

Ultrastructural and molecular characterization of *Tetraselmis* strains (Chlorodendrophyceae, Chlorophyta) isolated from Chile

Caracterización ultraestructural y molecular de cepas de *Tetraselmis* (Chlorodendrophyceae, Chlorophyta) aisladas de Chile

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ABSTRACT

Species of the genus *Tetraselmis* (Chlorodendrophyceae, Chlorophyta) have traditionally been used in aquaculture facilities as food for larval varieties of marine organisms. Recently, some strains with high lipid content have been considered as having potential for biofuel production. Although many *Tetraselmis* species are relatively well-characterized using light and electron microscopical methods, there are still taxonomic ambiguities among the species. DNA sequence analysis, which provides a reliable and more convenient tool for species delimitation, has been poorly used in *Tetraselmis*. In this study, three strains of *Tetraselmis*, isolated from coastal Chilean waters, have been characterized using an integrative approach (light and electron microscopy, molecular phylogeny of ITS, including secondary structure analysis). According to the pyrenoid ultrastructure of the strains investigated two of them (from Dichato and Coliumo) belong to the subgenus *Parviselmis*, while the Caldera strain corresponds to the subgenus *Tetrathele*. Even though it was not possible to identify the strains at species level, it was clearly demonstrated by phylogenetic analyses of ITS-1 and ITS-2 sequences and by ITS-2 secondary structure that the Dichato and Coliumo strains are the same species, and could belong either to *T. suecica* or *T. chui*. However, the Caldera strain might correspond to an undescribed species. As our study has shown, the genus *Tetraselmis* needs to be taxonomically revised using an integrative approach, which includes the investigations of authentic strains of this genus.

KEYWORDS: Cell ultrastructure, ITS region sequences, ITS-2 secondary structure, *Tetraselmis* strains.

RESUMEN

Especies del género *Tetraselmis* (Chlorodendrophyceae, Chlorophyta) han sido tradicionalmente usadas en centros acuícolas como alimento para larvas de una variedad de organismos marinos. Adicionalmente, algunas cepas con alto contenido de lípidos han sido recientemente consideradas como fuente potencial para la producción de biodiesel. Aunque muchas especies de *Tetraselmis* se encuentran morfológica y ultraestructuralmente bien caracterizadas, todavía hay ambigüedades taxonómicas al interior del género. El análisis de secuencias de ADN, una herramienta complementaria y conveniente para delimitar especies, ha sido pobremente utilizado en *Tetraselmis*. En este estudio, se caracterizaron tres cepas de *Tetraselmis*, aisladas de aguas costeras chilenas, utilizando un enfoque integrativo (morfológica, ultraestructura y filogenia molecular). Basado en la ultraestructura del pirenoide de las cepas estudiadas, dos de ellas (Dichato y Coliumo) pertenecen al subgénero *Parviselmis*, mientras que la cepa de Caldera corresponde al subgénero *Tetrathele*. Aunque no fue posible identificar las cepas a nivel específico, el análisis filogenético de las secuencias ITS-1 e ITS-2 y la estructura secundaria del ITS-2 demostraron claramente que las cepas de Dichato y Coliumo son la misma especie, y podrían pertenecer a *T. suecica* o a *T. chui*, mientras que la cepa de Caldera podría corresponder a una especie no descrita aún para la ciencia. Los resultados obtenidos demuestran la necesidad de llevar a cabo una revisión taxonómica del género *Tetraselmis*, utilizando un enfoque integrativo, el cual incluya las cepas auténticas de las especies.

PALABRAS CLAVE: Cepas de *Tetraselmis*, estructura secundaria del ITS-2, secuencias de la región ITS, ultraestructura celular.

INTRODUCTION

The genus *Tetraselmis* Stein (Chlorodendrophyceae, Chlorophyta) groups quadriflagellates unicellular organisms, widely recognized in aquaculture since they serve as food for a variety of marine organisms (DSouza & Kelly 2000, Brown 2002, Meseck *et al.* 2005, De la Pena & Villegas 2005). More recently, high lipid-containing strains of this genus have

been proposed for biofuel production (Montero *et al.* 2010, Grierson *et al.* 2012).

In *Tetraselmis*, the cells are usually compressed and the four flagella are located in opposite pairs. They are thick of equal length, shorter than the length of the cell, covered by hairs and scales, and are inserted into an apical cell depression. The flagellar apparatus of *Tetraselmis* consists of four zig-zag arranged basal bodies associated with microtubular roots

and two distinct rhizoplasts. Usually, two dictyosomes are restricted to the region surrounding the basal body complex. The cell is surrounded by a close-fitting periplast of fused scales. A single large chloroplast is present in each cell. The chloroplast is anteriorly lobed, and the posterior part may or may not be lobed, with one pyrenoid and with or without an eyespot. Cells reproduce asexually by simple division within the mother cell commonly forming two daughter cells usually in an inverted position from each other. Sexual reproduction is unknown (Graham & Wilcox 2000).

The genus is divided into four subgenera (*Tetraselmis*, *Prasinocladus*, *Tetrathele* and *Parviselmis*) containing 25 species (Hori *et al.* 1982). The identification of the subgenera depends on characteristics that are visible only with an electron microscope (EM), specially the pyrenoid ultrastructure (Hori *et al.* 1982, 1983, 1986). The described species differ mostly in cell size, cell shape, chloroplast morphology and the presence/absence and position of the eyespot. Nevertheless, most of these features are not good descriptors, so the taxonomy within the genus is confusing. Ultrastructural features such as flagellar scales and flagellar hairs had been studied by Melkonian (1982), Becker *et al.* (1990, 1994) and Marin *et al.* (1993), but no intraspecific variation of flagellar scales has been detected in *Tetraselmis*. However, Marin *et al.* (1993) found some intraspecific variation in the flagellar hairs and proposed that this feature may, to some extent, help to clarify the confused taxonomy within the genus when correlated with other ultrastructural and molecular characters.

Recent phylogenetic studies based on SSU rDNA sequences showed that the genus *Tetraselmis* is monophyletic (Nasare 2002, Lee & Hur 2009, Arora *et al.* 2013). However, it is striking that very little is known about molecular studies of *Tetraselmis* at species level.

