Eur. J. Phycol. (2013), **48(1)**: 61–78

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

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(Received 14 June 2011; revised 28 July 2012; accepted 23 August 2012)

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

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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 phycoplast (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 *Symsagittifera* (*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

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ISSN 0967-0262 (print)/ISSN 1469-4433 (online)/13/010061-78 © 2013 British Psycological Society
http://dx.doi.org/10.1080/09670262.2013.768357

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(Becker *et al.*, 1996, 2001; Wustman *et al.*, 2004). *Tetraselmis* includes about 26 currently accepted species, including taxa previously assigned to the genera *Platymonas*, *Prasinocladus* 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; Margalef, 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; Throndsen & 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, 1959; Fabregas *et al.*, 1984). The genus is widely used in aquaculture facilities as feed for juvenile molluscs, shrimp larvae and rotifers (Kim & Hur, 1998; Park & Hur, 2000; Cabrera *et al.*, 2005; Azma *et al.*, 2011). In addition, high lipid-containing strains have potential to be used in biofuel production (Laws & Berning, 1991; Montero *et al.*, 2011; Grierson *et al.*, 2012).

A survey of the nanoplankton from salt pans in Goa, India, revealed the presence of a hitherto undescribed species of *Tetraselmis*. The aim of this paper was to characterize this new species through light, electron and confocal microscopy, and assess its phylogenetic relationship by DNA sequence analysis. In addition genome size was estimated using flow cytometry.

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.

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.

Transmission electron microscopy

Cells were primarily fixed by rinsing several times 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.

Scanning electron microscopy

For scanning electron microscopy (SEM), cultured cells were sampled during the late exponential growth phase and fixed in 2% glutaraldehyde (TAAB) in cacodylate and seawater. Cells were allowed to settle on poly-l-lysine coated coverslips. The coverslips bearing the cell sample were placed in a coverslip holder and dehydrated in an ethanol series (10 min each in 25, 50 and 75% ethanol, followed by two 15 min steps in 100% ethanol at room temperature). The coverslips were

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 S40 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 microlitres 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 PCR 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 (*Chlorokybus* and *Mesostigma*), which were selected as outgroups. A second 18S dataset was used to examine the phylogenetic position of the new species within the Chlorodendrophyceae with more precision. This alignment was created as follows. First, all 18S sequences of Chlorodendrophyceae 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 treboux-iophycean 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 *Pseudo-nitzschia.* 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.

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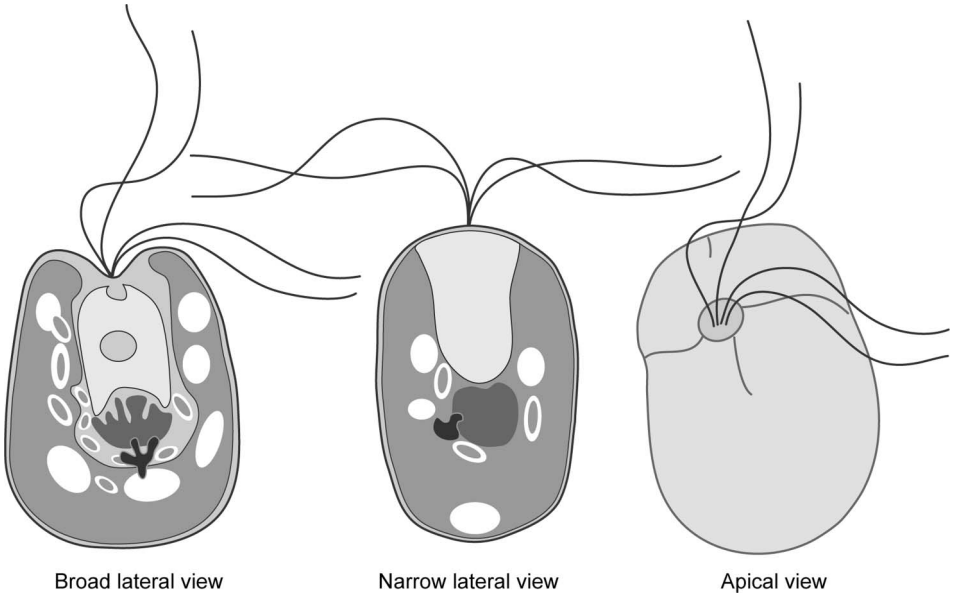

Fig. 1. Schematic drawings of cells of *Tetraselmis indica* in broad lateral view, narrow lateral view and apical view, indicating the position and arrangement of major cell components.

