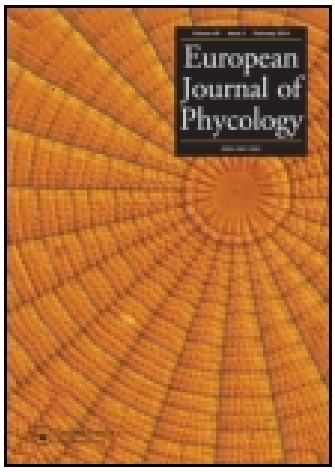

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Molecular diversity of green corticolous microalgae from two sub-Mediterranean European localities

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Molecular diversity of green corticolous microalgae from two sub-Mediterranean European localities

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Green algae in corticolous biofilms are simple coccoid cells or filamentous thalli with strikingly low morphological diversity. Consequently, microscopic identification of these organisms is difficult, and often possible only to higher taxonomic units. We investigated the taxonomic and phylogenetic composition of green microalgae isolated from biofilms growing on the bark of *Quercus pubescens* and *Pinus nigra*. The study was based on 122 partial sequences of the plastid-encoded *rbc*L gene. In total, 29 operational taxonomic units (OTUs), differing in their *rbc*L sequences, were encountered. Members of the Trebouxiophyceae formed 97.5% of the isolates; Streptophyta made up 2.5%. The most frequently occurring OTUs were in the genera *Coccomyxa, Parachloroidium* and *Stichococcus*. Within the *Watanabea* clade, we have probably discovered an as-yet undescribed generic lineage with chlorelloid morphology. OTUs belonging to the recently described trebouxiophycean genera *Kalinella, Leptochlorella* and *Xylochloris* were also encountered, which indicates that these genera are probably widely distributed in subaerial microhabitats, such as tree bark. The samples taken from oak trees were more diverse in their OTU composition than those taken from pine trees, but the average phylogenetic distances of OTUs in samples did not differ between the host tree taxa. Host tree species had a stronger effect on the community structure of algae than the sampling locality. This indicates that habitat filtering is important for the distribution of individual microalgal phylogenetic taxa.

Key words: chlorelloid microalgae, corticolous biofilms, distribution, diversity, phylogeny, *rbc*L, Trebouxiophyceae

Introduction

Phototrophic corticolous biofilms represent one of the most abundant microalgal assemblages in terrestrial habitats (Freystein & Reisser, 2010). They are chiefly composed of green algae and Cyanobacteria; diatoms, Xanthophyceae and Eustigmatophyceae are typically much less abundant (Hoffmann, 1989; Nakano *et al*., 1991; Ettl & Gärtner, 1995). Most microalgae occurring in these biofilms have a simple coccoid or filamentous morphology which is adapted to the frequent desiccation typically encountered in these subaerial microhabitats (Lüttge & Büdel, 2010). Consequently, microscopic identification of these organisms, which have strikingly low morphological diversity (Freystein *et al*., 2008; Neustupa & Štifterová, 2013), is difficult, and is often only possible to higher taxonomic units, such as genera or species complexes.

Several relatively well-defined green algal phylogenetic lineages frequently occur in subaerial habitats, such as Trentepohliales (Printz, 1939), Trebouxiales (Škaloud & Peksa, 2010), Klebsormidiales (Rindi *et al*., 2011), Prasiolales (Rindi *et al*., 2007), and Coccomyxaceae (Ettl & Gärtner, 1995). A number of new taxa of chlorelloid green microalgae, isolated from various corticolous biofilms, were recently described taxonomically and defined by molecular phylogenetic methods (e.g. Darienko *et al*., 2010; Ma *et al*., 2013; Neustupa *et al*., 2013*a*, *b*). Notably, the species and genera belonging to the phylogenetically defined *Watanabea* clade of Trebouxiophyceae seem to be especially abundant in various subaerial microhabitats. These algae typically have simple chlorelloid morphology and small cell dimensions, mostly not exceeding 10 µm in diameter. In this clade, three of the four currently recognized species of the genus *Chloroidium*, have, so far, only been found in subaerial habitats (Darienko *et al*., 2010). The sister genus *Parachloroidium* includes two recently described species that are only known from corticolous biofilms (Neustupa *et al*., 2013*b*). In addition, the genera *Kalinella* and *Heveochlorella* were described from tropical South-East Asian corticolous assemblages (Zhang *et al*., 2008; Neustupa *et al*., 2009; Ma *et al*., 2013). *Kalinella apyrenoidosa*, one of the two species of the genus *Kalinella*, was recently described from a sub-Mediterranean corticolous sample (Neustupa *et al*., 2013*a*). The life cycle of a peculiar and little-known genus *Phyllosiphon*, another member of the *Watanabea* clade, probably includes chlorelloid subaerial individuals, as well as siphonaceous parasitic populations

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thriving in the leaves of the subtropical vascular plant genus *Arisarum* (Aboal & Werner, 2011; Hallmann *et al*., 2013).

In addition to taxa belonging to the *Watanabea* clade, the class Trebouxiophyceae also includes several deeply branching phylogenetic lineages known exclusively or mostly from corticolous microhabitats, such as the mixotrophic genera *Apatococcus* and *Auxenochlorella*, and the well-known lichen photobionts *Asterochloris, Trebouxia* and *Dictyochloropsis* (Friedl & Rybalka, 2011). The genus *Xylochloris*, also forming a deep lineage with an unresolved phylogenetic position within Trebouxiophyceae, was originally described from tropical corticolous biofilms (Neustupa *et al*., 2011). However, additional members of this lineage were recently found on tree bark and in soil samples from various temperate European localities (Hodač *et al*., 2012). Similarly, the genus *Leptochlorella* was recently described from a sub-Mediterranean bark microhabitat (Neustupa *et al*., 2013*a*), but various published environmental sequences suggest that it is more widely distributed (De Wever *et al*., 2009; Wong *et al*., 2010).

