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Molecular, biochemical and morphological data suggest an affiliation of *Spongiochrysis hawaiiensis* with the Trentepohliales (Ulvophyceae, Chlorophyta)

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SUMMARY

The aeroterrestrial, unicellular green alga *Spongiochrysis hawaiiensis* had been included in the ulvophycean order Cladophorales based on small subunit (SSU) rDNA sequence data, and represents so far the only fully terrestrial member of this order. Other characteristics of *S. hawaiiensis* that are atypical for Cladophorales include the presence of large amounts of carotenoids and a budding-like mode of cell division. As the position of this terrestrial, unicellular alga in an order of aquatic, multicellular green algae is unusual, we re-evaluated the phylogenetic relationships of this enigmatic organism based on supplementary SSU rDNA sequences as well as novel large ribosomal subunit (LSU) rDNA and internal transcribed spacer (ITS rDNA) sequences. Additionally, we examined several morphological characters of *S. hawaiiensis*, as well as low molecular weight carbohydrate (LMWC) patterns of *S. hawaiiensis* and members of the Cladophorales and Trentepohliales as potential chemotaxonomic markers. We found *S. hawaiiensis* to be uninucleate. The analysis of the LMWC content detected the presence of the polyol erythritol in *S. hawaiiensis* and in the Trentepohliales, while this compound was missing in the Cladophorales. The phylogenetic analyses of the novel sequences placed *S. hawaiiensis* in the terrestrial Trentepohliales. This placement is supported by the aeroterrestrial habitat, the presence of large amounts of carotenoids, the uninucleate cells, and the presence of the polyol erythritol as a protective compound against water loss.

Key words: aeroterrestrial algae, Chlorophyta, Cladophorales, erythritol, molecular phylogenetics, *Spongiochrysis hawaiiensis*, systematics, Trentepohliales, Ulvophyceae.

INTRODUCTION

Spongiochrysis hawaiiensis Rindi, López-Bautista, Sherwood & Guiry has recently been described based on a peculiar unicellular alga growing on the bark of *Casuarina* trees on Hawai'i (Rindi *et al*. 2006). The genus was firmly placed in the Cladophorales based on phylogenetic analyses of small subunit (SSU) rDNA sequences. *Spongiochrysis* is an atypical member of the Cladophorales as it represents the only strictly terrestrial members of the order Cladophorales with a number of unusual features, such as a unicellular growth form (but occasionally producing short filaments) and a specialized form of budding-like cell division. Initial phylogenetic reconstructions placed *S. hawaiiensis* in the *Aegagropila* clade (Rindi *et al*. 2006), a clade of the Cladophorales consisting mainly of freshwater and brackish water species (presently split into the Pithophoraceae and the Pseudocladophoraceae, Boedeker *et al*. 2012). Based on this phylogenetic association, it was hypothesized that *S. hawaiiensis* might have evolved from a marine ancestor by the gradual reduction of the thallus and the production of carotenoid pigments as an adaptation to terrestrial habitats (Rindi *et al*. 2006).

Common adaptations of algae to the terrestrial realm include a simple morphology, such as coccoid cells; thick cell walls; the production of mucilage to retain moisture (Broady 1977; Shephard 1987; Rindi 2011); restriction of metabolic activity to periods of favorable conditions (e.g. Friedman *et al*. 1987); and the synthesis of metabolites that protect against high irradiation (carotenoids, e.g. Bidigare *et al*. 1993; Abe *et al*. 1998) and desiccation/water loss (polyols such as ribitol, sorbitol, glycerol, erythritol, e.g. Feige & Kremer 1980; Darienko *et al*. 2009; Gustavs *et al*. 2010).

Terrestrial algae are found in several of the major groups of green algae: in the charophyte lineage they occur in the Klebsormidiophyceae, and among the core

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chlorophytes they have been found in the Trebouxiophyceae, in several lineages of the Chlorophyceae, and within the Ulvophyceae (see Leliaert *et al*. 2012). Within the ulvophyte lineage, aeroterrestrial algae are mainly found in the strictly terrestrial order Trentepohliales (e.g. López-Bautista *et al*. 2002), but several other members of the Ulvophyceae such as *Desmochloris mollenhaueri* Darienko, Friedl et Pröschold (Darienko *et al*. 2009) and *Trichophilus welckeri* Weber-van Bosse, which grows on the hair of sloths (Suutari *et al*. 2010), are also terrestrial. Some freshwater species of *Ulothrix* (Ulotrichales) are also found on soil (Metting 1981).

The placement of the unicellular *S. hawaiiensis* in the Cladophorales is exceptional, as this order is distributed in marine, brackish or freshwater environments and is characterized by a siphonocladous organization, which means that multicellular thalli are composed of multinucleate cells (van den Hoek *et al*. 1995). However, several taxa are known to occur in semiterrestrial environments such as on moist stone and soil surfaces, for example *Wittrockiella calcicola* (F.E. Fritsch) Boedeker, *W. fritschii* (A.K. Islam) Boedeker and *W. sunderbanensis* (A.K. Islam) Boedeker (Fritsch 1944; Islam 1964). Furthermore, some members of *Arnoldiella* (former *Basicladia*, see Boedeker *et al*. 2012) that occur preferably on freshwater turtles can withstand extended periods of desiccation (Proctor 1958).

Spongiochrysis hawaiiensis was initially believed to be a member of the Trentepohliales based on its crustose occurrence on tree barks and the supposed production of carotenoids (Rindi *et al*. 2006). The Trentepohliales are characterized by filamentous thalli composed of uninucleate cells, and several other unique features such as the formation of sessile zoosporangia that detach and are dispersed, cell division by phragmoplast formation, transverse cell walls containing plasmodesmata, a multilayered structure in the flagellar root system, the presence of β-carotene and astaxanthin, and the synthesis of uncommon low molecular weight carbohydrates such as polyols (Feige & Kremer 1980; van den Hoek *et al*. 1995; Thompson & Wujek 1997; Chapman *et al*. 2001; López-Bautista *et al*. 2002). Furthermore, several species of the Trentepohliales are common photobionts in lichens. About 20–30% of lichen-forming fungi associate with trentepohlialean algae (Ahmadjian 1993; Nelsen *et al*. 2011).

