

A REASSESSMENT OF *GEMINELLA* (CHLOROPHYTA) BASED UPON
PHOTOSYNTHETIC PIGMENTS, DNA SEQUENCE ANALYSIS AND ELECTRON
MICROSCOPY

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ABSTRACT

A cultured microalgal strain (UTEX 2540) originally identified as *Heterotrichella gracillas* Reisigl (Xanthophyceae) was re-examined using various techniques. Morphological evidence, particularly the absence of dimorphic cells (one blunt, the other tapered to an acute point), indicate that strain UTEX 2540 has been misidentified. *Heterotrichella gracillas* is considered to be a member of the chlorophyll *a* and *c*-containing class Xanthophyceae (Chromista). However, HPLC analyses of photosynthetic pigments indicated the presence of chlorophylls *a* and *b*, β -carotene, lutein and violaxanthin while ultrastructural data revealed the presence of starch stored inside the plastid. These data, as well as small subunit (18S rRNA) gene sequence analysis, indicate that this alga belongs in the Chlorophyta, not the Xanthophyceae (Chromista). Further DNA sequence analyses suggest that UTEX 2540 is most closely related to *Geminella terricola* Petersen and certain *Microspora* species that are currently classified in the Ulotrichales. However, unlike other *Geminella* species, UTEX 2540 exists as single cells or forms poorly organized (2-8 celled) ephemeral pseudofilaments. A conspicuous extracellular mucilaginous sheath characterizes other *Geminella* species but this feature is lacking in UTEX 2540. Furthermore, our analyses convincingly demonstrate that *Geminella* and at least some isolates of *Microspora* do not belong in the Ulotrichales. These results suggest that (1) the generic concept for *Geminella* must be broadened to include unicellular species that lack an apparent mucilaginous envelope, (2) *Geminella* does not belong in the Ulotrichales, and, instead, (3) its closest relatives among other green algae are almost certainly found within the Trebouxiophyceae.

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I would like to thank my family for always encouraging me and seeing my potential. I am lucky to have such a strong support system; it has truly made all the difference in the world. A huge thanks to my best friend and roommate Dayna for always being there for me and making me smile and giving the best hugs when I am stressed. Thanks to my other best friend Kristen for being so understanding and loving, I so am lucky to have friends like you guys. I also have to thank Alex, who has been so supportive and so much fun throughout these last 4 months. Finding my partner in crime has given me so much to look forward to. Last but not least, thanks to my exceptionally sweet and silly Rottweiler, Maggie for always wanting to snuggle and provide comic relief.

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DEDICATION

I would like to dedicate this thesis to my father, Dr. Michael J. Durako for encouraging my curiosity about the world around me throughout my life. His enthusiasm for botany has fueled my interest in the subject and my quest to learn more in this field. He is an inspiration to me as a parent, as a scientist and as a person.

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INTRODUCTION

The Xanthophyceae includes a morphologically diverse assemblage of species that are found predominantly in freshwater or terrestrial environments (Bailey & Andersen, 1998). This class is distinguished from other heterokont taxa primarily by a distinctive combination of photosynthetic pigments and ultrastructural features of their swimming cells (Hibberd & Leedale, 1971; Goodwin, 1974; Norgård *et al.*, 1974; Bjørnland & Liaaen-Jensen, 1989; O'Kelly, 1989; Hibberd 1990; van den Hoek *et al.*, 1995). Xanthophytes are characterized by the chlorophylls *a*, *c*₁ and *c*₂ and the accessory pigments β -carotene, diatoxanthin, diadinoxanthin, heteroxanthin and vaucherioxanthin (Bjørnland & Liaaen-Jensen, 1989). Unlike other chromists, they lack the accessory pigment fucoxanthin, which makes them appear more similar in color to green algae, rather than their golden-hued chromistan relatives. The carbohydrate storage product for the xanthophyceae is commonly referred to as chrysolaminarin and is stored outside the chloroplast in a cytoplasmic vacuole (Dodge, 1973; Trainor, 1978, Bold & Wynne, 1985, Hibberd, 1990).

Systems of classification for the Xanthophyceae have undergone several revisions. The class presently includes roughly 600 species in over 90 genera (Ettl, 1978; Hibberd, 1990). The growth form ranges from coccoid, flagellate, filamentous, palmelloid, or siphonous with coccoid unicells making up approximately two-thirds of all described xanthophyte species (Hibberd, 1990). This diversity has led to problems in distinguishing these species from morphologically similar algae, especially those placed in the Estigmatophyceae (Heterokontophyta) and Chlorophyceae (Chlorophyta). Members within this class were once grouped with the green algae due to their yellow green appearance (Pringsheim, 1885; Sachs, 1882; Borzi, 1889). This

study focuses on the biology and systematics of a cultured algal strain identified as *Heterotrichella gracillas* Reisi gl.

Heterotrichella was erected by Reisi gl (1964) and includes only the type species, *H. gracilis*, which was first isolated from a soil sample taken in Austria. According to Reisi gl (1964), the thin-walled cells of *H. gracilis* are elongated (2-2.5 μm wide x 5.5-22 μm long), cylindrical, straight or slightly curved. One of the two ends of single cells is rounded (blunt), whereas the opposite end abruptly tapers to a more-or-less acute point (Ettl, 1978; fig. 518). Each cell possesses one or two band-like parietal chloroplasts that lack pyrenoids, and the cytoplasm is characterized by the presence of “many droplets” of unknown composition (Reisi gl, 1964; Ettl, 1978). Although unicells are most often encountered, short filaments composed of two to four cells have also been observed and, as with unicells, the two ends of the filament are dimorphic (i.e., one blunt, the other pointed). Asexual reproduction occurs by binary cell division or filament fragmentation; sexual reproduction is unknown. Reisi gl (1964) placed the species in the chromist algal class Xanthophyceae and, because of its filamentous nature, assigned it to the order Tribonematales, family Tribonemataceae (see also Ettl, 1978).

Strain UTEX 2540, identified as *Heterotrichella gracilis*, was obtained from the Culture Collection of Algae at the University of Texas at Austin (Starr & Zeikus, 1993). The UTEX isolate, deposited in 1990, was isolated from a tundra pool near Toolik Lake, Alaska, USA. Preliminary observations for this investigation indicated the morphology of the cultured alga differs significantly from the generic description.

The objective of this study was to re-examine the taxonomic and phylogenetic positions of strain UTEX 2540 using a suite of biochemical, DNA-based, and microscopic techniques.

MATERIALS AND METHODS

Culture methods

Heterotrichella gracilis Reisingl (strain UTEX2540), which had been collected and isolated from Toolik Lake, Alaska, USA was obtained from the Culture Collection of Algae at the University of Texas at Austin (Starr & Ziekus, 1993). Replicate unialgal cultures were subsequently maintained in DYIV medium (Andersen *et al.*, 1997) at 15° C under a 14:10 hr light:dark cycle or at 22-24°C under ambient light conditions. Some cultures were continuously shaken on a Yellow line® OS 2 oscillator (Yellowline®, Staufen, Germany). Cells were also grown on DYIV agar plates at 22-24°C.

In addition to UTEX 2540 we examined three other algal strains during the course of this study including SAG 53.94 (*Microspora* sp.), CCAP 348/1 (*Microspora amoena*), and CCAP 348/2 (*Microspora tumidula*). These strains were maintained in culture as describe above.

High Performance Liquid Chromatography (HPLC)

UTEX 2540 cells were prepared for HPLC using techniques described in Vidussi (1996). Briefly, 3 ml of cells were collected using a GF/F glass microfibre filter (Whatman Inc., Florham Park, NJ) and these cells were then added to a tube containing 3ml Methanol and sonicated for 30 seconds. The pigment solution was then passed through a GF/F filter, and 250 µl of ammonium acetate solution was added to 500 µl of the eluted filtrate. After 5 mins, 200 µl of this solution was injected through a C8 column (Supelco Mos-2 Hypersil, Bellefonte, Pennsylvania, USA) into a Hewlett Packard Series 1100 HPLC. The data were processed using ChemStation Rev A.10.02 software (Agilent Technologies, Foster City, CA, USA). The

pigments were identified according to their retention times as compared to those of pure standards (International Agency for ^{14}C Determination, Hørsholm, Denmark).