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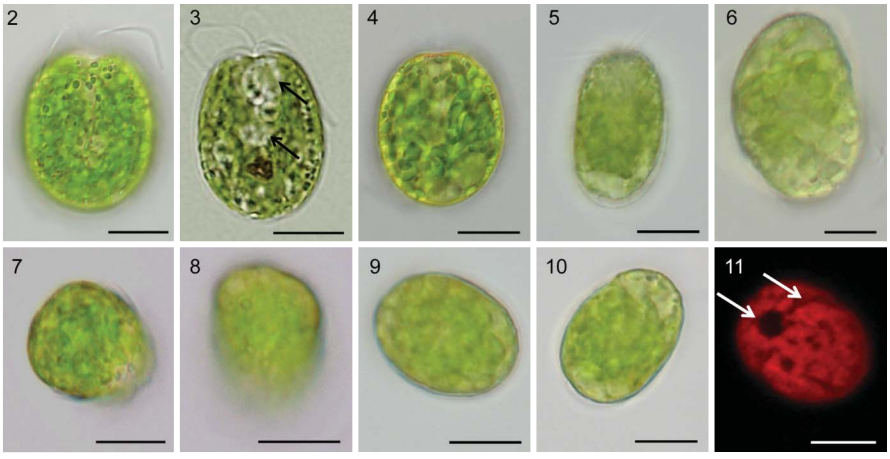
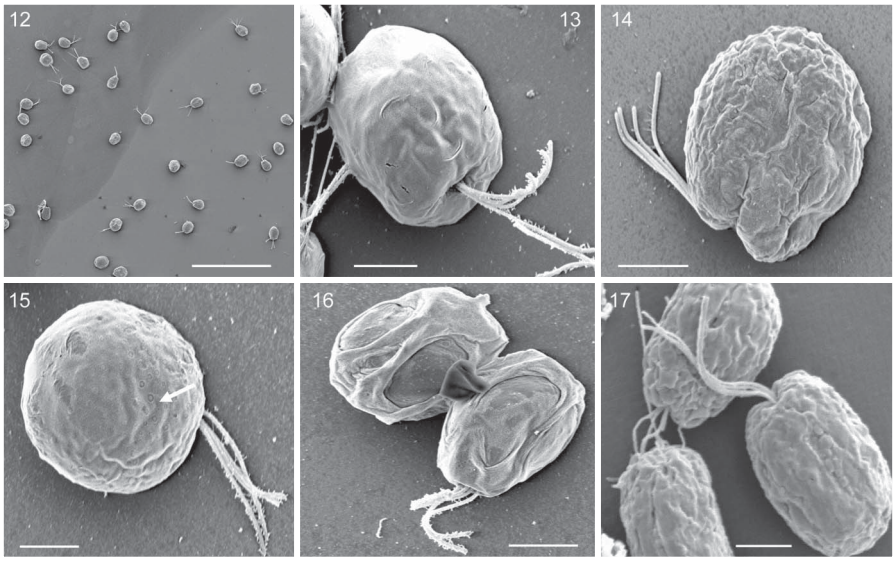
19), posterior to the nucleus and is traversed from several directions by cytoplasmic invaginations. The pyrenoid matrix measures 2.6–2.8 × 3.4–3.6 µm in longitudinal section and is surrounded by many biconvex or concave starch grains (Fig. 19). In addition to the starch grains appressed to the pyrenoid, 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



Figs 2–11. Light micrographs of *T. indica* in broad lateral view. **2.** Cell with emerging flagella. **3.** The positions of the nucleus (upper arrow), pyrenoid (lower arrow) and eyespot (red-brown granules) are visible. **4.** Resting cell. **5, 6.** Narrow lateral view. **7, 8.** Narrow lateral view of a cell attaching to the glass slide via the flagella. **9, 10.** Posterior view. **11.** Confocal micrograph of a cell showing flagellar aperture (left arrow) and distinct creases (e.g. at right arrow). Scale bars = 10 µm.



Figs 12–17. SEM of *T. indica*. **12.** Scatter of cells at low magnification. **13.** Narrow lateral view. **14.** Broad lateral view. **15.** Posterior view with scales (arrow). **16.** Dividing cells that are still enclosed by the parent cell wall. **17.** Individual cells. Scale bars = 200 µm (Fig. 12) or 10 µm (Figs 13–17).

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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.

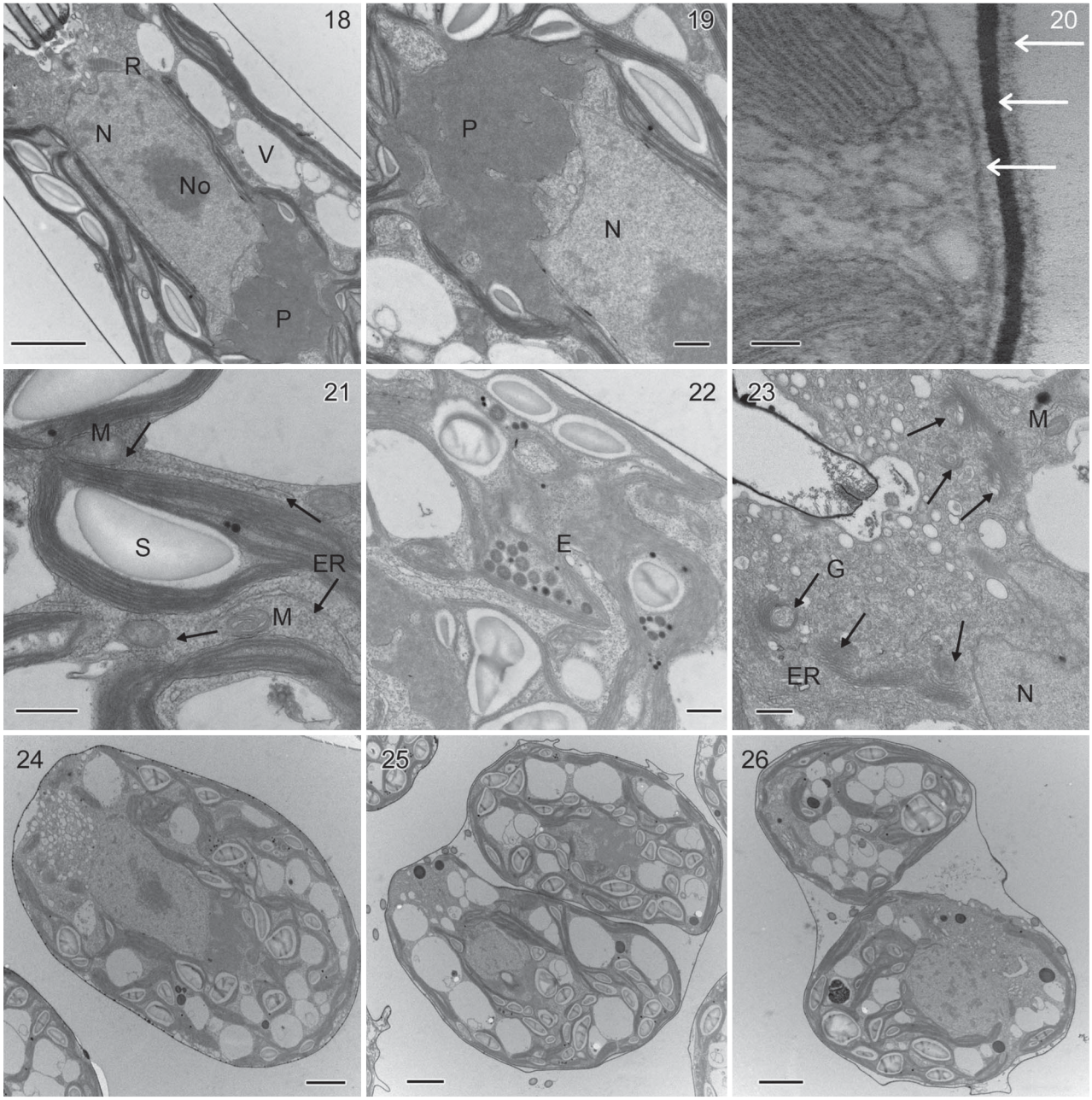
The alga survives during unfavourable periods as cysts, which are capable of rejuvenation and normal development with the return of favourable conditions (Figs 35–40). Apical papilla can be seen when the walls are cast off. Sexual reproduction has not been observed.