In Chile, only one record of *Tetraselmis suecica* (Kyllin) Butcher and *Tetraselmis tetrathele* (West GS) Butcher exist for the coast of Valparaíso (Catalán 1996), but their identification was based mostly on features observed under light microscopy that, considering the current information, raise some doubt about its taxonomic identity. The aim of this study was to characterize three new Chilean strains of *Tetraselmis* isolated from Dichato and Coliumo (Bio-Bio Region) and Caldera (Atacama Region) using morphological (light microscopy), ultrastructural (transmission and scanning EM) and molecular markers (ITS region sequences and ITS-2 secondary structure) in order to get a better understanding of the distribution of *Tetraselmis* in Chile. We used the Internal Transcribed Spacers of the rDNA because it is more variable in sequence than the SSU rDNA gene. In addition, the secondary structure of the ITS-2 for detection of complementary base changes (CBC) has often been taken into account when distinguishing between closely related species (Müller *et al.* 2007). Studies have shown that one CBC in a conserved region of the Helix II or III of the ITS-2 is in most cases associated to an inability for sexual reproduction (Coleman 2007, 2009).

MATERIALS AND METHODS

STRAINS COLLECTION AND ISOLATION

The strains were collected from water samples using the filtrate of a nylon mesh (33 µm diameter). The Coliumo and Caldera strains came from waters close to natural populations of the clam digger *Ensis macha* Molina, located in Coliumo Bay (36°32' S; 72°57' W), and of *Argopecten purpuratus* Lamarck in Caldera Bay (27°05' S; 70°82' W). The Dichato strain was collected from a tank located outside the hatchery of the University of Concepción (36° 33'S; 72° 56'W). Unialgal cultures of the three strains were obtained by either using the Pasteur pipette isolation and washing technique or streaking a drop sample on agar plates (Hoshaw & Rosowski 1973) with f/2 medium (Andersen 2005). Isolated cells were maintained in Walne medium (Andersen 2005). The strains were cultivated at a photon flux density of 15 – 20 µmol m⁻² s⁻¹, a temperature of 15 ±2°C and a photoperiod 16:8 (L:D) and maintained in the Microalgal Culture Collection at the University of Concepción (CCM-UDEC) (Concepcion, Chile) (see Table I).

STRAINS OBSERVATION UNDER LIGHT AND ELECTRON MICROSCOPY

Live cells were examined under an Olympus CX31 microscope fitted with bright field and phase contrast optics. The photomicrographs were taken with an Olympus C3040 camera attached to the microscope.

For Transmission Electron Microscopy (TEM), culture material of the strains was prepared according to the protocol of Moestrup & Thronsen (1988). Basically, the cells were harvested at 1000 rpm and fixed for one hour in equal volumes of sample with cold 2% (v/v) glutaraldehyde in 0.1M cacodylate buffer at pH 7. To avoid osmotic changes in ultrastructure of the cells, 0.2 M sucrose was added. After three rinses in buffer of decreasing sucrose concentration (0.2, 0.1 and 0.05M), the cells were treated for 3 h. in cold 2% osmium acid in 0.1M cacodylate buffer, followed by a wash in sucrose buffer and dehydrated in ethanol (30%, 50%, 70%, and twice in absolute ethanol) for 15 min each. Dehydration was completed by two 5-min treatments in propylene oxide and kept overnight in a mix of 1:1 propylene oxide and Spurr's resin. Finally, the samples were polymerized in Spurr's resin at 60°C. Ultrathin sections were obtained using a SORVALL MT 5000 (DU PONT) ultramicrotome, collected on slot grids and double-stained with uranyl acetate and lead citrate (Reynolds 1963). Observations were carried out with a JEOL JEM 1200EX-II transmission electron microscope. The photomicrographs were taken with a camera (Gatan Mod. 782, Gatan Inc. USA) attached to the microscope.

For Scanning Electron Microscopy (SEM), culture material of the strains was prepared following the protocol of Laza-Martínez (2012) with modifications. Ten ml of the algal suspension was centrifuged for 10 min at 1000 rpm and the pellet fixed in Lugol's solution overnight. Then the

TABLE I. List of the strains used in this study (*authentic strain).

TABLA I. Lista de las cepas utilizadas en este estudio (*cepa auténtica).

STRAIN NUMBER	SPECIES	ORIGIN	ACCESSION NUMBER	REFERENCE
WDCM NCC62	<i>T. suecica</i>	France	AY574382	Joubert <i>et al.</i> 2002 (Unpublished)
CCAP 8/6*	<i>T. chui</i>	Scotland, Millport, Clyde estuary, marine	-	Nasare 2002
CCAP 66/21B	<i>T. chui</i>	England, Yorkshire, marine.	-	Nasare 2002
CCM-UDEC 114	<i>Tetraselmis sp.</i>	Chile, coastal water at Coliumo Bay, Coliumo.	KF250348	this study
CCM-UDEC 109	<i>Tetraselmis sp.</i>	Chile, tank at University of Concepción hatchery, Dichato.	KF 250349	this study
CCM-UDEC 134	<i>Tetraselmis sp.</i>	Chile, coastal water at Caldera Bay, Caldera.	KF250350	this study
SAG 41.85*	<i>T. striata</i>	UK. N-Wales, oyster rearing tanks at Conway, marine.	HE610129	Marin 2012
SAG 17.86	<i>Scherffelia dubia</i>	UK, Essex, pond in Epping Forest.	HE610128	Marin 2012

cells were centrifuged as above and the pellet resuspended in Walne medium to continue with the dehydration in a series of increasing concentrations of ethanol (10%, 30%, 50%, 70%, 90%, 95%, 98% and 100%) for 15 min each. Afterward the cells were dried using the critical-point method with CO₂, then covered with gold, and observed with a JEOL JSM 6380LV scanning electron microscope.

MOLECULAR ANALYSIS

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total DNA was extracted according to the methodology described by Gómez & González (2001). The ITS region was amplified with the primers AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') and TW81 (5'-GTTTCCGTAGGTGAACCTGC-3'), as described in Goff *et al.* (1994). Purification of the amplified fragments was done using the purification kit "Wizard SV and PCR Clean-Up System" (PROMEGA, USA). The purified fragments were cloned using the kit PGEM-T Easy Vector kit PROMEGA. Plasmids of the cloned sequences were sent to MACROGEN (Korea) for automated sequencing.