Because of the unusual placement of *S. hawaiiensis* in the Cladophorales, a group with which it apparently shares few characters, and because some features such as the high pigment content and the terrestrial habitat are strikingly reminiscent of the Trentepohliales, we reinvestigated this alga using novel sequences and other approaches including fluorescent staining of nuclear DNA and analysis of the low molecular weight carbohydrates (LMWC).

MATERIALS AND METHODS

Studied organisms

The material of *Spongiochrysis hawaiiensis* investigated in this study (sample number G89, deposited as voucher WELT A031452 (National Museum of New Zealand Te Papa Tongarewa)) was collected at the type location (on the bark of *Casuarina equisetifolia* Linnaeus, Waimanalo Beach Park, Waimanalo, Oahu, Hawai'i) on 4 February 2006 by Alison Sherwood. The cells of *S. hawaiiensis* formed a thick, powdery layer of intense yellow color on the bark surface. For the molecular and biochemical analyses, the surface of especially thick parts of the yellow layer was scraped off with a scalpel into Petri dishes (approximately 10 mm^2 each), and the powder was transferred after microscopic inspection to 1.5-mL microfuge tubes.

To check for the presence of other (algal) organisms on the tree bark that could possibly lead to contaminating signals in the molecular and biochemical analysis, the surface of the bark was scraped with a scalpel (approximately 10 cm^2). The resulting material was fixed in 5% formaldehyde-freshwater solution and allowed to settle overnight. The sediment was then distributed with a pipette into three plastic Petri dishes (diameter 8 cm) with counting grids and the dishes subsequently checked using an inverted microscope (Wild M40, Wild Heerbrugg, Switzerland). All filamentous and biological structures were photographed with a digital camera (ColorView IIIu, Olympus Soft Imaging Systems, Münster, Germany).

Staining of nuclei

Cells of *S. hawaiiensis* were fixed in 5% formaldehyde-freshwater solution and stained using the fluorescent, DNA-binding dye Hoechst 33258 (Molecular Probes, Invitrogen, Eugene, OR, USA) and were photographed under exciting ultraviolet radiation (340–380 nm, XF06 filterset) using a Zeiss Axioplan2 Imager microscope (Carl Zeiss) with a Zeiss Axiocam MRc5 digital camera (Carl Zeiss) and Zeiss Axiovision software (Carl Zeiss MicroImaging GmbH, Germany).

Low molecular weight carbohydrate content

The LMWC content of members of the Cladophorales and Trentepohliales as well as *S. hawaiiensis* was examined (Table 1, see Appendix S1 in Supporting Information for complete dataset). LMWCs were extracted from dried algal material and the presence of arabitol,

Table 1. Content (μM g^{-1} dry weight) of selected low molecular weight carbohydrates (LMWCs) of *Spongiochrysis hawaiiensis* and some members of the Cladophorales and Trentepohliales (see Appendix S1 in Supporting Information for the whole dataset).

Species	No.	Erythritol	Trehalose	Mannitol	Sucrose
Trentepohliales					
Trentepohlia umbrina (Kützing) Bornet	G85	85.6	0	7.2	0
Trentepohlia abietana (Flotow) Hansgirg	G86	43.3	0	5.7	0
Trentepohlia aurea (Linnaeus) C.F.P. Martius	G87	14.9	0	10.9	0
Trentepohlia iolithus (Linnaeus) Wallroth	G88	14	0	0	0
Spongiochrysis hawaiiensis Rindi, López-Bautista, Sherwood et Guiry	G89	**21**	**7**	**0**	**143**
Cladophorales					
Pseudocladophora horii (C. Hoek et Chihara) Boedeker et Leliaert	D78	0	0	0	135
Aegagropila linnaei Kützing	B54	0	1	n.d.	11
Aegagropila linnaei	C01	0	0.3	n.d.	125
Pithophora roettleri (Roth) Wittr.	K01	n.d.	n.d.	0	84.3
Aegagropilopsis sterrocladia (Skuja) Boedeker	N84	0	3.5	n.d.	66
Arnoldiella kosterae (C. Hoek) Boedeker	K06	0	0	0	165
Wittrockiella lyallii (Harv.) C. Hoek, Duckert & Womersley	Q79	0	0	0	208
Wittrockiella salina V.J. Chapman	Q67	0	0	0	649
Wittrockiella amphibia (Collins) Boedeker & G.I. Hansen	N73	0	0	0	549
Wittrockiella calcicola (Fritsch) Boedeker	K92	0	0	0	692
Wittrockiella sp.	Q81	0	0	0	225
Anadyomene stellata (Wulfen) C. Agardh	J69	n.d.	n.d.	0	0
'*Cladophora*' *rugulosa* G. Martens	D60	n.d.	n.d.	0	119
'*Cladophora*' *prolifera* (Roth) Kützing	J63	n.d.	n.d.	0	0
Cladophora pseudopellucida Hoek	J59	0	0	n.d.	0
Cladophora battersii Hoek	J80	n.d.	n.d.	0	67.6
Cladophora sp. New Zealand (marine)	L01	0	0	n.d.	107

n.d., no data.

