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# Comparison of lichen-forming cyanobacterial and green algal photobionts with free-living algae

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**Abstract** – Cyanobacteria of the genus *Nostoc* and green algae in the Chlorophyceae are widespread in nature and may occur in symbiotic associations with lichen-forming ascomycetes or as free-living cyanobacteria. Recent findings for some groups of lichens suggest that special lichen-forming photobiont lineages may exist independent of the free-living lineages, but few comparisons on photobiont growth, pigment, and polysaccharide production have been made. The goal of this study was to isolate photobionts, confirm their identity, and characterize their growth, pigment and polysaccharide production relative to free-living lineages. Algal growth, pigment contents, and polysaccharide concentration was measured using standard methods. The identification of *Nostoc* species was determined using transfer RNA for Leucine (*trnL*) nucleotide sequences and green algae using the internal transcribed spacer 1 of ribosomal DNA (ITS rDNA) sequences. An additional heptanucleotide repeat was present in the trnL gene of the *Nostoc* strain that associates with *Leptogium rivulare*. The biomass of pigment and polysaccharide production was highest in the lichenized *Diplosphaera chodatii* but the specific growth rate was highest in the free-living green alga, *Chlorella vulgaris*. The specific growth of the free-living *Nostoc* was higher than the lichenized *Nostoc* but pigment production was similar and polysaccharide production was lower than some of the lichenized *Nostoc* isolates. It was further hypothesized that the rates of growth, polysaccharide and pigment production may be key factors in compatibility of lichen algae with the fungus.

**ITS rDNA / lichen photobiont / *Nostoc* / Percoll / trnL gene**

## INTRODUCTION

The photoautotrophic partner (photobiont) that associates with lichen-forming fungi (mycobiont) is either a cyanobacterium, mostly *Nostoc*, or a green alga in the Chlorophyta. The mycobiont may have developed symbiotic partnerships with photoautotrophs early in evolution with fossil records dating back to 400-600 million years ago (Taylor *et al.,* 1995; Yuan *et al.,* 2005). Even with the long evolutionary history, only about 100 species of green algae and cyanobacteria associate with 18,000 species of recognized lichen-fungi (Galun, 1988; Feuerer & Hawksworth, 2007). These photobionts are mostly in the class Trebouxiophyceae, and in the class Cyanophyceae, family Nostocaceae (Friedl &

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Büdel, 2008), nevertheless free-living Chlorophytes and *Nostoc* are also present in the surrounding habitat and are presumably in contact with the lichen fungus. Even though free-living algae are readily available, previous work suggested that lineages of symbiotic algae have evolved (Lücking *et al.,* 2009). Growth of the algae that associate with lichen fungi is slower than growth of most free-living algae (Friedl & Büdel, 2008), and links with polysaccharide production (Palmqvist *et al.,* 2008) and cell communication (Sacristán *et al.,* 2007) have been established. Studies on growth, pigments and carbohydrate production in lichen algae have been conducted (Tschermak-Woess, 1980; 1989a; 1989b; Bakor *et al.,* 1998; Kranner *et al.,* 2005; Balarinová *et al.,* 2013) showing variability within the genus *Trebouxia* (Voytsekhovich & Kashevarov, 2010). While differences between free-living and lichenized algae have been described (Richardson, 1973), quantitative comparison among lichen algae and some free-living forms will allow further understanding of algae that form lichen associations.

Studies on the growth and taxonomy of lichen-forming algae require algal isolation and subsequent cultivation on artificial medium. Many methods for isolating lichen photobionts have been developed using centrifugation (Richardson, 1971; Ascaso, 1980; Calatayud *et al.,* 2001), or other methods (Wiedeman *et al.,* 1964; Ahmadjian, 1967a, 1967b; Beck & Koop, 2001; Yoshimura *et al.,* 2002; Weissman *et al.,* 2005). Because of the challenge of isolating lichenized algae from other widely available algae in the surrounding habitat and on the thallus, the identification of the culture must be compared with that of *in situ* algae using molecular markers (Gielly & Taberlet, 1994; Hall *et al.,* 2010).

The objectives of this study were to 1) confirm the identity of the photobiont, and 2) to compare the growth curve, pigment content, and production of polysaccharides among symbiotic photobionts and to use free-living green algae and cyanobacteria as a benchmark to compare these parameters.

## MATERIALS AND METHODS

### Isolation of photobiont

Ten lichen species were collected from different locations (Table 1) in Manitoba and Ontario, Canada. All lichen specimens were deposited in the cryptogam division at the University of Manitoba Herbarium (WIN-C) as voucher specimens. We chose lichens with different photobionts to cover a range of photobionts, a cyanobacterium (*Nostoc*) and four green algae (*Trebouxia, Asterochloris, Diplosphaera*, and *Coccomyxa*), which were readily available as pure cultures. We also chose two green algae (*Coccomyxa* sp. and *Chlorella vulgaris* Beyerinck (Beijerinck)) and a cyanobacterium (*Nostoc muscorum* C. Agardh *ex* Bornet & Flahault) that are free-living and compared them to a more intensely studied free-living cyanobactium, *Spirulina platensis* (Norst.) Geitler, which produces high levels of polysaccharides. The lichen material was air-dried, in the shade at room temperature and the free-living algae were obtained from freshwater samples and rock scrapings (Table 1) and kept at 4°C until needed for experimentation or obtained from the Phycology lab, Faculty of Science, Tanta University, Egypt. The identification was carried out according to

Table 1. Collection number and location for lichen samples, showing collection information and identified photobionts

| *Lichen species* | *Coll. No.* | *Coll. location and date* | *Habitat and substrate* | *Expected photobiont and source* |
|---|---|---|---|---|
| Cyanobacteria | | | | |
| *Peltigera horizontalis* | Normore 10468 | Canada, MB, Payuk Lake. 2012 | Rocky shoreline; among moss. | *Nostoc* sp. (O'Brien *et al.,* 2005) |
| *Peltigera canina* | Normore 10489 | Canada, MB, Payuk Lake. 2012 | Rocky shoreline; among moss, soil, and plant debris. | *Nostoc* sp. (Paulsrud *et al., 1*998) |
| *Leptogium rivulare* | Brinker and Lewis 1086 | Canada, ON, southwest of Latta. 2012 | Spruce-Jackpine forest; on tree root. | *Nostoc* sp. in other *Leptogium* spp. (Wirtz *et al.,* 2003; Stenroos *et al.,* 2006; Olsson *et al.,* 2012) |
| Green algae | | | | |
| *Cladonia pleurota* | Normore 10434 | Canada, MB, Payuk Lake. 2012 | Rocky shoreline adjacent to Spruce-Jackpine forest; on soil. | *Asterochloris* sp. (Piercey-Normore and DePriest, 2001) |
| *Staurothele fissa* | Normore 10437a | Canada, MB, Payuk Lake. 2012 | Rocky shoreline at edge of water; on granite rock. | *Diplosphaera* sp. (Thues *et al.,* 2011) |
| *Dermatocarpon luridum* | Normore 10437b | Canada, MB, Payuk Lake. 2012 | Rocky shoreline at edge of water; on granite rock. | *Diplosphaera chodati* (Fontaine *et al.,* 2012) |
| *Peltigera aphthosa* | Normore 10459 | Canada, MB, Payuk Lake. 2012 | Spruce-Jackpine forest; on moss and soil. | *Coccomyxa* sp. (Cordeiro *et al.,* 2010) |
| *Stereocaulon paschale* | Normore 10461 | Canada, MB, Payuk Lake. 2012 | On soil with moss. | *Asterochloris* sp. (Bakor *et al.,* 2010; Nelsen and Gargas, 2006) |
| *Cladonia macrophylla* | Normore 10506 | Canada, MB, Payuk Lake. 2012 | Rocky shoreline adjacent to Spruce-Jackpine forest; on soil among moss. | *Asterochloris* sp. (based on other *Cladonia* species in Piercey-Normore and DePriest, 2001) |
| *Phaeophyscia sciastra* | Normore 10554 | Canada, MB, Payuk Lake. 2012 | Vertical rock face sheltered from lake by thin margin of Spruce and Birch trees; on rock. | *Trebouxia* sp. (Dahkild *et al.,* 2001) |