Brightfield microscopy

Cells were observed using a Zeiss Axio Imager.Z1 microscope and photomicrographic images were captured using a Zeiss Axio cam MRc5 camera. An AxioVision (Zeiss, Thornwood, NY, USA) software package was used to obtain digital images from which calibrated length and width measurements could be collected. Fifty length and width measurements were taken and means were calculated in a Microsoft Excel spreadsheet.

Transmission electron microscopy

Cells were collected using centrifugation (9000 rpm x 3 min), fixed on ice by adding a 3mL solution of 2.6% glutaraldehyde and 0.66M cacodylate buffer (pH 7) to an equal volume of the cell suspension, and followed 20 seconds later by adding 1mL 2% osmium tetroxide. After 1 hr, the cells were rinsed with distilled water and dehydrated through an ETOH series to 70% percent ETOH. Cells were left in 70% ETOH with 0.5% uranyl acetate overnight at 4°C. The following day dehydration was continued in the ETOH series to 100% ETOH followed by two changes of propylene oxide. Cells were infiltrated and embedded with Spurr's resin overnight at 70°C. Thin sections (900 nm) were cut using the Sorvall MT-1 Ultramicrotome and collected on Formvar-coated mesh copper grids. The grids were then sequentially stained 20 min each with uranyl acetate and lead citrate. Stained grids were observed on a Philips CM 12 transmission electron microscope. Digital images were processed using Adobe Photoshop Version 7.0.

DNA extraction, PCR amplification and DNA sequencing

Total cellular DNA was extracted from UTEX 2540 (*Heterotrichella gracilis*), SAG 53.94 (*Microspora* sp.), CCAP 348/1 (*Microspora amoena*), and CCAP 348/2 (*Microspora tumidula*) as described in Bailey *et al.* (1998) except that cells were mechanically broken using 0.5 mm glass beads and a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA). DNA was extracted from the CTAB buffer using chloroform:isoamyl alcohol and then concentrated using a GeneClean II kit (Q-Biogene, Irvine, CA, USA).

Overlapping portions of nuclear 18S rRNA target sequences were amplified using forward and reverse primers on a GeneAmp PCR System 9600 (Perkin Elmer, Wellesley, MA, USA). Amplifications were performed using primer pairs: GO1F, GO3F, GO4F, GO7R, GO9R (Saunders and Kraft, 1994) and primer pairs: P5F, P6F, P7R, and P10R (Medlin *et al.*, 1988). A novel primer, DC6F (5'-GAGGGACTTTTGGGTAATCA-3'), was used to amplify additional regions of the 18S rRNA gene. Amplifications were performed using the thermocycling profile described in Bailey *et al.* (1998) with an annealing temperature of 52°C for 1 minute.

PCR products were separated on 0.8% agarose gels and bands were visualized using a FOTO/Phoresis UV transilluminator (Fotodyne, Hartland, WI, USA). Amplification products were purified using a GeneClean II kit. Cleaned PCR template DNA was cycle sequenced (both strands) and the forward and reverse primers listed above using the Big Dye Version 2 terminator sequencing kit. These reactions were completed using 25 cycles of the following regime: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequencing reactions were purified using G-50 Sephadex columns (Amersham Biosciences, Uppsala, Sweden) and sequence data were determined using an ABI 3100 automated DNA analyzer (Applied

Biosystems, Foster City, CA, USA) and assembled using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA).

Phylogenetic analysis

Selected species representing the classes Chlorophyceae, Charophyceae, Ulvophyceae, and Prasinophyceae, as well as DNA data for all available trebouxiophyte genera were obtained from GenBank and included in the phylogenetic analyses (Table 1). Species representing a variety of growth forms within each class were selected to account for the diversity existing within each of these groups. The sequences were aligned automatically using Clustal X (Thompson *et al.*, 1997) and subsequently edited by eye in MacClade 4.0 (Maddison and Maddison, 2000). Two 18S rRNA sequence matrices were analyzed in this study. One included 34 green algal taxa, while the other included data for 166 taxa. Both trees were rooted on the 18S rRNA sequence for the embryophyte (moss) *Physcomitrella patens*. All phylogenetic analyses were performed using PAUP version 4.0 (Swofford, 2002) and solutions for 34 taxon data set were obtained under the optimality criteria of maximum parsimony and maximum likelihood; the 166 taxon matrix was analyzed using parsimony only. Parsimony analyses of each data set were conducted using two different models. Assumptions underlying Fitch parsimony (Fitch, 1971) were used to generate one set of hypotheses; trees were also generated under assumptions imposed by a TIM+I+G model of sequence evolution obtained using the ModelTest program (v. 3.06, Posada and Crandall, 1998). Bootstrap values (Felsenstein, 1985) for the parsimony trees were derived from analyses of 10,000 pseudoreplicate data sets using the “fast step-wise” option whereas values for the 34 taxon ML tree were based on 40 pseudoreplicates.

Table 1. Species included in the phylogenetic analyses named as they currently appear in GenBank and their accession numbers for their nuclear 18S rRNA gene sequences. Species used in the smaller (35 taxa) 18S analyses are indicated by an (*) beside the species name. Unpublished sequence is denoted by XXXXXX and was obtained during this study.

Species/ Taxon	Accession number
TREBOUXIOPHYCEAE	
<i>Auxenochlorella protothecoides</i> (Kruger) Kalina & Punochoava	X56101
<i>Chlorella ellipsoidea</i> Gerneck	X63520
<i>Chlorella kessleri</i> Fott & Novakova	X56105
↳ <i>Chlorella lobophora</i> Andreyeva	X63504
<i>Chlorella luteoviridis</i> Chodat *	X73997
<i>Chlorella luteoviridis</i> Chodat	AB006045
<i>Chlorella minutissima</i> Fott & Novakova	X56102
<i>Chlorella mirabilis</i> Andreyeva	X74000
<i>Chlorella saccharophila</i> (Kruger) Migula	X63505
<i>Chlorella sorokiniana</i> Shihira & Krauss Prag	X74001
<i>Chlorella vulgaris</i> Beijerinck	X13688

	<i>Chlorella</i> Beijerinck	AB058305
	<i>Chlorella</i> sp. Yanaqocha	Y14950
	Chlorophyte Isolate (endosymbiont of Ginkgo)	AJ302940
	<i>Choricystis minor</i> (Skuja) Fott	X89012
	<i>Choricystis</i> Suja sp.	AF357147/55
	<i>Choricystis</i> sp. 1	AF357148/56
	<i>Choricystis</i> sp. 2	AF357149
	<i>Choricystis</i> sp. 3	X81965
∞	<i>Closteriopsis acicularis</i> (G.M. Smith) J.H. Belcher & Swale	Y17470
	<i>Coenocystis inconstans</i> Hanagata & Chihara	AB017435
	<i>Dictyochloropsis reticulate</i> (Tschermak-Woess) Tschermak-Woess	Z47207
	<i>Eremosphaera viridis</i> de Bary	AF387154
	<i>Fusochloris perforate</i> (Lee & Bold) Floyd, Watanabe & Floyd	
	A.K.A. <i>Characium perforatum</i> (Lee & Bold) Floyd, Watanabe & Floyd	M62999
	<i>Gloeotila contorta</i> Chodat	AY422074
	<i>Gloeotila</i> Kutzing sp.	AY195976

<i>Koliella</i> Hindak sp.	AY352046
<i>Leptosira terrestris</i> (Fritz & John) Friedl A.K.A. <i>Pleurastrum terrestris</i> Fritz & John	Z28973
<i>Lobosphaera tirolensis</i> Reisigl	AB006051
<i>Marvania geminata</i> Hindak	AF124336
<i>Marvania</i> Hindak sp.	AY195977
<i>Micractinium pusillum</i> Fresenius	AF364101
<i>Microthamnion kuetzingianum</i> Nageli	Z28974
<i>Myrmecia biatorellae</i> (Tschermak-Woess & Plessl) Peterson	Z28971
6 <i>Myrmecia israelensis</i> (Chantanachat & Bold) Friedl A.K.A. <i>Friedmannia israelensis</i> (Chantanachat & Bold)	M62995
<i>Nannochloris atomus</i> Butcher	AB080305
<i>Nannochloris atomus</i> Butcher	AB080303
<i>Nannochloris bacillaris</i> Naumann	AB080300
<i>Nannochloris coccoides</i> Naumann	AB080301
<i>Nannochloris eucaryotum</i> (Wilhelm <i>et al.</i>) Menzel & Wild	AB080304
<i>Nannochloris maculatus</i> Butcher	AB080302

