Hylodesmus singaporensis gen. et sp. nov., a new autosporic subaerial green alga (Scenedesmaceae, Chlorophyta) from Singapore

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The algal flora of subaerial habitats in the tropics remains largely unexplored, despite the fact that it potentially encompasses a wealth of new evolutionary diversity. Here we present a detailed morphological and molecular characterization of an autosporic coccoid green alga isolated from decaying wood in a natural forest in Singapore. Depending on culture conditions, this alga formed globular to irregularly oval solitary cells. Autosporulation was the only mode of reproduction observed. The cell periphery was filled with numerous vacuoles, and a single parietal chloroplast contained a conspicuous pyrenoid surrounded by a bipartite starch envelope. The cell wall was composed of a thick inner layer and a thin trilaminar outer layer, and the cell surface was ornamented with a few delicate ribs. Phylogenetic analyses of 18S rRNA gene sequences placed our strain in the family Scenedesmaceae (Sphaeropleales, Chlorophyceae) as a strongly supported sister branch of the genus *Desmodesmus*. Analyses of an alternative phylogenetic marker widely used for the Scenedesmaceae, the ITS2 region, confirmed that the strain is distinct from any scenedesmacean alga sequenced to date, but is related to the genus *Desmodesmus*, despite lacking the defining phenotypic features of *Desmodesmus* (cell wall with four sporopolleninic layers ornamented with peculiar submicroscopic structures). Collectively, our results establish that we identified a novel, previously undocumented, evolutionary lineage of scenedesmacean algae necessitating its description as a new species in a new genus. We propose it be named *Hylodesmus singaporensis* gen. et sp. nov. A cryopreserved holotype specimen has been deposited into the Culture Collection of Algae of Charles University in Prague, Czech Republic (CAUP) as CAUP C-H8001.

INTRODUCTION

Coccoid green algae that reproduce solely by means of autosporulation represent one of the most difficult groups in terms of achieving natural taxonomy. Traditionally, these algae were classified as a subgroup of the green algal order Chlorococcales (Komárek & Fott, 1983), although it has been long recognized that they do not form a phylogenetically coherent assemblage. The revolutionary concept of green algal classification pioneered by Mattox & Steward (1984) and based on the ultrastructural features of the flagellar apparatus cannot be applied to taxa lacking flagellated stages. For this reason, gaining insight into the actual phylogenetic relationships of autosporic chlorophytes only became possible with the advent of molecular approaches (e.g. Huss & Sogin, 1990). Molecular phylogenetic studies, based primarily on 18S rRNA gene sequences, revealed that autosporic green algae form an array of unrelated lineages, the majority of which belong to the class Trebouxiophyceae or to the order Sphaeropleales within the class Chlorophyceae (e.g. Hanagata, 1998; Huss *et al.*, 1999; Hepperle *et al.*, 2000; Krienitz *et al.*, 2003; Wolf *et al.*, 2003a, b; Senousy *et al.*, 2004; Fawley *et al.*, 2005).

One of the most prominent lineages of the autosporic coccoid green algae is one that roughly corresponds to the traditional family Scenedesmaceae. The circumscription of

Abbreviations: LBA, long branch-attraction; ML, maximum-likelihood; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene–ITS2–5.8S rRNA gene–ITS2 region of *Hylodesmus singaporensis* strains CAUP H 8001 and E4-g are FJ436342 and FJ715936, respectively.

The predicted secondary structure of the ITS2 region of *Hylodesmus singaporensis* strain CAUP H 8001 and ML phylogeny of the ITS2 region of Scenedesmaceae are available with the online version of this paper.

this family, since it was established by Oltmanns (1904), has varied greatly depending on the author (Komárek & Fott, 1983; Ettl & Gärtner, 1995; Hegewald & Hanagata, 2000). Here, we understand the family Scenedesmaceae to be a monophyletic lineage within the order Sphaeropleales consisting of autosporic green algae of the genus *Scenedesmus* and its relatives. Following the most recent molecular phylogenetic studies and taxonomic revisions (Hegewald & Hanagata, 2000; Krienitz *et al.*, 2003), genera in this family now include: *Scenedesmus*, *Coelastrella*, *Neodesmus*, *Desmodesmus*, *Pseudodidymocystis* and *Hariotina*. Species attributed to the genera *Enallax*, *Coelastropsis*, *Pectodictyon* and *Coelastrum* have also been shown to cluster within the Scenedesmaceae lineage (Hegewald & Hanagata, 2000; Krienitz *et al.*, 2003), but their taxonomic status is uncertain at present (mostly due to lack of molecular data on the type species of the genera). In addition, Tsarenko raised the subgenus *Acutodesmus* of the genus *Scenedesmus* to the level of a separate genus (Tsarenko & Petlevanny, 2001), although this treatment has become controversial in light of more recent molecular evidence (Hegewald & Wolf, 2003). Nevertheless, further genera will doubtless be added to the Scenedesmaceae in the future by reclassification of known taxa, but also by the inclusion of novel organisms, not previously described.

An exceptionally rich source of novel phylogenetic diversity of coccoid green algae has been found in the subaerial habitats in tropical ecosystems (Rindi *et al.*, 2006; Neustupa *et al.*, 2007; Eliáš *et al.*, 2008; Zhang *et al.*, 2008; Neustupa *et al.*, 2009). In the course of our investigation into green algal cultures sampled from decaying wood in a natural forest in Singapore, we found a strain of a coccoid green alga occupying an interesting position in the 18S rRNA gene-based phylogenetic tree of the Scenedesmaceae. In order to learn more about this organism, we conducted a detailed morphological, ultrastructural and molecular phylogenetic study, and, based on our results, we describe a new genus and species, *Hylodesmus singaporensis* gen. et sp. nov. (Scenedesmaceae, Chlorophyta).

METHODS

Isolation and cultivation. The type strain of *H. singaporensis* (CAUP H 8001), along with a morphologically indistinguishable strain (E4-g) cloned out independently from a different colony, were isolated from a subaerial algal growth taken from a 10 × 10 cm site on decaying bare wood in a tropical forest in the Central Catchment Nature Reserve, Singapore (1°21′12.56′′N and 103°48′43.09′′E; altitude 60 m above sea level). For purposes of this study, the algae were cultivated on agar-solidified or liquid BBM medium (Bischoff & Bold, 1963), as well as in liquid CAUP OGM medium (http://botany.natur.cuni.cz/algo/caup.html). The cultures were maintained at 23 °C with illumination of 40 μmol m^{-2} s^{-1} provided by 18 W cool fluorescent tubes (Philips TLD 18W/33).

