**Tetraselmis indica** (Chlorodendrophyceae, Chlorophyta), a new species isolated from salt pans in Goa, India

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A new species of *Tetraselmis*, *T. indica* Arora & Anil, was isolated from nanoplankton collected from salt pans in Goa (India) and is described based on morphological, ultrastructural, 18S rRNA gene sequence and genome size data. The species is characterized by a distinct eyespot, rectangular nucleus, a large number of Golgi bodies, two types of flagellar pit hairs and a characteristic type of cell division. In nature, the species was found in a wide range of temperatures (48°C down to 28°C) and salinities, from hypersaline (up to 350 psu) down to marine (c. 35 psu) conditions. Phylogenetic analysis based on 18S rDNA sequence data showed that *T. indica* is most closely related to unidentified *Tetraselmis* strains from a salt lake in North America.

**Key words:** Chlorodendrophyceae; green algae; molecular phylogeny; morphology; pit hairs; Prasinophyceae; taxonomy; *Tetraselmis indica*; ultrastructure

**Introduction**

The Chlorodendrophyceae is a small class of green algae, comprising the genera *Tetraselmis* and *Scherffelia* (Massjuk & Lilitska, 2006; Leliaert et al., 2012). Although traditionally considered as members of the prasinophytes, these unicellular flagellates share several ultrastructural features with the core Chlorophyta (Trebouxiophyceae, Ulvophyceae and Chlorophyceae), including closed mitosis and a phyco-plast (Mattox & Stewart, 1984; Melkonian, 1990; Sym & Pienaar, 1993). A phylogenetic relationship with core Chlorophyta has been confirmed by molecular data (Fawley et al., 2000; Guillou et al., 2004; Marin, 2012).

The best-known members of the class are quadriflagellate unicells, but some species of *Tetraselmis* (originally considered to belong to the genus Prasinocladus) may form stalked colonies during some stage of the life cycle (Proskauer, 1950; Norris et al., 1980; Sym & Pienaar, 1993). The motile cells of Chlorodendrophyceae are generally laterally compressed, and bear four equal and homodynamic flagella, which emerge from an anterior pit of the cell. The cells are typically covered by a theca, which is a thin cell wall formed by extracellular fusion of scales (Manton & Parke, 1965; Sym & Pienaar, 1993). The flagella are covered by hairs and pentagonal scales (Melkonian, 1990). Cells generally have a single chloroplast, which includes a single conspicuous eyespot and a pyrenoid (only in *Tetraselmis*). Sexual reproduction is unknown in the class. Some species form vegetative thick-walled cysts, which may be extensively sculptured (McLachlan & Parke, 1967; Norris et al., 1980; Sym & Pienaar, 1993).

Most Chlorodendrophyceae are found as planktonic or benthic organisms in marine environments, where they sometimes occur in dense populations causing blooms in tidal pools or bays. A number of species occur in freshwater habitats (John et al., 2002). Some species have been described as endosymbionts of marine animals, including *Tetraselmis convolutae* which is a facultative symbiont of the acoel flatworm *Symagittifera (Convoluta) roscoffensis* (Parke & Manton, 1965; Provasoli et al., 1968; Serodio et al., 2011), and an undescribed *Tetraselmis* species that has been isolated from the radiolarian *Spongodrymus*.

*Tetraselmis* and *Scherffelia* are two relatively small genera. *Scherffelia*, a genus of about 10 described species, differs from *Tetraselmis* in lacking pyrenoids (Melkonian & Preisig, 1986). Molecular data from several species will be needed to test if the two genera form separate clades (Marin, 2012). *Scherffelia dubia* has been extensively used as a model organism for examining the structure and formation of the cytoskeleton, endomembrane system, cell wall and flagella...
(Becker et al., 1996, 2001; Wustman et al., 2004). Tetraselmis includes about 26 currently accepted species, including taxa previously assigned to the genera Platymonas, Prasinoclados and Aulacochlamys (Norris et al., 1980; Sym & Pienaar, 1993). Traditional species circumscriptions were based on light microscopical (LM) characteristics, such as cell size and shape, structure of anterior cell lobes, chloroplast morphology, position of the stigma, and shape and position of the pyrenoid (West, 1916; Kylin, 1935; Carter, 1938; Margsalef, 1946; Proskauer, 1950; Butcher, 1952, 1959). Many of these features were found to be variable and hence not useful for species identification. More recently, electron microscopical characters, including the ultrastructure of the pyrenoid and flagellar hair scales, have been proposed to distinguish between species (Parke & Manton, 1965; McLachlan & Parke, 1967; Melkonian, 1979; Melkonian & Robenek, 1979; Norris et al., 1980; Hori et al., 1982, 1983, 1986; Thronsden & Zingone, 1988; Becker et al., 1990, 1994; Marin et al., 1993, 1996; Marin & Melkonian, 1994).

Although many Tetraselmis species are relatively well characterized morphologically and ultrastructurally, correct species assignment is arduous because of complex methodologies for cellular characterization (e.g. the need for electron microscopic observations). DNA sequence analysis provides a reliable and more convenient tool for species delimitation in the genus. Genetic diversity has been studied based on 18S rDNA sequence data, especially from temperate regions (Lee & Hur, 2009). Diversity of Tetraselmis in the tropics has been much less explored. Several Tetraselmis species are economically important as they are ideal for mass cultivation because of their euryhaline and eurythermal nature (Butcher, 1952, 1959). Many of these features were found to be variable and hence not useful for species identification. More recently, electron microscopical characters, including the ultrastructure of the pyrenoid and flagellar hair scales, have been proposed to distinguish between species (Parke & Manton, 1965; McLachlan & Parke, 1967; Melkonian, 1979; Melkonian & Robenek, 1979; Norris et al., 1980; Hori et al., 1982, 1983, 1986; Thronsden & Zingone, 1988; Becker et al., 1990, 1994; Marin et al., 1993, 1996; Marin & Melkonian, 1994).

Light and confocal microscopy

For light microscopy (LM), living cells were observed using an Olympus BX 51 microscope equipped with an Olympus DP70 digital camera system and Image-Pro software. Cells were also observed under an Olympus Fluoview 1000-Confocal laser scanning microscope equipped with a multiline Argon laser.