Prescott (1978). The algal identification were also confirmed by comparing the ITS rDNA and trnL sequences with those in NCBI GenBank.

The photobionts were isolated following the micromethod of Gasulla *et al.* (2010) with some modification. The first part of the protocol was followed but after the 2-fold dilution with sterile distilled water and centrifugation at 10000×g for 10 min, there was no sonication as in Gasulla *et al.* (2010). The pellet was streaked on sterile 2% agar with BBM **(**Bischoff & Bold, 1963) and three times more nitrogen was used for the cyanobacteria. The Modified Detmer Medium (MDM; Watanabe 1960) was used for green algae. All the isolated photobionts and free-living algae and cyanobacteria were maintained on the appropriate agar media as slants at 4°C for future work.

**DNA extraction, PCR amplification and DNA sequencing**

Total genomic DNA was extracted from dried lichen thallus following the standard CTAB (cetyltrimethyl ammonium bromide) protocol modified from (Grube *et al.,* 1995), with minor modifications. Algal DNA was resuspended in sterile distilled water and amplified by polymerase chain reaction (PCR). The internal transcribed spacer 1 of ribosomal DNA (ITS rDNA) was amplified using the green algal-specific primer nr-SSU-1780-5`(50`-TGCGGAAGGATCATTGATTC-3`) (Piercey-Normore & DePriest, 2001) and ITS2R (5`-GCTGCGTTCTTCATCGATGC-3`) (White *et al.* 1990). However, the transfer RNA for Leucine (*trnL*) intron was used for the cyanobacteria with primers, trnL-F (5`-GGGGRTRTGGYGRAAT-3`) and trnL-R (5`-GGGGRYRGRGGGACTT-3`) (Paulsrud & Lindblad, 1998). PCR was performed in 20 µl reaction volumes using 1X PCR buffer (200mM Tris-HCl (pH 8·4), 500mMKCl), 200 µM of dNTP, 0.5 µM of primers, 0.5U Pfu DNA Polymerase, 2.5 µM of $MgCl_2$, 1 µl of DNA (10-50 ng). The PCR cycle for all algae started with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were purified and sequenced using BigDye v. 3.1 as previously described by Doering & Piercey-Normore (2009) on a 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequences were compared with other sequences by BLASTn in the NCBI GenBank database for gene similarity (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were assembled into full-length sequences using Sequencher v. 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Both strands were sequenced and they were aligned using ClustalX (Larkin *et al.*, 2007).

**Determination of growth**

Three types of growth media were used for the culture of algal species namely Modified Detmer Media (MDM; Watanabe, 1960) for cyanobacteria (blue-green algae), Zarrouk culture medium (Zarrouk, 1966) for *Spirulina platensis,* and 3N Bold's Basal medium (BBM) for the green algae (Chlorophyta). For growth, pigments and carbohydrate measurement, each algal strain was pre-cultured in 100-mL Erlenmeyer flasks at an initial O.D. 560 nm of 0.1 at 26 ± 1°C for free living algae either Cyanophyta or Chlorophyta except *Spirulina* cultivated at 31 ± 1°C. The photobionts were cultivated at 20 ± 1°C. The cultures were illuminated with continuous cool white fluorescent lights (60 µmol photons $m^{-2}$ $s^{-1}$). After stirring the algal culture for 30 min using a sterilized magnetic stirrer to avoid any multicellular aggregation, the growth phases were determined by monitoring changes in absorbance at 560 nm (O.D. 560nm) with a NanoDrop 2000c spectrophotometer (Wetherell, 1961).

**Dry weight**

The dry weight (DW) of algal cell suspension was calculated using standard curves of optical density vs. dry weight at a specific time in the exponential phase of the growth cycle. Five serial dilutions were made from this stock and the O.D at 560 nm was measured in triplicate. The DW of each dilution was measured in triplicate using a pre-weighed cellulose acetate filter paper and

kept in an oven at 60°C until constant. The specific growth rates of the algal species were calculated, as described by Wood *et al.* (2005) based on the formula: Specific Growth rate = (ln DWL – ln$DW_E$)/$t_L$ –$t_E$ with $DW_L$ representing the dry weight ($\mu gml^{-1}$) at days of late exponential phase ($t_L$) and $DW_E$ at days of early exponential phase ($t_E$), respectively.

**Estimation of photosynthetic pigments**

Photosynthetic pigments, chlorophyll-a, b, and carotenoids were determined by a spectrophotometer using the method recommended by Mckinney (1941). In this method, a known volume of algal culture was pipetted at the exponential growth phase and centrifuged at 3000 rpm for 10 min. The supernatant was decanted and a half volume of water-free methanol was added. The tubes were incubated in a water bath at 55°C for 15 min, and then centrifuged again. If the pellet was not white in color, the extraction was repeated. The supernatant, which contained the pigments, was measured against a blank of free methanol at 650, 665 and 452 nm. Methanol was used for pigment extraction, which was shown to be the best solvent for pigment extraction (Wright *et al.,* 1997; Henriques *et al.,* 2007). It is possible to determine the concentration of each pigment fraction of chlorophyll-a, b, and carotenoids as µg /ml of algal suspension using the following equations:

Chlorophyll-a = $10.3E_{665}$nm – $0.918E_{650}$nm

Chlorophyll-b = $1907E_{644}$nm – $3.87E_{663}$nm

Carotenoids = $4.2E_{452}$nm – (0.0246 chl-a + 0.426 chl-b)

Where E is the reading of the sample on the spectrophotometer.