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*



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).

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*.

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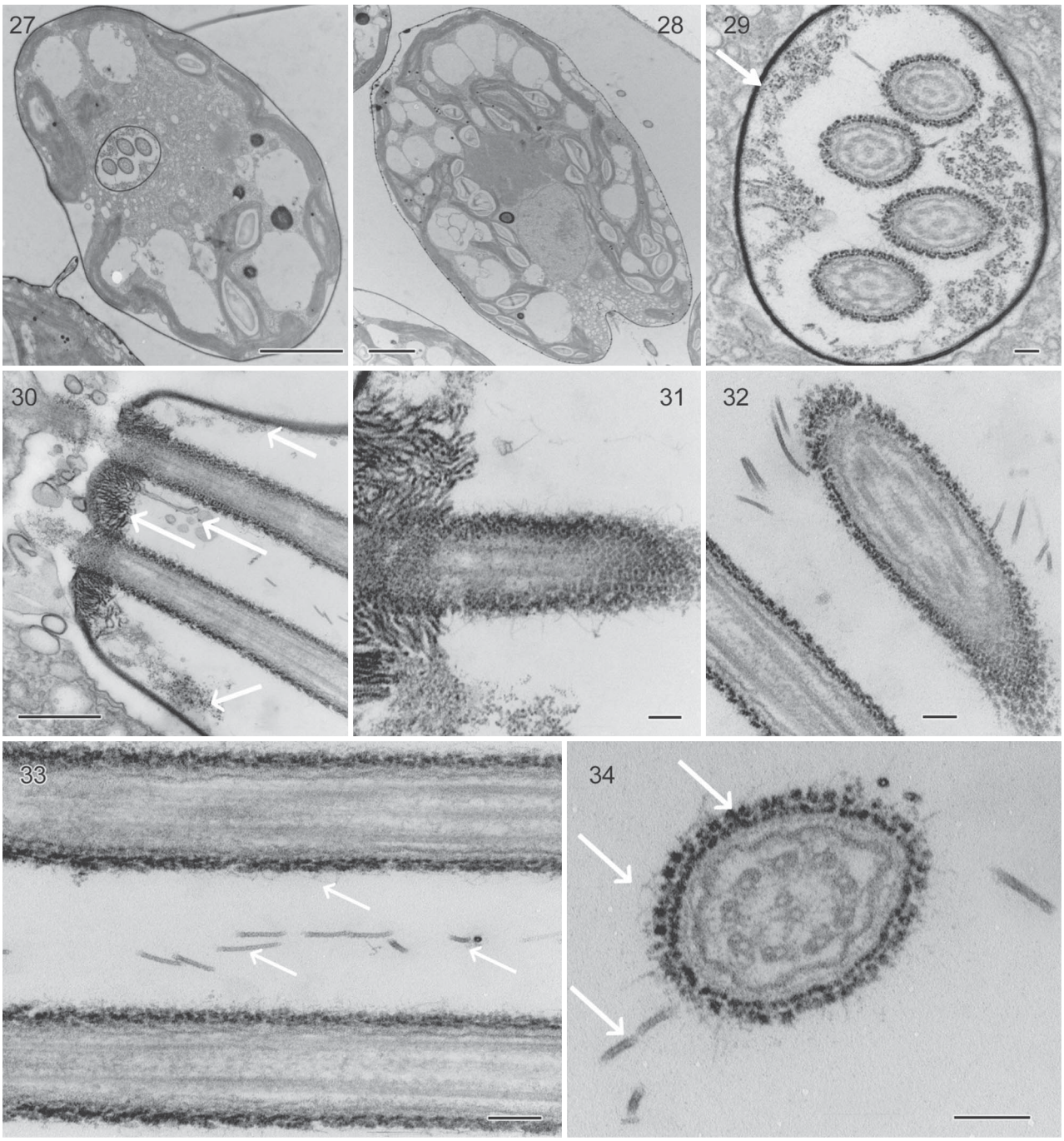
Discussion

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; Throndsen & 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).

