J Appl Phycol (2016) 28:113–121
DOI 10.1007/s10811-015-0571-z

Chlorella sorokiniana (formerly *C. vulgaris*) UTEX 2714, a non-thermotolerant microalga useful for biotechnological applications and as a reference strain

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Received: 11 February 2015 / Revised: 17 March 2015 / Accepted: 18 March 2015 / Published online: 2 April 2015

Abstract Molecular analyses employing sequencing of the complete ribosomal RNA cistron (18S rDNA, ITS1, 5.8S rDNA, ITS2, and 28S rDNA) and transcriptome analysis of the RuBisCO gene (*rbc*L) were done on *Chlorella vulgaris* UTEX 2714. The constructed phylogenetic trees showed that *C. vulgaris* UTEX 2714 is *Chlorella sorokiniana*. Growth analysis and production of chlorophyll *a* over a range of increasing cultivation temperatures (27–40 °C) showed that this strain is far less thermotolerant in comparison to a common *C. sorokiniana* strain. A change in the taxonomic designation of strain UTEX 2714 is proposed.

Keywords *Chlorella* · Reference strains · Systematics

This study is dedicated to the memory of the German/Spanish mycorrhizae researcher Dr. Horst Vierheilig (1964–2011) of CSIC, Spain.

Electronic supplementary material The online version of this article (doi:10.1007/s10811-015-0571-z) contains supplementary material, which is available to authorized users.

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Introduction

A strain of unicellular green microalgae was isolated from a secondary effluent of a wastewater treatment stabilization pond in Bogota, Colombia, in 1994, having average temperatures of 19 °C during daylight and 7 °C at night (Gonzalez et al. 1997). It was then identified as *Chlorella vulgaris* by microscopic observations and biochemical tests, according to the descriptions of Chlorophyceae in the literature (Round 1970; Reisser 1984). This isolate was deposited in UTEX, the culture collection of algae at the University of Texas at Austin in 2000 under accession no. 2714 http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=5235 (accessed 18 Nov 2014).

With time, this strain gained worldwide use as a model for basic science studies (Gonzalez-Bashan et al. 2000; Gonzalez-Bashan et al. 2000; Lebsky et al. 2001; de-Bashan et al. 2002a; 2005; 2008a; b; 2011; Meyer et al. 2006; de-Bashan and Bashan 2008; Ozkan and Berberoglu 2011; 2013a; b; c; Dewan et al. 2012; Gerken et al. 2013; Myers et al. 2013; Kim et al. 2014a,b; Leyva et al. 2014, 2015; Meza et al. 2015) biotechnological applications (Valderrama et al. 2002; de-Bashan et al. 2004; Hernandez et al. 2006; 2009; de-Bashan and Bashan 2010; Chinnasamy et al. 2010b; Perez-Garcia et al. 2010; 2011a; b; Wang et al. 2010, 2013; Heredia-Arroyo et al. 2011; Holland et al. 2011; Choix et al. 2012a; b; 2014; Covarrubias et al. 2012; Wileman et al. 2012; Aguirre and Bassi 2013, 2014; Coats et al. 2013; Cruz et al. 2013; Yoo et al. 2013; Asmare et al. 2014; Girard et al. 2014; Gultom et al. 2014; Hasan et al. 2014; Ma et al. 2014; Passero et al. 2014), and agricultural and environmental studies (de-Bashan et al. 2012; Cortés-Jiménez et al. 2014) and as a reference strain to compare its performance with newly isolated strains (Van Benthem et al.

2008; Zhou et al. 2011, 2012; Zhang et al. 2014). Its usefulness includes theses for academic degree (Castellanos 2013) and publication in patents (Chinnasamy et al. 2010a; Maor 2010; Shamzi et al. 2011; Das et al. 2014). Many more publications, where this strain was used, are in popular publications, non-reviewed publications, and graduate theses.