Microscopy. General vegetative and reproductive morphology was examined under an Olympus BX51 light microscope. Microphotographs were taken with Olympus Z5060 equipment using differential contrast. To visualize details of the cell wall, cells were stained with calcofluor white solution (Fritz & Triemer, 1985) and examined under an Olympus BX-41 microscope equipped with an Olympus U-RFL-T-200 epifluorescence illumination lamp. The UV filter was set at an excitation wavelength of 330–380 nm and an emission wavelength of 420 nm. For investigation of chloroplast morphology, cells were observed with a Leica TCS SP2 laser scanning confocal microscope. The microscope was equipped with an Ar-Kr laser using a 488 nm excitation line and an AOBS filter-free system collecting emitted light between 498 and 700 nm. A Leica 63 × /1.4 N.A. oil immersion or 63 × /1.2 water immersion objective fitted on a Leica DM IRE2 inverted microscope was used. A series of optical sections of chloroplasts was captured and used for 3D reconstruction. The autofluorescence of the chlorophyll was exploited for visualization of chloroplast structure. For the final processing of confocal images, various image analysis tools were applied using ImageJ 1.34p (Abramoff *et al.*, 2004) and Adobe Photoshop CS3 Extended. For transmission electron microscopy (TEM), samples were fixed in 2 % glutaraldehyde, post-fixed in 1 % osmium tetroxide in 0.05 M phosphate buffer and 1 % uranyl acetate in methanol. After dehydration in ethanol, the cells were embedded in Spurr's medium via isobutanol. Ultrathin sections, cut with a diamond knife on a Reichert–Jung Ultracut, were post-stained with lead citrate and examined with a JEOL 1011 TEM. Obtaining empty cell walls was achieved by mechanically breaking the cells by vortexing in a plastic tube with glass beads (0.5 mm diam.; Sigma). Cell walls were then collected by successive centrifugation. Washed walls were dried onto Formvar-coated grids and examined with the JEOL 1011 TEM. Samples for scanning electron microscopy (SEM) were processed using a procedure similar to that described by Poulíčková *et al.* (2007). In brief, fixed samples (2 % glutaraldehyde, 0.1 M phosphate buffer) were rigorously washed with 0.1 M cacodylate buffer and allowed to sediment onto poly-L-lysine-treated Nucleopore filters for 3 days at 4 °C. Filters with attached cells were dehydrated through a series of ethanol baths. Finally, the filters were coated with gold in a sputter-coater (Polaron). The specimens were examined in a Philips CM12/STEM electron microscope in SEM mode at 80 kV, spot size 10 nm.

Gene sequencing. For isolation of genomic DNA, cells were scraped from an agar plate with a clean spatula, transferred into an Eppendorf tube, resuspended in distilled water and harvested by centrifugation. Total DNA was extracted using the Invisorb Spin Plant Mini kit (Invitek). The sequence of the 18S rRNA gene–ITS1–5.8S rRNA gene–ITS2 region was obtained by PCR amplification, followed by the sequencing of two overlapping segments. The segment representing the 18S rRNA gene was amplified by using the universal forward (F) and reverse (R) primers according to Katana *et al.* (2001); the second segment comprising part of the 18S rRNA gene and the ITS1–5.8S rRNA gene–ITS2 region was amplified using the 1500af primer (Helms *et al.*, 2001) and a newly designed primer (5′-GTTCAGCGGGTAGCCTTGC-3′) matching the 5′ end of the 28S rRNA gene (amplification with the standard ITS4 primer failed). The PCR products were resolved by electrophoresis on a vertical 1 % agarose gel, bands of the expected size were excised and the DNA was purified using the QIAquick Gel Extraction kit (Qiagen). The first segment was sequenced from both ends using the amplification primers, internal sequencing primers from Katana *et al.* (2001) and a newly designed reverse primer (5′-GCATCGTTTATGGTTGAGAC-3′) that was required because of the presence of a long intron. The second segment was sequenced with the amplification primers and with the ITS1 primer (White *et al.*, 1990). Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analysed with the 3130 Genetic Analyzer (Applied Biosystems). Sequencing reads were assembled with the CAP3 assembler server (http://pbil.univ-lyon1.fr/cap3.php) and manually edited by visual inspection of sequencing chromatograms. We did not observe any differences in the sequences of the

overlapping regions of the two segments. The newly obtained sequence of the 18S rRNA gene–ITS1–5.8S rRNA gene–ITS2 region of *H. singaporensis* (excluding the regions representing the amplification primers) was deposited in GenBank with the accession number FJ436342 (strain CAUP H 8001) and FJ715936 (strain E4-g).

Phylogenetic analyses. A multiple alignment of the newly determined 18S rRNA gene sequence and other sequences selected from the GenBank/EMBL/DDBJ databases was built using CLUSTAL_X (Thompson *et al.*, 1997). This was then manually curated in GeneDoc (www.nrbsc.org/gfx/genedoc/) taking into account the secondary structure model for the *Chlamydomonas reinhardtii* 18S rRNA molecule available from the European rRNA Database (www.psb.ugent.be/rRNA/secmodel/Crei_SSU.html; Wuyts *et al.*, 2000). A widely sampled maximum-likelihood (ML) phylogenetic analysis demonstrated that *H. singaporensis* is firmly nested within the Scenedesmaceae clade (tree not shown). We therefore prepared a dataset combining the new sequence with all previously available, sufficiently complete scenedesmacean 18S rRNA gene sequences, which were selected from a comprehensive dataset of chlorophycean 18S rRNA gene sequences on the basis of a distance tree (BIONJ; Gascuel, 1997). Several sequences from representatives of the Hydrodictyaceae, Neochloridaceae and Selenastraceae (all Sphaeropleales) were included, and two representatives of the Volvocales were also included to provide a suitable outgroup (based on the preliminary BIONJ tree). The final alignment of the 18S rRNA gene sequences used for phylogenetic analyses presented herein comprised 74 sequences and 1747 unambiguously aligned positions. A multiple alignment of ITS2 sequences was constructed by eye, taking into account previously published secondary structure models of the scenedesmacean ITS2 region (van Hannen *et al.*, 2002; Hegewald & Wolf, 2003; Lewis & Flechtner, 2004; Hegewald *et al.*, 2005; Jeon & Hegewald, 2006). To guide the alignment, we also built a model of the ITS2 secondary structure of *H. singaporensis* (Fig. S1 in IJSEM online) using RNAstructure 4.5 (Mathews *et al.*, 2004) with the default parameter setting. For phylogenetic analyses we discarded ambiguously aligned regions, yielding a final alignment of 191 positions. Based on a preliminary analysis, we selected 60 representative sequences to infer the tree presented in Fig. S2 (in IJSEM online). All alignments used in this study are available upon request. An ML analysis of the 18S rRNA gene data was carried out with the RAxML-VI-HPC 2.2.3 program (Stamatakis, 2006) employing the GTRGAMMA substitution model (the most general model implemented by the program). ML bootstrap support was calculated from 100 bootstrap replicates. Evaluation of tree topology was also performed by calculating 1000 bootstrap BIONJ trees from distance matrices inferred by the DNADIST program (PHYLIP 3.6 package; Felsenstein 2004) employing the F84 model of nucleotide evolution, a transition/transversion ratio of 2.0, one rate category and no gamma correction. An ML analysis of the ITS2 data was done using the RAxML 7.0.4 program accessed through the CIPRES (Cyberinfrastructure for Phylogenetic Research) Portal (www.phylo.org/sub_sections/portal/). We employed a tree inference strategy that was recently demonstrated to be particularly effective in finding a tree with the highest likelihood, i.e. combining a new rapid bootstrap algorithm to infer 100 bootstrap trees with subsequent thorough ML inference on the original dataset utilizing the bootstrap trees for further optimization with the GTRGAMMAI model (Stamatakis *et al.*, 2008). Bayesian inference was performed using MRBAYES 3.1 (Huelsenbeck & Ronquist 2001). Two parallel MCMC runs were carried out for 2 million generations each with one cold and three heated chains employing the GTRGAMMAI evolutionary model (with parameters estimated from the data). Trees were sampled every 100 generations. The initial 6000 (18S rRNA gene dataset) or 500 (ITS2 dataset) trees of each run were discarded as 'burn-in' and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining trees. An ITS2 ML tree with a constrained tree topology was inferred with RAxML 7.0.4 employing the procedure used for the unconstrained ML tree. Per-site log-likelihoods for the alternative trees were computed with RAxML 7.0.3 (–f g option) and fed into CONSEL (Shimodaira & Hasegawa, 2001) to calculate *P* values of the approximately unbiased (AU) test (Shimodaira, 2002).