The morphological similarity of these different, relatively deep, trebouxiophycean lineages could be ascribed to strong positive selection for globular cell shapes with low surface-to-volume ratios in terrestrial conditions. In fact, almost all the coccoid green algae growing on tree bark share globular or ellipsoidal cell morphology, and usually reproduce by producing asexual autospores (Ettl & Gärtner, 1995). Therefore, morphology alone simply cannot be used for the precise identification of these phylogenetically defined taxa. Data on the distribution of aero-terrestrial microalgae are very limited, and some of the species thriving in tree bark microhabitats are still only known from their type locality. Environmental and spatio-temporal factors driving the structure of corticolous microalgal communities are also extremely unclear. Several lines of research have suggested that the effects of host tree specificity, varying pH, irradiation, air pollution or regional climatic factors may be important (Hedenås *et al*., 2007; Freystein *et al*., 2008; Neustupa & Štifterová, 2013). However, all the studies were based on morphological identification, so that similar or morphologically indiscernible chlorelloid lineages could not be taken into account.

In this study, we focus on the molecular diversity of coccoid green microalgae in corticolous biofilms. The study was conducted in the northern Adriatic coastal region, which has a typical sub-Mediterranean climate and relatively high annual precipitation (~ 1000 mm). In a previous study, based on microscopy of bark samples, we showed that the morphological diversity of microalgae in this region clearly surpasses that of temperate microhabitats with lower annual temperatures and precipitation (Neustupa & Štifterová, 2013). In addition, several previously unknown subaerial microalgal lineages, such as the genera *Parachloroidium* and *Leptochlorella*, were recently described from this region (Neustupa *et al*., 2013*a*, *b*). Consequently, we aimed to determine whether these new chlorelloid taxa could be found again, indicating their more frequent occurrence in sub-Mediterranean corticolous biofilms. Furthermore, we strived to ascertain whether any new sequences, unassignable to any described species or genus, were present. Finally, we evaluated the relationship between the phylogenetic diversity of individual samples, their host tree taxa and the sampling locality. Significant effects of host tree taxon on the community structure of biofilms would indicate strong filtering by local biotic or abiotic factors, such as bark pH, whereas significant differences between biofilms from different sampling localities from various tree taxa would suggest possible dispersal limitations at the regional spatial scale.

Materials and methods

Sampling and localities

The samples were collected in April 2012 from the bark of eight trees in two sub-Mediterranean localities (each *c*. 0.5 ha): Fiesa, Piran (Slovenia, altitude: 30–50 m a.s.l., coordinates: 45°31'18"N, 13°34'52"E) and Cernizza, Duino (Italy, altitude: 10–40 m a.s.l., coordinates: 45°46'42"N, 13°35'37"E). The flight distance between localities was 28.25 km. Four samples, consisting of 3 cm^2 of bark surface, were taken at each locality from two *Pinus nigra* and two *Quercus pubescens* trees with trunk diameters of at least 25 cm. The samples were taken from randomly selected spots on the northern side of trunks at a height of 140–160 cm. Microhabitats visibly covered by lichens were avoided, and any isolated lichen thalli were carefully removed prior to further analysis. The samples were put into sterile bags and processed carefully to prevent cross-contamination. Details of the climatic conditions at the sampling localities are provided by Neustupa & Štifterová (2013).

Cultivation of samples

The surface biofilm from each sample was scraped into two 1.5 ml Eppendorf tubes and shaken at 1500 rpm for 20 s with 1.0 ml of liquid medium and 0.5 cm^3 of sterile glass beads (diameter, 0.75 mm). Then, 40 µl of the suspension from each Eppendorf tube was placed in two replicate Petri dishes (diameter, 10 cm) containing agar-solidified Bold's Basal Medium (BBM; Bischoff & Bold, 1963; Andersen *et al*., 2005). The pH of the culture medium was measured using a WTW pH-330 meter with a flathead electrode (SenTix Sur); pH value was 5.3. The samples were cultured for 21 days at 24 °C, with illumination of 40 µmol m^{-2} s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33). Eight randomly selected algal microcolonies from each Petri dish were isolated with a sterile dissecting needle and placed into a drop of distilled water on a sterile microscope slide. Cells

from all microcolonies were then photographed with an Olympus BX51 light microscope with differential interference contrast, using an Olympus Z5060 camera (Olympus, Tokyo, Japan).

DNA isolation, amplification and sequencing

Microcolonies were moved from microscope slides into polymerase chain reaction (PCR) tubes. After centrifugation, the cells of microcolonies were transferred to 2 ml tubes filled with 80 µl of InstaGene matrix (Bio-Rad Laboratories). The cells were mechanically disrupted by shaking for 6 min in the presence of two to three glass beads (1.5 mm diameter; Sigma-Aldrich) in a Mixer Mill MM 400 (Retsch, Haan, Germany). Subsequent steps of DNA isolation followed the procedure described by Škaloud *et al*. (2012). The chloroplast large subunit of RuBisCO (*rbc*L) was amplified by PCR. The PCR solution contained 13.9 µl sterile Milli-Q water, 2.0 µl AmpliTaq Gold® 360 buffer 10× (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 2.0 µl $MgCl_2$ (25 mM), 0.4 µl dNTP mix (10 mM), 0.25 µl of forward and 0.25 µl of reverse primers (25 nM), 0.2 µl AmpliTaq Gold® 360 DNA polymerase and 1 µl DNA (not quantified). Combinations of different pairs of primers were used to obtain as many sequences as possible from microcolonies: forward – PRASF1 (Sherwood *et al*., 2000), rbcL-203F (Nelsen *et al*., 2011)/reverse – PRASR1 (Sherwood *et al*., 2000), PRASR2 (Sherwood *et al*., 2000), rbcL-991R (Nelsen *et al*., 2011), ellaR2 (Neustupa *et al*., 2013*b*). The amplification conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 90 s, and elongation at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Filamentous microcolonies were also amplified with rbcL-KF2 and rbcL-KR2 primers (Škaloud & Rindi, 2013) using PCR cycles according to the original publication of primers. PCR products were analysed by electrophoresis on 1% agarose gel, stained with ethidium bromide. Correctly amplified products were cleaned with the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Missouri, USA) in accordance with the manufacturer's specifications, or with the ethanol-sodium acetate precipitation method. To each PCR tube, 2 µl of sodium acetate and 50 µl of 96% ethanol were added. The tubes were vortexed, spun at 40 rcf for 1 min and held at room temperature for 15 min. After centrifugation (3700 rpm) for 30 min, the supernatants were pipetted off. Subsequently, 100 µl of 70% ethanol was added to the pellets, and tubes were spun at 3700 rpm for 10 min. The supernatants were pipetted off and the pellets were dried in a thermoblock at 65 °C. Finally, DNA was resuspended in 20 µl of redistilled water warmed to 65 °C. The purified amplification products were sequenced using Applied Biosystems at Macrogen in Seoul, Korea. Sequencing reads were assembled and edited by using SeqAssem software (Hepperle, 2004). The sequences are available in the EMBL Nucleotide Sequence Database under accession numbers: HG793061-HG793083.