<i>Picochlorum oculatum</i> (Droop) Henley, Hironaka, Guillou, Buchheim, Buchheim, Fawley, & Fawley	AY422075
<i>Nannochloris</i> Nauman sp.	AY195968
<i>Nannochloris</i> sp. 1	AY220081
<i>Nannochloris</i> sp.2	AY195983
<i>Nannochloris</i> sp.3	AJ131691
<i>Nannochloris</i> sp.4	AB080306
<i>Picochlorum</i> sp. 5	AY422076
<i>Nannochloris</i> sp. 6	AY560119
<i>Picochlorum</i> sp. 7	AY422077
<i>Nanochlorum eucarotum</i> Wilhelm <i>et al.</i> Mainz	X06425
<i>Nanochlorum</i> Wilhelm <i>et al.</i> sp.	AB058304
<i>Nanochlorum</i> sp. 8	AB058309
<i>Nanochlorum</i> sp. 9	AB058312
<i>Nanochlorum</i> sp. 10	AB058331
<i>Oocystis heteromucosa</i> Hegewald	AF228689
<i>Oocystis solitaria</i> Wittrock *	AF228686

	<i>Pabia signiensis</i> Friedl & O'Kelley	AJ416108
	<i>Paradoxia multiseta</i> Svirenko	AY422078
	<i>Parietochloris pseudoalveolaris</i> (Deason & Bold) Watanabe & Floyd	M63002
	<i>Picochlorum oklahomensis</i> Hironaka	AY422073
	<i>Picochlorum</i> Henley <i>et al.</i> sp.	AY526738
	<i>Planctonema</i> Schmidle sp.	AF387148
	<i>Prasiola crispa</i> (Lightfoot) Meneghini	AJ416106
	<i>Prasiola fluviatilis</i> (Summerfelt) Areschoug	AF189072
≡	<i>Prototheca wickerhamii</i> Tubaki & Soneda	X74003
	<i>Pseudochlorella</i> Lund sp.	AB006049
	<i>Raphidonema longiseta</i> Vischer	U18520
	<i>Raphidonema nivale</i> Lagerheim	AF448477
	<i>Stichococcus bacillaris</i> Nageli *	AB055866
	<i>Tetrachlorella alternans</i> Korsikov	AF228687
	<i>Trebouxia impressa</i> Ahmadjian	Z21551
	<i>Trebouxia magna</i> Ahmadjian	Z21552

<i>Watanabea reniformis</i> Hanagata <i>et al.</i>	X73991
<i>Prototheca zopfii</i> Krueger	AY973040
<i>Stichococcus deasonii</i>	DQ275460
<i>Coccomyxa pringsheimii</i> Jagg	AY762603
<i>Trichophilus welckeri</i> Weber-van Bosse	AY762601
<i>Prasiolopsis ramosa</i> Vischer	AY762600
<i>Parachlorella beyerinckii</i>	AY323841
<i>Didymogenes palatine</i> Schmidle	AY323840
<i>Didymogenes anomala</i> (Smith) Hindák	AY323839
<i>Dictyosphaerium sp.</i> Wolf	AY323835
<i>Kollielopsis inundata</i>	AY518591
<i>Prototheca ulmea</i> Pore	AB096929
<i>Botryococcus braunii</i> Kützing	AJ581911
<i>Pseudodictyosphaerium sp.</i> Itas	AY543066
<i>Trebouxia erici</i> Ahmadjian *	AB080310
<i>Parachlorella kessleri</i> (Fott & Nováková) Krienitz, E.H. Hegewald,	AB080309

Hepperle, V. Huss, T. Rohr & M. Wolf

Lagerheimia genevensis clone

AY122336

Viridiella fridericana Albertano, Pollio & Taddei

AJ439401

Muriella sp.

AY195969

Makinoella tosaensis Okada

AF228691

Amphikrikos sp. Hegewald

AF228690

Helicosporidium sp.

AF317893

Radiofilum transversale (Bréb.) Christensen

AF387161

Radiofilum conjunctivum Schmidle *

AF387155

Pseudochlorella subsphaerica Reisingl

AB006050

Friedmannia israelensis Chantanachat & Bold

M62995

***Geminella* & *Micropora* species**

UTEX 2540 *

XXXXXX

Geminella terricola Petersen *

AF387152

Geminella minor (Nägeli) Heering *

AF387151

Geminella sp. *

AF387158

Geminella sp. * AF387157

Microspora stagnorum (Kützing) Lagerheim * AF387153

Microspora sp. * AF387160

CHLOROPHYCEAE

Chlamydomonas noctigama Korschikov * AJ781311

Pediastrum tetras (Ehrenberg) Ralfs * AY780666

Hydrodictyon reticulatum (Linnaeus) Lagerheim * AY780660

Oedogonium stellatum Wittrock DQ115895

14

Chlorosarcina stigmatica Deason AB218711

Desmochloris halophila (Guillard, Bold & McEntee) Watanabe, Kuroda & Maiwa AB049416

Asterococcus korschikoffii Ettl AB175837

Coccomyxa glaronensis Jaag AY333645

Golenkinia longispicula Hegewald et Schnepf AF499923

Coelastrum astroideum var. *rugosum* Tsarenko AF388377

Dictyochloris fragrans Vischer isolate AF367861

Sphaeroplea annulina (Roth) Agardh AF302771

<i>Carteria olivieri</i> West	U70596
<i>Scenedesmus subspicatus</i> Chodat *	AJ249514
<i>Pteromonas angulosa</i> (Carter) Lemmermann	AF395438
<i>Bulbochaete hiloensis</i> (Nordstedt) Tiffany	U83132
<i>Chaetophora incrassate</i> (Hudson) Hazen	D86499
<i>Oedogonium cardiacum</i> Wittrock	U83133
<i>Aphanochaete magna</i> Godward *	AF182816

CHAROPHYCEAE

15

<i>Klebsormidium subtilissimum</i> (Rabenhorst) Silva, Mattox & Blackwell *	AF408241
<i>Chara connivens</i> Salzmänn ex Braun *	AF408223
<i>Coleochaete nitellarum</i> Jost *	AF497794
<i>Mesostigma viride</i> Lauterborn *	AJ250108
<i>Tolypella prolifera</i> (Ziz ex Braun) Leonhardi	AF408228
<i>Lamprothamnium macropogon</i> (Braun) Ophel	AF408224
<i>Nitella capillaris</i> (Krocker) Groves & Bullock-Webster	AJ250111
<i>Zygnema cylindricum</i> Transeau	AJ853451

Spirogyra sp. AJ549231

Desmidium swartzii Agardh ex Ralfs AJ428133

Xanthidium armatum (Brébisson) Rabenhorst ex Ralfs AJ428094

Gonatozygon kinahanii (Archer) Rabenhorst AJ553921

Closterium pleurodermatum West & West AF352238

Cosmarium isthmium var. hibernica West AJ428116

Staurastrum cristatum (Nägeli) Archer * AJ428110

ULVOPHYCEAE

15

Spongiochrysis hawaiiensis isolate DQ077806

Wittrockiella paradoxa Wille AB078732

Cladophora kosterae Hoek * AB078730

Microdictyon boergesenii Setchell Z35324

Ernodesmis verticillata (Kützing) Børgesen Z35321

Acrochaete viridis (Reinke) Nielsen AY303595

Percursaria percursa (Agardh) Rosenvinge * AY303589

Ulva californica Wille * AY303586

<i>Rhizoclonium hieroglyphicum</i> (Agardh) Kützing	AB062715
<i>Aegagropila linnaei</i> Kützing	AB062699
<i>Struvea plumosa</i> Sonder	AF510161
<i>Cephaleuros virescens</i> Kunze	AY220984
<i>Trentepohlia iolithus</i> (Linnaeus) Wallroth *	AY220983
<i>Acetabularia acetabulum</i> (Linnaeus) Silva *	AY165776
PRASINOPHYCEAE	
<i>Tetraselmis chuii</i> Butcher *	DQ207405
17 <i>Nephroselmis viridis</i> Inouye	AB214976
<i>Prasinopapilla vacuolata</i>	AB183649
<i>Crustomastix</i> sp.	MBIC10709
<i>Prasinoderma</i> sp.	AB183632
<i>Micromonas pusilla</i> (Butcher) Manton & Parke *	AB183589
<i>Nephroselmis pyriformis</i> (Carter) Ettl	AB158376
<i>Tetraselmis kochiensis</i>	AJ431370
<i>Pyramimonas aureus</i> *	AB052289