Scanning electron microscopy

For scanning electron microscopy (SEM), cultured cells were sampled during the late exponential growth phase and fixed in 2% glutaraldehyde (TAAB, Aldermaston, Berks) in seawater containing 0.1 M cacodylate buffer (pH 7.0). The cells were post-fixed overnight in 1% cold osmium tetroxide (Agar Scientific, Stansted, Essex) in 0.1 M cacodylate buffer, rinsed in buffer for 10 min and then dehydrated in an acetone series (30 min each in 25, 50 and 75% acetone, followed by 100% acetone for 1 h at room temperature). Following dehydration, cells were impregnated using an epoxy resin kit (TAAB) for 1 h each with 25, 50 and 75% resin (in acetone), followed by 100% resin for 1 h, with rotation overnight. The embedding medium was then replaced with fresh 100% resin at room temperature and the cells transferred 5 h later to an embedding dish for polymerization at 60°C overnight.

Sections were cut with a diamond knife mounted on a RMC MT-XL ultramicrotome. The sections were stretched with chloroform to eliminate compression and mounted on pioloform (Agar Scientific) filmed copper grids. Sections were stained for 20 min in 2% aqueous uranyl acetate (Leica UK, Milton Keynes) and lead citrate (Leica). The grids were examined using a Philips CM 100 Compustage (FEI) transmission electron microscope (TEM) and digital images were collected using an AMT CCD camera (Deben) at the Electron Microscopy Research Services facility, Newcastle University.

Materials and methods

Collection and culturing

Material was collected from a pool in salt pans at Panaji, Goa, India. The marine salt pans in this region are systems of interconnected ponds, in which there is a discontinuous salinity gradient. Salinity and maximum temperature become very high in these shallow salt pan pools and the species thrives well even in these conditions. For example, salinity in the salt pan from which T. indica was isolated reached as high as 350 psu and as low as 35 psu, while the temperature ranged between 48.2°C and 28.5°C.

A sample of the dark green water between the salt lumps in the pan was collected and diluted five-fold with autoclaved seawater. Unialgal clonal cultures were established by diluting the enriched crude culture and micropipetting single cells. These cultures were grown and maintained at the National Institute of Oceanography, India, in f/2 medium without silicate (Guillard & Ryther, 1962) at 25°C, with a photon flux density of 80 µmol photons m⁻² s⁻¹, and a 16 : 8 h light : dark cycle.
Tetraselmis indica sp. nov.

dried in a critical point dryer (BalTec, Reading, UK) and subsequently mounted on stubs with a silver DAG and carbon disc (Agar Scientific). Finally, the cells were sputtered with gold using a Polaron SEM coating unit and observed using a Stereoscan S400 Scanning Electron Microscope (Cambridge Instruments, UK) at the Electron Microscopy Unit of the Department of Biomedical Science, Newcastle University.

Flow cytometry

The DNA content of the species was estimated by flow cytometry. Nuclei were released by the injection of 50 µl of Tetraselmis culture into 450 µl of nuclei isolation buffer (NIB, described by Marie et al., 2000) twice diluted with distilled water. Five micro-litres of Micromonas pusilla (Mamiellophyceae) culture CCAP 1965/4 (CCMP 1545) were added as an internal standard of known genome size (15 Mb) (Moran & Armbrust, 2007). The nucleic acid specific stain SYBR Green I (Molecular Probes) was added at a final dilution of 1:10 000 of the commercial solution. Samples were incubated for 15 min before analysis by flow cytometry. Samples were run at a rate of 10 µl min⁻¹ on a FACS Aria II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) equipped with a 488 nm excitation laser and a standard filter setup. The data were acquired on a logarithmic scale due to the large difference in genome size between the sample and the reference, and the DNA concentration was estimated according to the method proposed by Marie et al. (2000).

Molecular phylogenetic analysis

Cultures were grown in 50-ml flasks for 1–2 weeks and cells recovered by centrifugation at 7000 × g for 10 min. Genomic DNA was extracted from cell pellets using an Invisorb® Spin Food Kit II, according to the manufacturer’s instructions. The 18S rRNA gene was amplified using universal eukaryotic primers (Medlin et al., 1988) and a QIAGEN Fast Cycling PCR Kit (Qiagen, USA) and Eppendorf Mastercycler PC machine (Eppendorf Scientific, USA). Dye terminator sequencing using the same primers as in the amplification step and an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, USA) were used to obtain nucleotide sequences. The 18S rDNA sequence of 1706 bp has been deposited in GenBank as accession HQ651184.

Two datasets were created for phylogenetic analyses. The first one was assembled to assess the phylogenetic position of the new species within the Chlorophyta. This alignment consisted of 44 18S rDNA sequences representing a broad range of Chlorophyta (Leliaert et al., 2012; Marin, 2012) and two Streptophyta (Chlororhabdos and Mesostigma), which were selected as outgroups. A second 18S dataset was used to examine the phylogenetic position of the new species within the Chlororhabdosphaecae with more precision. This alignment was created as follows. First, all 18S sequences of Chlororhabdosphaecea available in GenBank were downloaded, aligned using MUSCLE (Edgar, 2004) and a neighbour-joining tree was created using MEGA v5 (Tamura et al., 2011). Based on this tree, a reduced alignment was created by selecting 29 representative sequences from the main clades of Tetraselmis (with a preference for sequences obtained from identified strains of official culture collections), in addition to the single available sequence of Scherffelia, and six trebouxiophycean sequences as outgroups. The alignments used for this paper are available in the Supplementary information.

Sequences of the two datasets were aligned using MUSCLE (Edgar, 2004), and inspected visually in BioEdit 7.0.5.3 (Hall, 1999). Evolutionary models for the two alignments were determined by the Akaike Information Criterion in JModeltest (Posada, 2008). Both datasets were analysed under a GTR + I + G model with maximum likelihood (ML) using RAxML (Stamatakis et al., 2008) and Bayesian inference (BI) using MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Bayesian inference analyses consisted of two parallel runs of four incrementally heated chains each, and 5 million generations with sampling every 1000 generations. Convergence of log-likelihood and model parameters, checked in Tracer v. 1.4 (Rambaut & Drummond, 2007), was achieved after c. 50 000 generations for both datasets. A burn-in sample of 500 trees, well beyond the point of convergence, was removed before constructing the majority rule consensus tree.