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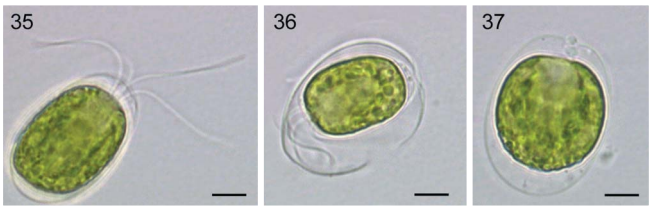
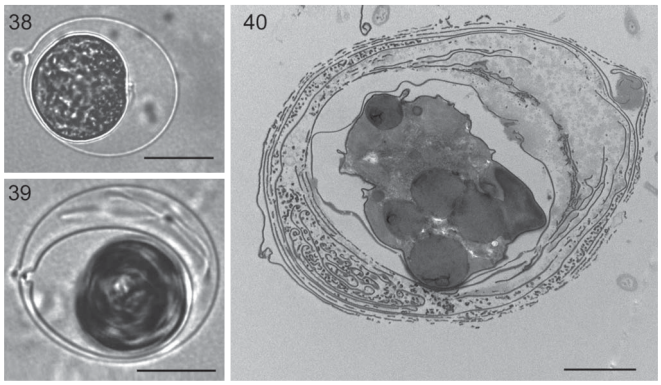


Figs 35–37. *Tetraselmis indica*: stages of transformation of a motile cell into a resting phase, LM. The only pore or opening in the wall is the slit at the base of flagellar pit; this part becomes everted and appears as a very short papilla when the walls are cast off. Scale bars = 5 μm.



Figs 38–40. *Tetraselmis indica*, resting cells. **38, 39.** LM showing formation of an apical papilla in resting cells. **40.** TEM showing concentric rings of discarded walls. Scale bars = 10 μm (Figs 38, 39) or 2 μm (Fig. 40).

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Tetraselmis indica Arora & Anil, ***sp. nov.***

Figs 1–40

Descriptio: 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 flavovirentes, 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 pyrenoidis per canaliculos invasa e cytoplasmate oriundos, 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)

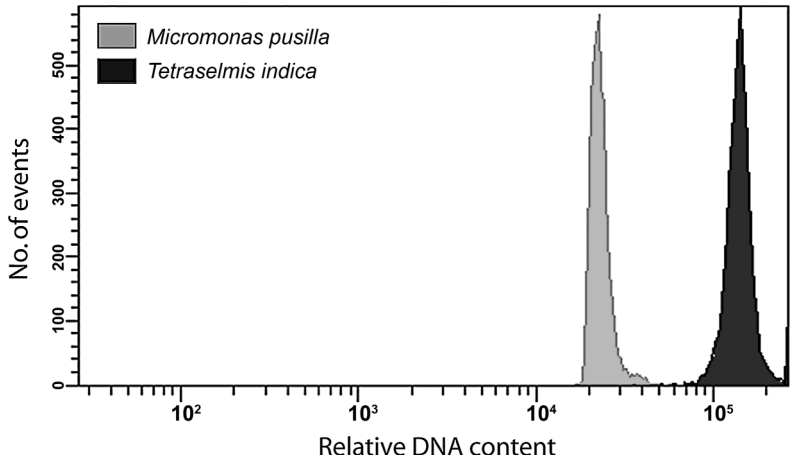

Fig. 41. Flow cytometry analysis of the DNA content of a cell nuclei of *T. indica*, compared to that of *Micromonas pusilla* CCAP 1965/4, which has a genome size of 15 Mbp. Relative DNA content (*x*-axis), no. of events (*y*-axis).