Considering the importance of this strain and because of intensification of molecular studies regarding *Chlorella* spp. in general and specifically this strain, it was essential to determine its molecular identity in detail. Furthermore, in recent laboratory studies, strain *C. vulgaris* UTEX 2714 lacked amplification with several primers based on sequences of *C. vulgaris* C-27, *Chlorella variabilis* NC64A, and of *C. vulgaris* C-169, considered as model species for molecular studies on *Chlorella* species (data not published). Surprisingly in 2012, after genome sequencing, *C. vulgaris* C-169 was reclassified as *Coccomyxa subellipsoidea* C-169 (Blanc et al. 2012; Joint Genome Institute 2012). Similarly, other species of *Chlorella*-like microalgae were revised after analysis of the 18S rDNA and the internal transcribed spacer (ITS2) sequences (detailed information in "Discussion"). These developments demanded confirmation of the taxonomic status of our strain by advanced methods.

Different species of microalgae prefer different growth temperatures. While the average growth temperature of *C. vulgaris* is 28–32 °C (Sorokin and Krauss 1958; Maxwell et al. 1995; Huss et al. 1999; Wilson and Huner 2000; Chinnasamy et al. 2009; Lv et al. 2010), *C. sorokiniana* prefer temperatures from 36 to 42 °C (Kessler and Huss 1992; Huss et al. 1999; Morita et al. 2000a,b) and grow well at temperatures >40 °C after a short adaptation period (de-Bashan et al. 2008c). Strain UTEX 2714 is routinely cultivated in the lower temperature range (Gonzalez and Bashan 2000; Valderrama et al. 2002; Choix et al. 2014). Attempts to grow it at temperatures higher than 35 °C failed (Fendrich. 2005).

As a consequence of the accumulated conflicting information regarding this strain, we hypothesized that *C. vulgaris* UTEX 2714 is another species of microalgae. Consequently, the following goals of this study were to: (1) precisely identify the strain by molecular methods; (2) determine if the identity conforms to the phenotypic and physiological characteristics of the species; (3) correct the identity of this strain in the literature if proven different from *C. vulgaris*, and this was attempted by phylogenetic analyses of the complete 18S rRNA, ITS1, 5.8S rRNA, and ITS2 gene sequence, as well as the *rbc*L sequence obtained by transcriptome analysis, which was used as an additional independent molecular marker; and (4) compare the growth temperature preference for strains UTEX 2714 and *Chlorella sorokiniana* UTEX 2805, whose identity was previously determined by its 18S rRNA gene sequence (de-Bashan et al. 2008c).

Materials and methods

Microorganisms and initial growth conditions

Two *Chlorella* strains were used and compared as follows: *C. vulgaris* Beijerinck (UTEX 2714, University of Texas, Austin, TX, USA) and *C. sorokiniana* Shihhara et Krauss (UTEX 2805). The microalgae were cultured for up to 10 days in mineral growth media (C30; Gonzalez et al. 1997) at 120 rpm in an orbital shaker and 80 μmol photons $m^{-2} \cdot s^{-1}$. To prepare the inoculum, six incubation days were used and, for testing growth, the incubation period was adjusted to 5 or 10 days. Growth was measured for at four cultivation temperatures: 27, 30, 35, and 40 (±1 °C). Because both strains were highly susceptible to sudden exposure to 40±1 °C, adaptation periods were conducted during the first 75 min of each 24-h incubation period. These included a period of gradual increase in temperature from 30 to 35 °C for 30 min, 15 min at 35 °C, and a slow increase of the temperature from 35 to 40 °C for 30 min. Incubation at 40±1 °C lasted for 5 h and then the temperature was gradually returned to 30 °C for 17.75 h. Then, the process was repeated for the next incubation period (de-Bashan et al. 2008c). Cell number of both microalgal strains were counted under a light microscope with a Neubauer hemocytometer (Gonzalez-Bashan et al. 2000) connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, USA).

Identification of strain UTEX 2714

The 18S rDNA gene sequence and the complete ITS region were determined as follows: The microalgae were cultivated in C-30 medium for 10 days, harvested by centrifugation, and re-suspended in saline solution. Cell disruption was done by vortexing the microalgal suspension with glass beads for 10 min and then heating the sample at 80 °C for 5 min. DNA isolation of and purification was performed by using the Promega Wizard Genomic DNA Purification Kit (no. A1120). PCR primers are listed in Ma et al. (2013). The amplified products were analyzed on a 1.8 % (*w*/*v*) gel stained agarose (GelRed Biotium, USA) and visualized under UV light (Bio-Rad Laboratories, USA). The PCR products were purified (QIAquick PCR purification kit, Qiagen, Germany) and cloned (Zero Blunt Topo PCR Cloning Vector Kit, Invitrogen, USA). The insert nucleotide sequences were determined with the universal M13 forward and reverse primers and with appropriate internal sequencing primers (Huss et al. 1999) by GATC Biotech (Germany).