Results

Light microscopical examination of the water revealed a dominance of motile Tetraselmis cells along with diatoms, including Amphora and Pseudonitzschia. Based on the sampling location, this species likely prefers hypersaline environments, although it cannot be excluded that it also occurs in other marine habitats. A clonal strain was examined by LM. A schematic presentation of cells is presented in different orientations (Fig. 1), as well as micrographs taken using LM (Figs 2–11) and SEM (Figs 12–17).

Cell body morphology and ultrastructure

Cells are slightly compressed, 10–25 µm long, 7–20 µm broad and 6.5–18 µm thick. A broad lateral view shows the cell shape to be oval with the posterior part wider than the anterior (Figs 2–4), and when viewed from the narrow side, cells are elliptical (straight in the middle with a markedly curved base and apex) (Figs 5, 6). Cells have distinct creases (Figs 11, 14 and video S1 of the supplementary material) that extend along much of the length of the cell wall. Cells contain a single lobed chloroplast and a nucleus located near the flagellar base (Fig. 18). The chloroplast is yellow-green in colour, cup-shaped. There is a conspicuous orange-red eyespot or stigma (Fig. 3). Two large rhizoplasts are present per cell, one associated with each pair of basal bodies. Each rhizoplast branches immediately adjacent to the basal body pairs (Fig. 18). The nucleus is positioned in the anterior half of the cell and is shield shaped (Fig. 18), 6.5–8.5 µm long and 3.5–4 µm broad, with a characteristic apical groove and a basal arch as seen in longitudinal sections. The nucleolus is very prominent (Fig. 18). A large pyrenoid is located in the chloroplast (Fig.
19), posterior to the nucleus and is traversed from several directions by cytoplasmic invaginations. The pyrenoid matrix measures 2.6–2.8 × 3.4–3.6 µm in longitudinal section and is surrounded by many biconvex or concave starch grains (Fig. 19). In addition to the starch grains appressed to the pyrenoid, the chloroplast contains numerous starch grains in the stroma (Figs 20, 21); the stigma is located at the level of the pyrenoid. Dictyosomes (Fig. 23), usually 2–8 in number are positioned in a circle near the anterior end of the nucleus and sometimes on the sides. The endoplasmic reticulum is widely distributed allowing the dictyosome forming face to be turned in a different direction in relation to the nucleus.

Numerous mitochondria are present (Fig. 23). Electron dense structures are present in the periphery of resting cells (Fig. 24) which appear as orange red globules under LM, possibly representing haematochrome bodies.

**Flagella and flagellar aperture**

The four anterior flagella emerging from the thecal slit in the bottom of the apical depression are slightly shorter than the cell. The flagellar pit is deep (Figs 27–30), up to about 0.5–1.8 µm, and grooved. It is covered with two types of well-developed pit hairs at distinct positions (Figs 29–31); the first type is present...
on the floor of the cavity and is striated, thick and rod shaped (Figs 30, 31), while the second type is present at the extension of the wall bordering the flagellar slit and is curly (Figs 29–31). The flagella emerge from the cell in two pairs, each pair ± parallel to the longitudinal flatter sides of the cell in the root position. The partners of the flagellar pair remain close to one another as they emerge from the pit, thereby lying on the middle part of the flat side of the cell (Fig. 18). The flagella are hairy and blunt ended and covered by a layer of pentagonal scales that are aligned in a compact layer next to the plasmalemma (Fig. 34). The flagellar scales in this layer have a low rim, a raised central point, and a tetragonal electron translucent zone on the floor of the scale surrounding the protuberance (Fig. 31). The scales are arranged in longitudinal rows. Each flagellum also bears rod-shaped scales or ‘man scales’ and fine hairs (Figs 31–34).

**Cell covering**

A theca of two layers covers the cell body, except for the flagellar grooves. Each of the two layers of the theca has a complex architecture (Fig. 20). A slit at the base of the flagellar pit becomes everted and appears as a very short papilla when the walls are cast off (Figs 35–40).

**Swimming behaviour**

Under LM, cells may swim for a few minutes before settling and attaching to the slide via the flagella. Cells usually swim rapidly in an almost straight line or in a slightly curved path, with the flagella at the forward end. They may resume movement in a new direction without pause. The cells show marked phototaxis.

**Reproduction**

Vegetative reproduction is by transverse division of the protoplast into two (Figs 25, 26), three or four daughter cells within the parental theca. Cells sometimes divide asymmetrically. Nucleolar and nuclear division are followed by cytoplasmic division. Daughter cells often develop flagella while still surrounded by the parental theca.

The alga survives during unfavourable periods as cysts, which are capable of rejuvenation and normal development with the return of favourable conditions (Figs 35–40). Apical papilla can be seen when the walls are cast off. Sexual reproduction has not been observed.

**Genome size**

Genome size was estimated by flow cytometry and compared to an internal standard (*Micromonas pusilla* CCAP 1965/4, genome size 15 Mbp) (Fig. 41). One peak was observed for *T. indica* of c. 90 Mbp.

**Phylogenetic analysis**

Analysis of the Chlorophyta 18S rDNA sequence alignment firmly placed *T. indica* in the Chlorodendrophyceae clade (Figs 42, 43). *Tetraselmis* was recovered as paraphyletic with respect to *Scherffelia*. Analysis of the Chlorodendrophyceae alignment resulted in a monophyletic *Tetraselmis*.
clade (although with low support) in which *T. indica* was placed on a long branch and close to two unidentified strains from the Great Salt Lake, Utah (USA). The 18S sequences from Utah, which were only 1358 bp (GenBank GQ243429) and 1255 bp (GenBank FJ546704) long, differed from the sequence from Goa by 10–12 bp, corresponding to an uncorrected p distance of 0.006–0.007. Sequences of *T. indica* and the North American strains were found to be very divergent from other *Tetraselmis* sequences with p distances up to 0.080, which exceeds sequence divergence among the other *Tetraselmis* strains (excluding our new species and the strains from Utah) (maximum p distance 0.040). The exact phylogenetic position of the Indian–American clade was uncertain, with only low support (ML bootstrap value 56%, Bayesian posterior probability 0.91) for a sister relationship with *T. cordiformis*.