ALIGNMENT AND PHYLOGENETIC ANALYSIS

The consensus sequences of the ITS region (ITS-1, 5.8S, ITS-2) obtained from the CLC Main Workbench program version 6.8.3 (CLC Bio, Denmark), were aligned using the Clustal W software (<http://www.genome.jp/tools/clustalw/>) and manually adjusted according to the secondary structure of both spacers (ITS-1 and ITS-2) (based on Coleman 2003, Mai & Coleman 1997). In this alignment, six further sequences were included, which were obtained from the GenBank (see Table I for details). The sister taxon of *Tetraselmis*, *Scherffelia dubia* was chosen as outgroup.

The resulting data of 9 strains with 488 aligned bases positions was used for the phylogenetic analyses. jModeltest 2 (Darriba *et al.* 2012) was used to determine the model of sequence evolution that fits best for the data set according to the Bayesian information criterion (BIC). Each spacer and the

5.8S gene were evaluated separately; in this case, a partitioning model was used. The best models for ITS-1, ITS-2 and 5.8S were K80, K80+G and JC, respectively. Bayesian phylogenetic analyses were conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), with 30 million generations Markov Chain Monte Carlo (MCMC) sampled every 1000 generations.

ITS-2 SECONDARY STRUCTURE

The helices II and III from ITS-2 rDNA secondary structure of all the strains examined were obtained by using the program mfold (Zuker 2003, <http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>), and the compensatory base pair changes (CBCs) have been manually identified.

RESULTS

Under light microscopy the cells of the three strains are similar in shape: slightly compressed, elliptical to ovate when viewed from the broad side. However, cells of the Coliumo strain when viewed from the narrow side are narrower at the base than the Dichato strain. On the other hand, cells from Caldera strain exhibited a slight torsion at the base when viewed from the same side. The sizes of the cells are different among the strains: Dichato strain is the smallest (10-12.5 µm length x 5.8-8.0 µm wide; Fig.1 A-C) while "Caldera" is the biggest (15-18.8 µm length, mainly 17.5 µm length x 8.5 – 12.5 µm wide; Fig.1 G-I). The strain from Coliumo exhibits intermediate sizes (12.5 - 15 µm length; mainly 15 µm length x 6.5 to 9.5 µm wide; Fig.1 D-F). Another feature clearly visible using light microscopy was the pyrenoid position within the chloroplast: mostly basal in the Dichato strain and sub-basal to sub-central in the Coliumo and Caldera strains (Fig. 1). The three strains have one eyespot with variable position within the chloroplast. Asexual reproduction was observed in the three strains; thus two daughter cells within the mother theca were seen in the strains from Dichato and Coliumo (Fig. 1 A, F), while the strain from "Caldera"

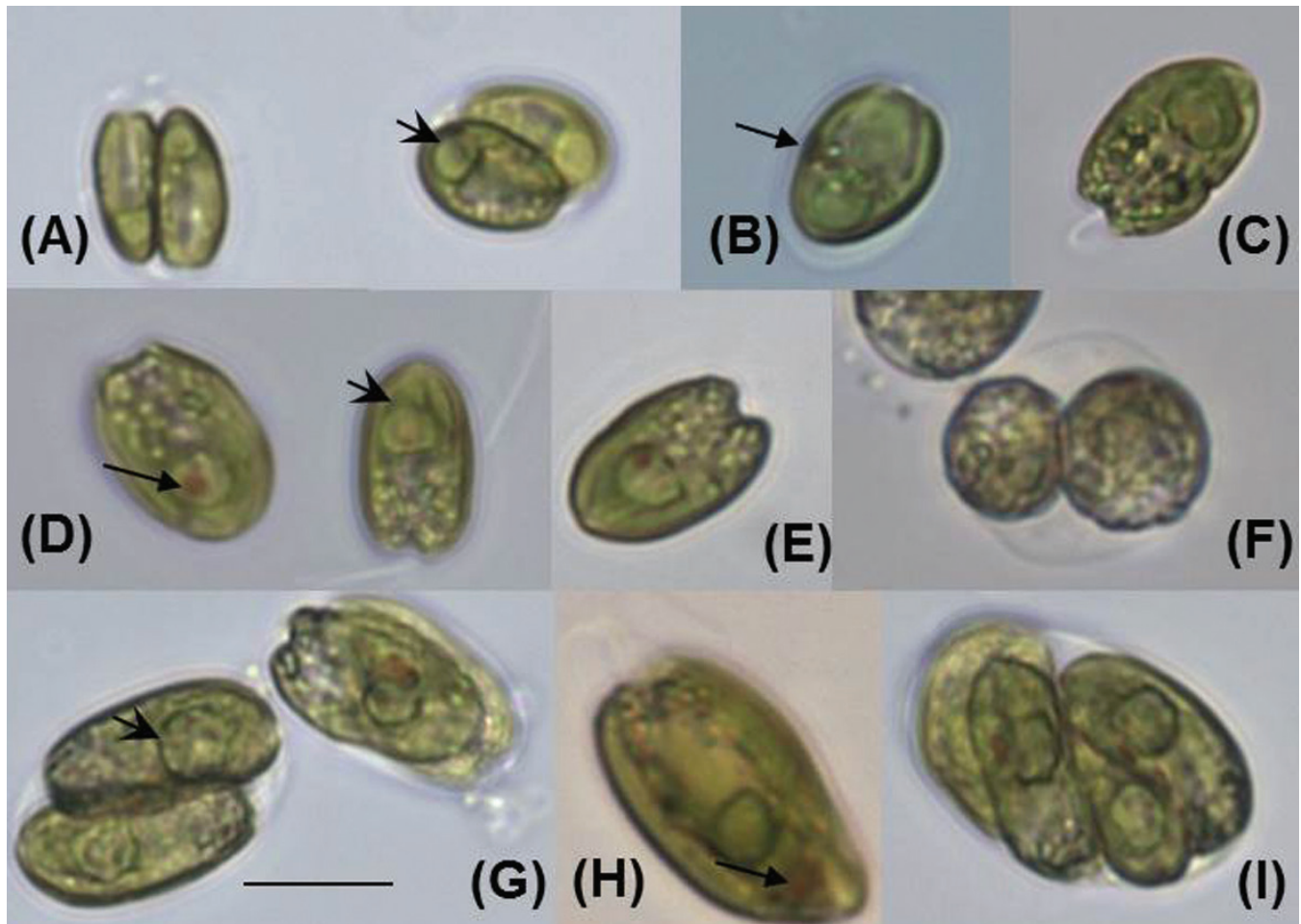


FIGURE 1. Light micrographs of the Chilean *Tetraselmis* strains. Dichato strain (A-C), Coliumo strain (D-F) and Caldera strain (G-I). Note the position of the pyrenoid (arrow head) and stigma (arrow). Scale bar = 10 μ m.