RESULTS

Hylodesmus Eliáš, Němcová, Škaloud & Neustupa, gen. nov.

Diagnosis latina. *Cellulae vegetativae singulares, sphericae vel ellipsoideae, uninucleatae. Paries cellularis in microscopio optico glabra; in microscopio electronico, superficies cum costis paucis persubtilibus, cum strato interno polysaccharato et cum externo trilaminari. Chloroplastus parietalis cum* 1 *pirenoide. Matrix pyrenoidis sine penetrantibus lamellis thylacoidum. Propagatio asexualis per autosporas. Substantiae carotenoides secundae absentes. A generibus ceteris familiae ordine nucleotidorum in* 18S rRNA *and* ITS2 *differt.*

Description. Vegetative cells solitary, spherical or ellipsoidal, uninucleate. The cell wall in an optical microscope appears smooth; in an electron microscope, the cell wall composed of an internal polysaccharide layer and an external trilaminar layer, the surface with a few delicate ribs. The chloroplast parietal with a single pyrenoid. The pyrenoid matrix is not penetrated by thylakoid lamellae. Asexual reproduction via autospores. Secondary carotenoids not produced. The sequences of the 18S rRNA gene and the ITS2 region differ from other genera of the family.

Type species. *Hylodesmus singaporensis* Eliáš, Němcová, Škaloud & Neustupa

Etymology. The genus name *Hylodesmus* (masc. n.) is derived from the Greek terms *hyle* (wood) and *desmos* (bond), analogous to several other generic names of the Scenedesmaceae family, and referring to the habitat where the first representative was collected.

Hylodesmus singaporensis Eliáš, Němcová, Škaloud & Neustupa, sp. nov.

Diagnosis latina. *Cellulae vegetativae singulares, uninucleatae. In agaro cellulae sphaericae,* (4.2)5.5–9.5(−11.0) μm *diametro. In medio liquido cellulae sphaericae vel ellipsoideae* (5.0–)5.5–10.8(−12.4) μm *longae et* (3.0–)4.0–7.5(−9.0) μm *latae. Chloroplastus parietalis cum* 1 *pyrenoide, interdum perforatus praedita. Propagatio asexualis per* 2–16 *autosporas. Autosporae interdum sphaericae,* 3–5 μm *in diametro.*

Description. Vegetative cells solitary, uninucleate. On agar media cells spherical, (4.2)5.5–9.5(−11.0) μm in diameter. In liquid media cells spherical or ellipsoidal,

(5.0–)5.5–10.8(−12.4) μm in length and (3.0–)4.0–7.5(−9.0) μm in width. Parietal chloroplast with one pyrenoid, usually perforated. Asexual reproduction via 2–16 autospores. Autospores usually spherical, 3–5 μm in diameter.

Holotype. Strain CAUP C-H8001 is permanently cryopreserved in the Culture Collection of Algae of Charles University in Prague, Czech Republic (CAUP, http://botany.natur.cuni.cz/algo/caup.html). CAUP C-H8001 is also maintained in an active culture as CAUP H 8001, from which the holotype was derived.

Type locality. Subaerial algal growth on decaying bare wood in a tropical forest in the Central Catchment Nature Reserve, Singapore (1°21′12.56′′N and 103°48′43.09′′E; altitude 60 m above sea level).

Etymology. The name *singaporensis* refers to the country where the type material was collected.

Morphology and ultrastructure

The morphological appearance of *H. singaporensis* varied depending on culture conditions. When grown on solid medium, it formed cells that were predominantly globular, with dimensions (4.2–)5.5–9.5(–11.0) μm in diameter (Fig. 1a–c; Fig. 2a). In liquid media (OGM or BBM), a proportion of cells assumed an irregularly oval shape of (5.0–)5.5–10.8(–12.4) μm in length and (3.0–)4.0–7.5(–9.0) μm in width (Fig. 1d–g). Autosporulation in oval or spherical sporangia was the only means of reproduction observed (Fig. 1h–i; Fig. 2b). The autospores were typically globular, 3–5 μm in diameter (Fig. 1j, k). The number of daughter cells per autosporangium generally differed in cultures grown on solid vs liquid media (2–4 or 4–16, respectively). Each cell possessed a single parietal chloroplast typically extended over most of the inner cellular surface (Fig. 1l). The chloroplast was perforated by apertures of varying shape (Fig. 1m, n). Before autosporogenesis, the chloroplast divided into several polygonal plates or strips (Fig. 1n). The chloroplast contained a conspicuous pyrenoid with a starch envelope composed, in most cases, of two halves (Fig. 1b, e; Fig. 2e, f). No thylakoids transecting the pyrenoid matrix were observed. The region between the plasma membrane and the chloroplast was occupied by a number of vacuoles of varying size (Fig. 2e, f, i). A different type of spherical vacuole, containing electron-dense granular material, was often seen in the region between the parietal chloroplast and the nucleus (Fig. 1b, o; Fig. 2e, f). The cell wall was composed of two main layers: a thick inner layer and a thin outer trilaminar layer. The cell wall surface was ornamented by two to five delicate, predominantly meridional ribs (Fig. 2a, c, d) formed by a thickening of the inner cell wall layer (Fig. 2 g–i). The loose and irregular granulation on the cell surface visible by SEM (Fig. 2a) was probably precipitation of inorganic material. Remnants of a shed parental cell wall were detected in the medium (Fig. 2f). Autospores often retained a part of the parental cell wall on their surface (Fig. 2h, j, k). Old cultures did not produce secondary carotenoids.