Figs 18–26. Thin sections of *T. indica*, TEM. 18. Detail of a cell sectioned vertically showing the characteristic shield-shaped nucleus (N) with its apical groove a basal arch and nucleolus (No), and the pyrenoid (P), chloroplast, vacuoles (V) and rhizoplast (R). 19. The pyrenoid (P) showing cytoplasmic channels. 20. Periphery of cell showing the two layers of the theca (two right arrows) surrounding the cell membrane (left arrow). 21. Starch grain (S) in a chloroplast surrounded by mitochondria (e.g. M) and endoplasmic reticulum (ER, arrows). 22. Eyespot (E). 23. Golgi bodies (G, arrows) and endoplasmic reticulum (ER) distributed on both sides of lobes, with nucleus (N) and a mitochondrion (M). 24–26. Dividing cells. Scale bars = 2 µm (Figs 18, 24–26), 500 nm (Figs 19, 21–23), or 100 nm (Fig. 20).
Discussion

The taxonomy and morphology of the genus *Tetraselmis* have been relatively well studied (Parke & Manton, 1965; McLachlan & Parke, 1967; Melkonian, 1979; Melkonian & Robenek, 1979; Norris, 1980; Norris *et al*., 1980; Hori *et al*., 1982, 1983, 1986; Thronsen & Zingone, 1988; Becker *et al*., 1990, 1991, 1994; Marin *et al*., 1993, 1996; Marin & Melkonian, 1994). This taxonomic framework allows a detailed comparison of morphological and ultrastructural features between the isolate from Goa and described *Tetraselmis* species (Table 1). The presence of several distinct morphological features, in combination with the results of the molecular phylogenetic analyses, supports the recognition of a new species of *Tetraselmis*.

Figs 27–34. Thin sections of *T. indica*, TEM. 27. Apical view of a cell showing the aperture through which flagella emerge. 28. Broad lateral view of the cell (anterior pointing downwards). 29. Transverse section close to the very top of the cell through the apical aperture, showing the four flagella with their 9 + 2 microtubular pattern, and hairs present on the walls bordering the flagellar pit (e.g. at arrow). 30, 31. Flagellar aperture and detail in longitudinal section, showing the characteristic three types of hairs (at arrows). 32–34. Oblique, longitudinal and cross-sections of flagella, showing the three types of scales (arrows); the scales of the outer layer overlap the gap between the scales of the inner layer and the two types of hair scales are present outside these two layers. Scale bars = 2 µm (Figs 27, 28), 500 nm (Fig. 30), or 100 nm (Figs 29, 31–34).
**Tetraselmis indica** Arora & Anil, *sp. nov.*

**Figs 1–40.** Algae planktonicae marinae, praeferens habitat hypersalinae. Cellulae in statu monadoide plerumque paulum compressae, 10–25 µm longae, 7–20 µm latae, 6–18 µm altae, bilateraliter symmetricae, a latere latiore ovales, faciebus latioribus sulco antico transverse conjunctis; a latere angustiore ellipticae. Species habet rugas distinctas. Chloroplasti flavovirventes, cyathiformes, lobis a dorso; forma chloroplasti formam loborum cellulae subsequitur. Nucleus in cellulae parte anteriore, peltatus, 6.5–8.5 µm longus, 3.5–4 µm latus, cum apicali canaliculo proprio suo et basi formae fornicatae apparentibus in sectionibus longitudinalibus multis; nucleolus maxime prominet. Pyrenoides in cellulae posteriore situm, matrix pyrenoidalis magna, 2.6–2.8 × 3.4–3.6 µm sectione longitudinali, circumdata granulis amylaceis multis plerumque biconvexis, aliquando latere uno concavis. Stigma unicum (interdum stigmata pluria)

**Fig. 41.** Flow cytometry analysis of the DNA content of a cell nuclei of *T. indica*, compared to that of *Micromonas pusilla* CCAP 1965/4, which has a genome size of 15 Mbp. Relative DNA content (x-axis), no. of events (y-axis).
aurantium conspicuum infra pyrenoidem positum. Corpuscula Golgiana plerumque 2–8, in circulo locata prope partem nuclei anteriorem et aliquando ab lateribus. Reticulum endoplasmaticum late diffusum. Mitochondria multa et admodum aucta. Propagatio non-sexualis fissione est effecta; cellulae filiales in theca parentis sunt bina vel terna vel quaterna.

**DESCRIPTION:** Marine planktonic green alga, preferring high salinities. Motile cells usually slightly compressed, 10–25 µm long, 7–20 µm wide, 6–18 µm thick, bilaterally symmetrical, oval in shape when viewed from broad side, with a single apical furrow passing from one broad face to the other; cells elliptical when viewed from the narrow side. The species has distinct creases. Chloroplasts yellow-green, cup-shaped, dorsoventrally lobed, the shape of the chloroplast closely following the shape of the cell lobing. Nucleus in the anterior half of the cell, shield-shaped, 6.5–8.5 µm long and 3.5–4.5 µm broad with a characteristic apical groove and a basal arch, both visible in many longitudinal sections; nucleolus very prominent. Cells containing a pyrenoid located in the posterior third of the cell; pyrenoid matrix traversed from several directions by cytoplasmic canaliculi, pyrenoid matrix large, 2.6–2.8 × 3.4–3.6 µm in longitudinal section, surrounded by many starch grains which are mostly biconvex and sometimes concave on one side; one or sometimes several conspicuous orange-red eyespots are located below the level of the pyrenoid.

**Fig. 42.** Maximum likelihood (ML) tree of the Chlorophyta inferred from 18S rDNA sequences, showing the phylogenetic position of *Tetraselmis indica* within the Chlorodendrophyceae. ML bootstrap values (> 50) and Bayesian inference (BI) posterior probabilities (> 0.80) are indicated at the branches, respectively.
Mitochondria well developed and many. Asexual reproduction by fission resulting in two, three or four daughter cells within the parental theca.