FIGURA 1. Fotomicrografías fotónicas de las cepas chilenas de *Tetraselmis*. Cepa de Dichato (A-C), cepa de Coliumo (D-F) y cepa de Caldera (G-I). Note la posición del pirenoide (punta de flecha) y estigma (flecha). Escala= 10 μ m.

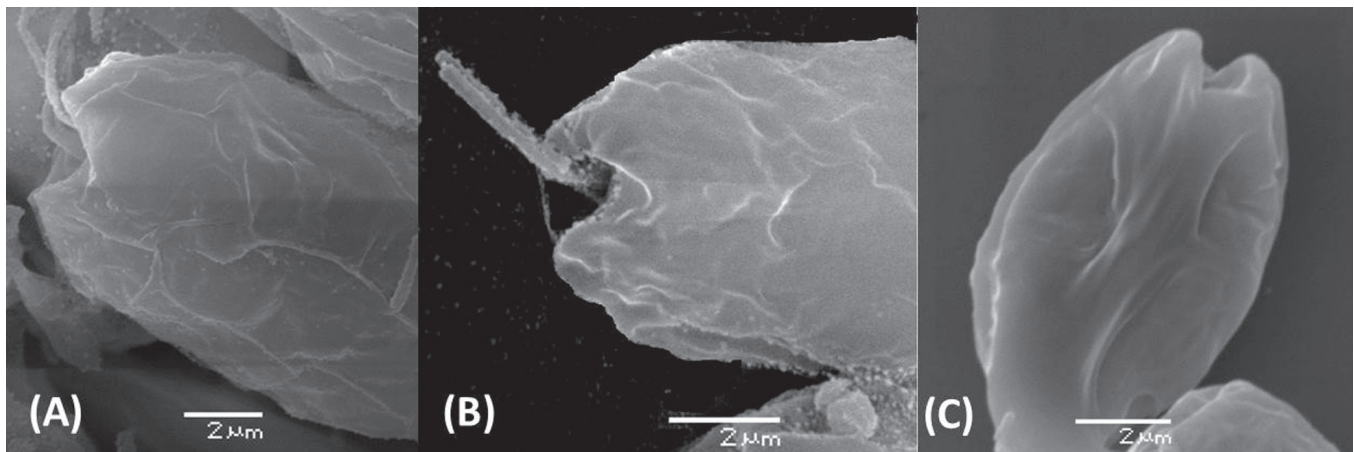


FIGURE 2. Scanning electron micrographs of the Chilean *Tetraselmis* strains. Cells of the Dichato (A), Coliumo (B) and Caldera (C) strains, denoting the presence of only two apical lobes, in each one.

FIGURA 2. Fotomicrografías electrónicas de barrido de las cepas chilenas de *Tetraselmis*. Células de las cepas de Dichato (A), Coliumo (B) y Caldera (C), mostrando la presencia de dos lóbulos apicales en cada una.

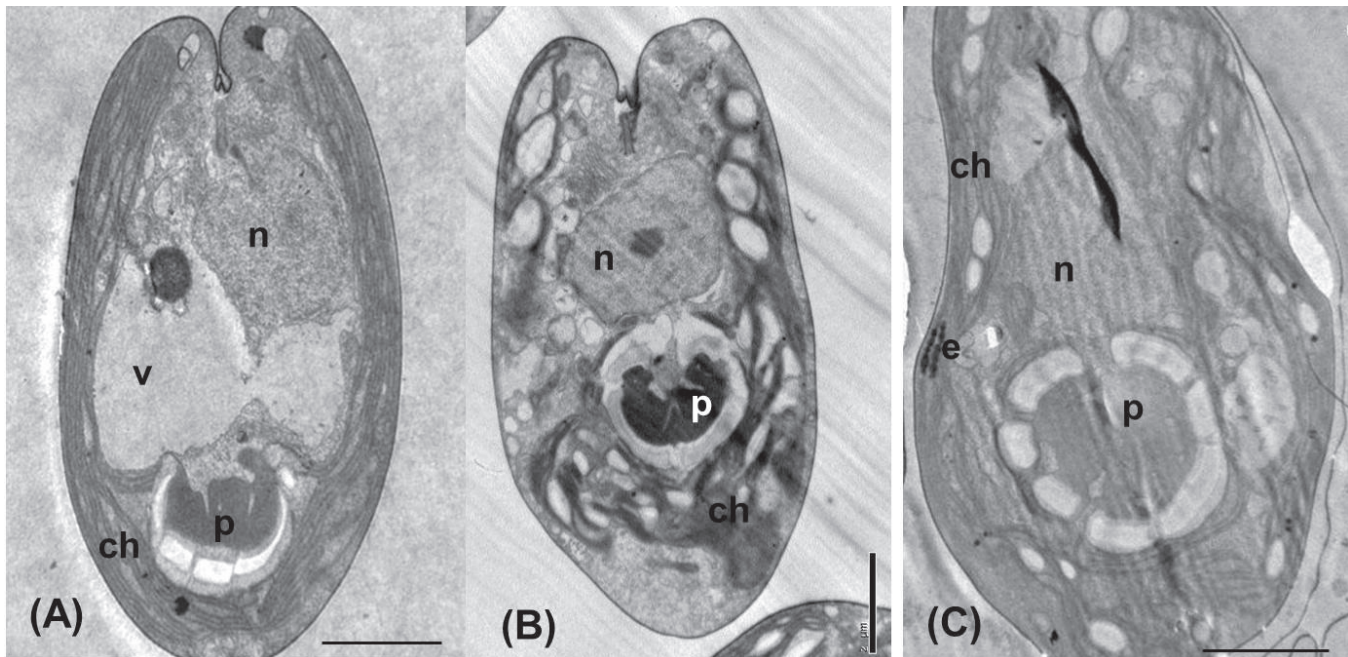


FIGURE 3. Transmission electron micrographs of the Chilean *Tetraselmis* strains. Longitudinal sections of cells from Dichato (A), Coliumo (B) and Caldera (C) showing the position of the pyrenoid (basal in A and sub-basal in B and C) and the lobed or non-lobed chloroplast at the cell posterior (B versus A). n = nucleus, p = pyrenoid, ch = chloroplast, e = eyespot, v = vacuole. Scale bars = 2 μ m.

