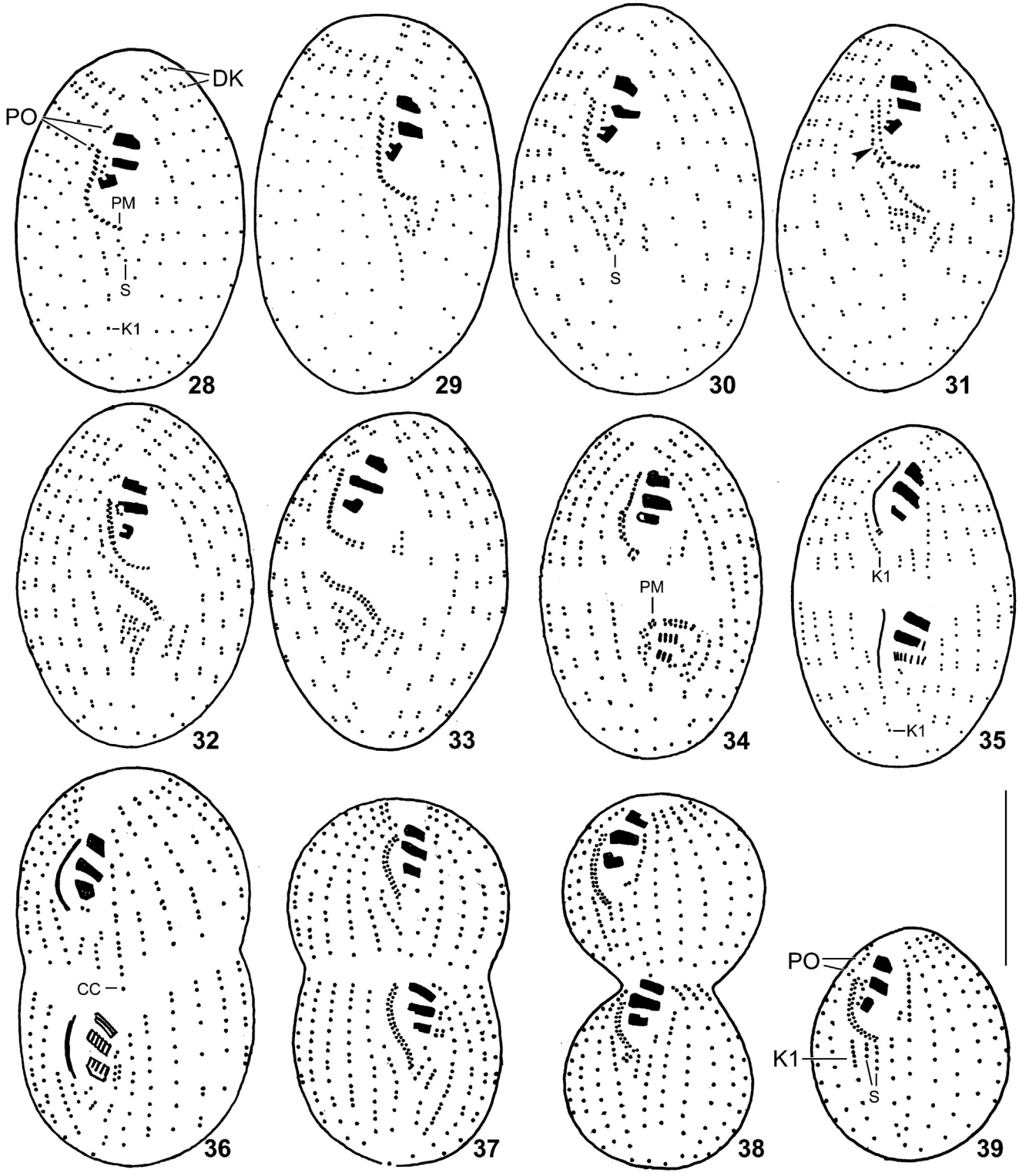
Then, a curious process commences, i.e., the long protomembranelle 1 splits into a left half that becomes adoral membranelle 1, and into a right half that forms the paroral of the opisthe; concomitantly, the posterior half of the proter paroral produces kinetids that migrate to the right to form a new kinety 1 (Figs 34, 35).