After the concentration of the pigments (Chl a, b and carotenoids) in the methanol extract was determined, the amount per gram dry weight was calculated as follows:

$$\text{Pigment} = \frac{\text{PC (µg/ml)} \times \text{Extract volume (ml)}}{\text{Weight of sample (mg)}}$$

Where PC is a pigment concentration that was a calculated value from the previous equation (µg/ml).

**Estimation of total carbohydrate productivity**

Total soluble carbohydrates were extracted from the algal cells with 1 N NaOH in a boiling water bath for 2 hours (Payne & Stewart, 1988) after pigment extraction. Quantitative total soluble carbohydrate was determined using the phenol-sulphuric acid method (Masuko *et al.,* 2005) with some modification. Thirty µl of 5% phenol were added to a 50 µl sample (1 N NaOH extract) in an Eppendorf tube, and then 150 µl concentrated sulphuric acid were added directly on the surface of the Eppendorf tube. After incubation for 5 min at 90°C in a water bath, the tubes were cooled to room temperature for 5 min in another water bath. The absorption was measured using a NanoDrop 2000/2000c spectrophotometer against a blank at 490 nm using a microcuvette (200-70 µl). The carbohydrate concentration of the unknown solution was estimated after preparation of a calibration curve using glucose as a standard. The total carbohydrate content was calculated per dry weight of the sample.

### Statistical and phylogenetic analyses

Results are presented as mean ± standard deviation of the mean, n = 3. The statistical analyses were carried out using the SPSS program, SPSS 10.0 software (SPSS, Richmond, VA, USA) as described by Dytham (1999). Data were analyzed to determine the degree of significance between treatments using one way analysis of variance (ANOVA) with Duncan's multiple range tests for comparison of the significance level between values at $P < 0.05$ level of significance. Aligned sequences were subjected to phylogenetic analyses using MEGA6 (Tamura *et al.*, 2013) using two sets of data; trnL gene from 17 cyanobacteria and the ITS rDNA region from 28 green algae. A dendrogram was constructed using the neighbor joining (NJ) algorithm (Saitou & Nei, 1987) based on parameter distance (PD) (Nei & Kumar, 2000), Maximum Likehood (ML) method based on the Kimura 2-parameter model (Kimura, 1980) and Maximum Parsimony (MP) analyses using the option for tree bisection and reconnection (TBR) branch swapping in MEGA6. The node support was conducted using 1000 bootstrap resamplings (Felsenstein, 1985). Bootstrap was done for NJ-PD, ML and MP in MEGA6 and values greater than 70 are reported in the trees.

## RESULTS

### Photobiont identity

Two phylogenetic trees show the placement of the sequences from both the *in situ* photobiont as well as the alga from culture with respect to similar GenBank sequences (Fig. 1). Sequences obtained from each of the thallus and cultured algae were identical or highly similar to one another. All samples were most similar to the expected photobiont from GenBank except two samples (algae from each of *C. macrophylla* (Schaerer) Stenh. and *P. sciastra* (Ach.) Moberg). Morphological examination of these two algal species showed that they are species belonging to the Chlorophyta with oval to cylindrical cell shapes and single chloroplasts. The algae that were isolated from *C. macrophylla* showed the cells to be ellipsoidal to nearly spherical, 3-4 X 2.5-3 μm in diameter with discoid chloroplasts. Therefore, this alga may be a species of *Stichococcus* according to Khaybullina *et al.* (2010). However, the alga that was isolated from *P. sciastra* had cylindrical to oval cells 2.5-2 X 1.25-1.5 μm in diameter with one elongate parietal chloroplast. Therefore, it may be a *Pseudococcomyxa* species according to Khaybullina *et al.* (2010). For this paper, these algae are hereafter called *Stichococcus*-like and *Pseudococcomyxa*-like, respectively.

The cyanobacterial sequence alignment consisted of 419 bp of the trnL gene containing an intron. The cyanobacterial analysis produced a NJ tree with a sum of branch length 0.69777827 for NJ-PD and log likelihood was (-1681.5536) in ML. The MP tree length was 221 changes, the CI was 0.741117, RI was 0.893750. The trnL intron contained four heptanucleotide repeats before and after the central loop in all samples analyzed except the thallus and culture from *Leptogium rivulare* (Ach.). *L. rivulare* contained a 24 bp insertion within the third heptanucleotide repeat and an additional heptanucleotide repeat after the eighth repeat. The NJ tree placed the *Nostoc* sequence isolated from *Peltigera canina* (L.) Willd. (MN-10489) with GenBank sequences representing other *Nostoc* sequences isolated from *Peltigera* species with 99, 94, and 99% bootstrap support for NJ-PD, ML and MP,

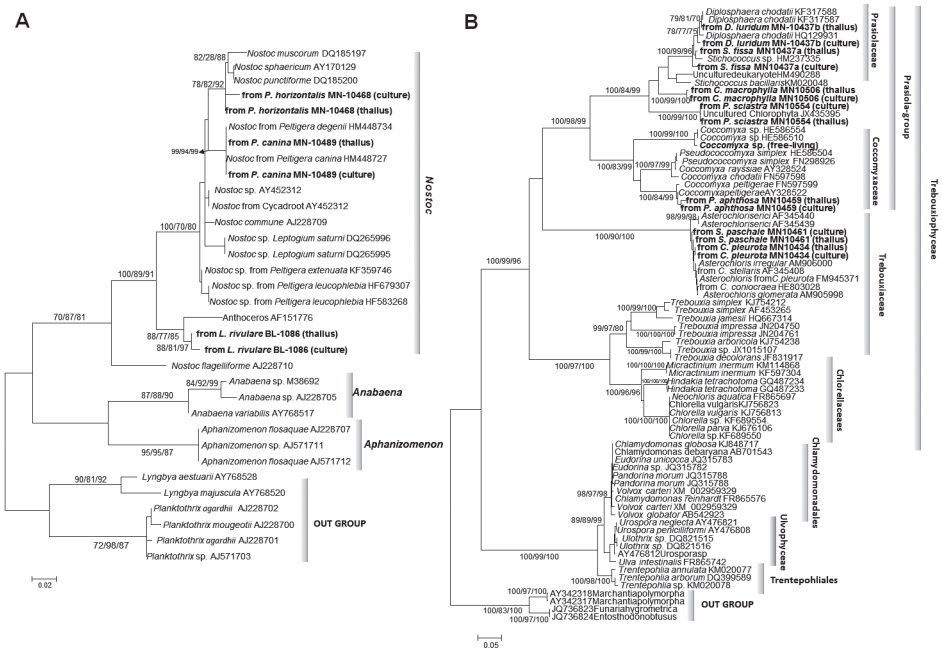


Fig. 1. Neighbor Joining (NJ) dendrograms showing **A.** cyanobacterial photobionts based on trnL nucleotide sequences and **B.** green algal photobionts based on ITS rDNA nucleotide sequences. Bootstrap values greater than 70 are shown on both trees in order of NJ distance (left), Maximum Likelihood bootstrap (in the middle) and Maximum Parsimony (MP) (right).

respectively. However, the *Nostoc* sequences isolated from *P. horizontalis* (Hudson) Baumg. (MN-10468) were placed with GenBank sequences of *Nostoc punctiforme* (Kütz.) Har., *Nostoc muscorum* and *Nostoc sphaericum* Vaucher with 78, 82, and 92% bootstrap support for NJ-PD, ML and MP, respectively. The photobiont from *Leptogium rivulare* is placed with a GenBank sequence of *Nostoc* from *Anthoceros* sp. with 88, 77, and 85% for NJ-PD, ML and MP, respectively.