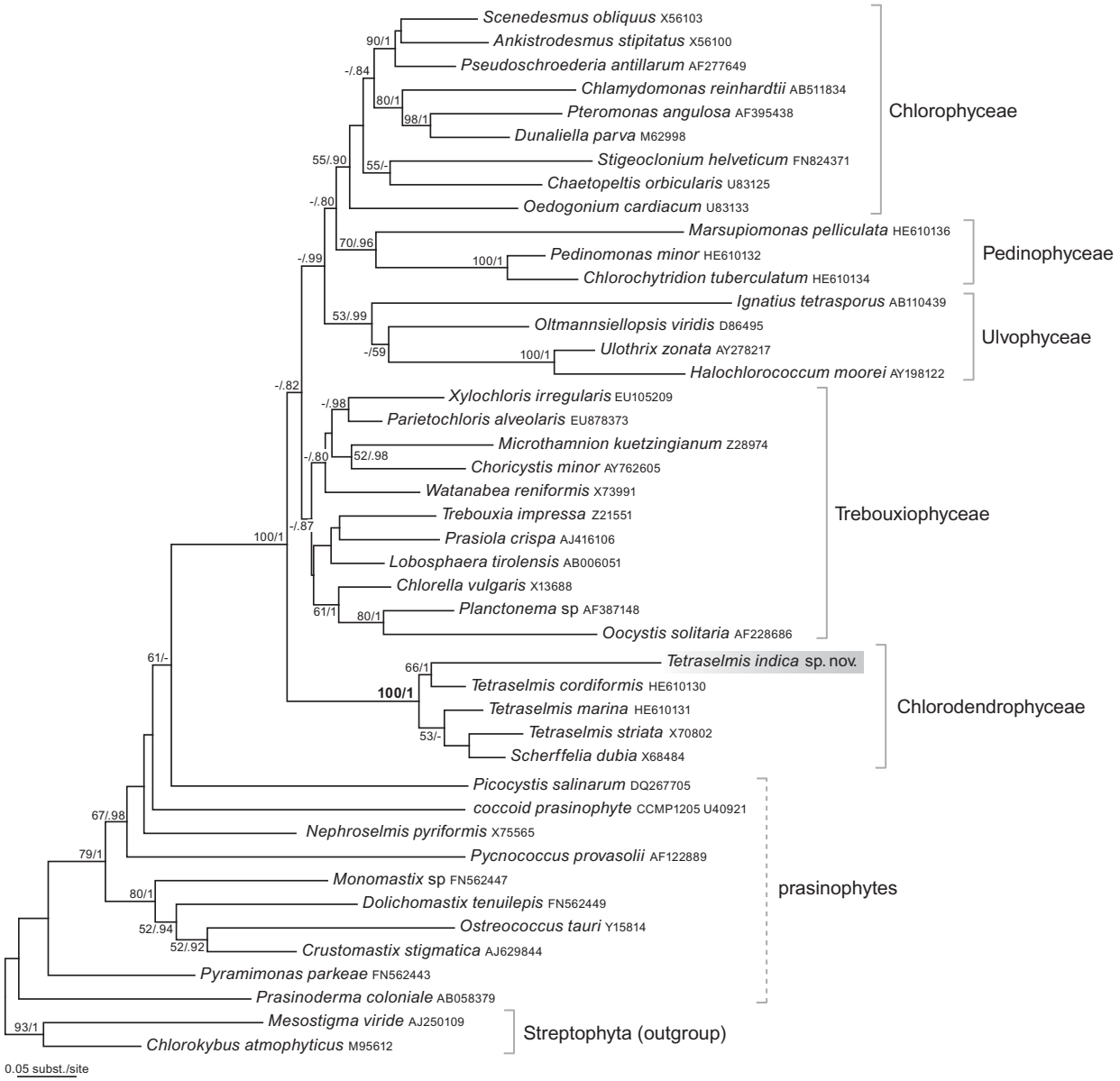

Fig. 42. Maximum likelihood (ML) tree of the Chlorophyta inferred from 18S rDNA sequences, showing the phylogenetic position of *Tetraselmis indica* within the Chlorodendrophyceae. ML bootstrap values (> 50) and Bayesian inference (BI) posterior probabilities (> 0.80) are indicated at the branches, respectively.

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aurantiacum 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 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. Dictyosomes usually 2–8, positioned in a circle near the anterior end of the nucleus and sometimes on the sides. Endoplasmic reticulum widely distributed.

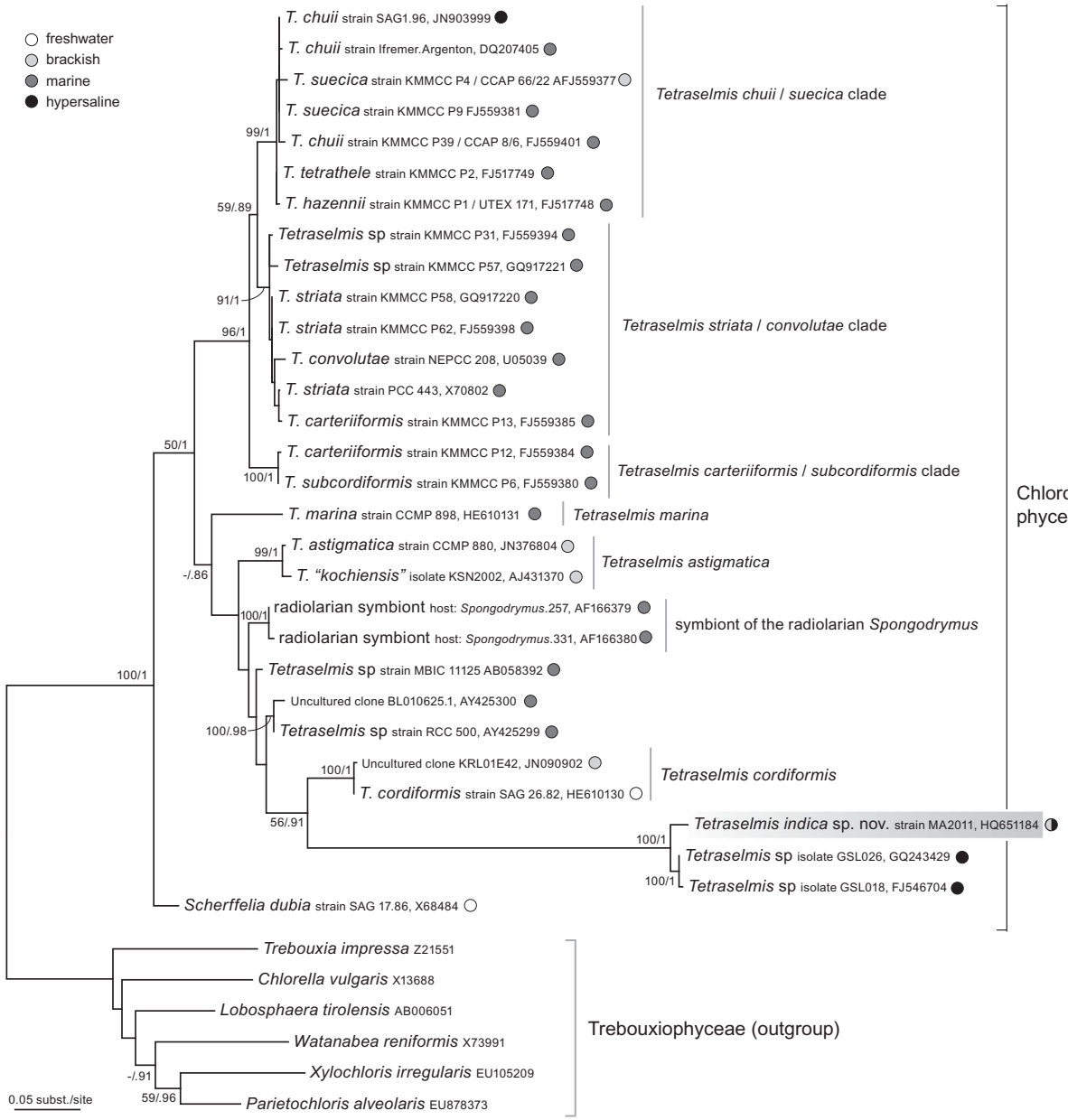

Fig. 43. Maximum likelihood (ML) tree of the Chlorodendrophyceae inferred from 18S rDNA sequences, showing the phylogenetic position of *Tetraselmis indica*. ML bootstrap values (> 50) and Bayesian inference (BI) posterior probabilities (> 0.80) are indicated at the branches, respectively. Species names are adopted from GenBank or the culture collections. Strain or sample information, and habitat type (freshwater/brackish/marine/hypersaline) is provided for each sequence.