From the sample of algal growth that provided the CAUP H 8001 strain of *H. singaporensis*, we also isolated another independent strain (E4-g), which under light microscopy appeared identical to strain CAUP H 8001 (data not shown), but differed at the molecular level (see below).

Molecular phylogenetic analysis

The determined sequence of the 18S rRNA gene–ITS1–5.8S rRNA gene–ITS2 region of *H. singaporensis* CAUP H 8001 comprises 3175 bp, including two group I introns (341 bp and 428 bp) in the 18S rRNA gene. BLASTN searches and ML phylogenetic analysis of a broader set of chlorophyte 18S rRNA gene sequences placed *H. singaporensis* firmly into the family Scenedesmaceae within the chlorophycean order Sphaeropleales (data not shown). In order to determine the position of *H. singaporensis* more precisely, we conducted a more focused analysis using all available sufficiently complete 18S rRNA gene sequences of representatives of the Scenedesmaceae plus several suitable outgroup species (Fig. 3). In this analysis, *H. singaporensis* branched with 100 %/100 % ML/BIONJ bootstrap support and 1.00 Bayesian posterior probability as a sister lineage of a strongly supported monophyletic (100 %/100 %/1.00) *Desmodesmus* clade. The *Hylodesmus*+*Desmodesmus* clade was in turn sister with varying support (64 %/85 %/1.00) to *Pseudodidymocystis planctonica*. The *Neodesmus* clade branched off immediately basal to the *Hylodesmus*+*Desmodesmus*+*Pseudodidymocystis* clade with statistical support from Bayesian inference only. The position of this larger clade, which corresponds to the subfamily Desmodesmoideae (Hegewald & Hanagata, 2000) within the Scenedesmaceae was unresolved in our analysis. In addition, our analysis strongly suggested a close relationship of *Dimorphococcus lunatus* and two *Scenedesmus arcuatus* varieties, and of *Hariotina reticulata* and *Pectodictyon pyramidale*. It further supported the existence of a clade corresponding to the genus *Coelastrella*, and of two distinct clades of traditional *Scenedesmus* species here referred to as *Scenedesmus* (*sensu stricto*) and *Acutodesmus* (*sensu stricto*). Support for the deepest nodes within the Scenedesmaceae was lacking, but the family as a whole was separated from other lineages of the order Sphaeropleales (here represented by families Hydrodictyaceae, Neochloridaceae and Selenastraceae), by a relatively long branch with the highest statistical support.

Because the ITS2 region is an important alternative marker for investigating the phylogenetic relationship within the Scenedesmaceae (An *et al.*, 1999; van Hannen *et al.*, 2002; Hegewald & Wolf, 2003), we analysed the phylogenetic position of *H. singaporensis* with respect to previously determined ITS2 sequences from the Scenedesmaceae. In BLASTN searches against the non-redundant nucleotide