The green algal alignment consisted of 340 nucleotides containing the complete ITS1 and 49 nucleotides of the 5.8S gene of the nuclear rDNA. The green algal analysis produced a NJ tree which had a 3.05503399 sum of branch length for NJ-PD and the log likelihood was (-6918.2902) in (ML). The MP tree length was 1607 changes, the CI was 0.671475, and the RI was 0.949845. The photobiont from *Dermatocarpon luridum* (With.) J. R. Laundon (MN-10437b) clustered with *Diplosphaera chodatii* Bialosukniá with 78, 77, and 75 % support for NJ-PD, ML and MP, respectively. The sister clade to this clade includes the photobiont from *S. fissa* (Taylor) Zwackh (MN-10437a) and *Stichococcus* which are all clustered together with 100, 99, and 96% support for NJ-PD, ML and MP, respectively. The photobiont of *Peltigera aphthosa* (L.) Willd. (MN-10459) clustered with *Coccomyxa peltigerae* Warèn with 99, 100, and 100% bootstrap for NJ-PD, ML and MP, respectively. The photobiont from *C. macrophylla* (MN-10506) is basal to the *Diplosphaera*/*Stichococcus* clade and basal to an uncultured Eukaryote. The photobiont of *P. sciastra* (MN-10554) clustered with sequences obtained by uncultured Chlorophyta with 100, 99, and 100% support for NJ-PD, ML and MP, respectively. The photobiont from *C. pleurota* (Flörke) Schaerer

(MN-10434) and *Stereocaulon paschale* (L.) Hoffm. (MN-10461) clustered with other *Asterochloris* sequences with 100, 90, and 100% bootstrap support for NJ-PD, ML and MP, respectively.

The identification of the algal strains in culture was confirmed by comparing their sequences with those of the thallus in the corresponding lichens and those of GenBank (Fig. 1 and Table 2). The cyanobacterial sequences were most similar to those of *Nostoc punctiforme*, *Nostoc* sp. HM448727 and *Nostoc* sp. AF151776 (Table 2). The green algae were genetically most similar to strains of *Diplosphaera chodatii*, *Stichococcus sp*., *Coccomyxa peltigerae* and *Asterochloris s*p. with ITS rDNA sequences deposited in GenBank. The cultured green algae showed significant matches with those of the thallus (Table 2).

Table 2. Sequence identity of cultured photobionts and photobionts within the thallus using a comparison with most identity GenBank accessions showing scores, percent coverage, e-value, and maximum identity with the most related species

| *Lichen species* | *Accession no.* | *Length (bp)* | *Max/Tot score* | *Query cover (%) /e-value /max identity (%)* | *Accession no.* | *Algal species* |
|---|---|---|---|---|---|---|
| Cyanobacteria | | | | | | |
| *Peltigera horizontalis* Culture | KJ690271 | 261 | 414/414 | 93/2e-112/96 | DQ185200 | *Nostoc punctiforme* |
| | | | 401/401 | 90/1e-108/96 | AY170129 | *Noctoc sphaericum* |
| | | | 477/477 | 89/3e-131/96 | HM448734 | *Nostoc* sp. |
| *Peltigera horizontalis* Thallus | KJ690274 | 263 | 473/473 | 82/4e-130/98 | DQ185200 | *Nostoc punctiforme* |
| | | | 464/464 | 80/2e-127/98 | AY170129 | *Noctoc sphaericum* |
| | | | 499/499 | 98/5e-123/97 | HM448734 | *Nostoc* sp. |
| *Peltigera canina* Culture | KJ690272 | 268 | 496/496 | 100/6e-137/100 | HM448727 | *Nostoc* sp. |
| | | | 496/496 | 100/6e-137/100 | HM448734 | *Nostoc* sp. |
| | | | 457/457 | 100/3e-125/97 | AY452312 | *Nostoc* sp. |
| | | | 446/446 | 100/6e-122/97 | HF583268 | *Nostoc* sp. |
| *Peltigera canina* Thallus | KJ690275 | 267 | 488/488 | 100/9e-135/99 | HM448727 | *Nostoc* sp. |
| | | | 488/488 | 100/9e-135/99 | HM448734 | *Nostoc* sp. |
| | | | 444/444 | 100/2e-121/97 | AJ228709 | *Nostoc commune* |
| | | | 438/438 | 100/1e-119/96 | HF583268 | *Nostoc* sp. |
| *Leptogium rivulare* Culture | KJ690273 | 298 | 466/466 | 100/5e-128/95 | AF151776 | *Nostoc* sp. |
| | | | 307/307 | 100/3e-80/87 | HF583268 | *Nostoc* sp. |
| | | | 298/298 | 100/2e-77/68 | DQ265996 | *Nostoc* sp. |
| | | | 298/298 | 100/2e-77/68 | DQ265996 | *Nostoc* sp. |
| *Leptogium rivulare* Thallus | KJ690276 | 299 | 477/477 | 100/2e-131/95 | AF151776 | *Nostoc* sp. |
| | | | 329/329 | 100/7e-78/88 | HF583268 | *Nostoc* sp. |
| | | | 331/331 | 100/2e-87/88 | DQ265995 | *Nostoc* sp. |
| | | | 331/331 | 100/2e-87/88 | DQ265996 | *Nostoc* sp. |
| Green Algae | | | | | | |
| *Cladonia pleurota* Culture | KJ690277 | 238 | 427/427 | 77/2e-116/99 | AM905999 | *Asterochloris irregularis* |
| | | | 427/427 | 77/3e-116/99 | HE803028 | *Asterochloris* sp. |
| *Cladonia pleurota* Thallus | KJ690285 | 236 | 414/414 | 77/2e-112/98 | AM905999 | *Asterochloris irregularis* |
| | | | 427/427 | 77/3e-116/99 | HE803028 | *Asterochloris* sp. |
| | | | 394/394 | 71/3e-106/99 | AM905998 | *Asterochloris glomerata* |