In late mid-dividers and in late dividers, most kinetids obtain their specific position and new kinetids are produced for kinety n, the paroral membrane, and the scutica in both proter and opisthe (Figs 36, 37). In very late dividers, the anterior girdle of dikinetids is formed, kinety 1 becomes dikinetidal in the anterior half, kinety 2 polymerizes anteriorly, and a new scutica develops (Fig. 38). The caudal cilium originates from kinety n−1 (Fig. 36). Very young post-dividers show isolated basal bodies right of membranelles 2 and 3 and thus possess the vegetative infraciliature while the body is much smaller and broadly ovate (Fig. 39).

Very likely, there is some "internal" reorganization of the parental infraciliature. This is indicated by a slight loosening of the basal bodies of the adoral membranelles and of the anterior girdle of dikinetids as well as by the disappearance of the isolated monokinetids right of membranelles 2 and 3 in early dividers (Figs 31–35).

Phylogenetic analyses

The 18S rRNA sequence of *Platynematum salinarum* is highly divergent from available 18S rRNA sequences deposited in the GenBank nucleotide database. The closest relative is the cinetochilid scuticociliate *Sathrophilus*



Figs 28–39. *Platynematum salinarum*, ontogenesis in protargol preparations. **28–30:** Very early stages. The scutica forms an anarchic field of dikinetids. **31–33:** Early stages, showing the split of proter's paroral (31, arrowhead) and the formation of three protomembranelles. **34:** Early mid-divider, showing the split of the anterior protomembranelle: the right half becomes the paroral (PM), the left adoral membranelle 1. **35:** A mid-divider, showing the origin of kinety 1. **36, 37:** Late dividers, showing cell furrowing and completion of the oral structures. **38:** Very late divider, showing the formation of the scutica and the dikinetid girdle. **39:** Very early post-divider looking like a miniaturized vegetative specimen. CC – caudal cilium, DK – dikinetid girdle, K1 – somatic kinety 1, PM – paroral membrane, PO – polymerization of kinetids in anterior region of kinety 2, S – scutica. Scale bar about 15 μm.

holtae with 92.7% sequence similarity (GenBank accession FJ868188.1, Zhang et al. 2010). In phylogenetic analyses (Fig. 27), cinetochilids are not monophyletic, and *P. salinarum* is sister to *Cinetochilum ovale*. However, there is no support for the position of the two Cinetochilidae clades in the tree topologies of ML and Bayesian inference analyses. The sister relationship of *P. salinarum* and *C. ovale* is only supported by Bayesian posterior probabilities (0.97) but not by ML bootstraps (53% support). To date, further sequences for the genera *Platynematum* and *Pseudoplatynematum* are not available.

Growth experiments

Platynematum salinarum grows well at salinities ranging from 140‰ to 300‰ with a maximum growth rate μ of 1.06 d^{-1} at 140‰ (Fig. 40). Growth occurs even at 300‰ ($\mu = 0.74$ cells d^{-1}). At 100‰ cells stop division but survive at stable population densities. Cultures die at salinities above 300‰ and below 60‰.

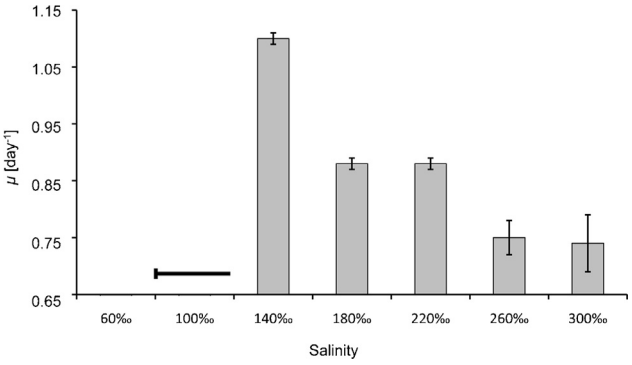


Fig. 40. Growth rates (μ) for *Platynematum salinarum* at different salt concentrations. Bar at 100 ‰ indicates survival but no growth. Cell death was recorded above 300‰ and at 60‰.

Discussion

Does the Portuguese population belong to *Platynematum*?