<i>Crustomastix stigmatica</i> Zingone	AJ629844
<i>Ostreococcus tauri</i> Courties & Chrétiennot-Dinet *	AY329635
<i>Pterosperma cristatum</i> Schiller *	AJ010407
<i>Dolichomastix tenuilepis</i> Throndsen & Zingone	AF509625
<i>Mantoniella antarctica</i> Marchant	AB017128
OUTGROUP	
<i>Physcomitrella patens</i> *	AF126289

RESULTS

Geminella (Turpin) Lagerhiem emend. Durako et Bailey

Synonym(s): *Hormospora* Nägeli non Brébisson, ?*Bachmanniella* Chodat (1933)

Coccoid cells enrobed by a thick, homogeneous mucilaginous sheath forming unbranched (uniseriate) pseudofilaments or free living coccoid cells lacking a conspicuous sheath or envelope. Cells longer than wide, cylindrical, ellipsoidal, oval or barrel-shaped with rounded ends. Cells in sheathed pseudofilaments sometimes widely separated, found in pairs, or adjacent to one another (touching) but do not share cell walls in common. Cells contain one, rarely two parietal, cup-shaped or laminate chloroplasts in which a pyrenoid may or may not be visible. Reproduction by fragmentation, simple cell division, or by brownish colored cysts ('akinetes').

Type species: *Geminella interrupta* Turpin (1828). *Mém. Mus. Hist. Nat.* **16**: 329

Pigment content

HPLC analysis indicated that UTEX 2540 contains photosynthetic pigments characteristic of green algae. The photosynthetic pigments identified were chlorophylls *a* and *b*, as well as β -carotene, lutein, and violaxanthin (Figure 1). Chlorophyll *c* was not detected.

Morphology: Brightfield microscopy

UTEX 2540 unicells range in shape from coccoid to elliptical with an average size of 7.2 μm by 4.4 μm . Cultured cells possess a birefringent cell wall and were observed singly or in pseudofilaments comprised of one to three cells (Fig 2,3). These cells lack an apparent

extracellular mucilaginous sheath. The chloroplast is parietal, cup-shaped and pyrenoids are visible in some, but not all, cells (Fig 5).

The ends of ellipsoidal cells and cells in pseudofilaments were always blunt (rounded) and never conspicuously tapered on either or both ends (Figs 2-5, 6-9). Cells cultured on DYIV agar (Andersen *et al.*, 1997) for over two months did not exhibit dimorphic ends (*cf.* Figs 2-5, 6-9). Most ellipsoid cells and pseudofilaments were straight; curved cells were rarely observed. Longer, larger and presumably older cells possessed several relatively large oil-like droplets in the cytoplasm (Figs 6-9). The cells undergo asexual reproduction by cytokinesis. Sexual reproduction and swimming cells (zoospores) were not observed. Reproduction in UTEX 2540 occurs by binary cell division (Fig 4).

Morphology: Ultrastructure

UTEX 2540 vegetative cells were surrounded by a thick, birefringent cell wall (Figs 10-13). Each cell contained a single nucleus and a single chloroplast. An average of two mitochondria were typically found per cell. Chloroplasts were parietal with pyrenoids found in some, but not all cells. When pyrenoids were present, starch grains were wrapped around them within the thylakoids of the chloroplast. A considerable volume of the cells for this alga was comprised of lipid droplets of unknown composition. Evidence of an invagination between dividing cells may suggest that UTEX 2540 forms a phycoplast, although no definitive evidence for this was observed (Figs 14-17).

Figure 1. A representative HPLC chromatogram for UTEX 2540 showing the photosynthetic peak assignments as compared to standards. Absorbance is represented by milli-absorbance units (mAU) at $\lambda=440\text{nm}$. Peak 1: violaxanthin; peak 2: lutein; peak 3: chlorophyll *b*; peak 4: chlorophyll *a*; and peak 5: β -carotene.

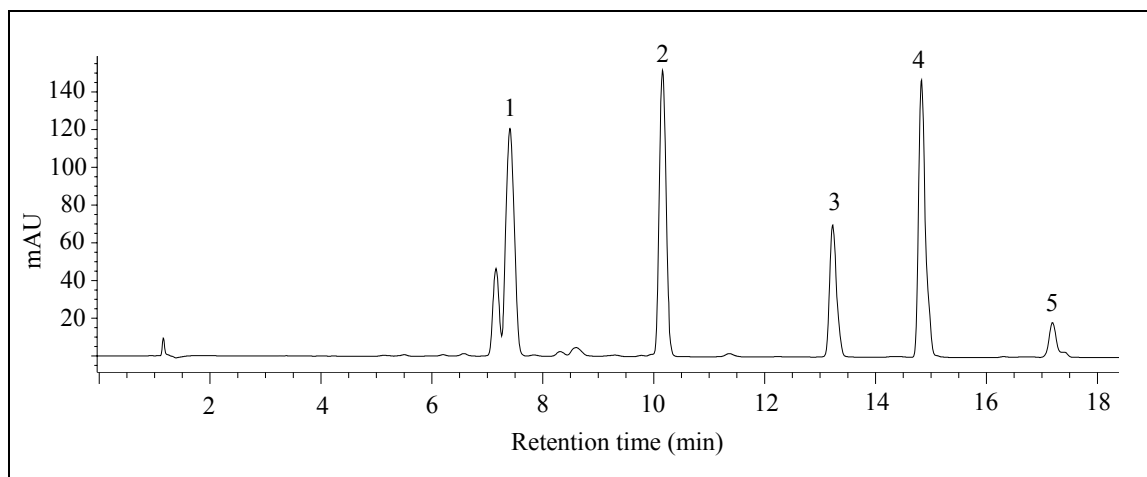


Plate 1:

Figures 2-5. Light micrographs representing UTEX 2540 cells. Note in figs 2 and 3 chains of three or four cells that have divided but not separated forming pseudofilaments (arrows). Dividing cells can be seen in fig 4 (arrow). Cells possessing pyrenoids (P) are shown in fig 3. Scale= 20 μ m.

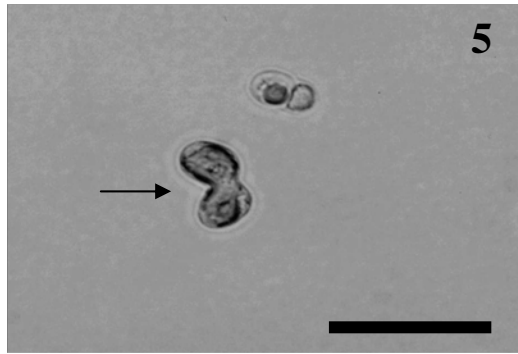
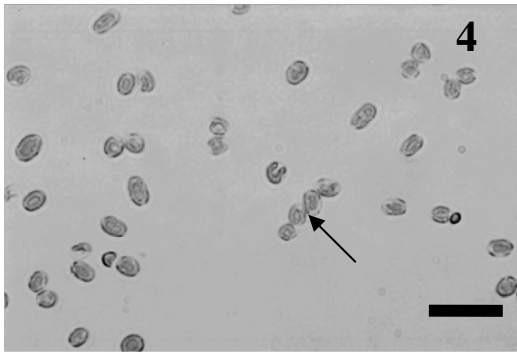
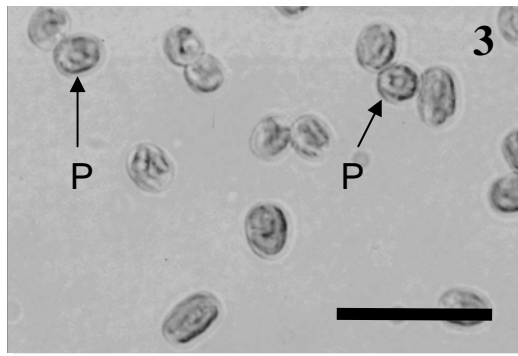


Plate 2:

Figs 6-9. Light micrographs representing UTEX 2540 cells. Lipid droplets (L) of unknown composition can be seen in most cells. Chloroplasts (C) are also visible in these cells. Scale bars: 10 μm (Fig 6) and 5 μm (Figs 7-9).