Phylogenetic and ecological data analyses

The manually edited sequence alignment consisted of 29 unique *rbc*L sequences (OTUs, operational taxonomical units) from 122 clonal isolates and 86 published sequences (the NCBI database, National Center for Biotechnology Information) that were selected on the basis of the genetic diversity of the OTUs. Partial *rbc*L sequences of isolates ranged in length from 545 to 1207 nucleotides. The final alignment with 1431 positions is available as supplementary material. The most appropriate substitution model was calculated in MEGA, ver. 5 (Tamura *et al*., 2011). The lowest value for the Bayesian information criterion was obtained by the general-time-reversible model with invariable sites and gamma distribution (GTR+G+I). The phylogenetic tree was inferred with the Bayesian inference (BI) method using MrBayes, ver. 3.1.2 (Huelsenbeck & Ronquist, 2001). Two parallel Markov chain Monte Carlo runs were carried out for 9 million generations, each with one cold and three heated chains. Convergence of the two cold chains was checked by the average standard deviation of split frequencies: the value was 0.013. Trees and parameters were sampled every 100 generations, and tree burn-in value was set to 1800. Bootstrap supports of individual phylogenetic lineages were calculated by maximum likelihood (ML) and weighted maximum parsimony (wMP) analyses. The ML bootstrapping was performed using *phangorn* package, ver. 1.7-4 (Schliep, 2011) in R, ver. 2.15.0 (R Development Core Team, 2012). The function *bootstrap.pml* was used for non-parametric bootstrap analysis (with 1000 replicates) of the individual tree nodes recovered by the ML analysis. The wMP bootstrapping (1000 replications) was performed using PAUP* version 4.0b10 (Swofford, 2002). Heuristic searches included 100 random sequence addition replicates, tree bisection reconnection swapping, and random addition of sequences (the number limited to 10 000 for each replicate). Character weights were assigned using the rescaled consistency index on a scale of 0 to 1000. New weights were based on the mean fit values for each character over all trees in the memory. The phylogenetic tree was graphically adjusted in FigTree ver. 1.3.1 (Rambaut, 2009) and Adobe Illustrator ver. CS4.

The abundances of OTUs in the samples, pooled data from two Petri dishes originating from a single sample, were used for two-dimensional non-metric dimensional scaling (NMDS) analysis with the Bray–Curtis index in PAST, ver. 2.15 (Hammer *et al*., 2001). The ordination diagram was created using SigmaPlot ver. 9.0 (Systat software). The effects of host tree species and locality on the composition of isolates from the biofilms were evaluated by permutational multivariate analysis of variance (Per-MANOVA), implemented by the function *adonis* of the *vegan* package (Oksanen *et al*., 2011) in R. The Bray–Curtis dissimilarities were used to create a distance matrix of the dependent variable (the abundances of OTUs in samples). The significance of the two factors and their interaction term was evaluated by randomization tests based on 999 permutations. The two-group permutation test, based on the Menhinick index, was used for statistical evaluation of the differences in alpha-diversity of the groups of samples (PAST, ver. 2.15). Tamura–Nei distances (Tamura & Nei, 1993) and uncorrected p-distances were calculated in MEGA to compare the phylogenetic distances between OTUs isolated from different tree species and localities. The obtained values were tested by permutation tests (999 permutations) for equitability of means between two groups of samples (tree or locality) using PAST software.

Results

Taxonomic composition

In total, 29 unique OTUs from 122 clonal isolates, differing in their partial *rbc*L gene sequences, were identified (Table 1, Fig. 1). Members of the Trebouxiophyceae were dominant, and the three most abundant trebouxiophycean OTUs (cort03, cort06 and cort15) represented more than 40% of the isolates. The OTU cort06, typified by its irregularly ellipsoidal coccoid cells and morphologically corresponding to the frequently occurring traditional genus *Coccomyxa* (Fig. 2), proved to be closely related to *Coccomyxa* sp. HQ287484, known from the bark of *Populus tremula* in northern Sweden (Muggia *et al*., 2011). In addition to cort06, four other OTUs belonged to the *Coccomyxa* lineage. In total, members of this trebouxiophycean lineage represented 21.3% of the isolates. Microcolonies with sequences identical to that of cort03 had minute oval to globular cells, 2.0–5.0 µm in diameter, which characteristically divided by binary fission and occasionally formed sarcinoid colonies (Fig. 3). Judging from these features, cort03 corresponded morphologically to the traditional genus *Diplosphaera.* This was confirmed by phylogenetic analyses of the *rbc*L sequences that placed it among various strains and natural populations identified as *Diplosphaera* spp. (Fig. 1). These taxa were part of the broader and well-supported *Prasiola* clade of the Trebouxiophyceae. In total, nine different OTUs, clustering in the *Prasiola* clade, formed 31.7% of the isolates. Three OTUs placed in this broadly defined lineage (cort02, cort05 and cort07), which were frequently encountered in samples from both tree species and in both localities, formed a well-supported independent clade without any close relatives among published *rbc*L sequences. Morphology of these OTUs generally corresponded to the traditionally defined genus *Stichococcus* (Fig. 4). The third most frequent OTU, cort15, had globular cells 2.5–7.5 µm in diameter, and reproduced by two to four oval autospores (Fig. 5). In most cases, a single relatively large autospore and several smaller autospores were formed within a single sporangium, demonstrating a pattern of autospore production well known in members of the trebouxiophycean *Watanabea* clade. Indeed, the *rbc*L gene sequences placed this OTU within this lineage: they were identical with sequence HF586462 from the type strain of *Parachloroidium laureanum*, CAUP H8501.