Table 2. Sequence identity of cultured photobionts and photobionts within the thallus using a comparison with most identity GenBank accessions showing scores, percent coverage, e-value, and maximum identity with the most related species *(continued)*

| *Lichen species* | *Accession no.* | *Length (bp)* | *Max/Tot score* | *Query cover (%) /e-value /max identity (%)* | *Accession no.* | *Algal species* |
|---|---|---|---|---|---|---|
| *Staurothele fissa* Culture | KJ690278 | 301 | 542/542 | 96/8e-151/99 | HM237335 | *Stichococcus* sp. |
| | | | 514/514 | 96/2e-142/98 | HQ129931 | *Diplosphaera chodatii* |
| *Staurothele fissa* Thallus | KJ690286 | 299 | 525/525 | 96/9e-146/98 | HM237335 | *Stichococcus* sp. |
| | | | 508/508 | 96/9e-141/97 | HQ129931 | *Diplosphaera chodatii* |
| *Dermatocarpon luridum* Culture | KJ690279 | 295 | 534/534 | 68/2e-148/99 | HQ129931 | *Diplosphaera chodatii* |
| *Dermatocarpon luridum* Thallus | KJ690287 | 285 | 416/416 | 100/4e-113/98 | HQ129931 | *Diplosphaera chodatii* |
| *Peltigera aphthosa* Culture | KJ690280 | 295 | 516/516 | 93/5e-143/98 | AY328522 | *Coccomyxa peltigerae* |
| | | | 156/156 | 74/1e-34/79 | HE586554 | *Coccomyxa* sp. |
| *Peltigera aphthosa* Thallus | KJ690288 | 236 | 431/431 | 88/2e-117/99 | AY328522 | *Coccomyxa peltigerae* |
| *Stereocaulon paschale* Culture | KJ690281 | 243 | 429/429 | 100/6e-117/98 | AF345408 | *Asterochloris* sp. |
| | | | 429/429 | 100/6e-117/98 | AM906000 | *Asterochloris irregularis* |
| | | | 418/418 | 100/1e-113/98 | HE803028 | *Asterochloris* sp. |
| *Stereocaulon paschale* Thallus | KJ690289 | 214 | 385/385 | 100/1e-103/99 | AF345408 | *Asterochloris* sp. |
| | | | 385/385 | 100/1e-103/99 | AM906000 | *Asterochloris irregularis* |
| | | | 375/375 | 100/6e-101/98 | HE803028 | *Asterochloris* sp. |
| | | | 375/375 | 100/6e-101/98 | AM905998 | *Asterochloris glomerata* |
| *Cladonia macrophylla* Culture | KJ690282 | 287 | 455/455 | 100/1e-124/93 | HM490288 | Uncultured eukaryote |
| | | | 315/315 | 94/2e-82/86 | HQ129931 | *Diplosphaera chodatii* |
| | | | 307/307 | 94/ 2e82/90 | JX435395 | Uncultured eukaryote |
| *Cladonia macrophylla* Thallus | KJ690290 | 287 | 411/411 | 99/2e-111/93 | HM490288 | Uncultured eukaryote |
| | | | 388/388 | 99/1e-104/91 | HQ129931 | *Diplosphaera chodatii* |
| | | | 363/363 | 99/1e-97/90 | JX435395 | Uncultured eukaryote |
| *Phaeophyscia sciastra* Culture | KJ690283 | 241 | 446/446 | 74/7e-122/100 | JX435395 | Uncultured Chlorophyta |
| | | | 263/263 | 99/8e-67/82 | HM490288 | Uncultured eukaryote |
| | | | 209/209 | 95/1e-50/79 | HQ129931 | *Diplosphaera chodatii* |
| *Phaeophyscia sciastra* Thallus | KJ690291 | 238 | 440/440 | 73/4e-120/100 | JX435395 | Uncultured Chlorophyta |
| | | | 248/248 | 96/2e-62/82 | HM490288 | Uncultured eukaryote |
| | | | 204/204 | 95/5e-49/79 | HQ129931 | *Diplosphaera chodatii* |
| Isolated free living algae | KJ690284 | 268 | 468/468 | 100/1e-128/98 | HE586554 | *Coccomyxa* sp. |

## Algal growth

In general, the exponential growth period of the chlorobionts and cyanobionts was much longer relative to the free-living algae. In the chlorobiont it ranged from 76 days for *Asterochloris*, 72 days for *Stichococcus* sp., 68 days for *Diplosphaera chodatii* and the *Stichococcus*-like alga, 64 days for *A. irregularis* and the shortest period was 56 days for *Coccomyxa peltigerae* and *Pseudococcomyxa*-like species. In contrast, the growth period for *Chlorella vulgaris* and *Coccomyxa* sp. as free-living green algae was 12 and 22 days, respectively. Regarding the cyanobionts, the exponential growth period was 32 days for *Nostoc* sp. AF151776, 40 days for *Nostoc* sp. HM448727 and 48 days for *N. punctiforme*. However, *Spirulina platensis* had a 14 day exponential growth period and *N. muscorum* had a 12 day exponential growth period.

The specific growth rates varied among the different species (Fig. 2). In general, the free-living Chlorophyta and Cyanophyta had higher growth rates compared with the photobionts. *Chlorella vulgaris* had the highest growth rate (0.16/day) for the Chlorophyta followed by *Coccomyxa* sp. (0.08/day). *Spirulina platensis* had the highest growth rate for the cyanobacteria followed by *Nostoc muscorum* (0.19, 0.09/day respectively). There were relatively small differences in the growth rates among the photobionts. *Diplosphaera chodatii* had the highest growth rate (0.04/day) and *Asterochloris irregularis* had the lowest growth rate among the chlorobionts (0.003/day). *Nostoc punctiforme* had the highest growth rate (0.045/day) followed by *Nostoc* sp. AF151776 and *Nostoc* sp. HM448727 (0.022, 0.011/day) for the cyanobionts.

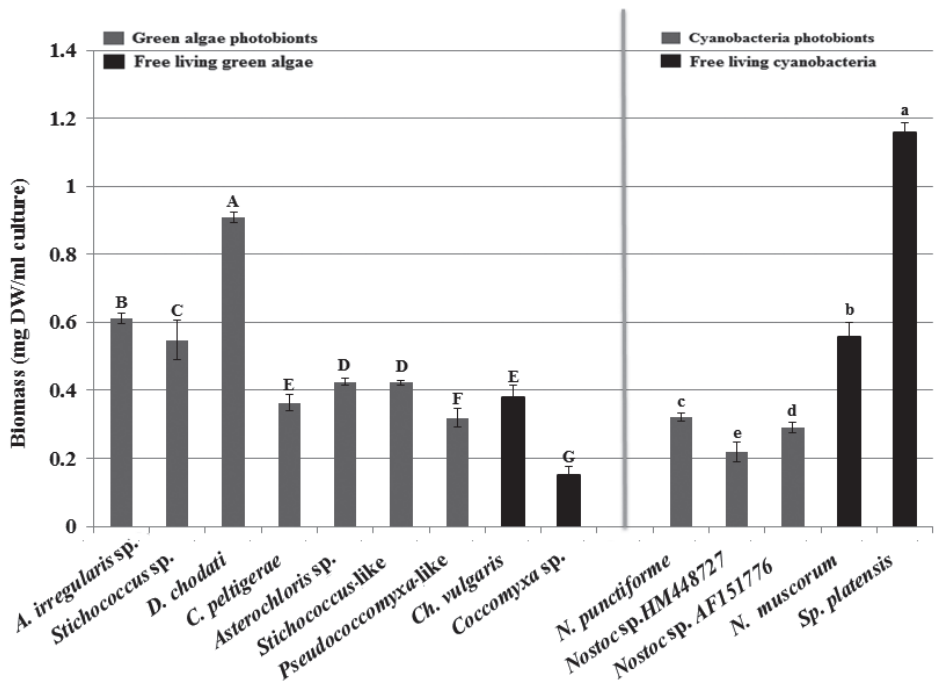


Fig. 2. Biomass productivity (Dry weight µg/ml culture) as a measure of growth of nine green algal taxa and five cyanobacterial taxa. Each point represents the mean value of three replicates; bars indicate standard deviations. Different letters represent significant differences at $P < 0.05$ (Duncans).