The sequence was manually aligned, using the conserved secondary structure of several *Chlorella* and *Chlorella*-related reference strains for comparison and identification. *Closteriopsis acicularis* and *Parachlorella kessleri* were used as outgroups. The phylogenetic analyses were essentially done, as described by Ma et al. (2013). For the final decision

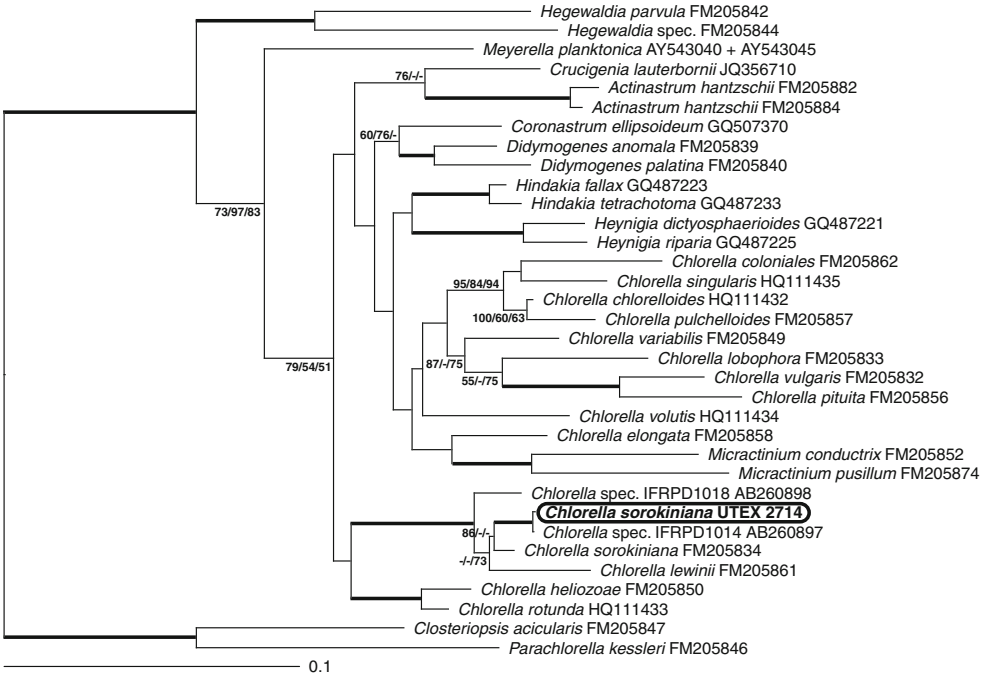

Fig. 1 Phylogenetic tree of 18S+ ITS sequence data of *Chlorella sorokiniana* UTEX 2714 and representatives of the Chlorellales. *Closteriopsis acicularis* and *Parachlorella kessleri* were used as outgroups. GenBank accession numbers are indicated after the taxon names. Tree topology was obtained by a maximum likelihood (ML) analysis, which was mostly consistent with trees calculated by maximum parsimony (MP) and neighbor joining (NJ). The *numbers at nodes* indicate bootstrap support of 1000 replicates for NJ/MP/ML. Statistical support of more than 90 % for all three methods is indicated by a *thick line*. Bootstrap values lower than 50 % are not shown

of species identity, the ITS2 secondary structure was modeled and checked for full (CBC) and hemi-compensatory base changes (hCBC), as described in Ma et al. (2013).

For the transcriptome analysis, cells at log growth phase were harvested and re-suspended in 100 μL of 0.85 % saline solution and processed immediately. Each sample contained 5 mL algae suspension (a mix from eight individual samples). The microalgal suspension was mixed with glass beads, frozen in liquid nitrogen, and pulverized by mortar and pestle. RNA isolation was performed by the Tri method (Fluka Sigma-Aldrich, Switzerland). Total RNA was pooled into two samples for further purification (RNeasy Mini Kit, no. 74104, Qiagen). cDNA synthesis and sequencing of the two samples were performed in an Illumina MiSeq platform at the Yale Center of Genome Analysis (USA). Paired-end reads were assembled by the Trinity RNA-seq assembler.