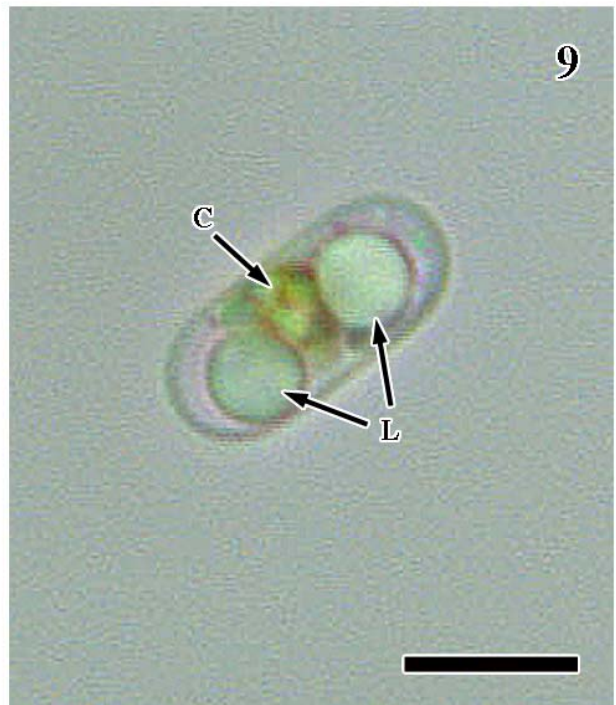
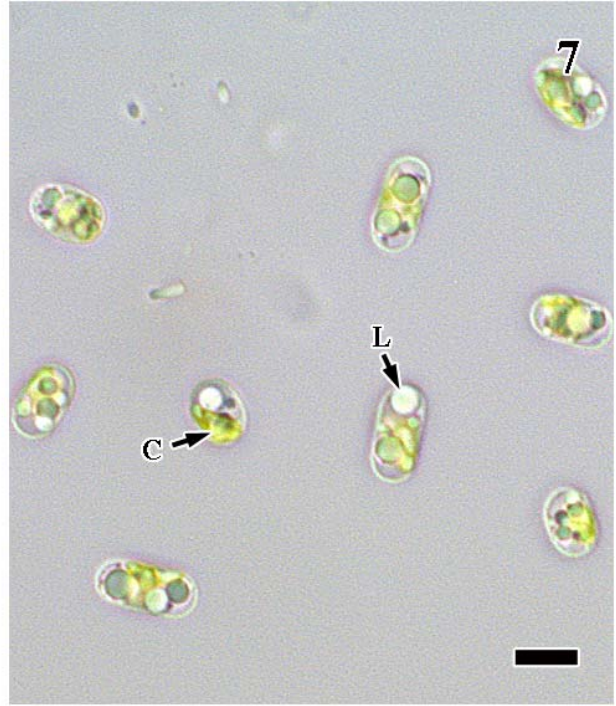
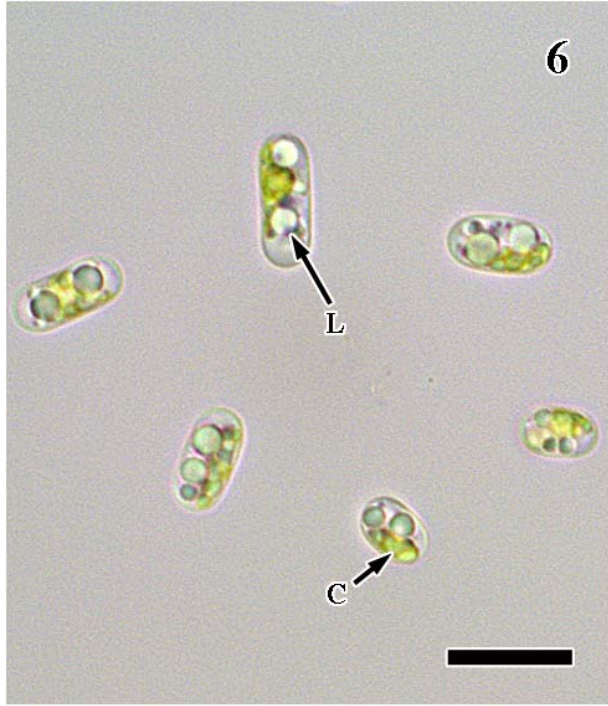


Plate 3:

Figs 10-13. TEM photomicrographs of whole cell images of UTEX 2540. Cells contain a single nucleus (N), mitochondria (M) and starch grains (S) stored inside of the thylakoids (T) of the chloroplast (C). A golgi apparatus (G) is visible in figs 11 and 13. The cells also possess a birefringent cell wall (CW) and noteworthy lipid droplets (L) of unknown composition. Scale bars: 1 μm .