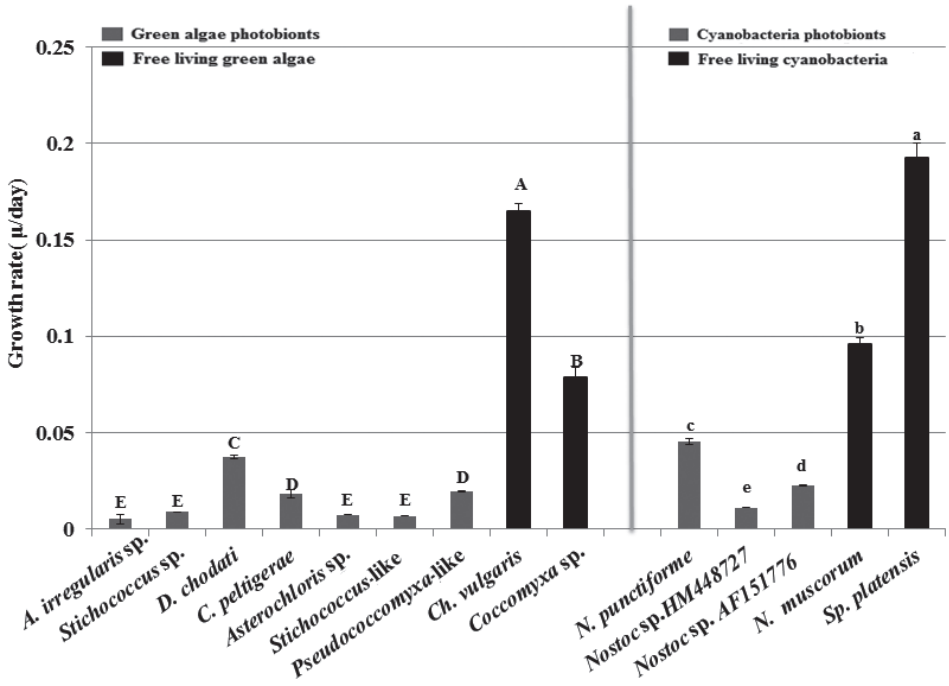


Fig. 3. The specific growth rate (µ/day) of nine green algal taxa and five cyanobacterial taxa. Each point represents the mean value of three replicates; bars indicate standard deviations.

## Biomass productivity

The biomass yield of the chlorobionts for each mL of algal suspension was higher than that of the free-living green algae (Fig. 2). *Diplosphaera chodatii* gave the highest biomass (0.90 mg compared to the free-living algae or photobiont, while the *Pseudococcomyxa*-like culture had the lowest biomass yield (0.31 mg). *Chlorella vulgaris* had biomass higher than *Coccomyxa peltigerae* (0.37, 0.15 mg, respectively). On the other hand, free-living cyanobacteria gave higher biomass than the cyanobionts. *Spirulina platensis* produced the highest biomass (1.15 mg) followed by *N. muscorum* (0.55 mg), *N. punctiforme* (0.32 mg), *Nostoc* sp. AF151776 (0.29 mg), and finally *Nostoc* sp. HM448727 (0.22 mg).

## Estimation of photosynthetic pigments

The results of the pigment analyses at the exponential phase (Fig. 4) revealed that Chlorophyll a and carotenoid contents of the Chlorophyta species were higher than the corresponding values of the cyanobacteria. The Chlorobionts had pigment contents (PC) higher than the free-living Chlorophyta. *Diplosphaera chodatii* had the highest total content of Chlorophyll a (15.07 µg/mg) and carotenoids (6.04 µg/mg) relative to other photobionts or the free-living Chlorophyta. The lowest total pigment content was in *Asterochloris irregularis* (Chlorophyll a 0.7 µg/mg, carotenoids 0.19 µg/mg). However, free-living *Chlorella vulgaris* had

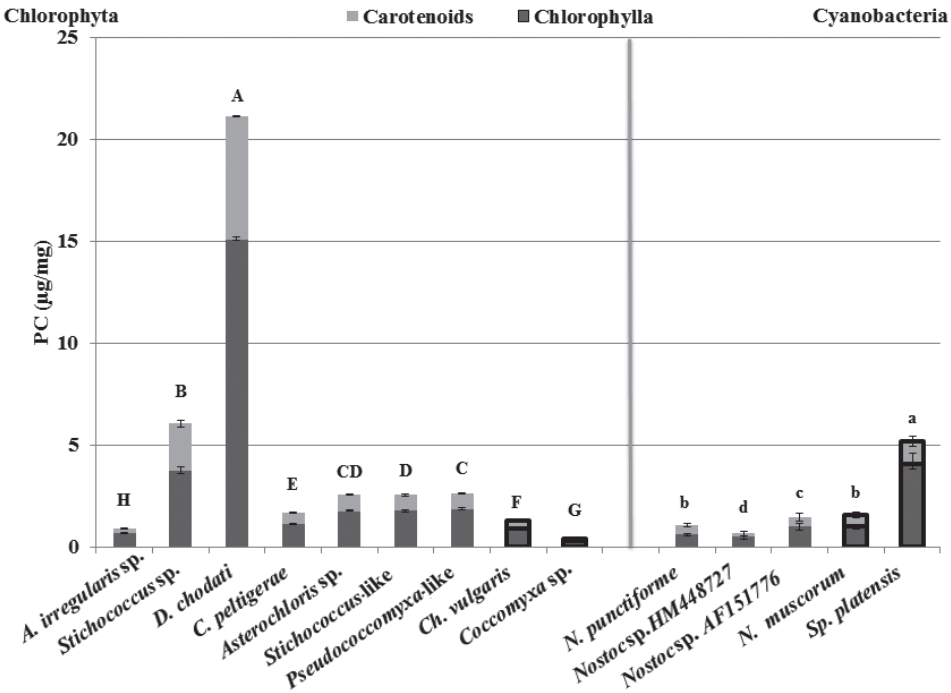


Fig. 4. Total pigment contents (µg/mg) of nine Chlorophyta taxa and five cyanobacterial taxa for chlorophyll a and carotenoids (the column with a black border refers to free-living green algae or cyanobacteria). Each point represents the mean value of three replicates; bars indicate standard deviations. Different letters represent significant differences at $P < 0.05$ (Duncans).