Estimated genome size: 90 Mbp.

**HOLOTYPE:** Permanently resin-embedded strain deposited in the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, Tamil Nadu, 620024, India. (Accession Number BDU GD001).

**TYPE LOCALITY:** Salt pan near Mandovi Bridge, Panaji, Goa (15.500623°N, 73.849046°E).

**HABITAT:** Hypersaline to marine (preferring hypersaline conditions); up to now known only from the type locality.

*Tetraselmis indica* can be distinguished from other *Tetraselmis* species on the basis of several features including its hypersaline habitat, the structure and position of the eyespot, nuclear shape, the kinds and positions of flagellar cavity hairs, numerous dictyosomes, sequence divergence and overall cell appearance (Table 1). Daughter cells often develop flagella while still surrounded by the parental theca. This feature is almost unique within the genus *Tetraselmis* and is a major aspect of the diagnosis. Notably, such a type of cell division is only known from two other species of *Tetraselmis*, *T. subcordiformis* and *T. impellucida* (Stewart et al., 1974; Trick, 1979). Typically, eight Golgi bodies are observed in apical cell sections, with a few more on the sides of the nucleus, which is

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**Fig. 43.** Maximum likelihood (ML) tree of the Chlorodendrophyceae inferred from 18S rDNA sequences, showing the phylogenetic position of *Tetraselmis indica*. ML bootstrap values (> 50) and Bayesian inference (BI) posterior probabilities (> 0.80) are indicated at the branches, respectively. Species names are adopted from GenBank or the culture collections. Strain or sample information, and habitat type (freshwater/brackish/marine/hypersaline) is provided for each sequence.
Table 1. Comparison of various species of the genus *Tetraselmis*. ND: information not available (certain species were described before the emergence of electron microscopy as a tool to describe ultrastructure, hence information is not available for those characteristics). Some species of *Tetraselmis*, such as *T. kochiensis*, *T. micropapillata* and *T. tetrabrachia* have not been included because detailed morphological characteristics are lacking.