Nucleotide sequences for *rbc*L were obtained by local BLAST (*e* value $\leq 1 \times 10^{-6}$) of the transcriptome against three acknowledged sequences of *Chlorella* species (*C. vulgaris*, *C. sorokiniana*, and *C. variabilis*). The complete gene was extracted from the transcriptome for phylogeny study, along with 11 representative sequences of *Chlorella*-related species obtained from the NCBI database. *P. kessleri* and *Dicloster acuatus* were used as outgroups. Multiple sequence alignment was performed with MUSCLE, allowing codon positions (Edgar 2004) in the MEGA 6 software (Tamura et al. 2013) and analyzed for phylogeny by the same program, using nucleotide substitution models (Verbruggen and Theriot 2008). Phylogenetic trees were inferred by maximum likelihood and neighbor joining methods with 1000 bootstrap replications for each method. Maximum likelihood was modeled with the general time-reversible model (GTR+G+I) that was selected from among 24 models by the AICc value (Akaike information criterion, corrected). This model used discrete gamma distribution (+G) (five categories, parameter=0.8933) and assumed that a certain fraction of sites are evolutionarily invariable (+I) (55.9071 % sites). The neighbor joining method is based on the Kimura two-parameter method (Kimura 1980) and gamma distribution (shape parameter=1).

Determination of chlorophyll *a*

To determine the quantity of chlorophyll *a*, extraction was according to Youngman (1978) with small modifications,

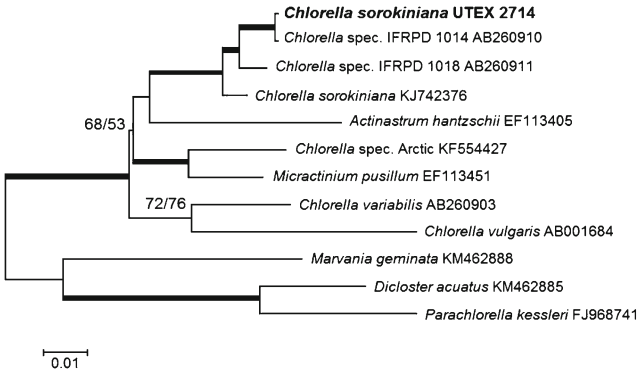

Fig. 2 Phylogeny of *Chlorella sorokiniana* UTEX 2714 based on *rbc*L transcript. Tree topology was obtained by a maximum likelihood (ML) analysis and was fully consistent with a neighbor joining (NJ) tree. *Parachlorella kessleri* and *Dicloster acuatus* were used as outgroup. The *numbers at nodes* indicate bootstrap support of 1000 replicates for NJ/ML. Bootstrap support ≥90 % is indicated in *bold branches*. Bootstrap values lower than 50 % are not shown

where 5 mL of freshly harvested culture were centrifuged for 10 min (6000×*g*). The supernatant was discarded and 5 mL 90 % methanol were added to the pellet and heated in a water bath for 10 min at 60 °C. After cooling, the samples were incubated in the dark for 24 h at 4 °C. Then, the samples were centrifuged for 10 min (4 °C; 6000×*g*) and absorbance was recorded in the supernatants at 655 and 750 nm. Quantification of chlorophyll *a* used the following equation: Chlorophyll *a* ($mg \cdot L^{-1}$)=13.9 ($OD_{655}-OD_{750}$) U/V, where *U*=final volume of methanol and *V*=volume of the sample.

Statistical analysis

For each microalgal strain, three 250-mL Erlenmeyer flasks containing 100 mL SGM were used, where each flask served as a replicate. Each experiment was repeated twice and average data of both trials were used for statistical analysis. Statistical analysis used Student's *t* test at $p<0.05$ (comparisons between strains in the same sampling time) or one-way ANOVA and LSD post hoc analysis at $p< 0.05$ (comparisons of growth of each strain with time). Linear regression models were calculated to compare growth and chlorophyll *a* content for each strain and for each growth temperature. All analyses used Statistica 8.0 software (StatSoft, USA).