In vivo, the Portuguese species hardly resembles the congeners and *Pseudoplatynematum* spp. because it lacks any spines and notches and the body is only slightly flattened; indeed, it is more similar to, e.g., *Uronema* and *Sathrophilus* (for a brief review, see Foissner et al. 1994). However, the oral and somatic ciliary pattern is as in *Platynematum* and *Pseudoplatynematum* (Fan et al. 2010, 2011; Foissner et al. 1994): the oral apparatus is tetrahymenid, the ciliary rows commence with two dikinetids, and kinety 2 has an anterior condensation of kinetids (Figs 2, 7, 14, 15, 23–26). Thus, we classify the Portuguese population into *Platynematum*. Molecular investigations on the type species of the genus, *Uronema sociale* Penard, 1922, will show whether this classification is correct.

Platynematum salinarum as a new species

Originally, we identified the Portuguese population as *Platynematum marinum* (Fig. 4), very briefly described by Kahl (1933), because blunt specimens, the single caudal cilium, body size, location of the oral apparatus, and the saline habitat (Kiel area, Germany) resemble that species. However, Kahl (1933, 1935) emphasized two features absent from our population: distinct frontal notches caused by the ciliary rows and a leaf-like flatten of the body. The first feature suggests that *P. marinum* has the ciliary rows curved anteriorly while those of the Portuguese specimens are straight. The second feature appears unimportant at first glance because body flattening often depends on the quantity of food ingested. However, this is very likely not the case in *Platynematum* and *Pseudoplatynematum* in which all described species are distinctly flattened (Bock 1952; Fan et al. 2010, 2011; Foissner et al. 1994; Kahl 1931, 1933, 1935; Penard 1922). Thus, we consider the Portuguese population as a new species.

Phylogeny

The scutica classifies *P. salinarum* into the Scuticociliatia Small, 1967, a highly diverse subclass consisting of 33 families in three orders (Lynn 2008): Philasterida (e.g. *Loxocephalus*), Pleuronematida (e.g. *Pleuronema*), and Thigmotrichida (e.g. *Ancistrum*). Much more difficult is the ordinal and familiar classification because, as yet, neither morphological nor molecular data have produced a stable system (Gao et al. 2012; Jankowski 2007; Li et al. 2006, 2010; Lynn 2008; Ma et al. 2005; de Puytorac 1994; Song et al. 2005; Zhang et al. 2010, 2011).

Fortunately, the familiar classification of *Platynematum* is not particularly difficult because morphology (Foissner et al. 1994; Figs 2, 7) and ontogenesis (Figs 28–39) classify the new species into the philasterine family Cinetochilidae, as already suggested by Jankowski (2007), Lynn (2008), and de Puytorac (1994). As concerns the ontogenesis, *Cinetochilum* and *Platynematum* match in generating adoral membranelles 2 and 3 by the scutica (de Puytorac et al. 1974; this study). *Platynematum* is unique in generating protomembranelle 1 by kinetids produced by the paroral membrane of the proter and of the scutica. This composite divides transversely: the right half becomes the paroral of the opisthe, the left half transforms to opisthe's membranelle 1 (Figs 31–35).

18S rRNA phylogenetic analyses neither reject nor solidly confirm the assignment to the Cinetochilidae. This is not very surprising, considering the high sequence divergence of *P. salinarum* to the closest deposited sequence in a public database and also considering the general difficulties with 18S rRNA phylogenies in the classification of scuticociliates (Gao et al. 2012; Li et al. 2006; Song et al. 2005;

Zhang et al. 2010, 2011). We here present the first 18S rRNA sequence of the genus *Platynematum*, making a step towards a more robust phylogeny in this difficult group.

Growth pattern in hypersaline conditions

Growth pattern at different salinities suggest that *P. salinarum* is a true halophilic ciliate, depending on high salt concentrations for survival. Even though ciliates are diverse and abundant in high-salt environments (e.g. Elloumi et al. 2009; Lei et al. 2009), metabolic adaptations are unknown in phagotrophic protists. Based on the growth pattern of *P. salinarum*, we speculate that it may adapt to hyper-saline conditions through the "salt-in strategy", which is the accumulation of molar concentrations of chloride and potassium. Proteins need to maintain their functional conformation and activity at high intracellular salt concentrations, which requires massive adaptations of the enzymatic machinery (Lanyi 1974). The proteomes of halophilic bacteria with a salt-in strategy are highly acidic and denature when suspended in low salt. If such a strategy is also common in ciliates, this could explain why *P. salinarum* thrives well at high salinities (100‰ and above) and cannot survive in salt concentrations below 60‰.

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