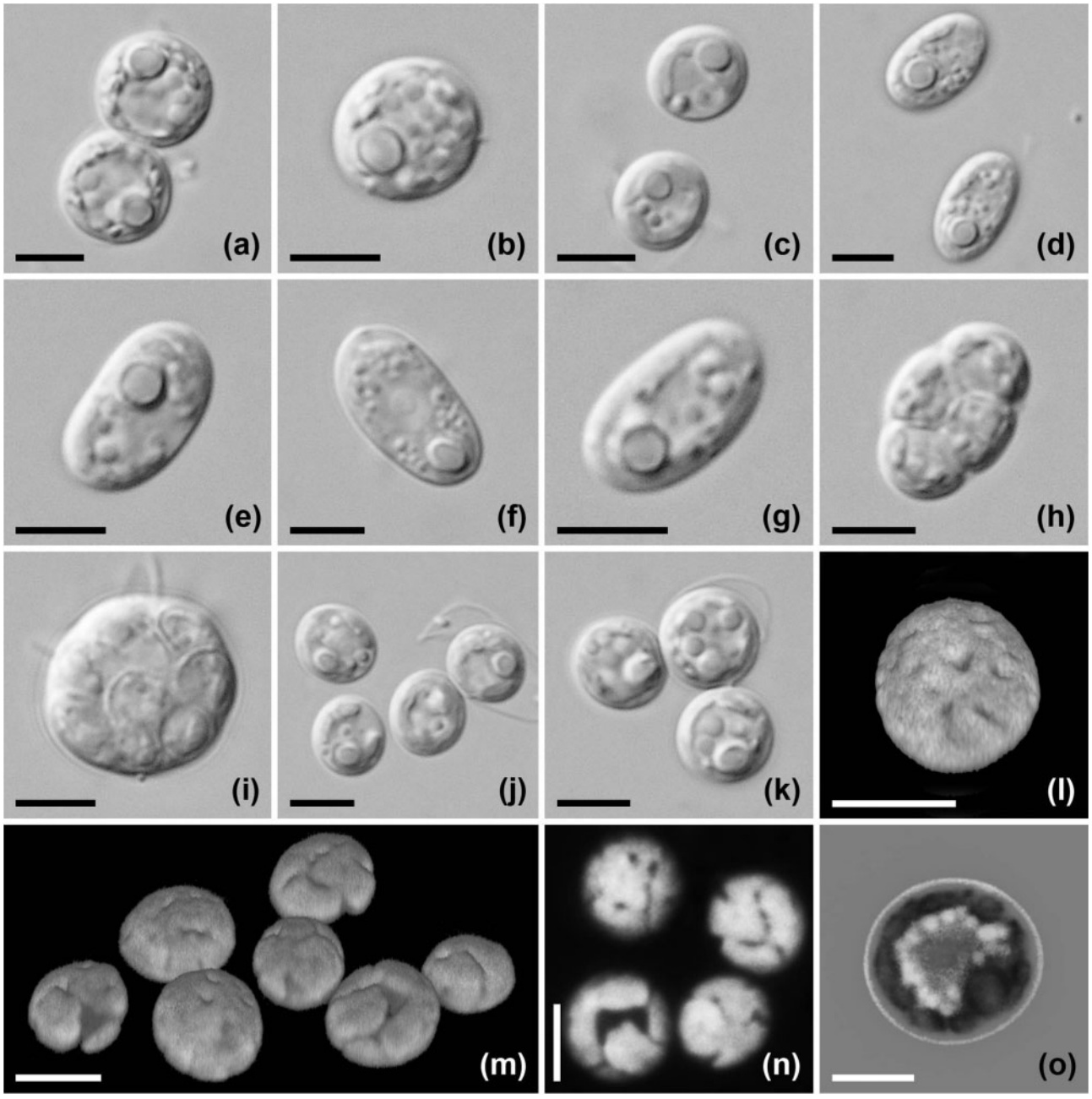

Fig. 1. Morphology of *Hylodesmus singaporensis* gen. et sp. nov. CAUP H 8001. (a–c) Globular vegetative cells growing on solid medium. (d–g) Oval vegetative cells in liquid medium; note the eccentrically positioned prominent pyrenoid. (h, i) Autosporangia of various shapes. (j, k) Released globular autospores. (l, m) 3D confocal reconstructions of the chloroplasts in mature vegetative cells; note several chloroplast perforations of various shapes. (n) Maximum projection of confocal sections through the chloroplasts; note the splitting of the compact parietal chloroplast into several polygonal plates before autosporogenesis (clearly visible in the bottom left cell). (o) A computer-processed epifluorescence image of a mature vegetative cell; note the parietal chloroplast (dark) spread along the inner cell surface and numerous spherical vacuoles (light) situated beneath the chloroplast layer. Bars, 5 μm.

database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the *H. singaporensis* ITS2 sequence, used as a query, returned ITS2 sequences from scenedesmacean species, and especially from *Desmodesmus* spp., as being the most similar, but no sequence shared more than 75 % identical positions. The ML tree calculated from ITS2 sequences of selected scenedesmacean representatives was not particularly well-resolved, owing to rather few (191) reliably

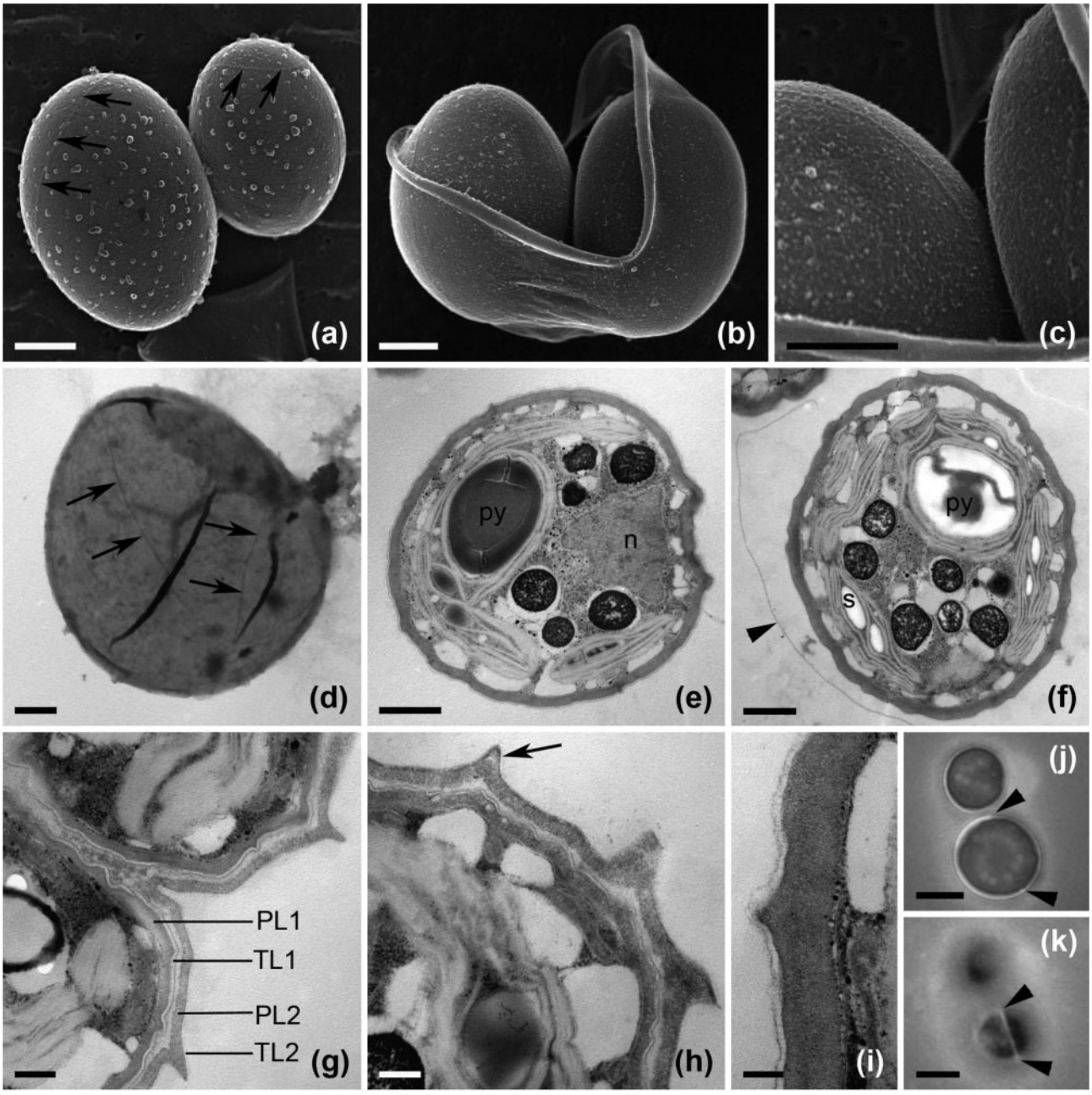

Fig. 2. Morphology and ultrastructure of *H. singaporensis* gen. et sp. nov. (a–c) *H. singaporensis* as observed by SEM. (a) The cell wall is ornamented by delicate, mostly meridional ribs (arrows). (b) Autospores enclosed in a parental cell wall. (c) A detailed view of the meridional rib. (d) An empty cell wall as viewed by TEM; the ribs are indicated by arrows. (e–i) TEM micrographs of cross-sections through cells of *H. singaporensis*. (e, f) The chloroplast with a pyrenoid surrounded by a bipartite starch envelope. Note the vacuoles of varying size subtending the plasma membrane. The remnant of a parental cell wall is indicated by an arrowhead; py, pyrenoid; n, nucleus; s, starch grain. (g) Two newly formed autospores still enclosed in a parental cell wall; PL1, polysaccharide layer of the daughter cell; TL1, trilaminar layer of the daughter cell; PL2, polysaccharide layer of the parental cell wall; TL2, trilaminar layer of the parental cell wall. (h) A cell partly covered by a parental cell wall. The ribs are formed by both the polysaccharide and the trilaminar layer (arrow). (i) Detail of the trilaminar layer composed of an electron-transparent lamella sandwiched between two electron-dense lamellae. (j, k) Different planes of focus on the same specimen of calcofluor white-labelled cells under fluorescence microscopy. The arrowheads point to the margin of the parental cell wall. Bars, (a–f), 1 μm; (g, h), 250 nm; (i), 100 nm; (j, k), 5 μm.