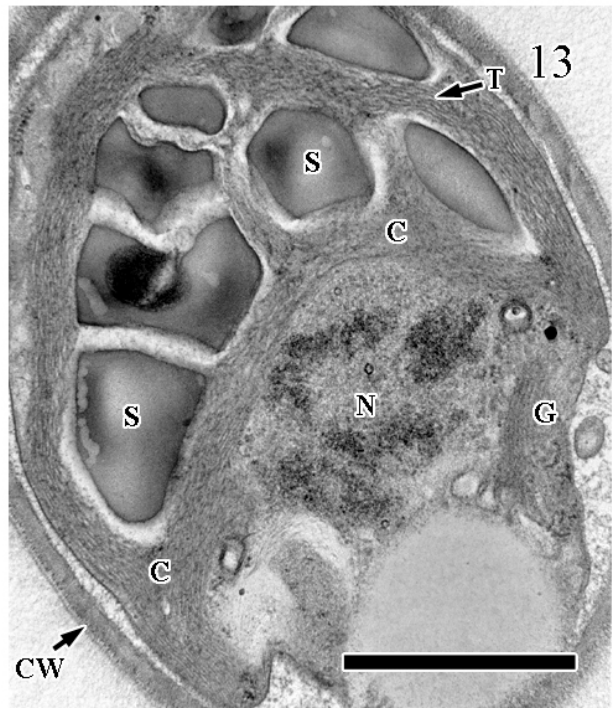
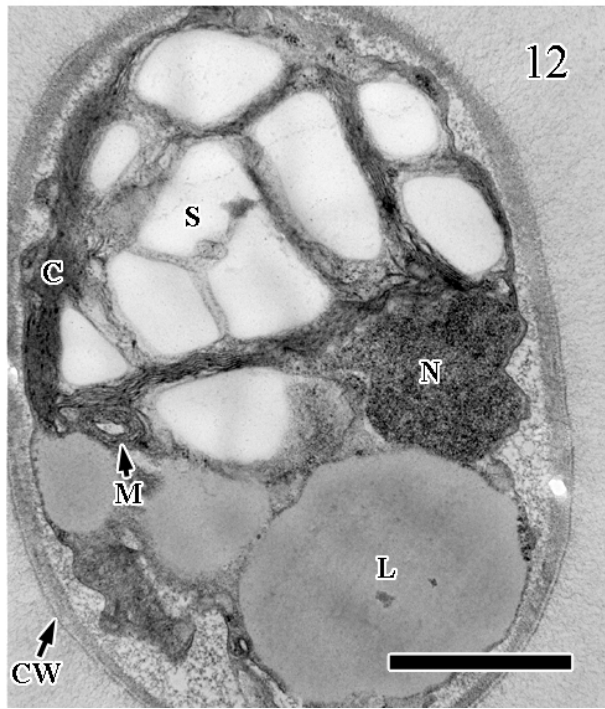
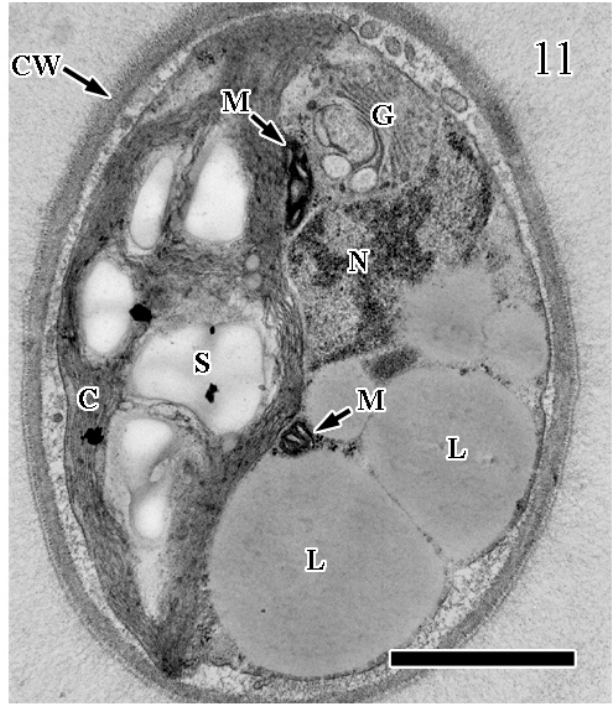
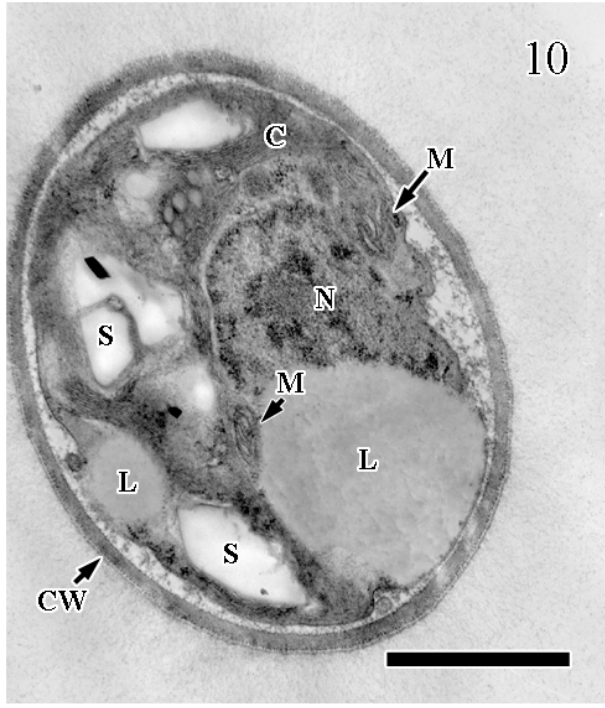
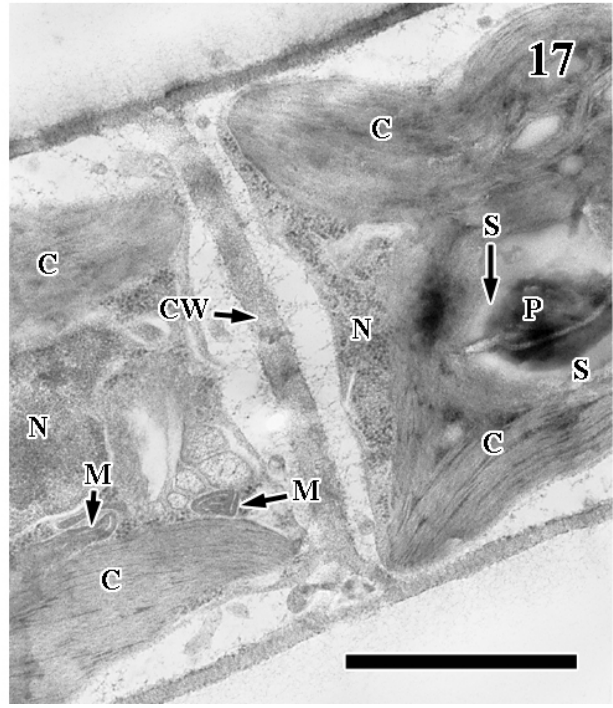
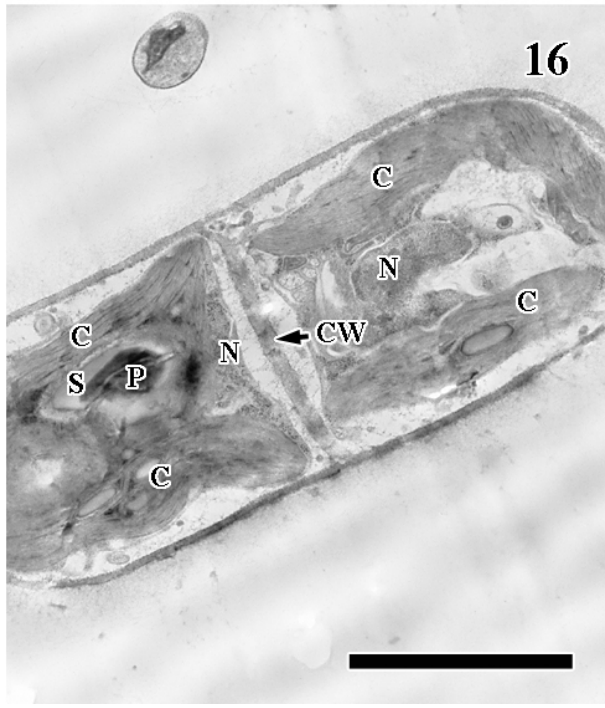
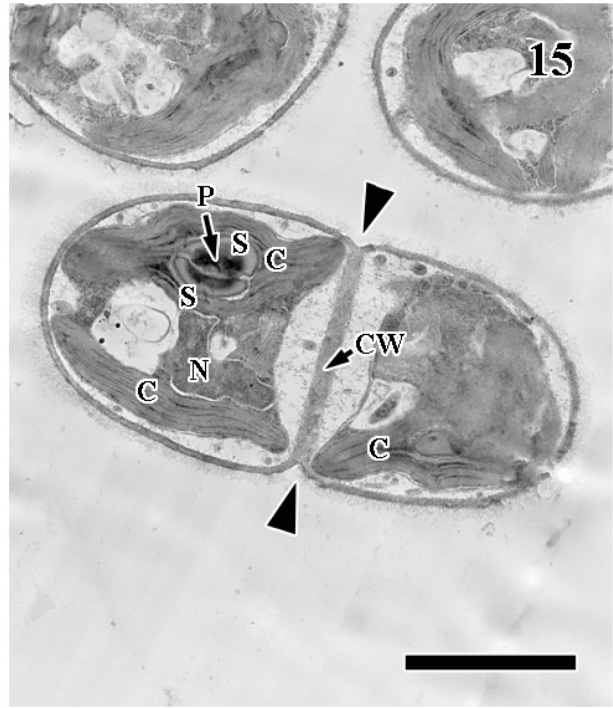
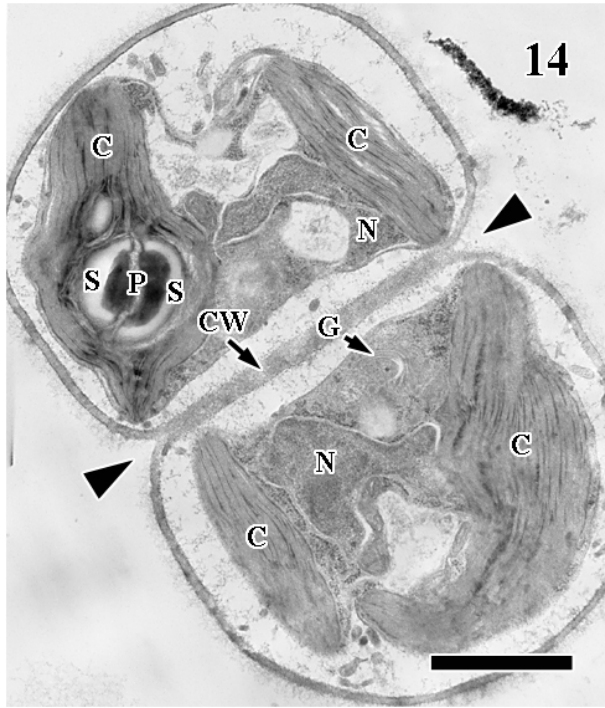


Plate 4:

Figs 14-17. TEM photomicrographs showing UTEX 2540 cell division. Daughter cells contain a nucleus (N), mitochondria (M), Chloroplasts (C) containing pyrenoids (P) transversed by one or two thylakoids and a birefringent cell wall (CW). Invaginations (arrowheads) are visible between some dividing cells (figs 14, 15). Scale bars: 1 μ m (Figs 14, 17) and 2 μ m (Figs 15, 16).