In total, seven OTUs, 30.3% of the isolates, belonged to the *Watanabea* clade. In addition to cort15, a single isolate, assigned as cort23, belonged to *Parachloroidium*, but its *rbc*L sequence differed from both described species of this genus. The *rbc*L sequence of cort24 (Fig. 6), which was found six times in two different samples (QC1, PF1), was identical to the sequence HF674885 of the type strain of *Chloroidium ellipsoideum*, CAUP H1904. Cort04, represented by three isolates from two *Q. pubescens*

Table 1. Identity and frequencies of operational taxonomic units (OTUs) from Petri dishes representing the samples of biofilms taken from the bark of *Pinus nigra* (P) and *Quercus pubescens* (Q) at localities Fiesa (F) and Cernizza (C).

Cort	Taxon name	PF1	PF2	QF1	QF2	PC1	PC2	QC1	QC2
01	*Kalinella* cf. *bambusicola*	1	3	-	1	-	1	-	1
02	*Stichococcus* sp.	-	-	1	3	1	-	-	-
03	*Diplosphaera* sp.	-	3	5	4	-	-	2	-
04	*Kalinella* cf. *apyrenoidosa*	-	-	-	2	-	-	-	1
05	*Stichococcus* sp.	-	1	2	4	-	-	2	1
06	*Coccomyxa* sp.	2	2	2	1	5	6	2	-
07	*Stichococcus* sp.	-	-	-	1	-	-	-	-
08	Trebouxiophyceae	-	-	-	-	-	-	-	1
09	*Auxenochlorella* sp.	-	-	-	-	-	-	-	2
10	*Klebsormidium* sp.	-	-	-	-	-	-	-	1
11	*Chlorokybus* sp.	-	-	-	-	-	-	-	2
12	*Diplosphaera* sp.	-	-	-	-	-	-	-	2
13	*Diplosphaera* sp.	-	-	1	-	-	-	-	1
14	*Coccomyxa* sp.	-	-	-	-	-	-	-	1
15	*Parachloroidium* cf. *laureanum*	-	-	-	-	7	6	1	1
16	*Coccomyxa* sp.	-	-	-	-	-	-	-	1
17	Trebouxiophyceae	-	-	-	-	-	-	-	1
18	*Coccomyxa* sp.	-	1	1	-	-	-	1	-
19	*Xylochloris* sp.	-	-	1	-	-	-	-	-
20	*Asterochloris* sp.	1	1	1	-	-	-	2	-
21	Trebouxiophyceae	-	-	-	-	-	-	2	-
22	Trebouxiophyceae	-	3	-	-	-	-	-	-
23	*Parachloroidium* sp.	1	-	-	-	-	-	-	-
24	*Chloroidium* cf. *ellipsoideum*	5	-	-	-	-	-	1	-
25	*Parachloroidium* sp.	-	-	-	-	1	1	-	-
26	*Leptochlorella* sp.	4	1	-	-	2	-	1	-
27	*Dictyochloropsis* sp.	-	1	-	-	-	-	-	-
28	*Diplosphaera* sp.	-	-	-	-	-	-	1	-
29	*Coccomyxa* sp.	-	-	-	-	-	1	-	-