dulcitol, erythritol, glycerol, mannitol, ribitol, sorbitol, sucrose and trehalose was checked. Dry algal samples each of 5–30 mg were extracted with 70% aqueous ethanol (v/v) in capped centrifuge tubes at 70°C in a water bath for 4 h according to Karsten *et al*. (1991). After centrifugation for 5 min at 5000 *g*, 700 μL of the supernatant was evaporated to dryness under vacuum (Speed Vac Concentrator SVC 100H, Savant Instruments Inc, Holbrook, NY, USA). Dried extracts were redissolved in 700 μL distilled water and vortexed for 30 s. Samples were analyzed with an isocratic Agilent HPLC system (Santa Clara, CA, USA) equipped with a differential refractive index detector. LMWCs were separated and quantified by two high performance liquid chromatography (HPLC) methods in order to maximize peak identification. Separation of polyols, mono-, and disaccharides was performed on a Bio-Rad resin-based column (Aminex Fast Carbohydrate Analysis, 100 × 7.8 mm, Bio-Rad Inc, Hercules, CA, USA) using a Phenomenex Carbo-Pb^{2+} (4 × 3 mm) guard cartridge. LMWCs were eluted with 100% HPLC grade water at a flow rate of 1 mL min^{-1} at 70°C (modified after Karsten *et al*. 1991). Separation of heterosides and polyols was performed on a Phenomenex resin-based column Rezex ROA-Organic Acid (300 × 7.8 mm) protected with a Phenomenex Carbo-H^+ (4 × 3 mm) guard cartridge (Phenomenex Inc, Torrance, CA, USA). On the latter column, LMWCs were eluted with 5 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1} at 75°C (modified after Karsten *et al*. 2005). LMWCs were identified by comparison of retention times with those of the commercial standard compounds (Roth, Karlsruhe, Germany; Sigma-Aldrich, St. Louis, MO, USA) prepared as 1 mM aqueous solutions and quantified by peak areas. All concentrations are expressed in micromole per gram dry weight.

Phylogenetic analyses

The partial small subunit (SSU) ribosomal DNA (rDNA), the partial large subunit (LSU) rDNA and the internal transcribed spacer (ITS) region of *S. hawaiiensis* were sequenced to clarify its phylogenetic position. Members of the Cladophorales (Pseudocladophoraceae and Pithophoraceae) and of the Trentepohliales were included in the SSU and LSU alignments, as well as outgroups (Ulvales and Dasycladales). The species and the EMBL/GenBank accession numbers of the SSU and LSU sequences are listed in Appendix S2, Supporting Information. As very few LSU rDNA and ITS rDNA sequences are publicly available for the Trentepohliales, we have generated some additional sequences for these markers for two *Trentepohlia* species (*Trentepohlia* cf. *arborum* (C. Agardh) Hariot,

sample no. D81, epilithic in dense forest (Umtamvuma Reserve, KwaZulu Natal, South Africa), voucher GALW015556 (National University of Ireland, Galway), ITS rDNA: Accession no. HE653921; *Trentepohlia* sp., sample no. H79, on tree bark (city center, Brussels, Belgium), voucher L0793308/9 (National Herbarium of the Netherlands, University of Leiden branch), ITS rDNA: Accession no. HE653920).

All primers used in the polymerase chain reactions (PCRs) were general primers that should amplify the target regions from almost all groups of eukaryotes. The following primer combinations were used in the amplifications: the LSU rDNA was amplified for all samples with the primers C'1/D2; the SSU rDNA was amplified for all samples using the two primer pairs SR1/SS11H and SSU897/18SC2; the ITS region of the Trentepohliales was amplified with ITS-5 m/ITS4; additionally, for *S. hawaiiensis*, the partial SSU rDNA and the partial ITS was amplified as one region with the two primer pairs SSU897/ITS2 and SSU897/ITS4, and the additional primer combination ITS3/ITS4 was used to verify the sequence of the ITS2 region (details of the primers are given in Appendix S3, Supporting Information).

DNA was extracted from silica-dried material. PCR amplifications were performed in a Biomed thermocycler with an initial denaturation step of 94°C for 5 min followed by 31 cycles of 1 min at 94°C, 1 min at 56–60°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C. The reaction volume was 25 μL and consisted of approximately 0.1–0.4 μg genomic DNA, 1.25 nmol of each dNTP, 6 pmol of each primer, 1× reaction buffer containing 1.5 mM $MgCl_2$ (New England Biolabs, Ipswich, MA, USA), 5 μL bovine serum albumin (2.5%), and one unit of *Taq* polymerase (New England Biolabs). Amplifications were checked by running them on 1% agarose gels and subsequent staining with ethidium bromide. PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Cleaned PCR products were sent to Macrogen, South Korea, for sequencing. The final consensus sequences were constructed with Geneious Pro v5.4 (Drummond *et al*. 2011).

Small subunit and LSU rDNA sequences were aligned using MUSCLE (Edgar 2004) and subsequently edited by eye in Se-Al v2.0a11 (Rambaut 2007). Evolutionary models of nucleotide substitution were determined by the Akaike Information Criterion for the LSU and SSU alignments with MrModeltest2 v2.3 (Nylander 2004). The SSU and LSU rDNA alignments were analyzed using maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML). The same models of nucleotide evolution were applied to both BI and ML analyses. BI was performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Analyses consisted of two independent, simultaneous runs of one cold and three incrementally heated chains, and 3×10^6 generations with sampling every 100 generations. Posterior probabilities were calculated using a Metropolis-coupled Markov chain Monte Carlo approach. The average standard deviation of the split frequencies of the two parallel runs approached zero in all analyses (<0.05), indicating that the tree samples became increasingly similar and that a stationary distribution was reached. The log files of the runs were checked with Tracer v1.4.1 (Rambaut & Drummond 2007) and a burn in sample of 5000 trees was removed before calculating the majority rule consensus trees in MrBayes. All bioinformatic analyses with MrBayes were carried out on the freely available Bioportal (http://www.bioportal.uio.no). ML analyses were performed with PhyML v3 (Guindon & Gascuel 2003) in Geneious Pro v5.4 (Drummond *et al*. 2011), with 100 nonparametric bootstrapping pseudoreplicates (Felsenstein 1985).

Sequences of the ITS region of *S. hawaiiensis* (Accession no. HE664127) and the Cladophorales were difficult to align, resulting in large ambiguously aligned regions, and the ITS sequences of the Cladophorales and Trentepohliales could not be aligned due to large sequence divergence between these taxonomic groups. Therefore no phylogenetic analyses were performed with the ITS sequences, instead the ITS sequences of *S. hawaiiensis* were compared to public sequence databases using BLAST (Basic Local Alignment Search Tool, blastn, http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 24 July 2012) and FASTA (http://www.ebi.ac.uk/Tools/sss, accessed 17 November 2012) to infer the systematic affinities based on retrieved significant matches.