DNA sequence analysis

BLASTn (Altschul *et al.*, 1997) analysis of the 18S rRNA gene sequences obtained for UTEX 2540 (= 1772 bp) indicated that the organism is a green alga, not a xanthophyte. BLASTn results further suggested that UTEX 2540 may, on the basis of these data, belong to the previously sequenced (= 1682 bp) green algal species *Geminella terricola* with the exception of one nucleotide substitution. In turn, BLASTn analyses confirm that strains UTEX 2540, SAG 53.94 and CCAP348/1 are green algae but reject the hypothesis for strain CCAP 348/2. The latter is not a green alga and its sequence suggests that it belongs in the xanthophyte genus *Tribonema*. Cladistic analyses of 34 green algal sequences under the TIM+I+G model yielded a single tree (L=2035, CI=0.49, RI=0.53) (Fig. 18). The Fitch parsimony tree obtained was topologically identical to the tree depicted in Fig. 18 and is therefore not shown. The ML tree is shown in Fig. 19 and, for the purposes of this study, is consistent with the parsimony trees. Cladistic analyses of 166 green algal sequences yielded 91,018 equally most parsimonious trees (L=6581, CI=0.30, RI=0.64) and the majority rule consensus of these trees is shown in Fig. 20.

Initial analyses conducted indicate that UTEX2540 is closely related to *Geminella* spp. as well as *Microspora* (Figs 18, 19, 20). These taxa form a robustly supported clade within the green algae, but it is unclear to which class these taxa should be assigned (Figs 18, 19, 20). A relationship between *Geminella* and *Microspora* has not previously been suggested. For this reason, we subsequently examined three cultures from two culture collections identified as *Microspora* spp. The objective was to help confirm or reject the *a posteriori* hypothesis that *Geminella* and *Microspora* are close relatives. DNA sequence analyses using BLASTn of the 18S rRNA gene indicate that two of the three cultures (CCAP 348/1 and 348/2) may not belong in the genus *Microspora*. CCAP 348/1, identified as *Microspora amoena*, probably belongs in

Figure 18. Parsimony 18S rRNA tree depicting relationships inferred among 34 chlorophyte species and one outgroup sample. This tree was generated under assumptions imposed by a TIM+I+G model of sequence evolution, is topologically identical to the tree obtained under assumptions of Fitch parsimony. Bootstrap values for nodes of the tree are shown above branches; the top value corresponds to that obtained using the model whereas the lower was derived using Fitch parameters. The positions of *Geminella* and *Microspora* isolates are highlighted. A & B: Trebouxiophyceae; C & E: Chlorophyceae; D: Ulvophyceae; F: Prasinophyceae; G: Charophyceae.

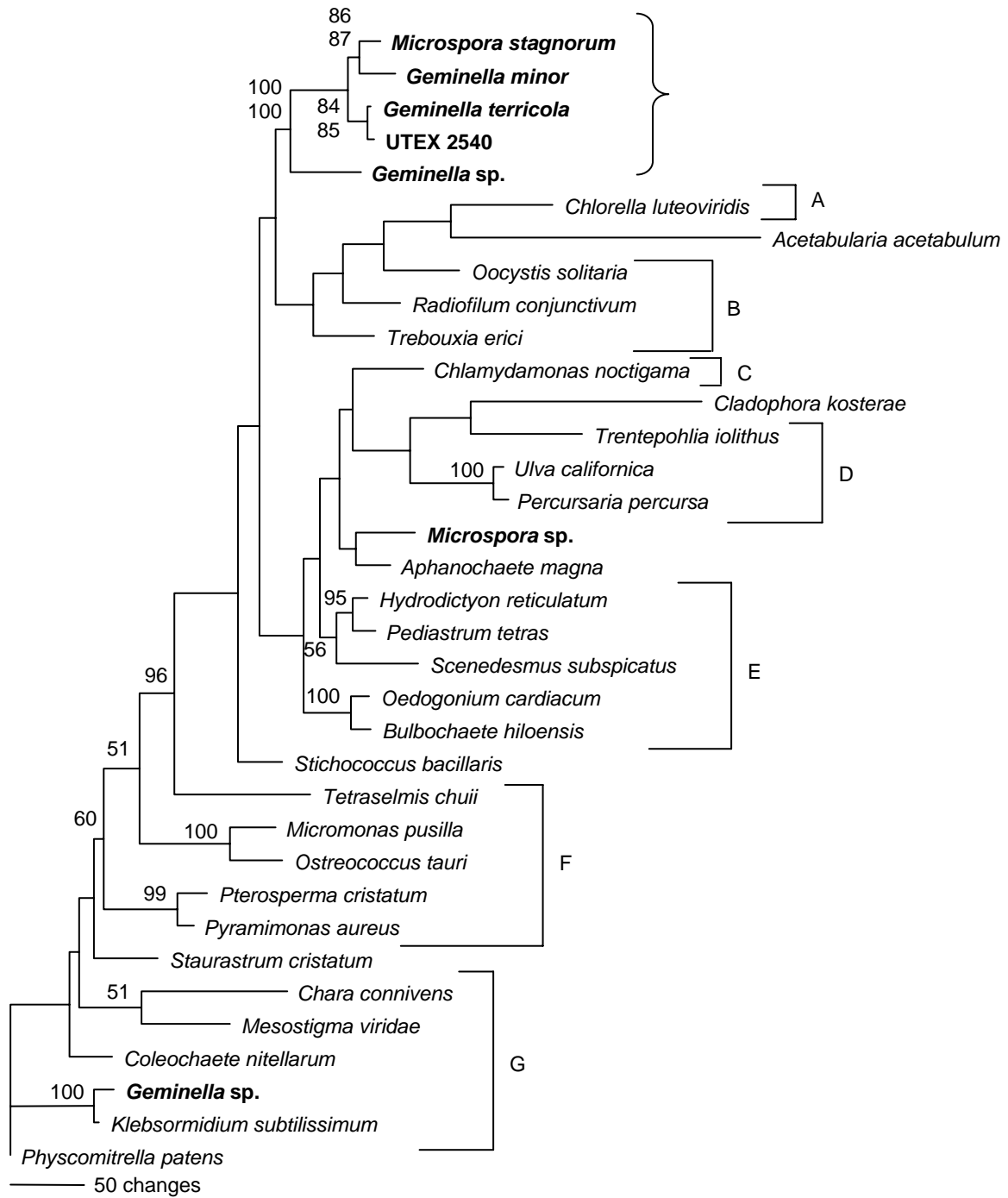


Figure 19. Maximum likelihood 18S rRNA tree for 34 chlorophyte species and one outgroup sample. The positions of *Geminella* and *Microspora* isolates are highlighted. Bootstrap values for nodes are based on 40 pseudoreplicates. These values are shown above branches. A & F: Prasinophyceae; B & E: Ulvophyceae; C: Trebouxiophyceae; D: Chlorophyceae; G: Charophyceae.