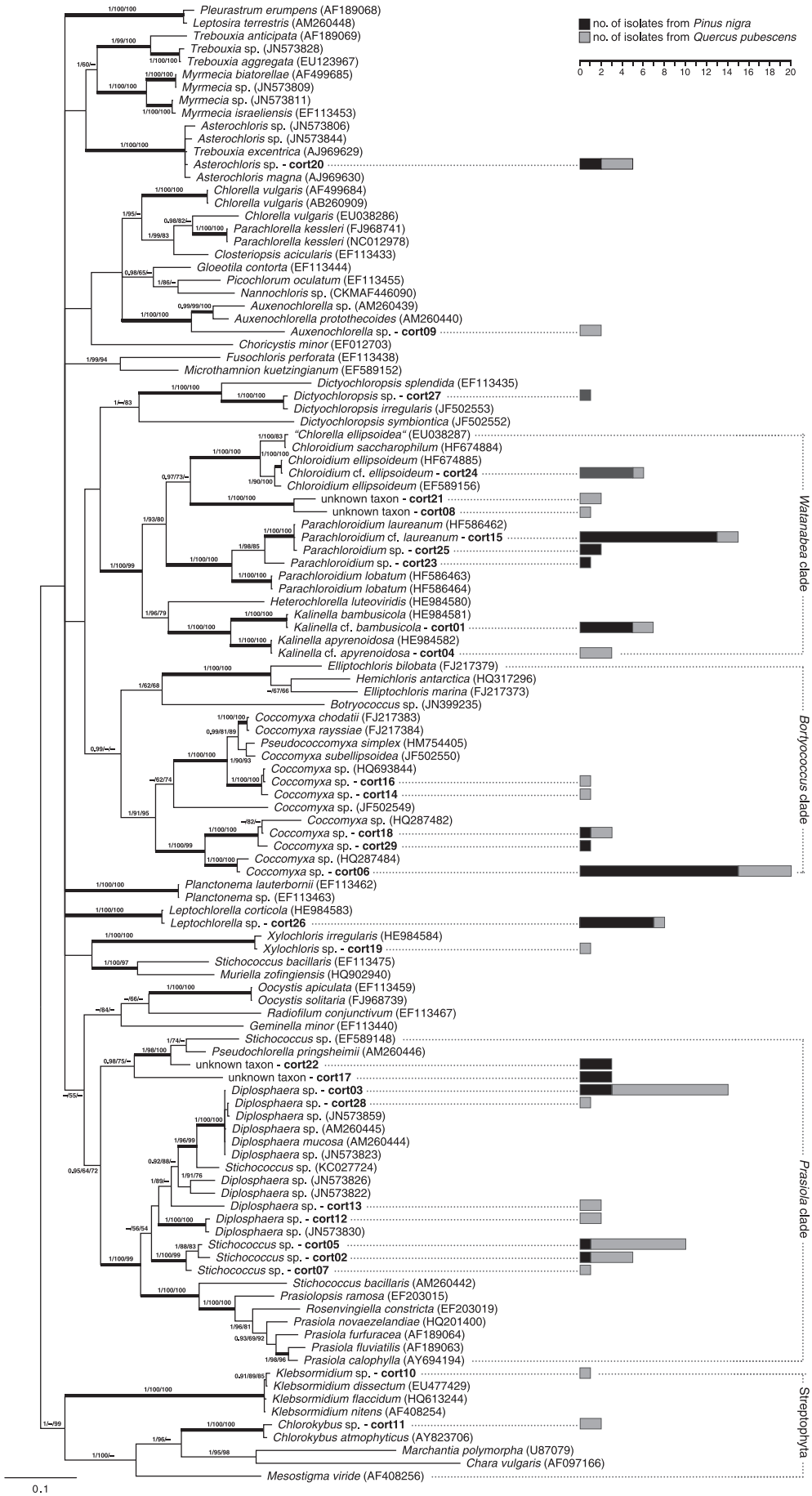
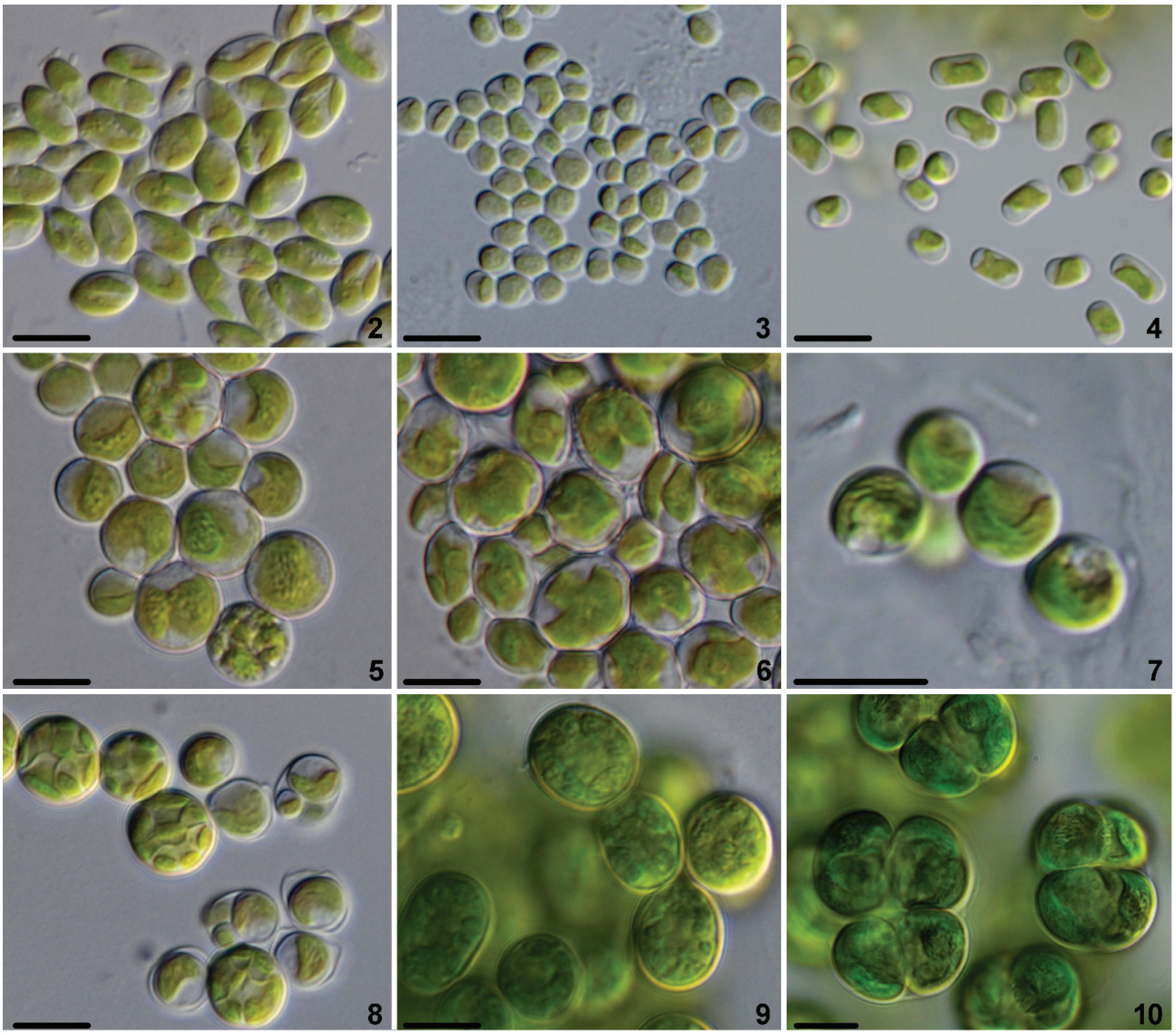


Fig. 1. Phylogenetic tree inferred from partial *rbc*L sequences of corticolous microalgae using Bayesian techniques, and bar charts representing the frequency of isolates from pine and oak trees at both localities. Numbers at nodes indicate statistical support (BI > 0.95/ML > 50%/wMP > 50%). Thick branches represent nodes receiving high statistical support. The scale bar shows the estimated number of substitutions per site.