Results

Identification of strain UTEX 2714

The complete 18S rDNA+ITS sequence of strain UTEX 2714 was submitted to the European Nucleotide Archive under accession number LK021940.

The phylogenetic tree of strain UTEX 2714 is presented in Fig. 1. The tree includes at least one representative of each acknowledged *Chlorella* species (Bock et al. 2011) and *Chlorella*-related genera. Strain UTEX 2714 is most closely related to *C. sorokiniana* (as a described species), but it is still closer to an undescribed strain designated as *Chlorella* sp. IFRPD 1018 (Hoshina and Imamura 2008). There is just one different position in the complete 18S+ITS sequence (namely in ITS1). In the 18S rDNA sequence, strain UTEX 2714 has a single different position compared with *C. sorokiniana* and *Chlorella lewinii* C. Bock, Krienitz and Pröschold. Therefore, the ITS sequence is indispensible for resolution at the species level (Bock et al. 2011). Based on the secondary structure-supported alignment of the ITS region, there are several differences between strain UTEX 2714 and the type strain of *C. sorokiniana* (23 in ITS1 and 52 in ITS2, mainly in length variation in variable regions). However, not a single CBC

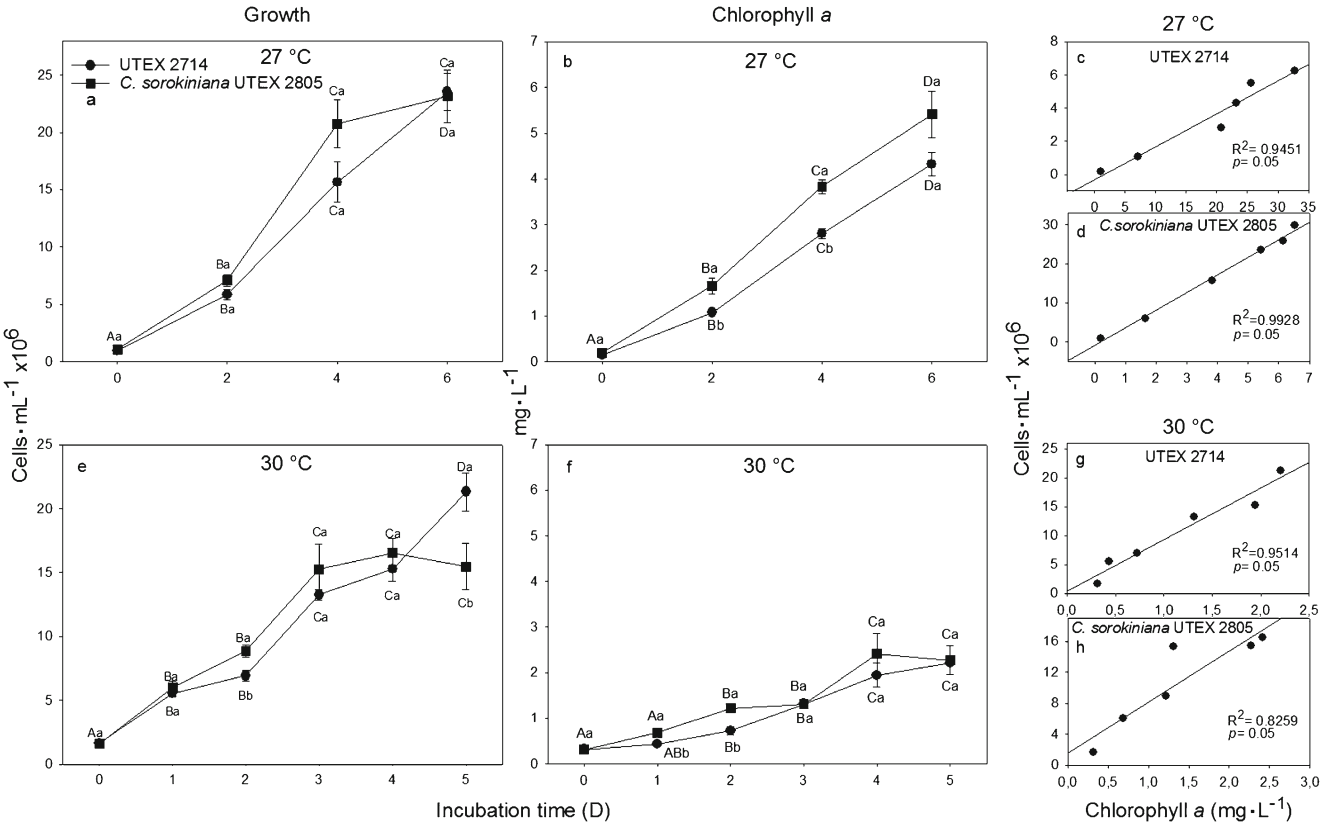

Fig. 3 Growth (**a**, **e**), chlorophyll *a* content (**b**, **f**) and linear correlation between growth and chlorophyll *a* content (**c**, **d**, **g**, **h**) of *C. sorokiniana* UTEX 2805 and strain UTEX 2714 at temperatures of 27 and 30 °C. Values of comparisons of growth of each strain with time denoted by *different capital letters* differ significantly by one-way ANOVA and LSD post hoc analysis at $p< 0.05$. Comparisons between strains in the same sampling time denoted by *different lower case letters* differ significantly by Student's *t* test at $p<0.05$

(compensating base change) or hCBC (hemi-compensating base change) was found in the conserved parts of the four helices of ITS2, which are used to define species delimitation (Coleman 2007). In contrast, one hCBC in helix 3 and four hCBCs and one CBC in helix 4 were found, compared to *C. lewinii*. Still, more CBCs distinguish strain UTEX 2714 from other *Chlorella* species.