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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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.

Species	Known geographical distribution and the type of habitat	Cell shape and size	Apical aperture hairs	Pyrenoid matrix	Starch grains surrounding the pyrenoid	Golgi bodies	Eyespot	Chloroplast	Nuclear shape, position and cell division	References
T. indica Arora & Anil	India (Goa); hypersaline to marine	Slightly compressed, elliptical to oval in outline, 10–25 × 7–20 × 6.5–18 µm. Distinct creases divide the cell into longitudinal segments	2 types of hairs, both are abundant.	Large, irregular in shape, penetrated by cytoplasmic strands	Convex, irregularly scattered	2–8, located around the flagellar base, a few may be present next to the nucleus	Situated below the level of pyrenoid, very conspicuous, red-orange	Cup shaped with 4 anterior lobes, more than 8 lobed posteriorly	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	This study
T. alacris Butcher	Europe and North America; marine	Compressed, cuneate in outline, 9–14 µm × 7–10.5 µm	Abundant;single type	Spherical	Concave convex	2, located around the flagellar base	Not conspicuous, up to 2 µm in diameter, located at the level of pyrenoid	Finely lobed	Spherical	Butcher (1959), Hori *et al*. (1986)
T. apiculata (Butcher) Butcher	Europe (France); marine	Slightly compressed, broadly elliptical to narrowly oval, 7.5–10.5 × 6.5 × 4.5–5 µm	ND	Large, spherical	ND	ND	Situated towards the anterior portion of cell	Deeply bilobed at the anterior end	ND	Butcher (1959)
T. arnoldii (Proshkina-Lavrenko) Norris, Hori & Chihara	Russia, western Ukraine and Spain); marine	Broad side elliptical to oval, narrow side stretched oval, posterior side wider, 12–15 × 9.6–12.5 µm	ND	Basal, Spherical, located in the posterior part of cell	ND	ND	Conspicuous, small, anterior, subcircular	Cup shaped, bilobed at the anterior end	Centrally placed.	Proshkina-Lavrenko (1945), Ettl (1983), Norris *et al*. (1980)
T. ascus (Proskauer) Norris, Hori & Chihara	Pacific coast of North America and Japan; marine, growing densely on rocks or on shells	Plants colonial and colony forming an aseptate stalk, cells elliptical, 19–30 µm × 8–16 µm	Hairs absent	Large, circular	Lens-shaped starch grains	5, surrounding the basal body.	Conspicuous, located in the anterior third of cell	Large, forming 4 lobes	Spherical and large	Proskauer (1950), Hori & Chihara (1974), Tanimoto & Hori (1975), Hori *et al*. (1983)
T. astigmatica Norris & Hori	Pacific coast of North America; brackish or marine (salt marsh)	Spherical, 11–19 × 7–16 µm	Sparse, single type	Large, located in the posterior part of the cell	Lens shaped	2–4, surrounding the basal body	Not present	Large, invaginated with cytoplasmic canaliculi in the posterior part	Lobed anteriorly	Hori *et al*. (1982)

(*continued*)

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Table 1. Continued

Species	Known geographical distribution and the type of habitat	Cell shape and size	Apical aperture hairs	Pyrenoid matrix	Starch grains surrounding the pyrenoid	Golgi bodies	Eyespot	Chloroplast	Nuclear shape, position and cell division	References
T. bolosiana Norris, Hori & Chihara	Spain; marine	Compressed, obovate, 15–22 × 10–16 × 6–10 µm	ND	Small, spherical, located in the posterior part of cell	ND	ND	Conspicuous, red, elongated	Anteriorly bilobed, and then irregular and perforated	ND	Margalef (1946), Norris *et al*. (1980)
T. chuii (*chui*) Butcher	Europe and N America; marine	Compressed, elliptical to obovate, 12–16 × 7–10 µm	Abundant,single type	Small, irregular in shape with angular outline	Concave convex	2	Large, conspicuous, located in the upper region of pyrenoid	Finely lobed	Lobed anteriorly	Butcher (1959), Hori *et al*. (1986)
T. contracta (N. Carter) Butcher	UK; marine	Compressed, broadly elliptical, 25 × 17 × 11 µm.	ND	Basal, medium, oval or irregular.	ND	ND	Central to anterior, small, conspicuous	Two large and two small apical lobes	ND	Carter (1937), Butcher (1959)
T. convolutae (Parke & Manton) Butcher	Europe and Japan; marine	Compressed, shape variable, occasionally curved, 8–13 × 6–10 × 4–6 µm. Theca not stratified	Hairs absent	Conspicuous, 2–4 µm, in posterior third of body, appearing eccentric	Concave towards pyrenoid	2–4	Exceptionally large, 1–2.3 µm, pale orange-red, oval to oblong, located in anterior third of body in one of the plastid lobes	Yellow green, companulate, with four lobes extending forward from just behind the middle of body	Central, immediately anterior to the pyrenoid	Butcher (1959), Parke & Manton (1967)
T. cordiformis (N. Carter) Stein	Cosmopolitan; brackish and fresh waters	Compressed, obovate, 17–19 × 13–16 × 8–11 µm	Hairs absent	Large, penetrated from all directions with canaliculi or cytoplasmic strands	Biconvex	2–4, around the basal body complex	Stigma located in the middle of one of the cell's broad sides	Large, highly reticulate in the posterior region	Spherical, lobed	Stein (1878), Hori *et al*. (1982)
T. desikacharyi Marin, Hoef-Emden & Melkonian	France; marine	Not compressed, elliptical in broad lateral view, 11–13 × 9–12 × 7–10 µm	Hairs absent	Located posteriorly, surrounded by a closed starch sheath and penetrated by cytoplasmic channels	Concave convex	2(–4), parabasal	Very large, 2–3 µm in diameter, located in the anterior part of cell	Cup shaped and divided into > 6 lobes anteriorly	Irregular in shape, non-spherical	Marin *et al*. (1996)
T. fontiana (Margalef) Norris, Hori & Chihara	Europe: Balearic Islands and Spain	Cells compressed, oval, 14–20 × 8–12 × 5–6 µm	ND	Located in the posterior third of cell, rounded, surrounded by amyloid (starch) cells	ND	ND	Conspicuous, small, subcircular, red, located adjacent to pyrenoid	4 anterior lobes and 4 short posterior lobes	ND	Margalef (1946); Norris *et al*. (1980), Ettl (1983)