Figs 2–10. Light micrographs of selected isolates from the subaerial corticolous biofilms. **Fig. 2.** *Coccomyxa* sp., cort06. **Fig. 3.** *Diplosphaera* sp., cort03. **Fig. 4.** *Stichococcus* sp., cort02. **Fig. 5.** *Parachloroidium* cf. *laureanum*, cort15. **Fig. 6.** *Chloroidium* cf. *ellipsoideum*, cort24. **Fig. 7.** *Kalinella* cf. *apyrenoidosa*, cort04. **Fig. 8.** An unknown taxon of the *Watanabea* clade, cort21. **Fig. 9.** *Dictyochloropsis* sp., cort27. **Fig. 10.** *Chlorokybus* sp., cort11. Scale bar = 7 µm.

samples (QC2, QF2), was identical to the type strain of *Kalinella apyrenoidosa*, CAUP H7902, a species recently described from a biofilm sample growing on the bark of *Laurus nobilis* (Neustupa *et al*., 2013*a*). The characteristic chlorelloid morphology of these isolates corresponded with the taxonomic description of this species (Fig. 7). Isolates identical to the second species of the genus *Kalinella*, described originally as *K. bambusicola* from subaerial biofilms in Singapore (type strain CAUP H7901, HE984581), were also present among the microcolonies isolated in this study (cort01). In total, this OTU was isolated seven times, both from *P. nigra* and from *Q. pubescens* bark samples. Finally, two rarely encountered OTUs (cort08 and cort21), from the bark of *Q. pubescens*, formed an independent and probably new lineage within the *Watanabea* clade. Phylogenetic Bayesian inference placed this lineage in a sister position with the genus *Chloroidium*, but this relationship was only weakly supported by statistical analyses (BI 0.97/ML 73/wMP not supported). Both these OTUs shared chlorelloid morphology and had globular cells 2.0–7.5 µm in diameter. The cells possessed a single parietal chloroplast, without any visible pyrenoids (Fig. 8), and reproduced by two to eight oval autospores. In total, eight identical isolates from four different samples, originating from both *P. nigra* and *Q. pubescens* biofilms, were identified as *Leptochlorella* sp. The *rbc*L sequence of these isolates (assigned as cort26) differed by a single substitution near the 3' end of the sequenced region from the sequence HE984583 of the type strain of *L. corticola*, a species described from a corticolous biofilm found in the same region (Neustupa *et al*., 2013*a*). Cort20, isolated as five microcolonies from four different trees, was closely related to members of the genus *Asterochloris*, a

widely distributed terrestrial alga, mostly occurring as a photobiont of various lichens, including numerous corticolous taxa.

A single member of the Chlorellales, assigned tentatively to cort09, was isolated from two samples taken from *Q. pubescens*. It was placed with high statistical support (BI 1.0/ML 100/wMP 100) with the sequences AM260439 and AM260440, representing the genus *Auxenochlorella*, and including the type strain of *A. protothecoides*, SAG 211-7a. Cort27 occurred in a single sample taken from *P. nigra*. Its *rbc*L sequence was 99% identical to the sequence JF502553 of *Dictyochloropsis irregularis*, strain SAG 2036. Typical features of the cellular morphology of cort27, such as plastid shape, corresponded with this identification (Fig. 9). Likewise, cort19, isolated from a single *Q. pubescens* sample, showed 99% identity with sequence HE984584 of *Xylochloris irregularis*, type strain CAUP H7801, which was originally described from tropical corticolous biofilms (Neustupa *et al*., 2011).

In total, 2.5% of the isolates belonged to the Streptophyta, which was represented by two different OTUs isolated from a single *Q. pubescens* sample. Cort10, identified morphologically as *Klebsormidium* sp., clustered into subclade 2 of clade E2 within the broadly defined genus *Klebsormidium*, as demonstrated by Rindi *et al.* (2011) and Škaloud & Rindi (2013). Members of this lineage characteristically occur on various natural substrates in subaerial conditions. Finally, two isolates (cort11) were 99% identical to *Chlorokybus atmophyticus*, strain SAG 34.48, GenBank accession AY823706. The cells of these isolates formed mucilaginous sarcinoid colonies, in which individual cells were 8–16 µm in diameter (Fig. 10). These morphological characteristics closely correspond with those of *C. atmophyticus*, a species occurring in various terrestrial habitats.

Diversity and community structure

On average, 9.50 different OTUs were found in samples taken from *Q. pubescens*, and 6.25 OTUs in samples from *P. nigra*. Likewise, the mean Menhinick index, used to quantify the local diversity of samples, was 2.43 for the oak and 1.60 for the pine biofilms. These differences indicate that the samples from oak trees may contain more diverse algal communities than samples from pines. However, differences between the two tree species were statistically not significant.

The two-dimensional NMDS ordination plot of the samples, based on their differences in OTU composition (Fig. 11), indicates that the effect of host tree species was largely correlated with the first NMDS axis ($R^2 = 0.50$). The biofilm samples taken from *P. nigra* had mostly negative scores, whereas the samples from *Q. pubescens* had positive scores on the first axis of the NMDS analysis. Conversely, the two sampling localities differed mostly in their position on the second axis ($R^2 = 0.34$).