Phylogeny on the *rbc*L gene is shown in Fig. 2. Based on the limited number of complete sequences available, we determined that UTEX 2714 is clearly not *C. vulgaris*, but most closely related to *C. sorokiniana* and two undescribed strains designated as *Chlorella* spec. IFRPD 1014 and *Chlorella* spec. IFRPD 1018 (Hoshina and Imamura 2008). Based on these data and the rDNA analyses, strain UTEX 2714 can unambiguously be assigned to *C. sorokiniana*.

Growth and chlorophyll *a* content of strain UTEX 2714 and *C. sorokiniana* UTEX 2805 at increasing temperatures

Culturing *C. sorokiniana* and strain UTEX 2714 with increasing temperatures from 27 to 40 °C yielded decrease in growth, although both strains can grow at each temperature (Figs. 3a, e and 4a, e). Apart from similar growth at the optimal temperature of 27 °C, *C. sorokiniana* UTEX 2805 always grew significantly better than strain UTEX 2714 (Figs. 3a, e and 4a, e; lower case analysis). Strain UTEX 2805 grew similarly at 27 and 40 °C, while significant reduction in growth occurred in strain UTEX 2714 at 40 °C (compare Figs. 3a and 4e). The same pattern was obtained when chlorophyll *a* was recorded (Figs. 3b, f and 4b, f). A direct linear correlation was found between growth and chlorophyll *a* content in both strains at each temperature (Figs. 3c, d, g, h and 4c, d, g, h).

To confirm that strain UTEX 2714 is less thermotolerant than UTEX 2805, an additional experiment was conducted for 10 days at 40 °C (twice the time of the other experiments). During this extended incubation, UTEX 2805 continued to grow exponentially, while growth and chlorophyll *a* production of UTEX 2714 was inhibited, but not completely stopped (Fig. S1, supplemental material).

Discussion

Precise identification of a microbial strain is crucial for culture collections. Well-defined strains allow other researchers to