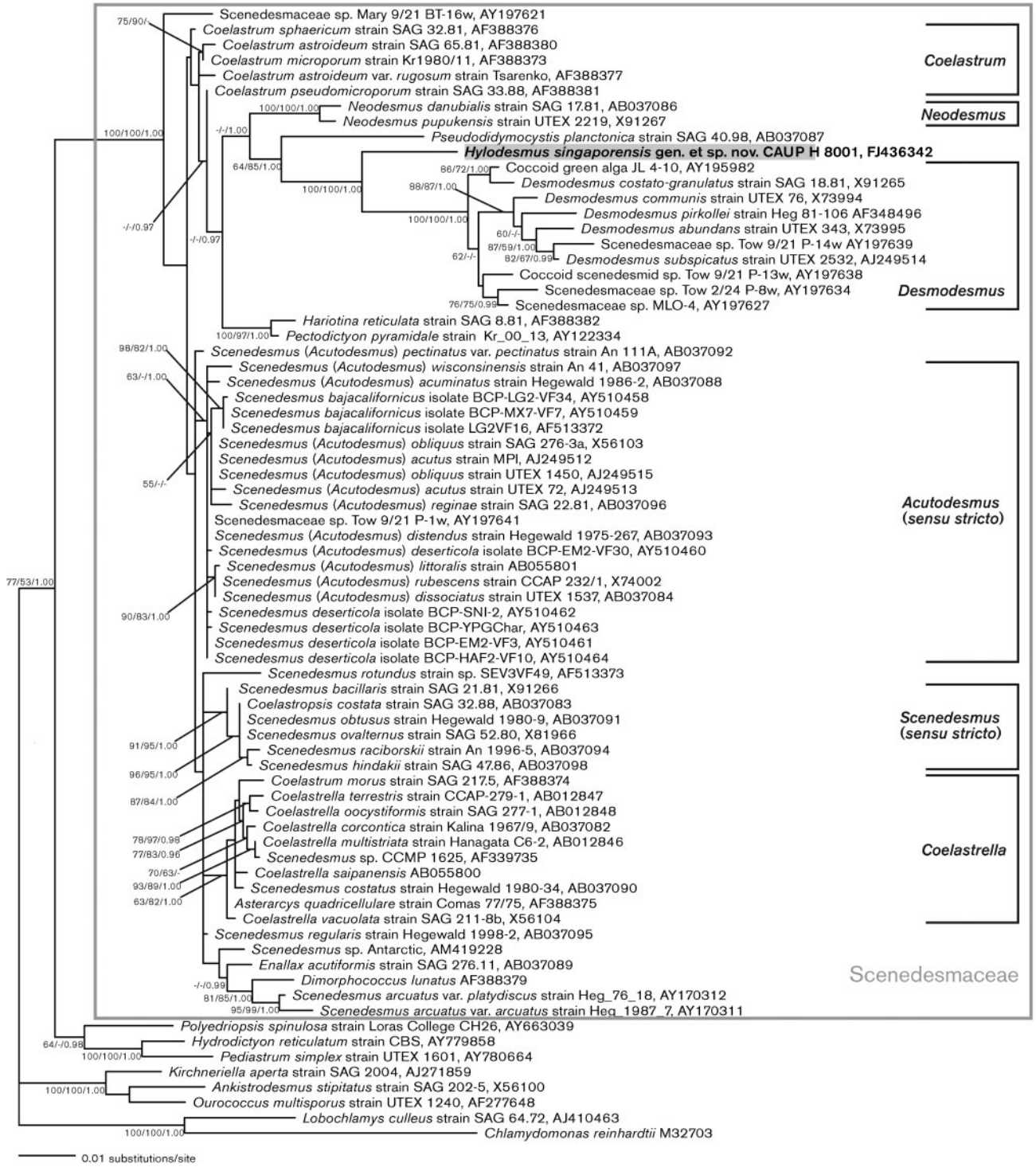

Fig. 3. Phylogenetic position of *H. singaporensis* gen. et sp. nov. CAUP H 8001 based on 18S rRNA gene sequence data. The ML tree shown (GTR+Γ4, loglk=−6903.361986, α=0.111494) was constructed with RAxML-VI-HPC 2.2.3 (see Methods). ML/BIONJ bootstrap values/Bayesian posterior probabilities are indicated for branches when higher than 50 %/50 %/0.95. The root of the tree is placed on a branch separating *Chlamydomonas reinhardtii* and *Lobochlamys culleus* (Volvocales) and the remainder of the tree (Sphaeropleales). GenBank/EMBL/DDBJ accession numbers are provided for each sequence following the taxon name. The names of scenedesmacean taxa used in the figure often differ from the names provided in the respective GenBank entries, because we took subsequent taxonomic revisions into account (Hegewald & Hanagata, 2000; Lewis & Flechtner, 2004).

aligned positions available for the phylogenetic analysis, and a highly uneven rate of evolution in different taxa. *H. singaporensis* was placed as an isolated branch within a broader clade of generally very divergent sequences attributed to the genus *Desmodesmus* (Fig. S2). To test whether the inclusion of the *H. singaporensis* ITS2 sequence within the latter genus was robust, we inferred the best ML tree while constraining the monophyly of the genus *Desmodesmus* to the exclusion of *H. singaporensis*. This tree, showing *H. singaporensis* in a sister position to the *Desmodesmus* monophylum (as in the 18S rRNA gene tree), was not found to be statistically less likely (*P* value of 0.214) than the unconstrained tree (*P* value of 0.786), according to the Approximately Unbiased (AU) test (Shimodaira, 2002). Furthermore, we found that all available ITS2 sequences from the genus *Desmodesmus* have a single nucleotide deletion in the conserved helix III (Fig. S1) at a position occupied by a highly conserved 'U' residue in essentially all non-*Desmodesmus* scenedesmacean ITS2 sequences, including *H. singaporensis* (and ITS2 sequences from at least some other Sphaeropleales, e.g. Hydrodictyaceae).

To assess the molecular diversity of the morphotype represented by *H. singaporensis* strain CAUP H 8001, we sequenced the 18S rRNA gene–ITS1–5.8S rRNA gene–ITS2 region of the morphologically identical strain E4-g, independently isolated from the original collected sample. Compared with the CAUP H 8001, E4-g contained within its 18S rRNA gene two more group I introns (four in total) and multiple substitutions or indels in the two shared introns (data not shown), but the sequence of the 18S rRNA gene itself was identical. The 5.8S rRNA gene sequences of these two strains were also identical, but there were differences in the ITS1 region at five positions (data not shown), and in the ITS2 region at two positions (one in a loop in the conserved helix I and one near the 3′ end of the ITS2 region; Fig. S1).