Chlorophyll a (0.90 µg/mg) and carotenoids contents (0.36 µg/mg) higher than *Coccomyxa* sp. (Chlorophyll a 0.29 µg/ mg, carotenoids 0.13 µg/ mg, respectively).

Free-living cyanobacteria had pigment contents higher than the cyanobionts. *S. platensis* had the highest pigment content (Chlorophyll a 4.09 µg/mg, Carotenoids 1.06 µg/mg) of the tested cyanobacteria followed by *N. muscorum*. However, *Nostoc* sp. AF151776 had pigment contents higher than *N. punctiforme* and *Nostoc* sp. HM448727, respectively. We observed a highly significant difference in pigment content between algae by the one-way ANOVA at $p < 0.05$.

## Carbohydrate contents

In general, the carbohydrate content (CC) for Chlorophyta was higher than that of the cyanobacteria (Fig. 5). Within the Chlorophyta, *D. chodatii* showed the highest carbohydrate content (34.84 µg/mg) relative to other green algal symbionts or the free-living green algae (*Chlorella vulgaris* and *Coccomyxa s*p. HE586554). The *Stichococcus* sp. had the next highest CC (12.37 µg/mg), followed by the lichenized *Asterochloris irregularis, Pseudococcomyxa*-like, *Coccomyxa peltigerae,* and *Asterochloris* sp. The lowest carbohydrate content was recorded for *Stichococcus* sp. (6.81 µg/mg).

*Spirulina platensis* had the highest CC for all the cyanobacteria with 18.53 µg/mg followed by *N. punctiforme* (6.89 µg/mg), *Nostoc* sp. AF151776

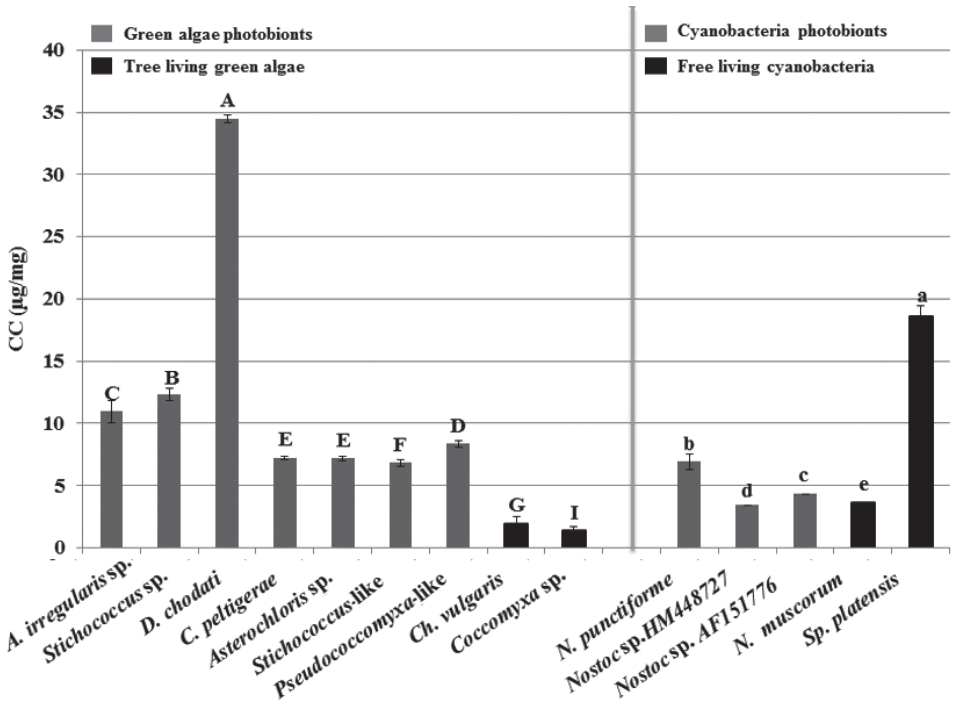


Fig. 5. Total carbohydrate contents (ug/mg) of nine Chlorophyta taxa and five cyanobacterial taxa. Each point represents the mean value of three replicates; bars indicate standard deviations. Different letters represent significant differences at $P < 0.05$ (Duncans).

(4.30 µg/mg), *N. muscorum* (free living) (3.63 µg/mg), and *Nostoc* sp. HM448727 (3.42 µg/mg). We observed a highly significant effect of the different algae on carbohydrate productivity in the one-way ANOVA at $p < 0.05$ (Fig. 5).

## DISCUSSION

### Photobiont selection and isolation

The modified Percoll centrifugation method in this study was successful in isolating the photobionts of lichens (nevertheless see results for *Cladonia macrophylla* and *Phaeophyscia sciastra*) proving to be an effective method for both cyanobacterial and green algal isolation. This is the first time that this method is used to isolate cyanobacterial symbionts. The fungal symbionts were absent from all cultures after only two subculturing events. However, after plate spreading the diverse algal taxa were present in some cultures.

While **t**his study confirmed the identity of eight of ten lichen photobionts using a phylogenetic evaluation of the cultured and the *in situ* algae (see references in Table 1), there were two anomalous findings. Photobiont isolates from *Cladonia macrophylla* (growing on thin soil within rock crevices; Hinds and

Hinds (2007)) and *Phaeophyscia sciastra* (growing directly on rock; Hinds and Hinds (2007)), were not as expected. The unexpected findings may be explained by at least three scenarios even though some may be unlikely scenarios. 1) These lichens may have contained other superficial undetermined chlorophytes on the surface of the thallus or in crevices within the thallus. Other studies have shown organisms to be prevalent within the lichen thallus such as bacteria (Bates *et al.,* 2011), lichenicolous fungi (Lawrey & Diederich, 2003), and even green algae within the thallus of cyanobacterial lichens (Henskens *et al.,* 2012). 2) The photobionts were a mixture of both *Asterochloris*/*Trebouxia* and the *Stichococcus*-like/*Pseudococcomyxa*-like algae throughout the lichen thallus. Multiple algae within a thallus have been shown previously (Piercey-Normore, 2006; Bakor *et al.*, 2010) and closely related species of lichens may share photobionts (Fontaine *et al.,* 2012). If *Stichococcus*-like/*Pseudococcomyxa*-like PCR product dominated the PCR reactions, the dominant chromatographic peaks in the sequences would show the *Stichococcus*-like/*Pseudococcomyxa*-like sequences. 3) The *Stichococcus*-like and *Pseudococcomyxa*-like algae may be the actual photobiont in the thallus of these species. Even though species of *Cladonia* are reported to associate with *Asterochloris* (Piercey-Normore and DePriest, 2001; Yahr *et al.,* 2006; kaloud & Peksa, 2010) and *Phaeophyscia* species associate with *Trebouxia (*Dahkild *et al.,* 2001; Hinds and Hinds, 2007), these species or specimens may be exceptions to this general rule of association. The fact that both thallus and algal culture show the same algal sequence but a different taxon than those expected supports the finding of these photobionts (*Stichococcus*-like/*Pseudococcomyxa*-like) in these lichens. However, this result needs further investigation for confirmation of photobiont identity.