RESULTS

Microscopic observations

The material of *S. hawaiiensis* consisted of thick-walled unicells ranging from 10–20 μm in diameter or short (pseudo)filaments containing yellow or orange pigments (Fig. 1a), the layer of cells was bright yellow on the exposed parts of the tree bark and greenish in cracks. DNA staining showed that the cells contained a single nucleus (Fig. 1b). Some cells were dividing asymmetrically (Fig. 1c), with nuclei visible in the mother and smaller daughter cells (Fig. 1d). Many of the larger cells of *S. hawaiiensis* were found to have small, colorless structures (2–3 μm in diameter) closely adhered to the outside cell wall (Fig. 2), indicating fungal filaments. The fungal nature of these filaments was confirmed by DNA sequencing (data not shown).

The yellow layer on the surface of the tree bark was up to 1 mm thick and consisted of >99% of *S. hawaiiensis* cells and very few other organisms were

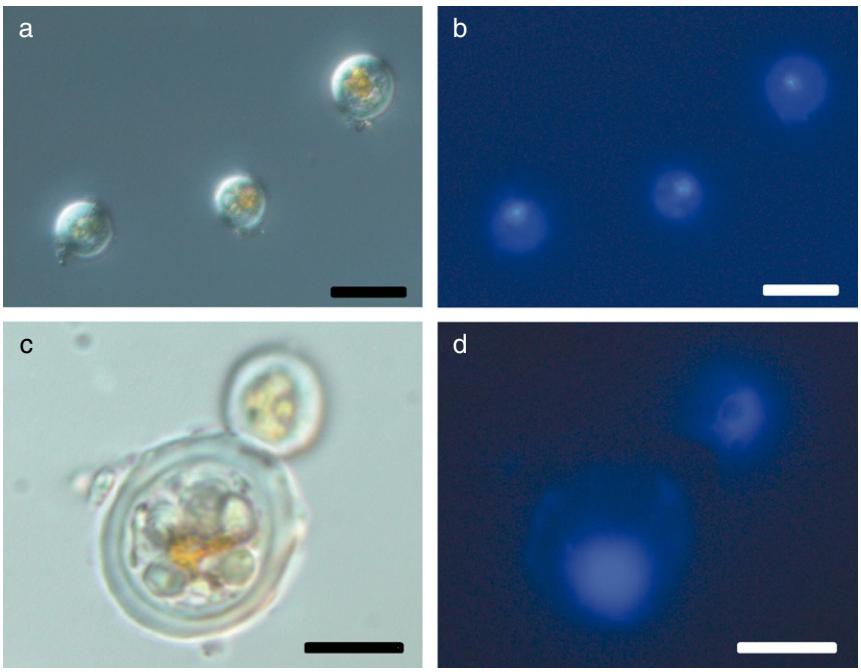


Fig. 1. Cells of *Spongiochrysis hawaiiensis* (WELT A031452). (a,b) Individual unicells. Scale = 20 μm. (c,d) Cell division, a daughter cell is produced and cut off by 'budding'. Scale = 12 μm. The light microscopy images on the left show the same fluorescent DNA-stained cells displayed on the right side. A single nucleus per cell is seen.

encountered in scraped samples, except for the fungal filaments attached to the cells of *S. hawaiiensis*. These included unidentified multicellular assemblages (probably of fungal origin), cyanobacteria, and one single uniseriate green algal filament (Appendix S5, Supporting Information). This thick-walled filament had long cells with a diameter of 30–65 μm and showed the typical branching mode of the Cladophorales, with slightly subterminal insertion as in the Pithophoraceae. No recognizable members of the Trentepohliales were found.

Low molecular weight carbohydrate content

Several LMWCs were examined in members of the Cladophorales and Trentepohliales as well as in *S. hawaiiensis* (Table 1 and Appendix S1 in Supporting Information). Only the LMWCs with the potential to be chemotaxonomically informative are mentioned here. Sucrose and trehalose were not present in the Trentepohliales, but were found in *S. hawaiiensis* and some taxa of the Cladophorales. While trehalose was only present in trace amounts in the Cladophorales (max. 3.5 μM g^{-1} dry weight) and in low amounts in *S. hawaiiensis* (7 μM g^{-1} dry weight), sucrose was detected in high amounts in *S. hawaiiensis* (143 μM g^{-1} dry weight) and in some Cladophorales (0–165 μM g^{-1} dry weight). The polyol mannitol was detected in some but not all Trentepohliales (5.7–10.9 μM g^{-1} dry weight; *Trentepohlia iolithus* (Linnaeus) Wallroth did not contain mannitol), and was not present in *S. hawaiiensis* and the Cladophorales. The polyol erythritol was present in all Trentepohliales (14–86 μM g^{-1} dry weight) and in *S. hawaiiensis* (21 μM g^{-1} dry weight), but not in the Cladophorales. The observed high values in the *Spongiochrysis* sample indicate that the detected erythritol originates from *S. hawaiiensis* and not from trace contamination from other organisms.

Phylogenetic analyses

Details of the SSU and LSU rDNA alignments including the number of in- and outgroup taxa, alignment length and number of variable sites, as well as estimated parameters of nucleotide substitution are given in Appendix S4, Supporting Information.

The SSU rDNA analysis yielded a phylogenetic tree with two well-supported main clades (Fig. 3), one clade including all Cladophorales and the other clade including all Trentepohliales. The previously published sequence of *S. hawaiiensis* (DQ077805, Rindi *et al*. 2006) was placed in a subclade of Cladophorales, grouping together with very low support with *Pseudocladophora horii* and *P. conchopheria*. Amplifying the SSU rDNA with the primers SSU897/18SC2 resulted in the same partial sequence as obtained by Rindi *et al*. (2006). However, the partial SSU sequence amplified with the primers SSU897/ITS2 (GenBank accession no. HE664127) was placed with high support within the Trentepohliales. The phylogenetic relationships of *Trentepohlia annulata* (DQ399588), *T.* cf. *arborum* (HE664130) and *S. hawaiiensis* (HE664127) with the three main clades of Trentepohliales was not resolved. *Spongiochrysis hawaiiensis* was recovered on an exceptionally long branch. This