FIGURA 3. Fotomicrografías electrónicas de transmisión de las cepas chilenas de *Tetraselmis*. Células en sección longitudinal de las cepas de Dichato (A), Coliumo (B) y Caldera (C) mostrando la posición del pirenoide (basal en A y subbasal en B y C) y la presencia o no de lobulación del cloroplasto en la parte posterior de la célula (B versus A). n = núcleo, p = pirenoide, ch = cloroplasto, e = estigma, v = vacuola. Escalas = 2 μ m.

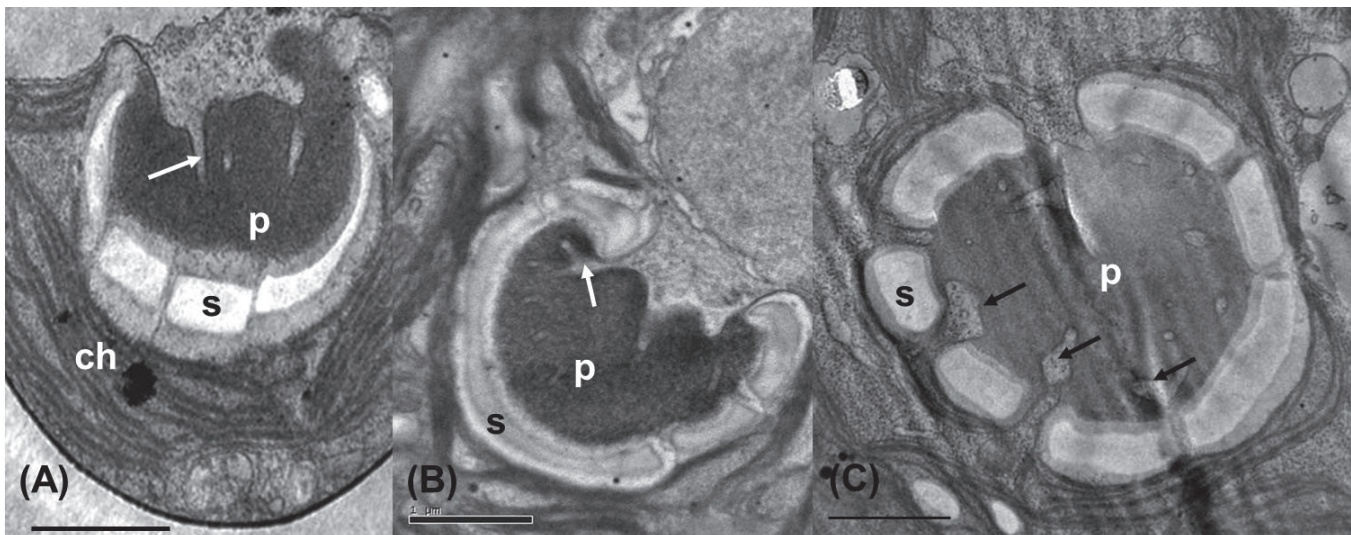


FIGURE 4. Transmission electron micrographs of the Chilean *Tetraselmis* strains. Close up of cells showing the ultrastructure of the pyrenoids (p) in (A) Dichato, (B) Coliumo and (C) Caldera strains. Note the characteristic “horseshoe” shape in A and B and a cavity from which cytoplasmic canaliculi extend and end blindly (white arrows). In C the pyrenoid is rounded and its matrix is transversed by various cytoplasmic channels (black arrows). ch = chloroplast, s = starch. Scale bars = 1 μ m.

FIGURA 4. Fotomicrografías electrónicas de transmisión de las cepas chilenas de *Tetraselmis*. “Close-up” celular mostrando la ultraestructura del pirenoide (p) en las cepas de Dichato (A), Coliumo (B) y Caldera (C). Note la forma característica tipo “herradura” en A y B y la cavidad del pirenoide con canaliculos citoplasmáticos que terminan ciegos (flechas blancas). En C, el pirenoide es redondo y su matriz se encuentra atravesada por varios canales citoplasmáticos (flechas negras). ch = cloroplasto, s = almidón. Escalas = 1 μ m.