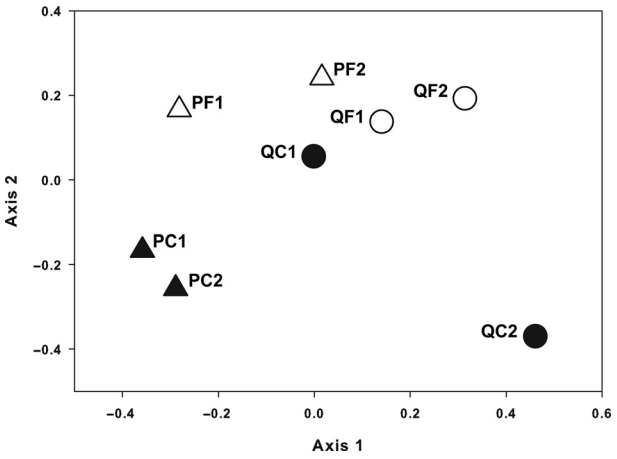


Fig. 11. Two-dimensional non-metric dimensional scaling (NMDS) ordination plot, based on the Bray–Curtis index, comparing corticolous assemblages from bark of *Quercus pubescens* (circle) and *Pinus nigra* (triangle) sampled from four sites at two localities, Fiesa (empty symbols) and Cernizza (filled symbols). Stress value = 0.08.

Table 2. Results of permutational multivariate analyses of variance to evaluate effects of the host species and sampling locality on the composition of OTUs in samples. The tests are sequential, which means that the terms are evaluated sequentially in the order in which they appear in the formula.

Factor	Df	Sums of squares	Mean squares	F	P
Locality	1	0.43	0.44	2.36	n.s.
Host species	1	0.50	0.50	2.71	*
Locality × Host species	1	0.33	0.33	1.82	n.s.
Residuals	4	0.74	0.18		

*: $0.01 < P < 0.05$; n.s.: $P > 0.05$

The samples taken at Fiesa had distinctly higher scores on this axis than most of the samples taken at Cernizza, which were typically positioned at the lower end of this axis. In the Per-MANOVA analyses, the effect of host tree species proved to be significantly related to the structure of the biofilms, as illustrated by the taxonomic composition of samples (Table 2). Conversely, the samples taken from both localities were statistically similar ($P > 0.05$). Comparisons of phylogenetic distances (Tamura–Nei/uncorrected p-distances) between samples showed that the samples from Cernizza were phylogenetically less closely related than those from Fiesa ($P < 0.01/P < 0.05$). Phylogenetic distances between samples taken from *P. nigra* and *Q. pubescens* were not significantly different.

Discussion

Our data suggest that bark algal biofilms growing on *Quercus pubescens* are more diversified than those on *Pinus nigra*. The significance levels of the statistical tests evaluating differences in diversity of samples taken from different host tree species were only slightly above the 0.05 value and more sampling would be

expected to show that the diversity values of both groups differ, as oaks have considerably higher bark pH than pines (Barkman, 1958). Increasing diversity of corticolous microalgae with increasing bark pH was previously illustrated by means of microscopic morphological identification (Neustupa & Štifterová, 2013). However, such a pattern was not observed in our analyses of phylogenetic distances: these did not differ between the biofilm samples taken from different host tree species. Consequently, our results suggested that an OTU isolated from either host tree species had a similar probability of belonging to a deeply phylogenetically isolated lineage.

Subaerial coccoid green algae are generally well adapted to desiccation (Gustavs *et al*., 2010; Lüttge & Büdel, 2010). Given their typically small cell dimensions, frequent air-mediated dispersal of populations might prevent any pattern in the distribution of individual taxa among host tree taxa or in different localities. However, our results do not confirm a neutral pattern in green algal community structure in corticolous biofilms. The host tree species proved to be the most important factor in structuring species composition of microalgal communities. This indicates that local factors, such as the specific physico-chemical conditions of the bark surface that differ between tree taxa, shape community structure, whereas macroscale factors, differing among localities, are less important. Several recent studies of phototrophic corticolous biofilms based on microscopic identification of morphotypes or major taxa (Nakano *et al*., 1991; Hedenås *et al*., 2007; Neustupa & Škaloud, 2008; Neustupa & Štifterová, 2013) illustrated similar non-neutral patterns. The principal drivers of biofilm community structure can therefore be detected at various identification levels, ranging from the major taxa, such as Cyanobacteria, Trentepohliales and coccoid green algae (Hedenås *et al*., 2007; Neustupa & Štifterová, 2013), to phylogenetic OTUs defined by molecular genetic data (this study).

The isolates from our biofilm samples mostly belonged to Trebouxiophyceae. Although Chlorophyceae include a number of terrestrial species and genera, such as *Bracteacoccus* and *Chromochloris* (Fučíková & Lewis, 2012), *Pseudomuriella* (Fučíková *et al*., 2011) and *Scenedesmus* (Lewis & Flechtner, 2004), no representatives of the Chlorophyceae were detected in our samples. Many Chlorophyceae recorded from terrestrial microhabitats are characteristic members of soil surface crust communities (Lewis & Flechtner, 2002; Büdel *et al*., 2009; Flechtner *et al*., 2013). We assume that most terrestrial chlorophyceans are better adapted to relatively high irradiation levels than members of the Trebouxiophyceae, which typically dominate in more shaded microhabitats, such as on tree bark and inside buildings (Hallmann *et al*., 2013).