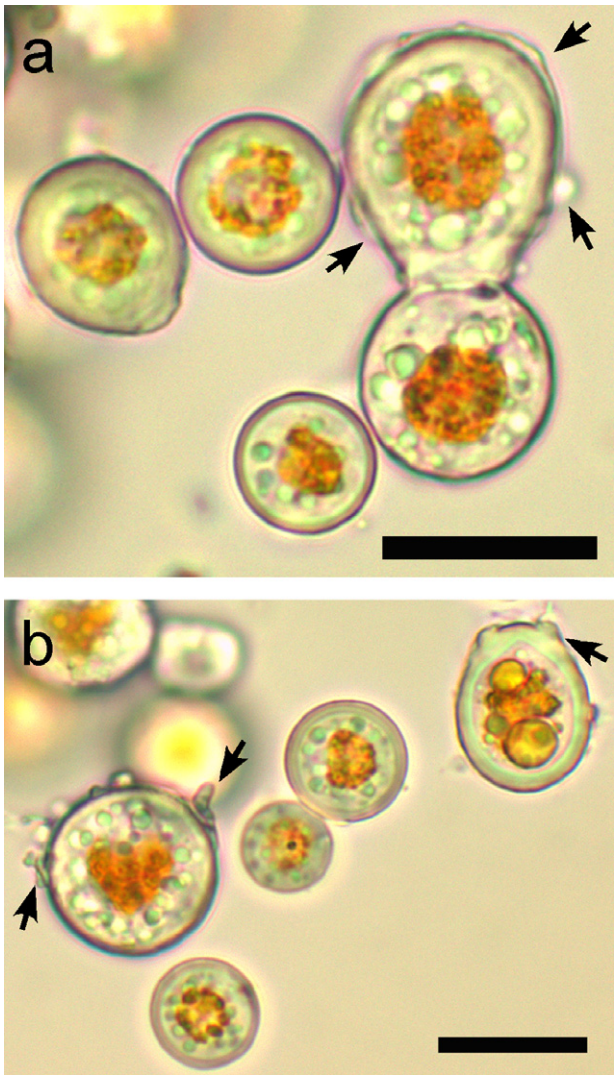


Fig. 2. Cells of *Spongiochrysis hawaiiensis* (WELT A031452) showing fungal hyphae in close association. (a) A larger cell in the process of 'budding' covered with fungal hyphae (arrows). (b) Two cells with fungal hyphae on the outside (arrows). Scale = 20 µm.

branch was very long in both the BI analysis (0.11 substitutions/site) and in the ML analysis (0.8 substitutions/site, data not shown).

Similar to the SSU analysis, the LSU rDNA analysis (Fig. 4) yielded a tree with well-supported Cladophorales and Trentepohliales clades. The sequence of *S. hawaiiensis* (Accession no. HE664128) was included in the Trentepohliales clade.

The results of the BLAST and FASTA searches of the ITS sequence of *S. hawaiiensis* are shown in Appendix S6, Supporting Information. All significant matches are ITS sequences of members of the Trentepohliales (including the newly generated ITS sequences of *Trentepohlia*), with all top hits originating from trentepohlialean lichen photobionts. No matches to any member of the Cladophorales were retrieved in the first 100 BLAST and first 50 FASTA hits.