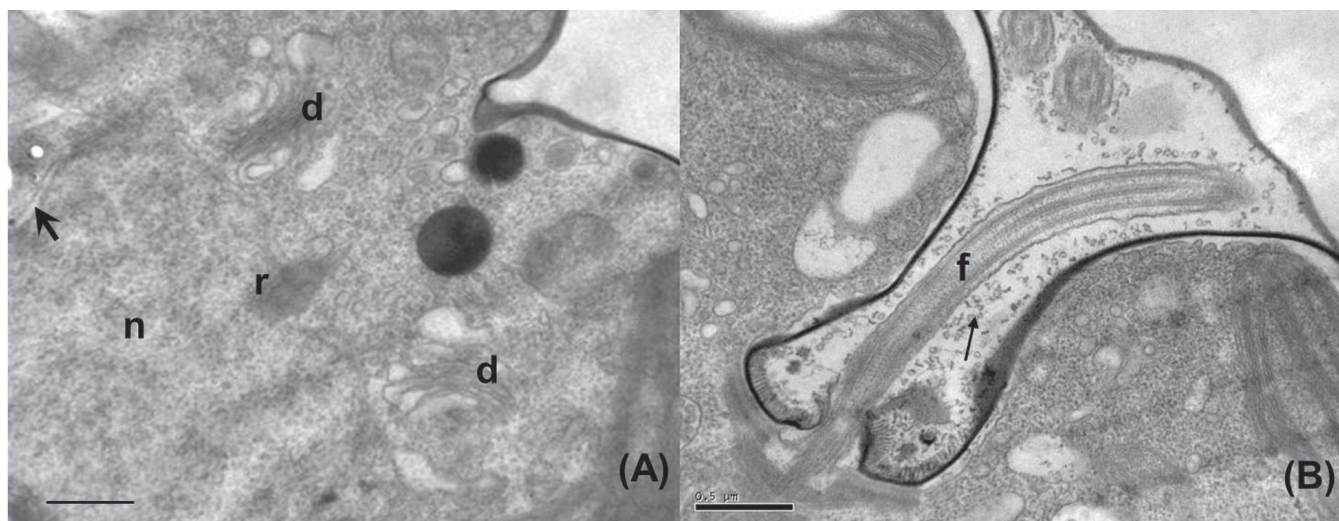


FIGURE 5. Transmission electron micrographs of the Coliumo strain of *Tetraselmis*. Longitudinal section of the apical portion of the cell. In A note the two dictyosomes (d), part of the rhizoplast (r) and the nucleus (n). In B the apical depression, one emerging flagellum (f) and numerous scales (arrow). Arrow head = nuclear membrane. Scale bars = 0.5 μ m.

FIGURA 5. Fotomicrografías electrónicas de transmisión de la cepa Coliumo de *Tetraselmis*. Sección longitudinal de la porción apical de la célula. En A note la presencia de dos dictiosomas (d), parte del rizoplasto (r) y el núcleo (n). En B se observa la depresión apical, uno de los flagelos (f) y la presencia de numerosas escamas (flechas). Punta de flecha= membrana nuclear. Escalas=0.5 μ m.

exhibited 2 or 4 daughter cells within the mother theca (Fig. 1 G, I). The cells of the three strains exhibited a rather shallow two-lobed apical furrow that was clearly seen in SEM observations (Fig. 2 A-C).

Under TEM the cells of the three strains are covered with a smooth theca and have one cup shaped chloroplast (Fig. 3). The chloroplast of the Coliumo strain is lobed at the posterior part of the cell while the chloroplast of the Dichato strain is not lobed (Fig. 3 A, B). The pyrenoid in the Coliumo and Dichato strains of around 2.2 μ m in diameter is surrounded by starch grains which are concave-convex in shape (Fig. 4 A, B). The pyrenoid cavity is usually open towards the nucleus and has cytoplasmic channels which end within the matrix as shown in Figure 4 A and B. Due to the presence of this cavity, the pyrenoid has generally a horseshoe-shape when viewed in longitudinal section. The nucleus is located at the anterior middle of the cell (Fig. 3 A, B) and two dictyosomes are present at each side of the flagellar base (Fig. 5 A).

In spite of similarities in chloroplast shape and pyrenoid location as described above, the strain from Caldera exhibited a quite different pyrenoid shape and ultrastructure. The pyrenoid is spherical, 3.0 -3.5 μ m in diameter. The pyrenoid cavity is open to the nucleus and its matrix is invaded by several cytoplasmic channels that transverse it in various directions (Figs. 3 C and 4 C). The morphological and ultrastructural characters of the examined strains of *Tetraselmis* are summarized in Table II.

The phylogenetic analysis of the nuclear ribosomal internal transcribed spacers ITS-1 and ITS-2 sequences presented in Figure 6 revealed that the Coliumo and Dichato

strains group together forming the clade III (Fig. 6) containing the species of the subgenus *Parviselmis*: *T. chui* (CCAP 8/6, CCAP 66/21B) and *T. suecica* (WDCM NCC62). The ITS-1 and ITS-2 sequences of the Coliumo strain differ only in one base compared to those of the Dichato strain. However, when the secondary structure of ITS-2 is observed, specifically the helix III, no differences in sequence are observed neither between both strains nor with the strains of *T. chui* and *T. suecica* included in this study. We get the same result by analyzing the helix II of the same spacer; except for one nucleotide difference in the Coliumo strain (Figs. 7 and 8).

The Caldera strain, on the other hand, appears alone, forming its own clade in the tree (see clade II in Fig. 6). ITS-1 and ITS-2 sequences of this strain differ in 18 and 45 nucleotides compare to Coliumo and Dichato strains, and to *T. chui* and *T. suecica* sequences, respectively. Most of the sequence differences were located in helix III and helix II of the ITS-2.

Interestingly, the strain of *T. striata* included in this study, which also belong to the subgenus *Parviselmis* form its own clade (clade I in Fig. 6) separated from other clades, which contain the Chilean strains.

DISCUSSION

The strains of *Tetraselmis* from Dichato (CCM-UDEC 109) and Coliumo (CCM-UDEC 114) shared the typical pyrenoid ultrastructure defined for the subgenus *Parviselmis* by Hori *et al.* (1983), which is the pyrenoid cavity open towards the nucleus and the presence of cytoplasmic channels without

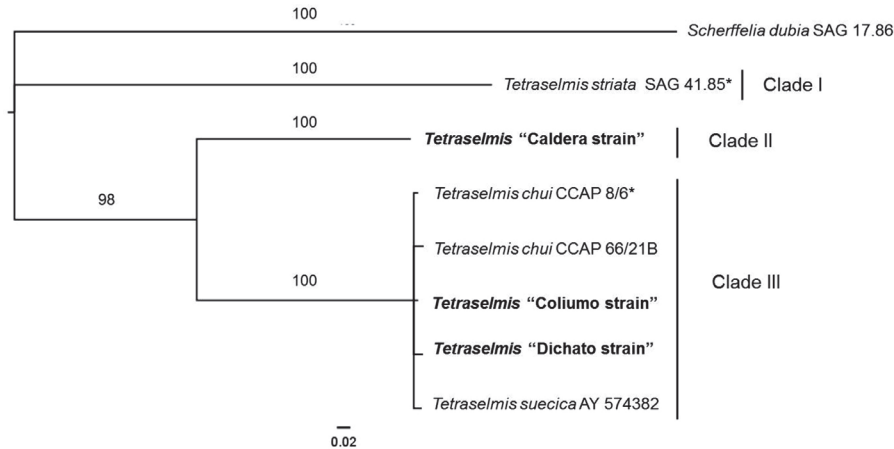


FIGURE 6. Molecular phylogeny of the *Tetraselmis* strains included in this study. Bayesian phylogenetic tree (50% majority rule consensus), as inferred from ITS-1, ITS-2 and 5.8S gene of nuclear rDNA sequences. Numbers above branches indicate the Bayesian values (posterior probability). Scale bar= substitutions per site. *= authentic strains.