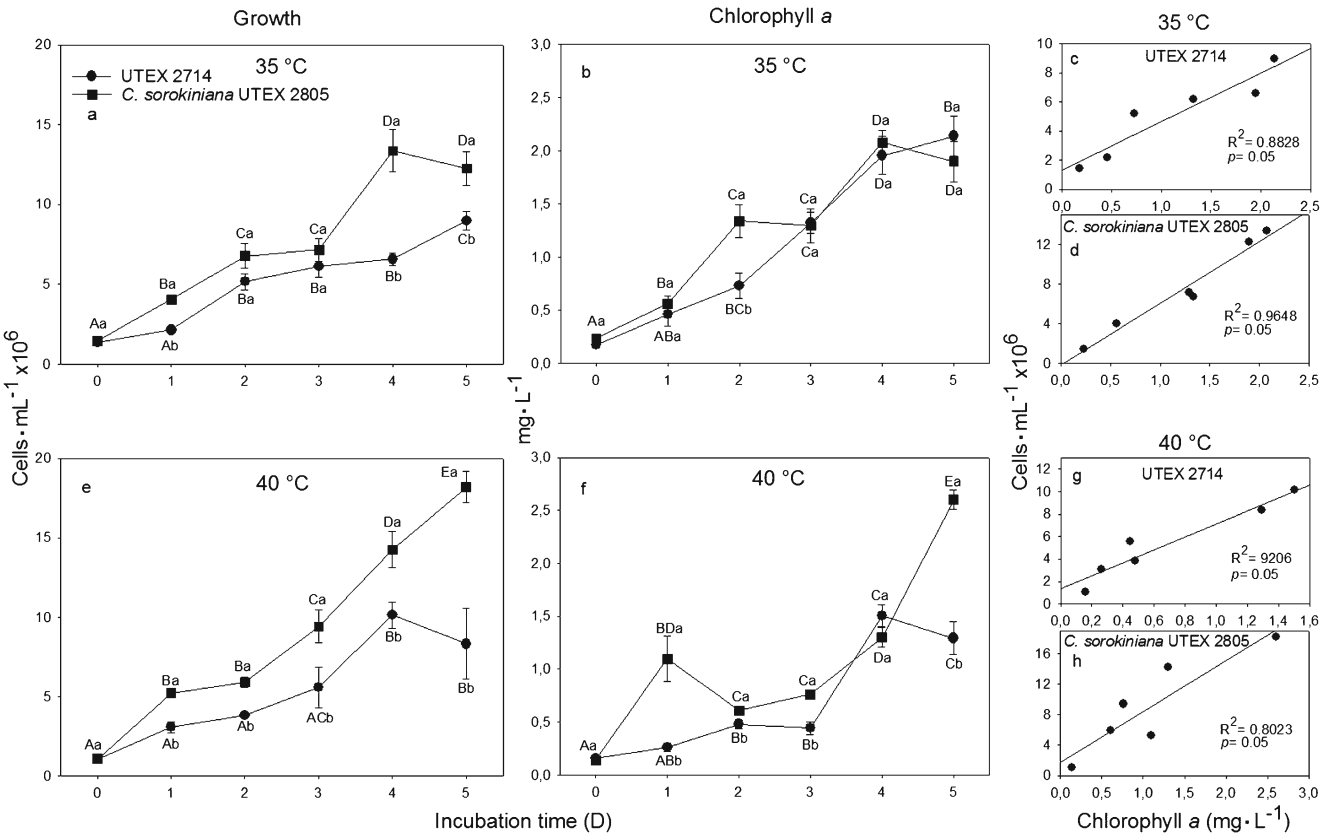

Fig. 4 Growth (**a**, **e**), chlorophyll *a* content (**b**, **f**) and linear correlation between growth and chlorophyll *a* content (**c**, **d**, **g**, **h**) of *C. sorokiniana* UTEX 2805 and strain UTEX 2714 at temperatures of 35 and 40 °C. Values of comparisons of growth of each strain with time denoted by *different capital letters* differ significantly by one-way ANOVA and LSD post hoc analysis at $p < 0.05$. Comparisons between strains in the same sampling time denoted by *different lower case letters* differ significantly by Student's *t* test at $p < 0.05$

repeat results done far from their laboratory. The genus *Chlorella* (Trebouxiophyceae, Chlorophyta) is one of the most important commercial microalgae and perhaps the most studied (Krienitz et al. 2015). Since the description of the type species *C. vulgaris* by the Dutch microbiologist Beijerinck (1890), more than a hundred species of *Chlorella* have been isolated from freshwater, marine, and terrestrial habitats, and as symbionts from lichens, protozoa, and invertebrates (Krienitz et al. 2004). Their assignment to *Chlorella* was done primarily on the basis of morphological similarities and biochemical tests (Shihira and Krauss 1965; Pröschold and Leliaert 2007). The challenge in the past was how to distinguish species when high phylogenetic diversity occurred, combined with a limited amount of morphological characters. Based on morphology and biochemical characteristics, UTEX 2714 was originally identified as *C. vulgaris* and deposited in the UTEX alga culture collection. During the last four decades, it was shown that *Chlorella* includes species of similar morphology but of polyphyletic origin (Huss et al. 1999; Darienko et al. 2010; Luo et al. 2010; Krienitz et al. 2015). Consequently, a revision of the existing systematics of *Chlorella* was needed and proposed by Bock et al. (2011). Currently, the genus *Chlorella* contains 48 species and varieties, including the type species *C. vulgaris* (Guiry and Guiry 2015).