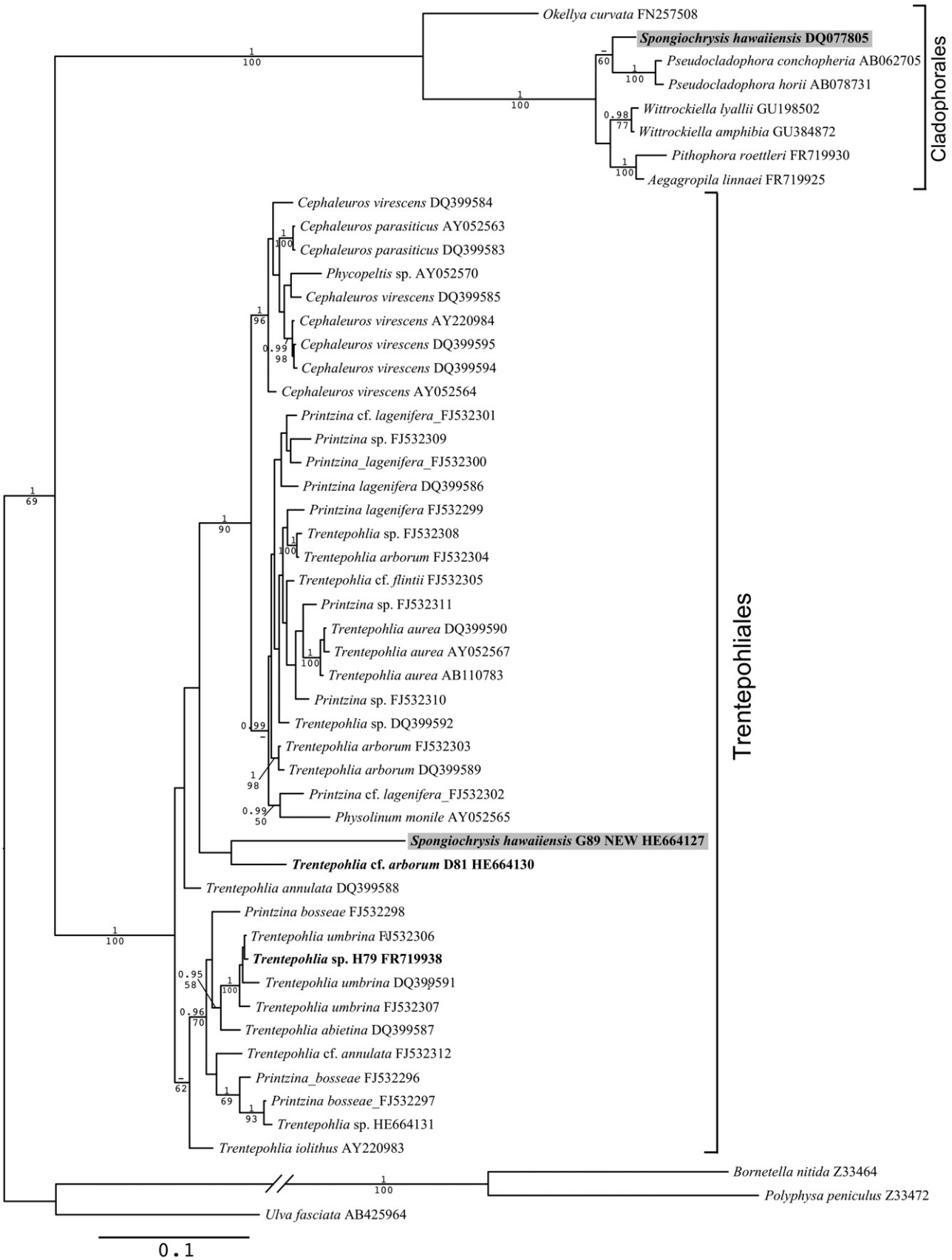
DISCUSSION

The original placement of the unicellular, aeroterrestrial *S. hawaiiensis* in the multicellular, predominantly aquatic order Cladophorales by Rindi *et al*. (2006) was unexpected, especially in the light of striking similarities of some biological features of *S. hawaiiensis* to the terrestrial Trentepohliales. We re-sequenced nuclear ribosomal DNA, and evaluated morphological features as well as LMWC patterns of *S. hawaiiensis*. Our data indicate a possible relationship of *S. hawaiiensis* with the Trentepohliales.

Microscopic observations

Fluorescent DNA staining revealed that *S. hawaiiensis* is uninucleate, which is atypical for Cladophorales. The siphonocladous organization (i.e. thalli composed of multinucleate cells) is one of the principal characters of this order, and members of the Cladophorales typically contain dozens to hundreds of nuclei per cell (van den Hoek *et al*. 1995). In thin filaments of the genus *Rhizoclonium*, a minimum of only two nuclei per cell can be found, and numbers can vary depending on environmental conditions (Parodi & Caceres 1993). Early stages such as germlings or very young filaments of the Cladophorales can be uninucleate (Koorders 1902; Wille 1910), as they originate from a zygote or zoospore containing a single nucleus. None of the cells of *S. hawaiiensis* contained multiple nuclei, regardless of size. Rindi *et al*. (2006) reported that pyrenoids could not be observed in *S. hawaiiensis* (possibly due to the presence of large amounts of carotenoids), which would also be atypical for the Cladophorales. The only known member of the Cladophorales without pyrenoids is the marine filamentous *Okellya curvata* (Printz) Leliaert et Rueness (Leliaert *et al*. 2009), which forms a

Fig. 3. Bayesian inference (BI) phylogram of the Cladophorales and Trentepohliales based on small subunit (SSU) rDNA sequences showing the ambiguous phylogenetic position of *Spongiochrysis hawaiiensis* (sequence from Rindi *et al*. (2006) and newly generated sequence, both highlighted in grey). Trentepohlialean specimens that are also used in the large subunit (LSU) tree are indicated in bold. Posterior probabilities (PP) from BI larger than 0.94 are indicated above the branches, maximum likelihood (ML) bootstrap values larger than 50 are indicated below. The branch leading to the Dasycladales (outgroup) was shortened by 50%. The scale bar represents substitutions per site.



