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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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; Bjøornland & 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 (Bjøornland & 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* Reisigl.

Heterotrichella was erected by Reisigl (1964) and includes only the type species, ***H. gracilis***, which was first isolated from a soil sample taken in Austria. According to Reisigl (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 (Ettyl, 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 (Reisigl, 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. Reisigl (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 Reisigl (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.

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 ß-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 unkown 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 λ= 440nm. Peak 1: violaxanthin; peak 2: lutein; peak 3: chlorophyll *b*; peak 4: chlorophyll *a*; and peak 5: β-carotene.

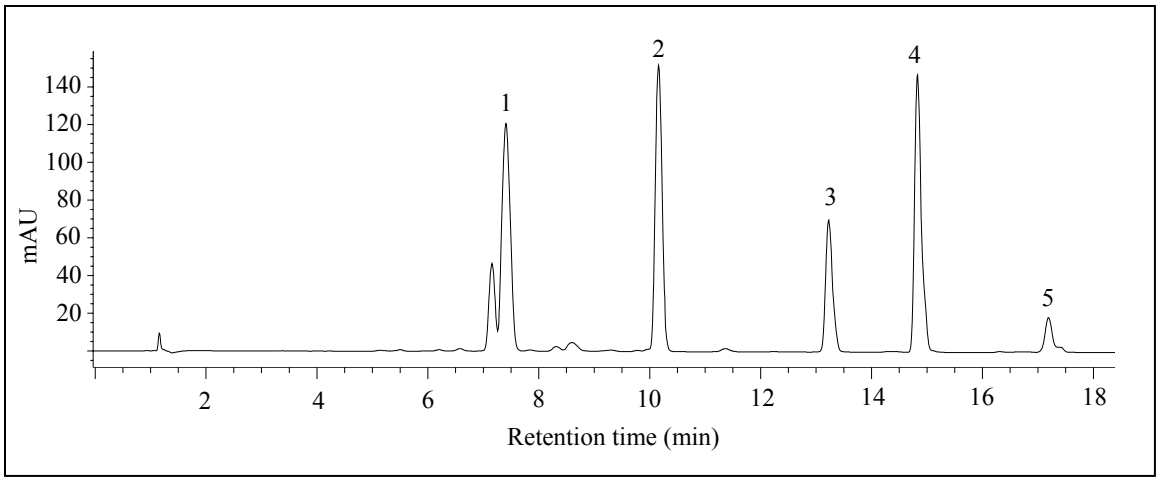
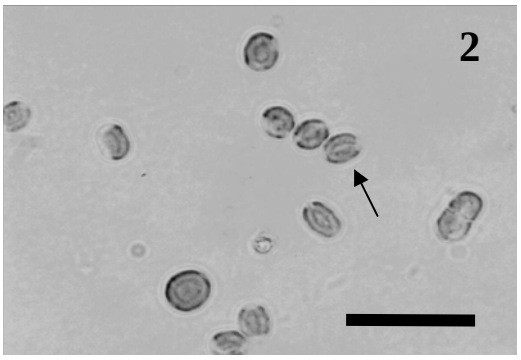

mAU
140
120
100
80
60
40
20
1
2
3
4
5
2
4
6
8
10
12
14
16
18
Retention time (min)

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 μm.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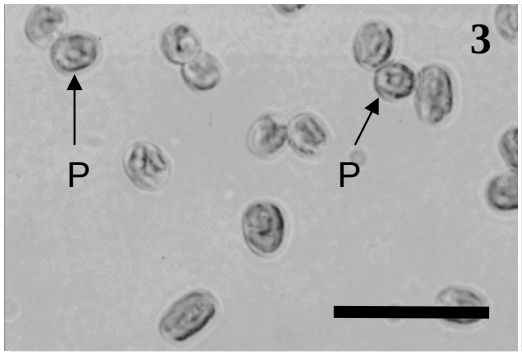
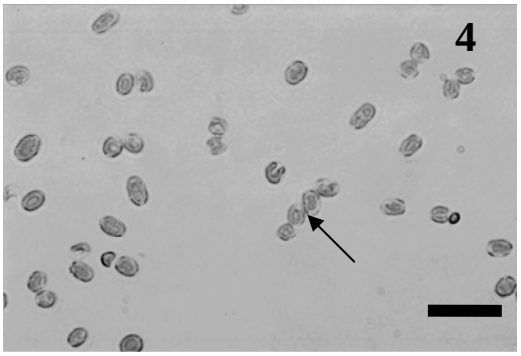

3
P
P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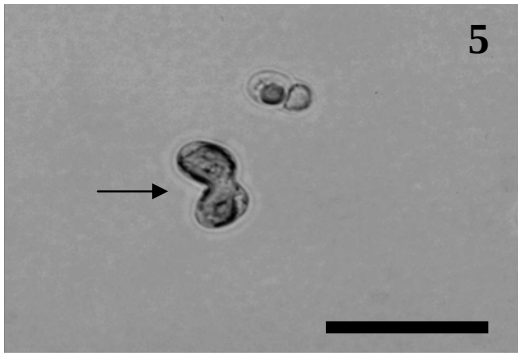
5

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).