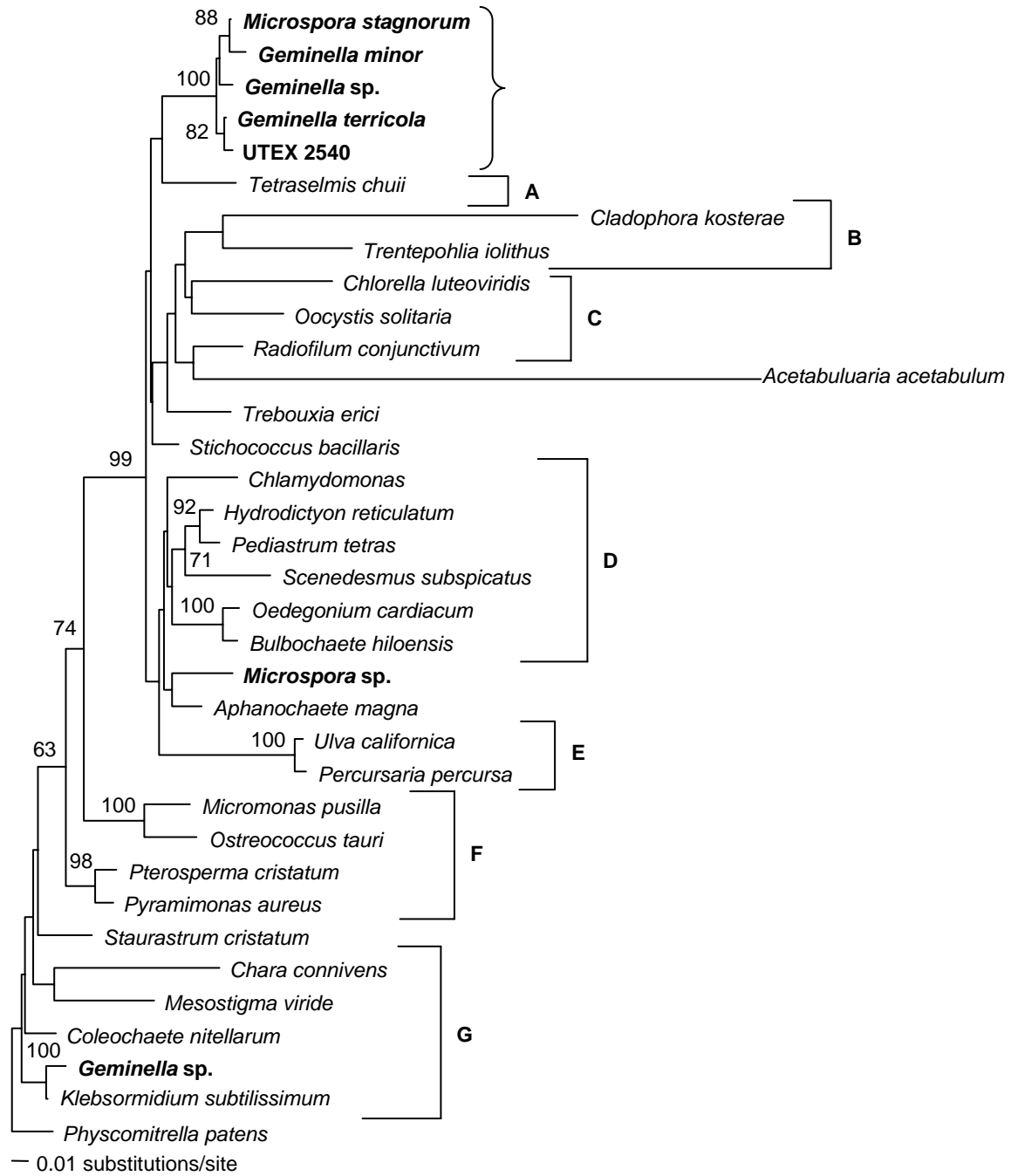


Figure 20. Majority rule consensus tree depicting relationships inferred among 166 chlorophyte species based upon cladistic analysis of 18S rRNA gene sequences. Bootstrap values ($\geq 51\%$) are shown above or below associated nodes of the tree. A: Ulvophyceae; B & C: Chlorophyceae; D, E & F: Trebouxiophyceae; G & H: Prasinophyceae; I: Charophyceae. The *Geminella-Microspora* clade is indicated by an asterisk.

genus *Ulothrix* whereas CCAP 348/2 is an undoubted member of the genus *Tribonema* (Xanthophyceae, Heterokontophyta).

Our 18S rRNA sequence for UTEX 2540 differs from a previously available sequence for *Geminella terricola* by only a single substitution and we conclude that the two isolates are conspecific.

DISCUSSION

A number of different lines of evidence indicate that UTEX 2540 has been misidentified; the alga is not *Heterotrichella gracilis* and does not belong in the Xanthophyceae (Heterokontophyta). According to Reising (1964), the ends of *H. gracilis* cells or filaments are dimorphic; one end is blunt or rounded whereas the other tapers to an acute point. This feature is absent in UTEX 2540 and HPLC and DNA sequence analyses unequivocally indicate that the alga is a member of the green algal lineage.

The phylogenetic analyses of 18S rRNA gene sequence data indicate that UTEX 2540 is most closely related to previously sequenced isolates identified as belonging to the genera *Geminella* or *Microspora*. UTEX 2540 cannot, though, be placed in *Microspora*: TEM images confirm that this strain does not possess H-shaped cell wall pieces that are characteristic of *Microspora* spp. (Figs 10-17). Instead UTEX 2540 is referred to *Geminella terricola*; this action, however, requires that the circumscription of *Geminella* be emended. *Geminella* was erected by Turpin (1828) and typified by *Geminella interrupta* Turpin. According to Turpin and subsequent authors, *Geminella* species are characterized as uniseriate, unbranched filaments with cells enclosed in a thick, mucilaginous sheath (John *et al.*, 2002). The cells are cylindrical, ellipsoidal or round and form loose rows, sometimes in pairs, or are otherwise positioned end to

end within the mucilaginous envelope. *Geminella* is, by some authors, termed filamentous (e.g., Smith 1950), but because daughter cells do not share any portions of their cell walls in common and in most instances are not truly in contact the alga is more accurately described as ‘pseudofilamentous’. In fact, only when cells are frequently dividing are adjacent cells observed to touch (Hindák, 1982). *Geminella* cells usually have one parietal plate-like chloroplast per cell containing a single pyrenoid and reproduces by fragmentation and/or the formation of thick brownish akinetes. UTEX 2540 differs from the above description of *Geminella* in that: (1) the predominant form in culture is that of coccoid unicells that (2) lack an apparent mucilaginous sheath. Despite these differences, the results presented here suggest that UTEX 2540 should be classified as *Geminella terricola* and that the generic concept for *Geminella* should be broadened to include unicellular organisms lacking a conspicuous extracellular mucilaginous sheath.

In a previous study, Hindák (1996a) transferred several species of *Geminella* and *Gloeotila* lacking visible pyrenoids and mucilaginous sheaths to *Stichococcus*. Our results provide no support allying *Stichococcus bacillaris* [the type of *Stichococcus*; Naegeli (1849)] with *Geminella* and our morphological evidence clearly indicates that “true” *Geminella* may be unicellular in form and lack pyrenoids and a sheath. For this reason, the species moved from *Geminella* to *Stichococcus* by Hindák (1996a) should be re-evaluated as should the limits of the generic concept for *Stichococcus*.

Phylogentic positions of *Geminella* and *Microspora* within green algae

Nuclear 18S rRNA gene sequences for two isolates, one identified as *Geminella* sp. and the other as *Microspora* sp., were included in our analyses. Our results show that *Geminella* sp. (AF387157) belongs to the charophyte genus *Klebsormidium*. The phylogentic position of

Microspora sp. (AF387160) could not be definitively determined, but it is clear that it is not a member of the *Geminella-Microspora* clade *sensu stricto*. These strains are misidentified and need not be discussed further here.

Our 18S rRNA gene sequence analyses indicate that *Geminella* and *Microspora* (*s.s.*) are sister taxa (Figs 18, 19). Both genera were once placed in the order Ulotrichales which includes unbranched green filaments (Hindak, 1996a, 1996b). Our results indicate that these genera do not belong in the Ulotrichales nor can they be placed in the class Ulvophyceae. Using TEM, Lockhorst and Star (1999) reconstructed the flagellar apparatus of the biflagellate zoospores of *Microspora quadrata*. They found that these cells possess unequal length flagella that originate subapically (below the papillar region), and that the orientation of the basal bodies is parallel. According to these authors, the flagellar apparatus of *M. quadrata* is most similar to species placed in the chlorophycean order Chlorococcales. However, the *Geminella-Microspora* clade is not definitively placed among the Chlorophyceae in any of the DNA sequence trees. In fact, it is not obvious to which of the five currently recognized classes these genera belong. *Geminella* and *Microspora* are excluded from the Ulvophyceae (as described above) and, on the basis of our phylogenetic analyses, from the Charophyceae as well (i.e., Charales, Desmidiiales, Zygnematales, et al.: McCourt *et al.*, 1995, 1996). These green algae are obviously not members of the Prasinophyceae, a class that includes motile, scale covered green monads possessing one or more flagella (Steinkotter *et al.*, 1994).

Thus, there are two remaining possibilities for taxonomic assignment of *Geminella* and *Microspora* at the rank of class, *viz.* the Chlorophyceae and Trebouxiophyceae. Unfortunately, these classes are resolved as polyphyletic in the 18S rRNA trees. The *Geminella-Microspora*

clade may represent a new lineage of green algae, but until further data are available we recommend treating these genera as *incertae sedis* within the Chlorophyta.

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