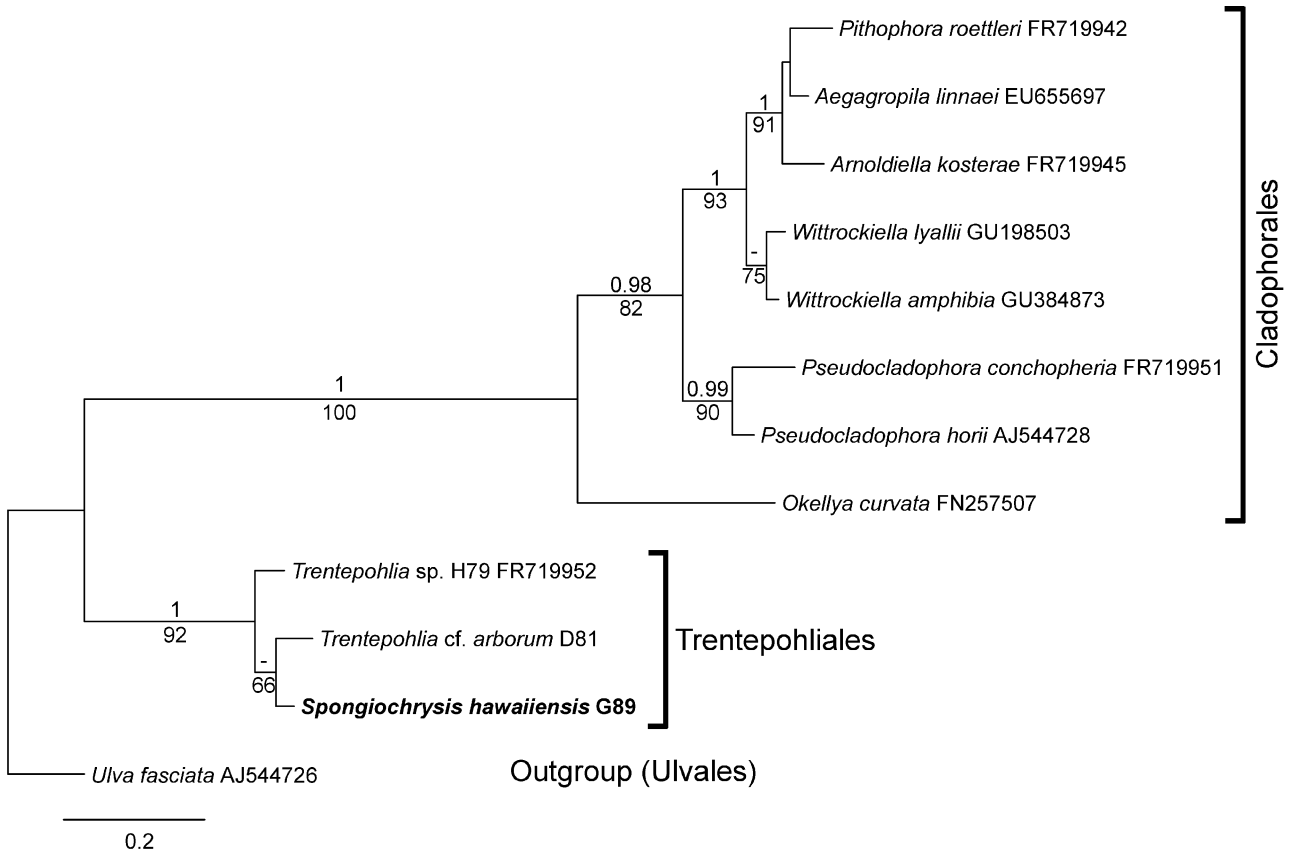


Fig. 4. Bayesian inference (BI) phylogram of the Cladophorales and Trentepohliales based on large subunit (LSU) rDNA sequences showing the phylogenetic position of *Spongiochrysis hawaiiensis*. Posterior probabilities (PP) from BI larger than 0.94 are indicated above the branches, maximum likelihood (ML) bootstrap values larger than 50 indicated below. The scale bar represents substitutions per site.

sister lineage to all other Cladophorales. The cells of *S. hawaiiensis* are strongly colored by large amounts of carotenoid pigments (Rindi *et al*. 2006 and this study). While carotenoids are also known from the Cladophorales (Yoshii *et al*. 2004), they do not generally occur in amounts that mask the chlorophylls leading to visible yellow or red coloration of the cells.

We observed fungal filaments closely adhering to the cells of *S. hawaiiensis*. This may simply be co-occurrence, or parazitation of the alga by the fungus, but it could also indicate lichenization. Several members of the Trentepohliales, including the genera *Cephaleuros*, *Trentepohlia*, *Phycopeltis*, *Physolinum* and *Printzina*, are common phycobionts in lichens (Matthews *et al*. 1989; Ahmadjian 1993; Nelsen *et al*. 2011). The occurrence of unicells as well as short moniliform filaments in *S. hawaiiensis* is reminiscent of trentepohlialean phycobionts, where the algal filaments can considerably change appearance when in contact with the mycobiont and are frequently reduced to short filaments or single cells (e.g. Uyenco 1965; Ahmadjian & Hale 1973; Davis & Rands 1993). Furthermore, the most significant BLAST and FASTA hits of the ITS2 sequence of *S. hawaiiensis* were from trentepohlialean lichen photobionts. However, much more work is required to investigate the relationship of *S. hawaiiensis* and the observed fungal filaments.

The mode of asexual reproduction exhibited by *S. hawaiiensis*, termed 'budding-like autosporulation', was regarded as one of the most peculiar features of this organism by Rindi *et al*. (2006), and this special mode of autosporulation is so far only known from few green algae, such as the trebouxiophytes *Marvania* (Sluiman & Reymond 1987) and *Pseudomarvania* (Eliáš & Neustupa 2009), the prasinophytes *Prasinoderma* (Jouenne *et al*. 2011) and *Pycnococcus*, and in the Palmophyllales (O'Kelly 1988; Zechman *et al*. 2010).

Low molecular weight carbohydrate data

Only the disaccharides trehalose and sucrose and the polyols mannitol and erythritol yielded patterns that could be potentially informative with regards to the systematic placement of *S. hawaiiensis*. However, mannitol does not seem to be a suitable diagnostic chemotaxonomic marker as it was neither present in the Cladophorales, nor in *S. hawaiiensis* nor in *T. iolithus*.

Trehalose was present in *S. hawaiiensis* and two cladophoralean species while it was absent from the Trentepohliales and four species of Cladophorales, but this result is not significant as these low amounts are near the detection limit of the HPLC and thus do not allow verification of the presence or absence of this LMWC (Yang *et al*. 2010). It has been shown that trehalose is not a suitable chemotaxonomic marker in the red algae, while polyols generally have diagnostic power (Karsten *et al*. 2007).

It is interesting that sucrose was found in *S. hawaiiensis*, as all Trentepohliales are known to produce glucose as a photoassimilate rather than sucrose (Feige & Kremer 1980). Sucrose was also found in about two-thirds of the Cladophorales analyzed in this study (Appendix S1, Supporting Information). However, this disaccharide is of little chemotaxonomic value as it is quickly metabolized, for example, into starch, and is present in the primary metabolism of all algal cells and frequently co-occurs with glucose, fructose and glycerol (e.g. Nagashima & Fukuda, 1981). Among the Cladophorales, *Cladophora rivularis* (Kamenarska *et al*. 2004) and *Wittrockiella salina* (Boedeker & Karsten, unpubl. data, 2011) contain glucose in addition to sucrose.

The polyol erythritol was present in comparable amounts in the four analyzed isolates of *Trentepohlia* and in *S. hawaiiensis*, indicating that it does not originate from contaminants. Polyols are generally rare and chemotaxonomically more informative than mono- and disaccharides. Erythritol is a compound rarely encountered in the green algae (and all other algal divisions) but is typical for the Trentepohliales (Kremer 1980; Feige & Kremer 1980). The only other alga in which erythritol has been found is the aeroterrestrial trebouxiophyte *Apatococcus lobatus* (Chodat) Boye (Gustavs *et al*. 2010). Erythritol has also been found in lichens such as *Rocella* that contain *Trentepohlia* as photobiont, where it is believed that erythritol is produced by the photobiont (Lewis & Smith 1967). Thus, while the presence of erythritol is not entirely exclusive for the Trentepohliales, this finding seems to further indicate that *S. hawaiiensis* is a member of the Trentepohliales as polyols have never been detected in the Cladophorales (this study; Kremer 1980). The presence of Erythritol, Trehalose and Sucrose and the absence of Arabitol and Mannitol represent an unusual biochemical signature, stressing that *S. hawaiiensis* is a very unique member of the Trentepohliales.