<table>
<thead>
<tr>
<th>Species</th>
<th>Known geographical distribution and the type of habitat</th>
<th>Cell shape and size</th>
<th>Apical aperture hairs</th>
<th>Pyrenoid matrix</th>
<th>Starch grains surrounding the pyrenoid</th>
<th>Golgi bodies</th>
<th>Eyespot</th>
<th>Chloroplast</th>
<th>Nuclear shape, position and cell division</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. indica</em> Arora &amp; Anil</td>
<td>India (Goa); hypersaline to marine</td>
<td>Slightly compressed, elliptical to oval in outline, 10–25 × 7–20 × 6.5–18 µm. Distinct creases divide the cell into longitudinal segments</td>
<td>2 types of hairs, both are abundant.</td>
<td>Large, irregular in shape, penetrated by cytoplasmic strands</td>
<td>Convex, irregularly scattered</td>
<td>2–8, located around the flagellar base, a few may be present near the nucleus</td>
<td>Situated below the level of pyrenoid, very conspicuous, red-orange</td>
<td>Cup shaped with 4 anterior lobes, more than 8 lobed posteriorly</td>
<td>Present in the anterior half of the cell, rectangular, shield shaped with an apical groove and a basal arch. Cells develop flagella while surrounded by the parental theca. Cells occasionally divide asymmetrically</td>
<td>Butcher (1959), Hori et al. (1986)</td>
</tr>
<tr>
<td><em>T. alacris</em> Butcher</td>
<td>Europe and North America; marine</td>
<td>Compressed, cuneate in outline, 9–14 µm × 7–10.5 µm</td>
<td>Abundant; single type</td>
<td>Spherical</td>
<td>Concave convex</td>
<td>2, located around the flagellar base</td>
<td>Not conspicuous, up to 2 µm in diameter, located at the level of pyrenoid</td>
<td>Situated towards the anterior portion of cell</td>
<td>Deeply bilobed at the anterior end</td>
<td>Spherical</td>
</tr>
<tr>
<td><em>T. apiculata</em> (Butcher) Butcher</td>
<td>Europe (France); marine</td>
<td>Slightly compressed, broadly elliptical to narrowly oval, 7.5–10.5 × 6.5–4.5–5 µm</td>
<td>ND</td>
<td>Large, spherical</td>
<td>ND</td>
<td>ND</td>
<td>Not present</td>
<td>Large, invaginated with cytoplasmic canaliculi in the posterior part</td>
<td>Lobed anteriorly</td>
<td>Proshkina-Lavrenko (1945), Ettl (1983), Norris et al. (1980)</td>
</tr>
<tr>
<td><em>T. arnoldii</em> (Proshkina-Lavrenko) Norris, Hori &amp; Chihara</td>
<td>Russia, western Ukraine and Spain; marine</td>
<td>Broad side elliptical to oval, narrow side stretched oval, posterior side wider, 12–15 × 9.6–12.5 µm</td>
<td>ND</td>
<td>Basal, Spherical, located in the posterior part of cell</td>
<td>ND</td>
<td>ND</td>
<td>Conspicious, small, anterior, subcircular</td>
<td>Cup shaped, bilobed at the anterior end</td>
<td>Centrally placed.</td>
<td>Proshkina-Lavrenko (1945), Ettl (1983), Norris et al. (1980)</td>
</tr>
<tr>
<td><em>T. ascus</em> (Proskauer) Norris, Hori &amp; Chihara</td>
<td>Pacific coast of North America and Japan; marine, growing densely on rocks or on shells</td>
<td>Plants colonial and colony forming an aseptate stalk, cells elliptical, 19–30 µm × 8–16 µm</td>
<td>Hairs absent</td>
<td>Large, circular</td>
<td>Lens-shaped starch grains</td>
<td>5, surrounding the basal body.</td>
<td>Conspicuous, located in the anterior third of cell</td>
<td>Large, forming 4 lobes</td>
<td>Spherical and large</td>
<td>Proskauer (1950), Hori &amp; Chihara (1974), Tanimoto &amp; Hori (1975), Hori et al. (1983), Hori et al. (1982)</td>
</tr>
<tr>
<td><em>T. astigmatica</em> Norris &amp; Hori</td>
<td>Pacific coast of North America; brackish or marine (salt marsh)</td>
<td>Spherical, 11–19 × 7–16 µm</td>
<td>Sparse, single type</td>
<td>Large, located in the posterior part of the cell</td>
<td>Lens shaped</td>
<td>2–4, surrounding the basal body</td>
<td>Not present</td>
<td>Large, invaginated with cytoplasmic canaliculi in the posterior part</td>
<td>Lobed anteriorly</td>
<td>(continued)</td>
</tr>
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<tr>
<td><em>T. bolosiana</em> Norris, Hori &amp; Chihara</td>
<td>Spain; marine</td>
<td>Compressed, obovate, 15–22 × 10–16 × 6–10 µm</td>
<td>ND</td>
<td>Small, spherical, located in the posterior part of cell</td>
<td>ND</td>
<td>ND</td>
<td>Conspicuous, red, elongated</td>
<td>Anteriorly bilobed, and then irregular and perforated</td>
<td>ND</td>
<td>Margalef (1946), Norris <em>et al.</em> (1980)</td>
</tr>
<tr>
<td><em>T. chuii</em> (chui) Butcher</td>
<td>Europe and N America; marine</td>
<td>Compressed, elliptical to obovate, 12–16 × 7–10 µm</td>
<td>Abundant, single type</td>
<td>Small, irregular in shape with angular outline</td>
<td>Concave convex 2</td>
<td>2</td>
<td>Large, conspicuous, located in the upper region of pyrenoid</td>
<td>Finely lobed</td>
<td>Lobed anteriorly</td>
<td>Butcher (1959), Hori <em>et al.</em> (1986)</td>
</tr>
<tr>
<td><em>T. contracta</em> (N. Carter) Butcher</td>
<td>UK; marine</td>
<td>Compressed, broadly elliptical, 25 × 17 × 11 µm</td>
<td>ND</td>
<td>Basal, medium, oval or irregular.</td>
<td>ND</td>
<td>ND</td>
<td>Central to anterior, small, conspicuous</td>
<td>Two large and two small apical lobes</td>
<td>ND</td>
<td>Carter (1937), Butcher (1959)</td>
</tr>
<tr>
<td><em>T. convolutae</em> (Parke &amp; Manton) Butcher</td>
<td>Europe and Japan; marine</td>
<td>Compressed, shape variable, occasionally curved, 8–13 × 6–10 × 4–6 µm. Theca not stratified</td>
<td>Hairs absent</td>
<td>Conspicuous, 2–4 µm, in posterior third of body, appearing eccentric</td>
<td>Concave towards pyrenoid</td>
<td>2–4</td>
<td>Exceptionally large, 1–2.3 µm, pale orange-red, oval to oblong, located in anterior third of body in one of the plastid lobes</td>
<td>Yellow green, companulate, with four lobes extending forward from just behind the middle of body</td>
<td>Central, immediately anterior to the pyrenoid</td>
<td>Butcher (1959), Parke &amp; Manton (1967)</td>
</tr>
<tr>
<td><em>T. cordiformis</em> (N. Carter) Stein</td>
<td>Cosmopolitan; brackish and fresh waters</td>
<td>Compressed, obovate, 17–19 × 13–16 × 8–11 µm</td>
<td>Hairs absent</td>
<td>Large, penetrated from all directions with canaliculi or cytoplasmic strands</td>
<td>Biconvex 2–4, around the basal body complex</td>
<td>4</td>
<td>Large, highly reticulate in the posterior region</td>
<td>Spherical, lobed</td>
<td>Stein (1878), Hori <em>et al.</em> (1982)</td>
<td></td>
</tr>
<tr>
<td><em>T. desikacharyi</em> Marin, Hoef-Emden &amp; Melkonian</td>
<td>France; marine</td>
<td>Not compressed, elliptical in broad lateral view, 11–13 × 9–12 × 7–10 µm</td>
<td>Hairs absent</td>
<td>Located posteriorly, surrounded by a closed starch sheath and penetrated by cytoplasmic channels</td>
<td>Concave convex 2–4, parabasal</td>
<td>4</td>
<td>Very large, 2–3 µm in diameter, located in the anterior part of cell</td>
<td>Cup shaped and divided into &gt; 6 lobes anteriorly</td>
<td>Irregular in shape, nonspherical</td>
<td>Marin <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>T. fontiana</em> (Margalef) Norris, Hori &amp; Chihara</td>
<td>Europe: Balearic Islands and Spain</td>
<td>Cells compressed, oval, 14–20 × 8–12 × 5–6 µm</td>
<td>ND</td>
<td>Located in the posterior third of cell, rounded, surrounded by amyloidal (starch) cells</td>
<td>ND</td>
<td>ND</td>
<td>Conspicuous, small, subicular, red, located adjacent to pyrenoid</td>
<td>4 anterior lobes and 4 short posterior lobes</td>
<td>ND</td>
<td>Margalef (1946); Norris <em>et al.</em> (1980), Ettl (1983)</td>
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<tr>
<td><em>T. gracilis</em> (Kylin) Butcher</td>
<td>Europe; marine</td>
<td>Compressed, broadly to narrowly elliptical, 8–9 × 5.5–7.5 × 5–6.5 µm</td>
<td>ND</td>
<td>Conspicuous, sub-basal, large, spherical with a U-shaped starch sheath</td>
<td>Concave convex, large starch grains</td>
<td>ND</td>
<td>Large, conspicuous, red orange, situated in the anterior half of the cell and well above pyrenoid</td>
<td>Uniformly and markedly rugose, yellow green, axile</td>