(*continued*)

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Table 1. Continued

Species	Known geographical distribution and the type of habitat	Cell shape and size	Apical aperture hairs	Pyrenoid matrix	Starch grains surrounding the pyrenoid	Golgi bodies	Eyespot	Chloroplast	Nuclear shape, position and cell division	References
T. gracilis (Kylin) Butcher	Europe; marine	Compressed, broadly to narrowly elliptical, 8–9 × 5.5–7.5 × 5–6.5 µm	ND	Conspicuous, sub-basal, large, spherical with a U-shaped starch sheath	Concave convex, large starch grains	ND	Large, conspicuous, red orange, situated in the anterior half of the cell and well above pyrenoid	Uniformly and markedly rugose, yellow green, axile	ND	Butcher (1959)
T. hazeni Butcher	Europe: Spain, and USA; marine	Compressed, elliptical to oval, 13–17 × 7–8 × 4–5 µm	ND	Basal, cup shaped, rather large	ND	ND	Small, sub-central, usually situated in the upper part of the pyrenoid	Bright green, cup shaped, with 4 anterior lobes but non posterior	ND	Butcher (1959)
T. helgolandica (Kylin) Butcher	Helgoland; marine	Compressed, oval, 21–24 × 14–15 × 7–9 µm	ND	Sub-central to sub-basal, conspicuous, spherical	Large	ND	Stigma 3–6, scattered	Bright green, axile, with a sinus reaching down to pyrenoid, a shorter posterior lobe, and two longitudinal lateral lobes	ND	Butcher (1959)
T. impellucida (McLachlan & Parke) Norris, Hori & Chihara	Puerto Rico; marine	Slightly compressed, shape variable, 14–23 × 8–17 µm	Sparse, single type	Inconspicuous by light microscopy, lying posterior to nucleus, penetrated by cytoplasmic canaliculi	Pyrenoid lacking a starch shell	ND	Stigma pale, orange-red, usually single but may be multiple, irregular in outline, position variable but never in anterior part of the body	Yellow green, cup shaped, covering the peripheral region with a slit from cell apex to middle of the body	Large, rounded	McLachlan & Parke (1967)
T. incisa (Nygaard) Norris, Hori & Chihara[1]	Russia, Ukraine; marine	Slightly compressed, 13.5–17.5 × 10.5–13.5 × 7–9 µm	ND	Lacks a pyrenoid	ND	ND	Conspicuous, round, small	ND	Central	Nygaard (1949), Norris *et al*. (1980), Ettl (1983), Massjuk & Lilitskaya (1999), Massjuk & Lilitska (2006)

(*continued*)