Phylogenetic data

Analyses of the SSU, LSU and ITS rDNA sequences generated in this study supported the position of *S. hawaiiensis* in the Trentepohliales. This suggests that the SSU sequence of Rindi *et al*. (2006), which was also found in this study when using the general primer pair SSU897/18SC2, belongs to a cladophoralean species co-occurring with *Spongiochrysis*. Rindi *et al*. (2006) used general primers designed for higher plants (Hamby *et al*. 1988). The primer SSU897 has a 2 basepair (bp) mismatch to the Trentepohliales and the primer 18SC2 has a 1 bp mismatch to the Trentepohliales, so it is possible that a cladophoralean contaminant is preferably amplified even when present in very small amounts. When we co-amplified the SSU and ITS region by using the primer combinations SSU897/ITS2 and SSU897/ITS4, we were able to get data showing the present trentepohlialean placement. The general primers used in this study sometimes amplified fungal DNA for all three markers. That no cladophoralean ITS and LSU sequences were obtained is most likely due to the scarcity of the cladophoralean organism on the tree bark.

While several of the features of *S. hawaiiensis* appear to fit the Trentepohliales better than the Cladophorales, this organism would also be an unusual member of the Trentepohliales, for example, with regard to the budding-like cell division, the thallus organization and the biochemical patterns. *Spongiochrysis hawaiiensis* was recovered on a very long phylogenetic branch in our SSU analyses (Fig. 3; see also Results). Currently, five or six are accepted for the Trentepohliales (*Trentepohlia*, *Cephaleuros*, *Phycopeltis*, *Printzina*, *Stomatochroon* and *Physolinum*, depending on whether *Physolinum monile* De Wildeman is accepted as a separate genus or as a synonym of *Trentepohlia rigidula* (J. Müller) Hariot (López-Bautista *et al*. 2002; Rindi *et al*. 2009). Representatives of all these genera (except *Stomatochroon*, for which no molecular data are available) were included in our phylogenetic analyses; however, *S. hawaiiensis* did not show any phylogenetically supported affiliation to any Trentepohliales (the most comprehensive dataset available of Rindi *et al*. (2009) was used), confirming its uniqueness. *Spongiochrysis hawaiiensis* occurs mainly as unicells but may form short moniliform filaments of dividing cells (Rindi *et al*. 2006), a mode of growth that is reminiscent of the ease of fragmentation in some moniliform species of the Trentepohliales (e.g. *T. rigidula* (J. Müller) Hariot (= *Physolinum monile*), *Trentepohlia umbrina* (Kützing) Bornet or *Printzina lagenifera* (Hildebrand) R.H. Thompson & D.E. Wujek and several other species) in which the adjacent cells are connected by a very small area (Cribb 1970; Davis & Rands 1993; Ettl & Gärtner, 1995; Rindi *et al*. 2005; Rindi & López-Bautista 2007), but the phylogenetic analyses did not confirm any relationship with moniliform species of *Trentepohlia*. No other obvious morphological similarities exist between *S. hawaiiensis* and any other genera of the Trentepohliales. Additional taxon sampling and molecular data are clearly required to establish the relationships of *S. hawaiiensis* to other

members of the Trentepohliales and to assess the validity of its placement in a separate genus.

The Trentepohliales have a phragmoplast-mediated cell division (Chapman *et al*. 2001), which is otherwise only known in the Streptophyta, and the phragmoplastin gene has been sequenced for the Trentepohliales (López-Bautista *et al*. 2003). Therefore, we tried to confirm the presence of the phragmoplastin gene in *S. hawaiiensis* by using the primers from López-Bautista *et al*. (2003). We amplified a 341 bp-long fragment for *S. hawaiiensis* and a 380 bp-long fragment for *Trentepohlia* sp. H79 (data not shown). However, neither sequence resulted in a BLAST match with previously published phragmoplastin sequences, including those of López-Bautista *et al*. (2003), and meaningful alignment of the nucleotide and translated amino acid sequences was not possible. The phragmoplastin nucleotide sequences of López-Bautista *et al*. (2003) ranged from 250 to 495 bp, and none of them was a BLAST match to any higher plant phragmoplastin sequence, or to each other, or to our sequences. More molecular studies are clearly needed to evaluate the phragmoplastin gene in Chlorophyta.

We analyzed environmental samples that clearly contain both a trentepohlialean and cladophoralean alga (ambiguous 18S results), thus the problem of linking the dominant alga ('*Spongiochrysis*') with the 18S data arose. Is it possible that we sequenced a trentepohlialean contaminant present in the sample in addition to *S. hawaiiensis*? *Spongiochrysis hawaiiensis* was clearly the dominant organism on the tree bark, with the yellow powdery layer being essentially pure. The observed amounts of erythritol in *S. hawaiiensis*, a characteristic chemotaxonomic marker for the Trentepohliales, were comparable to the amounts found in the four analyzed isolates of *Trentepohlia*, indicating it does not originate from a rare contaminant. Together with our other observations that imply an affiliation of *S. hawaiiensis* with the Trentepohliales, we believe it is not likely that our sequences originate from a contaminating source.

In conclusion, the aeroterrestrial habitat, the presence of large amounts of carotenoids, the uninucleate cells, the presence of the polyol erythritol and the new molecular data suggest an affiliation of *S. hawaiiensis* with the Trentepohliales. Even so, the data of Rindi *et al*. (2006) provided irrefutable molecular evidence for a terrestrial species of Cladophorales, which must have been present in two locations (Kailua Beach Park and Waimanalo Beach Park, Oahu, Hawai'i–c. 5 km apart). This raises the question as to which organism the cladophoralean SSU rDNA sequence of Rindi *et al*. (2006) belongs. When checking for the presence of other algae on the bark (see Materials and Methods), we found a fragment of one single filament that could represent an undescribed, corticolous member of the Cladophorales (Appendix S5, Supporting Information). Further sampling of Cladophorales from (semi)terrestrial environments could reveal additional unknown species.

Additional studies of *S. hawaiiensis* are clearly necessary, including single-cell amplifications, investigation of its reproduction and life history, ultrastructure of the budding-like cell division and the identification of the carotenoid pigments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Low molecular weight carbohydrate content.

Appendix S2. LSU and SSU rDNA sequences.

Appendix S3. Primers used for PCR amplifications.

Appendix S4. Specifications of the sequence alignments

Appendix S5. Image of potentially cladophoralean filament.

Appendix S6. ITS rDNA BLAST & FASTA results.