<td>ND</td>
<td>Butcher (1959)</td>
</tr>
<tr>
<td><em>T. hazeni</em> Butcher</td>
<td>Europe: Spain, and USA; marine</td>
<td>Compressed, elliptical to oval, 13–17 × 7–8 × 4–5 µm</td>
<td>ND</td>
<td>Basal, cup shaped, rather large</td>
<td>ND</td>
<td>ND</td>
<td>Bright green, cup shaped, with 4 anterior lobes but non posterior</td>
<td>ND</td>
<td>Butcher (1959)</td>
<td></td>
</tr>
<tr>
<td><em>T. helgolandica</em> (Kylin) Butcher</td>
<td>Helgoland; marine</td>
<td>Compressed, oval, 21–24 × 14–15 × 7–9 µm</td>
<td>ND</td>
<td>Sub-central to sub-basal, conspicuous, spherical</td>
<td>Large</td>
<td>ND</td>
<td>Stigma 3–6, scattered</td>
<td>Bright green, axile, with a sinus reaching down to pyrenoid, a shorter posterior lobe, and two longitudinal lateral lobes</td>
<td>ND</td>
<td>Butcher (1959)</td>
</tr>
<tr>
<td><em>T. impellucida</em> (McLachlan &amp; Parke) Norris, Hori &amp; Chihara</td>
<td>Puerto Rico; marine</td>
<td>Slightly compressed, shape variable, 14–23 × 8–17 µm</td>
<td>Sparse, single type</td>
<td>Inconspicuous by light microscopy, lying posterior to nucleus, penetrated by cytoplasmic canaliculi</td>
<td>Pyrenoid lacking a starch shell</td>
<td>ND</td>
<td>Stigma pale, orange-red, usually single but may be multiple, irregular in outline, position variable but never in anterior part of the body</td>
<td>Yellow green, cup shaped, covering the peripheral region with a slit from cell apex to middle of the body</td>
<td>Large, rounded</td>
<td>McLachlan &amp; Parke (1967)</td>
</tr>
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<tr>
<td>T. inconspicua Butcher</td>
<td>Europe; marine</td>
<td>Slightly compressed, oval in front, elliptical in lateral view; 4.5–7 × 4.5–6 × 3.5–4 µm</td>
<td>ND</td>
<td>Basal, very small, globular, with a continuous starch sheath</td>
<td>ND</td>
<td>ND</td>
<td>Conspicuous, in the region of pyrenoid, reddish orange</td>
<td>Anterior two lobed to the centre of the cell</td>
<td>ND</td>
<td>Butcher (1959)</td>
</tr>
<tr>
<td>T. levis Butcher</td>
<td>England; marine</td>
<td>Compressed, ovate, 9–12 × 6–7.5 µm</td>
<td>Poorly developed and scanty</td>
<td>Small, irregular in shape with angular outline</td>
<td>Biconvex 2</td>
<td>ND</td>
<td>Same size as of pyrenoid, located at about the same level</td>
<td>Finely lobed</td>
<td>ND</td>
<td>Non-spherical</td>
</tr>
<tr>
<td>T. maculata Butcher</td>
<td>Europe, collected from salt marsh pools and apparently not common; marine</td>
<td>Slightly compressed, ovate in front, elliptical in lateral view; 5.5–7.5 × 5–6.5 µm</td>
<td>ND</td>
<td>Basal, medium or small, usually with a discontinuous starch sheath</td>
<td>Small</td>
<td>ND</td>
<td>Stigma large, conspicuous, at least half the size of, and close to pyrenoid, irregularly rounded, diffuse, orange</td>
<td>Yellow green, rugose or finely granular, anterior two lobed, sinus wide, reaching up to pyrenoid</td>
<td>ND</td>
<td>Butcher (1959)</td>
</tr>
<tr>
<td>T. marina (Cienkowski) Norris, Hori &amp; Chihara</td>
<td>Europe, North America and Japan; marine</td>
<td>Plants unicellular or colonial with a septate stalk, cells elliptical, 16–20 × 7–8 µm</td>
<td>Hairs absent</td>
<td>Large, almost spherical</td>
<td>Concave on the side adjacent to the pyrenoid matrix</td>
<td>Usually 5, in a circle near the anterior end of the nucleus</td>
<td>ND</td>
<td>Stigma conspicuous, located peripherally at a level between the nucleus and the pyrenoid</td>
<td>Massively cup shaped, located peripherally with 4 anterior lobes, irregularly lobed posteriorly</td>
<td>Irregular, with a lobe penetrating the pyrenoid matrix. Longitudinal division</td>
</tr>
<tr>
<td>T. mediterranea (Lucksch) Norris, Hori &amp; Chihara</td>
<td>France; marine</td>
<td>Cells flattened dorsi-ventrally, rounded base, 15–25 × 10–20 µm</td>
<td>ND</td>
<td>Spherical to elliptical, in the rear third of cell</td>
<td>ND</td>
<td>ND</td>
<td>Stigma small, conspicuous, at the same position as pyrenoid</td>
<td>Coat-shaped, on the one side a narrow column releases that passes until to the rear end of the cell</td>
<td>Positioned in the anterior end before the middle part of the cell</td>
<td>Lucksch (1932), Ettl &amp; Ettl (1959), Norris et al. (1980), Ettl (1983), Butcher (1959)</td>
</tr>
<tr>
<td>T. rubens Butcher</td>
<td>Europe; marine</td>
<td>Compressed, 8–11 × 5–8 × 4.5–5 µm. (Similar to T. verrucosa except reddish due to haematochrome)</td>
<td>ND</td>
<td>Basal, medium, globular with a U-like starch sheath</td>
<td>Concave convex ND</td>
<td>ND</td>
<td>Conspicuous, in the anterior to middle region, orange, dense, large</td>
<td>ND</td>
<td>Positioning of the posterior end of the cell</td>
<td>Hori et al. (1984)</td>
</tr>
<tr>
<td>T. striata Butcher</td>
<td>Europe and Japan; marine</td>
<td>Compressed elliptical, 7–11 × 5.5–7.2 µm</td>
<td>Poorly developed and scanty</td>
<td>Small, circular</td>
<td>ND</td>
<td>2</td>
<td>Conspicuous, larger than pyrenoid matrix, located lateral to the pyrenoid</td>
<td>ND</td>
<td>Irregular</td>
<td>Hori et al. (1986)</td>
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<td><em>T. subcordiformis</em></td>
<td>Norway; marine</td>
<td>Compressed, elliptical, 11–17 × 8–10 µm</td>
<td>ND</td>
<td>Sub-central to sub-basal, large, spherical, conspicuous</td>
<td>Large</td>
<td>ND</td>
<td>In lower part of the cell near the pyrenoid</td>
<td>Bright green, axle, a shorter posterior lobe and two lateral lobes</td>
<td>ND</td>
<td>Butcher (1959)</td>
</tr>
<tr>
<td><em>T. suecica</em> (Kylin)</td>
<td>Widely distributed; brackish, marine</td>
<td>Compressed, elliptical to obovate, 6–11 × 4–8.5 µm</td>
<td>Single type</td>
<td>Spherical, large</td>
<td>Concave convex 2</td>
<td>ND</td>
<td>Not conspicuous</td>
<td>Cup shaped, usually simple, rarely bilobed at the posterior part</td>
<td>Spherical</td>
<td>Hori <em>et al.</em> (1986)</td>
</tr>
<tr>
<td><em>T. tetrathele</em> (West)</td>
<td>Europe, widely distributed and common; marine</td>
<td>Compressed, elliptical, 10–16 × 8–11 × 4.2–5 µm</td>
<td>ND</td>
<td>Pyrenoid conspicuous, large, sub-central to sub-basal, spherical</td>
<td>Large</td>
<td>ND</td>
<td>Sub-median, usually situated in the region of upper part of pyrenoid, a shorter posterior lobe and two lateral lobes</td>
<td>Bright green, axle with a narrow sinus reaching to pyrenoid, a shorter posterior lobe and two lateral lobes</td>
<td>ND</td>
<td>West (1916), Carter (1937), Butcher (1959)</td>
</tr>
<tr>
<td><em>T. verrucosa</em> Butcher</td>
<td>Europe and Japan; marine</td>
<td>Compressed, elliptical in front view with a deep apical furrow in lateral view, 8.5–10 × 6–6.5 × 4.5–6 µm. Warty appearance due to irregularly scattered plastids, starch grains and other irregular refractive bodies</td>
<td>Poorly covered with hairs or bare, single type</td>
<td>Spherical, sub-basal, or at times central, small, with a starch sheath of uniform outline</td>
<td>Concave on the side adjacent to pyrenoid matrix</td>
<td>ND</td>
<td>Usually 2, rarely three</td>
<td>Conspicuous and variable in position but usually located at or above the middle of the cell, orange</td>
<td>Bright, yellow-green, massive, with 2 anterior lobes near the pyrenoid and 4 or more sublobes in anterior region, not lobed posteriorly</td>
<td>ND</td>
</tr>
<tr>
<td><em>T. wettsteinii</em></td>
<td>Gulf of Naples; marine</td>
<td>Cells strongly compressed, heart shaped, broader than long, 7–9 × 11–12 µm. Cells have a characteristic median yellowish accumulation body</td>
<td>Hairs absent</td>
<td>2 or more asymmetrically positioned pyrenoids surrounded by starch sheath</td>
<td>ND</td>
<td>Usually 2</td>
<td>A faint eyespot located eccentrically in the middle of the cell</td>
<td>Single green chloroplast</td>
<td>Present at the anterior part of the cell</td>
<td>Schiller (1913), Ettl <em>et al.</em> (1959), Throndsen <em>et al.</em> (1988)</td>
</tr>
</tbody>
</table>