DISCUSSION

The relatively simple morphology of coccoid autosporic algae is generally a poor indicator of phylogenetic relationships, but *H. singaporensis* does exhibit a suite of features indicating its phylogenetic provenance. These features include the spherical parietal chloroplast, the conspicuous simple pyrenoid surrounded by a starch envelope, and the cell wall composed of a thick, presumably polysaccharide, layer and a thin trilaminar, presumably sporopolleninic, layer; all these characters are typical for representatives of the family Scenedesmaceae (Hegewald, 1978; Komárek & Fott, 1983; Kalina & Punčochářová, 1987; Ettl & Gärtner, 1995). Indeed, our analysis of 18S rRNA gene sequences places *H. singaporensis* into a strongly supported monophyletic lineage of autosporic taxa that can be equated with the traditional family Scenedesmaceae (Fig. 3). The content of this clade corresponds well to analyses by Hegewald & Hanagata (2000) and Krienitz *et al.* (2003), but is expanded by a number of sequences not included by (or not available to) these authors, especially sequences attributed to *Dimorphococcus lunatus*, *Asterarcys quadricellulare* and several *Coelastrum* species. Hence, the diversity of the Scenedesmaceae is apparently greater than that conceived in the latest revision of this family (Hegewald & Hanagata, 2000; see also Krienitz *et al.*, 2003), and, although *H. singaporensis* is the latest, it will certainly not be the last arrival in this family.

However, despite the morphological hints as to the familial assignment, defining the specific phylogenetic position of *H. singaporensis* within the Scenedesmaceae would be difficult, if not impossible, without molecular data. With the notable exception of the *Desmodesmus* lineage, exhibiting an obvious synapomorphy in the form of a cell wall with four sporopolleninic layers ornamented with peculiar submicroscopic structures (Hegewald, 1978; An *et al.*, 1999), other higher-level monophyletic subgroups within the Scenedesmaceae evaded recognition prior to the era of molecular phylogenetics (Hegewald & Hanagata, 2000). For example, a strain originally identified as *Chlorella minutissima* (Fott & Nováková, 1969) was later described as a new genus and species *Kermatia pupukensis* in the subfamily Scotiellocystoideae (Kalina & Punčochářová, 1987), but 18S rRNA gene sequences finally showed its very close relationship to *Neodesmus danubialis* in the Scenedesmaceae, leading to its taxonomical re-evaluation as *Neodesmus pupukensis* (Hegewald & Hanagata, 2000). Similarly, the genus *Coelastrella*, as currently conceived, comprises species that had been previously classified in other genera (*Scenedesmus*, *Chlorella*, *Scotiellopsis*, *Graesiella*) and that were reassigned to *Coelastrella* only upon a phylogenetic analysis of 18S rRNA gene sequences (Hegewald & Hanagata, 2000). Finally, neither *Scenedesmus* nor *Acutodesmus* (*sensu* Tsarenko) is monophyletic in the light of molecular data (Hegewald & Wolf, 2003; see also Fig. 3). Thus, the interpretation of the morphological evolution of the Scenedesmaceae is difficult owing to a high degree of plasticity or convergence.

Nevertheless, *H. singaporensis* does display a combination of features suggesting that it probably represents an evolutionary lineage of its own, separate from species described previously. From the genus *Desmodesmus*, its sister group according to 18S rRNA gene phylogeny, it is in all likelihood distinguished by the plesiomorphic trilaminar outer cell wall layer, and by the lack of structures comparable to 'ornaments' (granules, spines) formed by the outermost sporopolleninic layer in *Desmodesmus*. The genus *Pseudodidymocystis*, in contrast to *H. singaporensis*, lacks a polysaccharide cell wall layer, but forms unique bowl-shaped structures on the cell wall surface (Hegewald & Deason, 1989). In addition, shed mother cell wall, which persists in the medium of *H. singaporensis* cultures (Fig. 2f), does not exhibit the typical scrolling observed in the cell walls of *Pseudodidymocystis planctonica*. The two known species of the genus *Neodesmus* are characterized

by heteropolar cells (Hegewald & Hanagata, 2000), and at least one of these species (*N. pupukensis*) possesses a unique claviform pyrenoid embedded in a homogeneous starch envelope, unlike *H. singaporensis* (Kalina & Punčochářová, 1987). Species of the genus *Scenedesmus* (*sensu lato*) generally form multicellular coenobia (Komárek & Fott, 1983; Hegewald & Hanagata, 2000), often have acute cell poles or polar thickenings of the cell wall (especially in the *Acutodesmus* group), or are surrounded by mucilage (Komárek & Fott, 1983; Hegewald & Hanagata, 2000) – all features not seen in *H. singaporensis*. Finally, *Enallax acutiformis*, *Hariotina reticulata*, *Pectodictyon pyramidale* and species of the genus *Coelastrum* are also all morphologically dissimilar to *H. singaporensis* (Komárek & Fott, 1983; Hindák, 1990; Ettl & Gärtner, 1995; Hegewald *et al.*, 2002; Krienitz *et al.*, 2003).

Among the known scenedesmacean taxa, *H. singaporensis* perhaps most closely resembles species currently classified into the genus *Coelastrella*. It also shares with most *Coelastrella* species the subaerial lifestyle (Ettl & Gärtner, 1995; Hegewald & Hanagata, 2000), so it is possible that the morphological similarities result from convergence due to similar selection pressures. However, most *Coelastrella* species are characterized by numerous prominent ribs on the cell surface (Kalina & Punčochářová, 1987; Ettl & Gärtner, 1995; Hegewald & Hanagata, 2000), whereas *H. singaporensis* possesses only a few barely visible fine ribs (Fig. 2a, c, d). It is true that *Coelastrella* (=*Graesiella*) *vacuolata* also has only fine, if any (see Hegewald & Hanagata, 2000) surface ribs, but it differs from *H. singaporensis* by generally larger cells, the absence of the peripheral vacuoles, and the production of secondary carotenoids in old cultures (Kalina & Punčochářová, 1987). Finally, *H. singaporensis* may be similar to a species recently described from North American desert soil as *Scenedesmus rotundus* (Lewis & Flechtner, 2004), although it falls outside *Scenedesmus* (*sensu stricto*) clade in the 18S rRNA gene phylogeny (Fig. 3). Unfortunately, the ultrastructure of *S. rotundus* has not been reported, so the extent of similarity, beyond features visible under light microscopy, is unknown. Regardless, *S. rotundus* is a taxon clearly distinct from *H. singaporensis* based on both the 18S rRNA gene and ITS2 sequences (Fig. 3, Fig. S2).