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Table 1. Continued

Species	Known geographical distribution and the type of habitat	Cell shape and size	Apical aperture hairs	Pyrenoid matrix	Starch grains surrounding the pyrenoid	Golgi bodies	Eyespot	Chloroplast	Nuclear shape, position and cell division	References
T. inconspicua Butcher	Europe; marine	Slightly compressed, oval in front, elliptical in lateral view, 4.5–7 × 4.5–6 × 3.5–4 µm	ND	Basal, very small, globular, with a continuous starch sheath	ND	ND	Conspicuous, in the region of pyrenoid, reddish orange	Anterior two lobed to the centre of the cell	ND	Butcher (1959)
T. levis Butcher	England; marine	Compressed, ovate, 9–12 × 6–7.5 µm	Poorly developed and scanty	Small, irregular in shape with angular outline	Biconvex	2	Same size as of pyrenoid, located at about the same level	Finely lobed	Non-spherical	Hori *et al.* (1986)
T. maculata Butcher	Europe, collected from salt marsh pools and apparently not common; marine	Slightly compressed, ovate in front, elliptical in lateral view, 8–9 × 5.5–7.5 × 5–6.5 µm	ND	Basal, medium or small, usually with a discontinuous starch sheath	Small	ND	Stigma large, conspicuous, at least half the size of, and close to pyrenoid, irregularly rounded, diffuse, orange	Yellow green, rugose or finely granular, anterior two lobed, sinus wide, reaching up to pyrenoid	ND	Butcher (1959)
T. marina (Cienkowski) Norris, Hori & Chihara	Europe, North America and Japan; marine	Plants unicellular or colonial with a septate stalk, cells elliptical, 16–20 × 7–8 µm	Hairs absent	Large, almost spherical	Concave on the side adjacent to the pyrenoid matrix	Usually 5, in a circle near the anterior end of the nucleus	Stigma conspicuous, located peripherally at a level between the nucleus and the pyrenoid	Massive cup shaped, located peripherally with 4 anterior lobes, irregularly lobed posteriorly	Irregular, with a lobe penetrating the pyrenoid matrix. Longitudinal division	Norris *et al.* (1980), Hori *et al.* (1983)
T. mediterranea (Lucksch) Norris, Hori & Chihara	France; marine	Cells flattened dorsiventrally, rounded base, 15–25 × 10–20 µm	ND	Spherical to elliptical, in the rear third of cell	ND	ND	Stigma small, conspicuous, at the same position as pyrenoid	Coat-shaped, on the one side a narrow column releases that passes until to the rear end of the cell	Positioned in the anterior end before the middle part of the cell	Lucksch (1932), Ettl & Ettl (1959), Norris *et al.* (1980), Ettl (1983)
T. rubens Butcher	Europe; marine	Compressed, 8–11 × 5–8 × 4.5–5 µm. (Similar to *T. verrucosa* except reddish due to haematochrome)	ND	Basal, medium, globular with a U-like starch sheath	Concave convex	ND	Conspicuous, in the anterior to middle region, orange, dense, large	Green, anterior deeply 2 lobed, sinus long and narrow, presence of haematochrome	ND	Butcher (1959)
T. striata Butcher	Europe and Japan; marine	Compressed elliptical, 7–11 × 5.5–7.2 µm	Poorly developed and scanty	Small, circular	ND	2	Conspicuous, larger than pyrenoid matrix, located lateral to the pyrenoid	ND	Irregular	Hori *et al.* (1986)

(*continued*)

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Table 1. Continued

Species	Known geographical distribution and the type of habitat	Cell shape and size	Apical aperture hairs	Pyrenoid matrix	Starch grains surrounding the pyrenoid	Golgi bodies	Eyespot	Chloroplast	Nuclear shape, position and cell division	References
T. subcordiformis (Wille) Butcher	Norway; marine	Compressed, elliptical, 11–17 × 8–10 µm	ND	Sub-central to sub-basal, large, spherical, conspicuous	Large	ND	In lower part of the cell near the pyrenoid	Bright green, axile, a shorter posterior lobe and two lateral lobes	ND	Butcher (1959)
T. suecica (Kylin) Butcher	Widely distributed; brackish, marine	Compressed, elliptical to obovate, 6–11 × 4–8.5 µm	Single type	Spherical, large	Concave convex	2	Not conspicuous	Cup shaped, usually simple, rarely bilobed at the posterior part	Spherical	Hori *et al.* (1986)
T. tetrathele (West) Butcher	Europe, widely distributed and common; marine	Compressed, elliptical, 10–16 × 8–11 × 4.2–5 µm	ND	Pyrenoid conspicuous, large, sub-central to sub-basal, spherical	Large	ND	Sub-median, usually situated in the region of upper part of pyrenoid, large, red orange	Bright green, axile with a narrow sinus reaching to pyrenoid, a shorter posterior lobe and two lateral lobes	ND	West (1916), Carter (1937), Butcher (1959)
T. verrucosa Butcher	Europe and Japan; marine	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	Poorly covered with hairs or bare, single type	Spherical, sub-basal, or at times central, small, with a starch sheath of uniform outline	Concave on the side adjacent to pyrenoid matrix	Usually 2, rarely three	Conspicuous and variable in position but usually located at or above the middle of the cell, orange	Bright, yellow-green, massive, with 2 anterior lobes near the pyrenoid and 4 or more sublobes in anterior region, not lobed posteriorly	Irregularly lobed at the posterior part, the lobe invading the pyrenoid	Butcher (1959), Hori *et al.* (1983)
T. wettsteinii (Schiller) Throndsen	Gulf of Naples; marine	Cells strongly compressed, heart shaped, broader than long, 7–9 × 11–12 µm. Cells have a characteristic median yellowish accumulation body	Hairs absent	2 or more asymmetrically positioned pyrenoids surrounded by starch sheath	ND	Usually 2	A faint eyespot located eccentrically in the middle of the cell	Single green chloroplast	Present at the anterior part of the cell	Schiller (1913), Ettl *et al.* (1959), Throndsen *et al.* (1988)

[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. Guillou *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.

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Acknowledgements

This document is an output from the UKIERI (UK–INDIA Education and Research Initiative) project entitled 'Development of Methodology for Biological Assessment of Ballast Water Management Systems' funded by the British Council, the UK Department for Education and Skills (DfES), Office of Science and Innovation, the FCO, Scotland, Northern Ireland, Wales, GSK, BP, Shell and BAE for the benefit of the India Higher Education Sector and the UK Higher Education Sector. The views expressed are not necessarily those of the funding bodies. We wish to convey our gratitude to Christine Campbell from the CCAP algal culture collection for comparing this new organism with the existing strains and to Dr Gary Caldwell for the algal culturing facility. We express our sincere thanks to Professor Jakob Wisse, for his kind help in preparing the Latin version of the diagnosis and to Marina Tumanina for English translation of the Ukrainian species descriptions. We thank our colleagues at CSIR–National Institute of Oceanography (NIO) and Newcastle University, especially V. D. Khedekar, Ian Harvey, Nithyalakshmy Rajarajan, Sneha Naik, Carol Barnett, Ravidas Naik, Priya D'Costa and Jonathan Rand for their help and support. This work was supported by the Council of Scientific and Industrial Research (CSIR), India and British Council, UK. This is a NIO contribution no. 5321.

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.

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