This study also reported the unusual *Nostoc* trnL intron differences according to the structure reported in Costa *et al.* (2004). The unique repeat region in this species is notable since the cyanobacterial symbiont sequences from other species of *Leptogium* are present in the alignment but none of them have these additional repeat regions. The trnL intron of the cyanobacterial symbionts from the aquatic lichen, *Peltigera hydrotheria* Miadl. & Lutzoni, has a reduced number of repeats. It should be noted that both lichens are considered to be rare (COSEWIC: (http://www.cosewic.gc.ca/eng/sct1/SearchResult_e.cfm?commonName=andscience Name=leptogium+rivulareandSubmit=Submit). The use of the trnL region is contentious as a marker for cyanobacteria but it has been accepted in plants (Gielly & Taberlet, 1994). The intron in the trnL is as variable as the ITS rDNA regions and as an intron it is susceptible to horizontal transfer but it has been used for identification of cyanobacteria in some *Peltigera* species (Linke *et al.,* 2003).

While the nucleotide sequences were highly similar between thallus and culture pairs for each of the cyanobacterial and green algal trees, some were less similar such as those from *S. paschale* (MN-10461) and from *P. horizontalis* (MN-10468). The green alga from *S. paschale* (MN-10461) has 198 missing positions between culture and thallus sequences. The cyanobacterium from *P. horizontalis* (MN-10468) has 175 missing positions between culture and thallus sequences. The other differences in the tree may be explained by shorter regions of ambiguous or missing positions within the DNA sequence or near the ends of the sequences. Additionally, it cannot be discounted that the ITS2 rDNA contains intragenomic polymorphisms (Song *et al*., 2012), which may also explain some of the variability in topology. Another explanation for variation in topology is that if an alga like *Diplosphaera* is common as a free-living alga (Handa *et al.,* 2001), it may have sequence differences from the lichenized alga and have been present on the rocks when these lichens were collected. This would be consistent with the findings of

Hallmann *et al.* (2011) and Ragon *et al.* (2012) who demonstrated the occurrence of *Diplosphaera* on stone substrates.

**Comparison among free-living and lichenized algae**

The free-living genera, *Spirulina* and *Chlorella,* are considered to be high carbohydrate producers and have been successful in producing commercial products such as polysaccharides (Pugh *et al.,* 2001). Comparison of growth, pigment, and polysaccharide production by these free-living genera with lichen-forming algae illustrate the difference in growth and productivity between lichenized and non-lichenized conditions. However, the amount of algal pigment present may also vary with environmental conditions, the habitat occupied by the alga, and the life phase of the alga. Except for *Spirulina* (which requires specific growing conditions) we used the same growth media for all algae and measured the pigment and polysaccharide production at the same life phase, which is the exponential growth phase. Polysaccharide production is important to serve as an indirect measure of cell growth since photosynthesis provides the majority of carbon available for metabolism. The significantly larger amount of pigments produced by the lichenized *D. chodatii* than any other green alga, even the free-living *C. vulgaris,* provides an advantage over other algae for increased photosynthetic capacity. While its growth was not as rapid as the free-living *Chlorella vulgaris*, it was faster than any other lichenized alga and its carbohydrate content was higher than any green alga making this species the most productive despite its slower growth rate. This may be explained if *D. chodatii* can better utilize the nitrogen source in 3N BBM media than *C. vulgaris* to produce photosynthetic pigments. *C. vulgaris* occurs as a rock epiphyte like *Diplosphaera chodatii* (Hallmann *et al*., 2011a; Ragon *et al.,* 2012) and some species prefer nitrogen-rich habitats (Friedl, 2002). Also Ettl & Gärtner (1995) reported that some algae from the Prasiolales, are able to rapidly colonize nitrogen rich sites in mixed excreta and feces of birds, and produce photosynthetic pigments that cause staining of stone substratum. However, the colonization of nitrogen rich sites is typical of the genera, *Prasiola* and *Rosenvingiella,* but it may not be common with other genera (Rindi *et al.,* 1999). While the lichenized cyanobacteria had slower growth rates and less pigment production than the free-living species (except *Nostoc* sp. AF151776), the carbohydrate content was higher than the free-living *Nostoc*, again suggesting a high level of productivity in the lichenized cyanobionts.

While this study was not successful in isolating *Trebouxia* species, only one lichen that was cited to associate with *Trebouxia* (Hinds and Hinds, 2007) was investigated, *Phaeophyscia sciastra*. The growth of *Trebouxia* species has been examined previously (Balarinova *et al.,* 2013) as well as pigment production in other lichen algae (Voytsekhovich & Kashevarov, 2010), which were comparable to those in this study. Voytsekhovich & Kashevarov (2010) reported pigment production for other genera of lichen algae, and they implied that algal ecology, where obligate lichen-forming algae grow more slowly than facultative genera such as *Trentepohlia,* would have an effect on the ability to become lichenized. While this study examined different genera from those examined by Voytsekhovich & Kashevarov (2010), both studies came to a similar conclusion. The present study further suggests that the growth rate of the lichen algae, and the rates of pigment and carbohydrate production, may be important factors in determining the ability for algae to form lichens. It is noteworthy to mention that Athukorala *et al.* (2014) observed that slow growing cultures of *Chlorella vulgaris* showed signs of recognition in the first stage of the interaction, but there was no recognition in the faster growing cultures, suggesting a role of growth rates in the interaction.

## CONCLUSION

In conclusion, we observed that the micromethod for a Percoll gradient was an effective way to isolate both green algae and cyanobacteria from the lichen thallus. While the selection of algae after plate streaking produced multiple algae in some cultures, this procedure of algal selection may reveal larger numbers of potential lichen algae that are available for fungal ascospore symbiosis as shown in this study, and a potential new method for studying algal diversity and selection. Another finding in this study is the additional heptanucleotide repeat in the trnL gene of the *Nostoc* strain that associates with *Leptogium rivulare*, a threatened species. Further study may correlate the heptanucleotide repeat with the rarity of the species. A more significant finding of this study was that, while productivity of the free-living *Chlorella vulgaris* was higher than any other green alga, the pigment and polysaccharide production was highest in the lichenized *Diplosphaera chodatii.* High pigment and polysaccharide production in this lichen alga was unexpected and may be related to both the free-living capacity of the alga and its ability to lichenize, nevertheless further research is needed to further understand the biology of *D. chodatii*. Growth of the free-living *Nostoc* was higher than the lichenized *Nostoc* but pigment production was similar and polysaccharide production was lower than some of the lichenized *Nostoc* isolates. The rates of growth, polysaccharide and pigment production may be key factors in compatibility of lichen algae with the fungus but this possibility requires further study.

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