We applied a polyphasic approach to identify and characterize strain UTEX 2714 more precisely. This included complete sequencing of the 18S+ITS rDNA transcriptome analysis of a major enzyme in microalgae (RuBisCO) and growth pattern and chlorophyll *a* content at a variety of temperatures. For comparison, we used the thermotolerant *C. sorokiniana* strain UTEX 2805, whose identity was determined by 18S rDNA analysis (de-Bashan et al. 2008c) and has proven to be useful for several biotechnological applications (Hunt et al. 2010; Trejo et al. 2012). Transcriptome data, in combination with other high-throughput genomic data, are now more frequently used to elucidate phylogenetic questions, especially phylogenies of understudied groups (Lemmon and Lemmon 2013; Stefanik et al. 2014). The comparison to *C. sorokiniana* was chosen for two reasons: (1) a study on anaerobic digested effluent from cattle manure referred to strain UTEX 2714 as *C. sorokiniana*, despite its official designation by the alga culture collection as *C. vulgaris*, but without explanation (Kobayashi et al. 2013), and (2) in a study on lipid accumulation in *Chlorella* species, it is stated that *C. vulgaris* UTEX 2714 is a mismatched species (Rosenberg et al. 2014). Yet, no further analysis was done on this strain and its classification has not been changed in the alga culture collection.

Our study indicates that strain UTEX 2714 is *C. sorokiniana*. The 18S+ITS sequence (as well as the *rbc*L sequence) was most closely related to *Chlorella* sp. IFRPD 1018 (Hoshina and Imamura 2008). These authors claim that there is one CBC in helix 2 of ITS2 between IFRPD 1018 and *C. sorokiniana*, and therefore, IFRPD 1018 should be a different species. However, this CBC is not in the conserved part of helix 2 and cannot be unequivocally aligned. Even if transcriptome data are not intended for phylogenetic purposes, our phylo-transcriptomic analysis constituted a useful tool to support the taxonomic designation of *C. vulgaris* UTEX 2714 as *C. sorokiniana* UTEX 2714.

Many *C. sorokiniana* strains are well known to grow at high temperatures of 40 °C or higher (Sakai et al. 1995; Huss et al. 1999; Morita et al. 2000a,b; de-Bashan et al. 2008c; Wan et al. 2012; Li et al. 2013; Zheng et al. 2013). Our growth data and chlorophyll *a* content over an increasing range of temperatures indicated that both strains (UTEX 2714 and UTEX 2805) were negatively affected by higher temperatures (Figs. 3 and 4). Yet, growth and chlorophyll *a* content of *C. sorokiniana* UTEX 2714 were significantly less at high temperatures, compared to *C. sorokiniana* UTEX 2805. Therefore, strain UTEX 2714 provides a unique opportunity in comparative studies of thermotolerant strains of *C. sorokiniana*.

In conclusion, molecular analyses and temperature-dependent growth performance indicate that *C. vulgaris* UTEX 2714 is a non-thermotolerant strain of *C. sorokiniana*. We recommend that the UTEX alga culture collections change the designation of this strain.

Acknowledgments We thank Raul Llera-Herrera at the Research Center for Food and Development (CIAD, Mazatlan, Mexico) for assembling the transcriptome. At CIBNOR, we thank Paulina Adams and Juan-Pablo Hernandez for technical assistance and microalgae cultivation and Ira Fogel for providing English editing and editorial suggestions. This study was supported by Consejo Nacional de Ciencia y Tecnologia of Mexico (CONACYT-Basic Science-2009, contracts 130656 and 164548) and time for writing by The Bashan Foundation, USA. This is contribution 2015–001 from the Bashan Institute of Science, USA.

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