A notable ultrastructural feature of *H. singaporensis* is the system of peripheral vacuoles (Fig. 2e, f, h). Their functional significance is unclear, but similar (homologous?) peripheral vacuoles have been observed in a TEM section of *Coelastrella corcontica* (=*C. multistriata* var. *corcontica*; Kalina & Punčochářová, 1987), so they may not be unique to *H. singaporensis*. Likewise, structures similar to what we interpret as vacuoles containing electron-dense granular material (Fig. 2e–f) were also found in other scenedesmacean representatives, including *Coelastrella* (*Graesiella*) species and *Neodesmus* (=*Kermatia*) *pupukensis*, and in more distantly related taxa such as '*Chlorella*' (=*Mychonastes*) *zofingiensis* (Kalina & Punčochářová, 1987). The identity of these structures is unknown, but we speculate that they represent some type of reserve substance. Interestingly, we observed that in *H. singaporensis* a part of the parental cell wall frequently remains appressed to the surface of autospores released from the autosporangium (Fig. 2h, j, k). We are currently unaware of such a feature in other scenedesmacean species, but it could simply have been overlooked; therefore, further careful investigation is necessary before we can claim that this is a character idiomatic to *H. singaporensis*.

The distinctiveness of *H. singaporensis* from other scenedesmacean taxa is immediately apparent from the 18S rRNA gene tree (Fig. 3). The overall topology of the tree is generally in accord with previous analyses (Hegewald & Hanagata, 2000), although the clear-cut division of the family into two subfamilies (Scenedesmoideae and Desmodesmoideae) is no longer supported following expanded sampling. *H. singaporensis* is placed into a portion of the tree comprising species with accelerated evolution of the 18S rRNA gene (note the length of the branches compared with the rest of the tree), and is firmly resolved as a lineage most closely related to the *Desmodesmus* clade. The common *Hylodesmus*+*Desmodesmus* node is separated from the deepest divergence within the *Desmodesmus* clade by a relatively long and strongly supported branch, reflecting the evolutionary gulf between *H. singaporensis* and the genus *Desmodesmus*. However, to date the 18S rRNA gene sequence has been determined for only a very few *Desmodesmus* species; therefore, with a wider sampling within this genus, it is possible that the length of this branch may become shorter. The 18S rRNA gene sequences of both *H. singaporensis* and representatives of the *Desmodesmus* clade are highly divergent compared with sequences from most other scenedesmacean taxa, thus suggesting that the inferred *Hylodesmus*+*Desmodesmus* clade might result from the well-known long branch attraction (LBA) artefact (Felsenstein, 1978). However, the methods of phylogenetic inference employed in our study (maximum-likelihood and Bayesian methods with complex models of substitution) are relatively insensitive to this artefact (see Philippe *et al.*, 2005). In addition, inspecting the multiple alignment of the 18S rRNA gene sequences revealed a number of substitutions synapomorphic for the *Hylodesmus*+*Desmodesmus* clade (data not shown), a finding not expected under the LBA scenario.

A much more comprehensive sampling of the diversity within *Desmodesmus* has been achieved with an alternative genetic marker, the ITS2 region (An *et al.*, 1999; Hegewald *et al.*, 2002, 2005; Tsarenko *et al.*, 2005; Jeon & Hegewald, 2006; Johnson *et al.*, 2007; Vanormelingen *et al.*, 2007). Although our ML analysis of ITS2 sequences placed the *H. singaporensis* sequence within the clade of the genus *Desmodesmus*, the topology should be interpreted with caution. First, all sequences of this clade are highly divergent compared with most other scenedesmacean ITS2 sequences, indicating the possibility of LBA affecting this part of the tree. Second, the number of positions of the

ITS2 multiple alignment suitable for phylogenetic analysis is so small that stochastic error necessarily plays a role in shaping the inferred tree. Indeed, we found that an alternative topology, one with the *H. singaporensis* sequence branching off in a sister position to the monophyletic *Desmodesmus* clade, was not statistically less likely. Finally, the two major subclades of *Desmodesmus* shown in the ITS2 tree (Fig. S2) both have representatives in the 18S rRNA gene tree (*D. costato-granulatus* from one subclade; and *D. communis*, *D. pirkollei* and *D. subspicatus* from the other subclade; see Fig. 3), where they belong, contrary to the ITS2 tree, to a strongly supported monophyletic branch excluding *H. singaporensis*. The monophyly of *Desmodesmus* to the exclusion of *H. singaporensis* is further supported by the apparently *Desmodesmus*-specific deletion of a conserved residue in helix III of the ITS2 structure (Fig. S1). Considering also the fact that *H. singaporensis* lacks morphological features characteristic of the genus *Desmodesmus*, these results indicate that the former is best classified in a genus separate from *Desmodesmus*.

Sequence variability in the 18S rRNA gene or the ITS regions within morphotypes (morphospecies) of diverse unicellular eukaryotes is well-established, and has also been documented for species of the family Scenedesmaceae (van Hannen *et al.*, 2002; Lewis & Flechtner, 2004; Jeon & Hegewald, 2006; Vanormelingen *et al.*, 2007). Differences in the presence/absence of introns in the 18S rRNA gene has also been previously reported for different strains attributed to the same species of green algae (e.g. Friedl *et al.*, 2000). It is, therefore, unsurprising to find such variability in the 18S rRNA gene and ITS sequences of the two strains examined in this study, even though they are indistinguishable at the light-microscopic level. Since the sequences of the 18S rRNA gene itself are identical between strains CAUP H 8001 and E4-g, and the ITS2 region differs at only two positions without any compensatory base changes, it is probably best to consider both strains as representatives of the same species, *H. singaporensis*. It is nevertheless very interesting that we found this genetic variation in strains isolated from the same sample (a small spot of subaerial algal growth). Very little is known about the extent and structure of genetic diversity within populations of subaerial unicellular green algae, but the case of these two *H. singaporensis* strains indicates that genetically closely related, yet nevertheless different, clones might coexist *in situ*, without competitive exclusion of one clone by another. Further investigation is needed to evaluate the significance of these findings.

In summary, our microscopic observations and phylogenetic analyses of the 18S rRNA gene and ITS2 sequences indicate that *H. singaporensis* represents a novel phylogenetic lineage within the family Scenedesmaceae, and must be given a generic and species status separate from previously known taxa. Although the genus *Hylodesmus* remains monotypic at present, we expect that much greater species diversity will be revealed in future samplings, especially in those from poorly explored tropical ecosystems. Further field surveys are also necessary to ascertain the place of this organism in nature, including its geographical range, habitat distribution and ecological importance.

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