FIGURA 6. Filogenia molecular de las cepas de *Tetraselmis* en estudio. Arbol filogenético de inferencia Bayesiana (consenso, regla de la mayoría 50%) basado en las secuencias de los espaciadores ITS-1, ITS-2 y gen 5.8S del DNA ribosomal nuclear. Los números sobre las ramas indican los valores Bayesianos (probabilidad posterior). Escala=sustituciones por sitio. *= cepas auténticas.

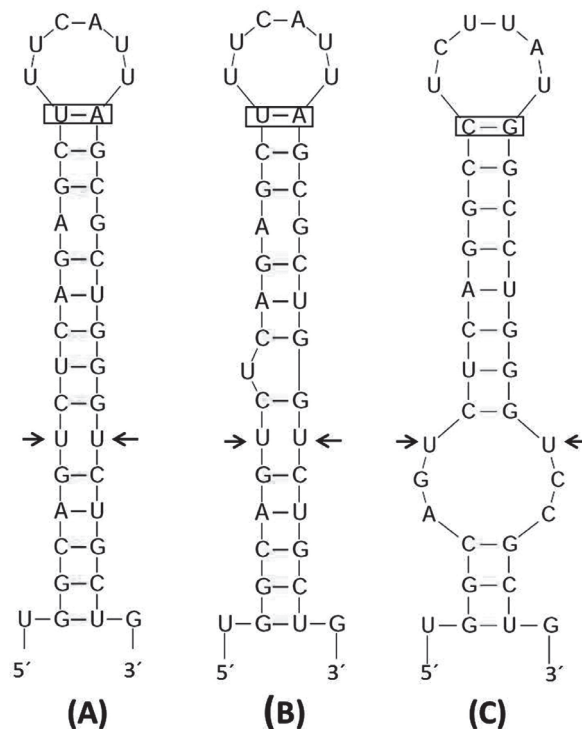


FIGURE 7. Predicted secondary structures of nuclear ITS-2 helix II (clockwise from 5' termini to 3' termini) from *Tetraselmis chui*, *T. suecica* and the Chilean strains under study. (A) Dichato strain, *T. chui* CCAP 8/6 and CCAP 66/21B and *T. suecica* AY574382.1, (B) Coliumo strain and (C) Caldera strain. The compensatory base changes are highlighted in boxes. The arrows mark the putative RNA processing sites.

FIGURA 7. Estructuras secundarias predichas de la hélice II del espaciador nuclear ITS-2 (del terminal 5' al 3') de *Tetraselmis chui*, *T. suecica* y las cepas chilenas de *Tetraselmis* en estudio. (A) Cepa Dichato, *T. chui* CCAP 8/6 y CCAP 66/21B y *T. suecica* AY574382.1, (B) cepa Coliumo y (C) cepa Caldera. Los cambios compensatorios de bases están marcados en rectángulos. Las flechas indican los supuestos sitios de procesamiento del RNA.

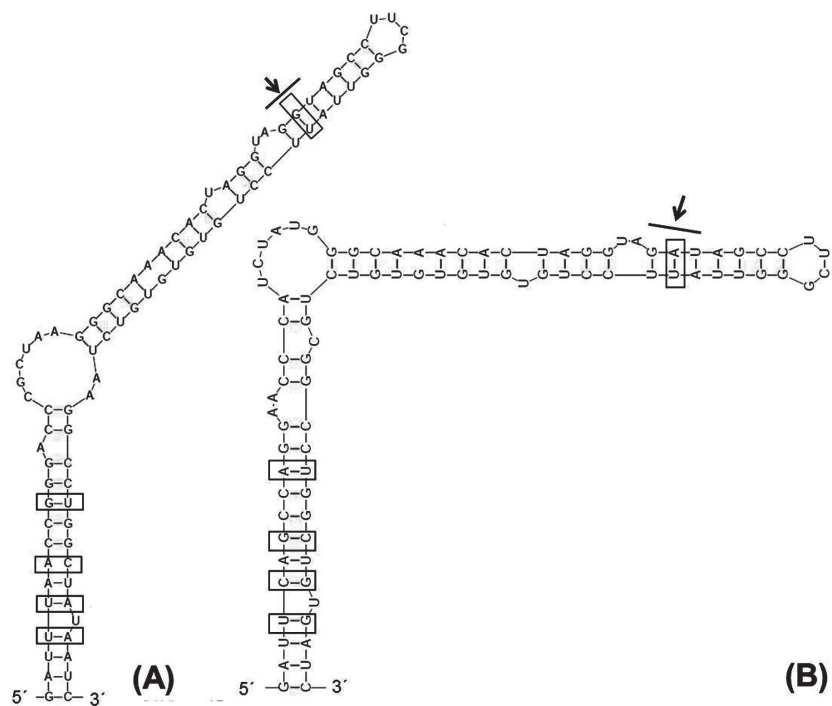


FIGURE 8. Predicted secondary structures of the nuclear ITS-2 helix III (clockwise from 5' termini to 3' termini) from *Tetraselmis chui*, *T. suecica* and the Chilean strains under study. (A) Dichato strain, Coliumo strain, *T. chui* CCAP 8/6 and CCAP 66/21B and *T. suecica* AY574382.1 and (B) Caldera strain. The compensatory base changes are highlighted in boxes. The arrows marked the putative RNA processing sites.

FIGURA 8. Estructuras secundarias predichas de la hélice III del espaciador nuclear ITS-2 (del terminal 5' al 3') de *Tetraselmis chui*, *T. suecica* y las cepas chilenas de *Tetraselmis* en estudio. (A) Cepa Dichato, cepa Coliumo *T. chui* CCAP 8/6 y CCAP 66/21B y *T. suecica* AY574382.1 y (B) cepa Caldera. Los cambios compensatorios de bases están marcados en rectángulos. Las flechas indican los supuestos sitios de procesamiento del RNA.

dissection of the pyrenoid matrix. Moreover, the cells of both strains have two dictyosomes present in the vicinity of the flagellar base which is a feature of most of the species assigned to the subgenus *Parviselmis* according to Hori *et al.* (1986).