1 This species has been transferred to the genus *Scherffelia*, as *Scherffelia incisa* (Nygaard) Massjuk & Lilitska.
much more than the number reported in other Tetraselmis taxa (which generally have 2–4 Golgi bodies). A widely distributed endoplasmic reticulum allows the Golgi bodies to be more widely placed and allows flexibility in the direction of their forming faces.

Although the taxonomy of the genus has been well studied, a comprehensive systematic revision of the genus, combining morphological and molecular data of a large number of species and isolates, is lacking. Such a study will be indispensable to assess the validity of morphology-based species circumscriptions and to examine possible morphological variation across taxa. Phylogenetic studies based on 18S sequence data have shown that different morphospecies (e.g. T. chuii, T. hazenii, T. suecica and T. tetrathele) cluster in a single clade of nearly identical sequences, indicative of intraspecific morphological variation (Lee & Hur, 2009; present study). However, it is likely that these 18S clades may in fact comprise multiple species as 18S sequences have been shown to be too conservative to assess planktonic eukaryotic diversity (Piganeau et al., 2011). More variable molecular markers, such as the rDNA internal transcribed spacer regions or protein coding genes (Verbruggen et al., 2007; Leliaert et al., 2009; McManus & Lewis, 2011; Friedl & Rybalka, 2012; Krienitz & Bock, 2012) will be needed to assess species boundaries within Tetraselmis.

Our phylogenetic analyses showed a very close relationship between T. indica and unidentified Tetraselmis strains from the Great Salt Lake, Utah (USA) (Posewitz et al., unpublished GenBank data). The position of this clade could not be determined with high certainty, although low support was provided for a sister relationship with T. cordiformis (the type species of Tetraselmis). As has been revealed in previous phylogenetic studies (e.g. Guilhou et al., 2004), the position of Scherffelia dubia is unstable based on 18S rDNA sequence data. Our analysis of the Chlorophyta alignment placed Scherffelia within the Tetraselmis clade, while denser taxon sampling resulted in a sister position of Scherffelia to Tetraselmis (although strong support was lacking). A well-supported monophyletic Tetraselmis clade has been recovered based on analyses of complete nuclear- and plastid-encoded rRNA operons (Marin, 2012).

The disjunct geographical distribution of T. indica and the closely related, undescribed species from Utah, both occurring in hypersaline habitats, is notable. However, the current distribution data are likely a result of undersampling. Additional collecting in the tropics, especially in atypical or extreme environments such as hypersaline water bodies, will be required to better understand the diversity, phylogenetic relationships and geographical distributions of Tetraselmis species.

Acknowledgements

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Supplementary information

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article’s online at http://dx.doi.org/10.1080/09670262.2013.768357

Supplementary Video, showing the position of the flagellar groove with respect to distinct creases and the overall appearance of the cell of T. indica.

Nexus files of the alignments used in the phylogenetic analysis.

References


Tetraselmis indica sp. nov.