Based on the morphological and molecular data obtained from this study both strains could belong either to *T. suecica* (Kyllin) Butcher or to *T. chui* Butcher. The described differences between both species according to Butcher (1959) and Hori *et al.* (1986) are the slightly difference in cell size and shape, as well as, in chloroplast shape and the locations of the pyrenoid and eyespot within the cell. However, there is incongruence in the descriptions provided by Butcher (1959) and Hori *et al.* (1986) concerning the position of the pyrenoid and the shape of the chloroplast at the posterior of the cell in these two species. Thus, Hori *et al.* (1986) described *T. suecica* as having an unlobed chloroplast at the posterior end, and that the location of the pyrenoid is basal. This is in contrast to *T. chui*, which has a finely lobed chloroplast at the posterior part of the cell and its pyrenoid is located sub-basal to sub-centrally. Butcher (1959), however, stated that the pyrenoid position in *T. chui* (in contrast to *T. suecica*) is basal

and that the chloroplast is not lobed at the posterior end of the cell. The morphological variability within *Tetraselmis* needs to be proven in comprehensive comparative study using the authentic strains of the species.

The ITS-1 and ITS-2 sequence data show the close phylogenetic relationship of the Coliumo and the Dichato strains to sequences of *T. chui* and *T. suecica*. Moreover, a comparison of the secondary structure of the ITS-2, showed no compensatory base changes in the conserved region of the ITS-2 (specifically in helix II and III) of the strains belonging to clade III. The CBCs in the secondary structure of the ITS-2 have often been taken into account when distinguishing between closely related species (Luo *et al.* 2006, Krienitz *et al.* 2010, Bock *et al.* 2010, 2011, Pröschold *et al.* 2011). Studies have shown that one CBC in the conserved region of the helix II or III of the ITS-2 is, in most cases, associated with an inability for sexual reproduction (Coleman & Mai 1997, Coleman 2007, 2009). Therefore, based on these results, we can conclude that the strains from Coliumo and Dichato (CCM-UDEC 114, CCM-UDEC 109), as well as the other strains (WDCM NCC 62, CCAP 8/6, CCAP 66/21B)

TABLE II. Morphological and ultrastructural characters of the examined strains of *Tetraselmis*.

TABLA II. Caracteres morfológicos y ultraestructurales de las cepas de *Tetraselmis* examinadas.

STRAINS	CELL SIZE (µm)	CELL SHAPE	Nº LOBES IN CELL APEX	CHLOROPLAST SHAPE AT CELL BASE	POSITION OF THE PYRENOID	ULTRASTRUCTURE OF PYRENOID
Coliumo (CCM- UDEC 114)	12.5-15 length x 6.5-9.5 wide	In lateral view : narrower at the cell base.	2	Lobed	Sub-basal to sub-central	Horseshoe-shape. Cytoplasmic channels end within the pyrenoid matrix.
Dichato (CCM- UDEC 109)	10-12.5 length x 5.8-8.0 wide	In lateral view: rounded at the cell base	2	Not -lobed	Basal	Horseshoe-shape. Cytoplasmic channels end within the pyrenoid matrix.
Caldera (CCM- UDEC 134)	15-18.8 length x 8.5-12.5 wide	In lateral view: narrower and curved at the cell base	2	Lobed	Sub-basal to sub-central	Spherical in shape. Cytoplasmic channels transverse the pyrenoid matrix

included in this study, belong to the same species. At present it cannot be decided which species name should be chosen (*T. suecica* or *T. chui*). However, the strain CCAP 8/6 of *T. chui* is the authentic strain of this species, whereas the authentic strain of the other species is not available in public culture collection. If an epitype of *T. suecica* will be designated, this species name has priority according to the International Code of Nomenclature for Algae, Fungi and Plants. To solve this problem, it will be necessary to include more strains in comparative studies using an integrative approach as indicated by Pröschold & Leliaert (2007).

Based on pyrenoid ultrastructure (pyrenoid cavity opening towards the nucleus and cytoplasmic channels traversing its matrix in different directions), the strain of *Tetraselmis* from Caldera (CCM UDEC 134) belongs to the subgenus *Tetrathele* (Hori *et al.* 1983). Despite that many strains of *T. tetrathele* are available in public culture collections, no morphological and molecular study of this species has been published so far. Only Butcher (1959) compared the morphology of this species with the others of this genus.

The comparison of the morphology of the Caldera strain shows similarities to those published by Butcher (1959) for *T. tetrathele*: cell size, shape (in lateral view: the posterior part of the cell acute, slightly twisted and curved) and the position of the pyrenoid (sub-central to sub-basal). Butcher also mentioned the spherical shape of the pyrenoid, which was also observed in cells of the Caldera strain under TEM. Caldera strain cells differ from the Butcher's description in the presence of only two apical lobes (instead of four) and the absence of a lateral deep longitudinal furrow. Until now there is no ITS-1 and ITS-2 rDNA sequences available in the GenBank of strains belonging to this species. Therefore, it is difficult to make a conclusion about taxonomic identity of the Caldera strain.

Summarizing our results, the Coliumo and Dichato strains correspond to the same species, which is identical to the authentic strain of *T. chui* Butcher. The Caldera strain

showed some morphological differences to the original description of *T. tetrathele* (as emended in Butcher 1959), and can therefore represent an undescribed species within the subgenus *Tetrathele*. However, the taxonomic revision of this genus is necessary by including of more strains.

In addition, the subdivision into subgenera as introduced by Hori *et al.* (1986) is probably artificial and needs further studies. According to Hori *T. striata* belongs to the same subgenus *Parviselmis* like the strains of *T. chui*. However, both species form separate clades in our phylogeny (Fig. 6).

Furthermore, the authentic strain of *T. striata* (which belongs to the subgenus *Parviselmis*) is phylogenetically more distant to clade III than the Caldera strain, which fits within the subgenus *Tetrathele* according to the pyrenoid ultrastructure. These results highlight the need for testing the validity of the pyrenoid ultrastructure as the predicting attribute used to recognize subgenera within the genus *Tetraselmis*. This issue has not been taken into account in any molecular phylogenetic studies previously published on this genus (Nasare 2002, Lee & Hur 2009, Arora *et al.* 2013).

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