Most of the trebouxiophycean OTUs belonged to well-supported phylogenetic lineages that were previously described as genera, or higher infraclass taxa (such as the *Prasiola* clade). In total, 30.3% of the isolates belonged to the genera *Leptochlorella, Kalinella, Parachloroidium* and *Xylochloris*. These relatively new genera were recently reported and taxonomically described from similar microhabitats (Neustupa *et al*., 2009, 2011, 2013*a*, *b*; Wong *et al*., 2010; Hodač *et al*., 2012); they were not found in desert soil samples (Flechtner *et al*., 2013). Members of these genera may in fact be widely distributed in specific microhabitats (tree bark, hypolithon); however, because of their simple chlorelloid morphology, they cannot be recognized and taxonomically defined by morphological characters.

Although a unique *rbc*L gene sequence does not necessarily represent a separate species (Rindi *et al*., 2011), several recent studies have demonstrated that variability of the *rbc*L gene of Trebouxiophyceae generally corresponds well to the variation of the 18S rDNA sequences, and even slight differences among sequences usually correlate with relatively well-supported lineages (Novis &Visnovsky, 2012; Neustupa *et al.,* 2013*a*, *b*). Therefore, it is likely that several of the corticolous OTUs represent previously undescribed species or genera of various known lineages, or species without any published sequence data for this marker. Nevertheless, taxonomic status cannot be established without additional genetic data and detailed morphological investigation of isolates.

Notably, the OTUs cort02, cort05 and cort07, with typical *Stichococcus*-like morphology, formed an independent lineage within the *Prasiola* clade. However, this lineage was not monophyletic with other published *rbc*L sequence data for strains identified morphologically as *Stichococcus* spp.; this confirms the convincing paraphyly of this genus highlighted by Eliáš & Neustupa (2009). The members of this genus, which are phylogenetically closely related to taxa identified as species of the genera *Diplosphaera* (Thüs *et al*., 2011), *Pseudomarvania* (Eliáš & Neustupa, 2009) and *Desmococcus* (Gärtner & Ingolić, 2003), form one of the most common terrestrial microalgal groups (Ettl & Gärtner, 1995; Uher, 2008; Rindi *et al*., 2010). Taxonomic revision of this group, which may result in the description of several monophyletic genera, would greatly benefit further research on microalgal diversity in subaerial habitats. Similarly, five OTUs were placed in the well-supported lineage encompassing taxa with *Coccomyxa* and *Pseudococcomyxa* morphology. These microalgae are widely distributed in various terrestrial habitats (Ettl & Gärtner, 1995). In addition, they occur as lichen photobionts (Zoller & Lutzoni, 2003) and as parasites and endophytes of vascular plants and invertebrates (Rodríguez *et al*., 2008). Given their phylogenetic and ecological heterogeneity, they should probably be divided into several genera in the future. However, morphological characteristics of these phylogenetically defined taxa may not correlate with the traditional

distinction between *Pseudococcomyxa* and *Coccomyxa* (Krienitz & Bock, 2012).

The OTU cort23 was firmly nested within the genus *Parachloroidium* of the *Watanabea* clade. Given the substantial differences between the *rbc*L sequence of this OTU and those of both described species of this genus, it probably represents a third, previously undescribed, *Parachloroidium* species. Other putative new taxa, OTUs cort08 and cort21, formed a new well-supported lineage within the *Watanabea* clade that did not include any previously published *rbc*L sequences and may represent previously undescribed genera. Isolates with *rbc*L sequences identical to both species of the genus *Kalinella* were found. Interestingly, seven isolates were identical to *K. bambusicola*, a species previously only known from the type locality in Singapore (Neustupa *et al*., 2009). Therefore, it seems likely that this species is widely distributed outside the tropical zone.

Surprisingly, we did not detect any isolates that could be identified as members of the trebouxiophycean genus *Apatococcus*, which is a dominant component of subaerial biofilms (Ettl & Gärtner, 1995; Hallmann *et al*., 2011, 2013). Gustavs (2010) reported that the genus *Apatococcus* is a mixotroph that may be unable to grow on media without suitable organic substances. Therefore, *Apatococcus* populations may in fact have been present in our samples, but not identified using our methodology as microcolonies were grown on standard inorganic BBM medium. In addition, members of several other genera, such as *Desmococcus, Jenufa, Myrmecia, Prasiola* or *Trentepohlia*, which have previously been recovered from subaerial habitats (Ettl & Gärtner, 1995; Gärtner & Ingolić, 2003; Němcová *et al.*, 2011), were also not recorded. These taxa may have been present in our sub-Mediterranean bark samples, but they can be overlooked due to their low abundances in samples or unsuccessful amplification of the *rbc*L gene.

Streptophyta were rarely encountered among the isolates. They occurred in a single sample from *Q. pubescens*, and belonged to two separate streptophytan lineages. *Chlorokybus atmophyticus* is frequently reported from various terrestrial habitats (Hoffmann, 1989); our data diverged slightly from the reference *rbc*L sequence (AY823706), which indicates possible cryptic species differentiation within this ancient and phylogenetically isolated lineage (Leliaert *et al*., 2012). The single *Klebsormidium* isolate belonged to subclade no. 2 of the E2 clade of this diversified and heterogeneous genus. Other members of this subclade have been reported from various natural rock and soil microhabitats (Škaloud & Rindi, 2013). Consequently, our isolate represents the first record of this lineage from a corticolous biofilm.

In conclusion, the present study demonstrated that many generic lineages of the trebouxiophycean coccoid green algae, described originally from various subaerial habitats worldwide, can repeatedly be found by detailed molecular analysis of corticolous biofilms growing on two tree species in a single European region. This indicates that most of these genera may in fact have cosmopolitan distributions in suitable microhabitats, such as on tree bark. At the species level, fast-evolving markers may reveal additional, fine-grained phylogenetic structure of infrageneric taxa with restricted geographic distributions. Nevertheless, our data suggested that the number of commonly occurring deep trebouxiophycean lineages of coccoid green microalgae in subaerial biofilms may not be much higher than that described so far.

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Supplementary information

The following supplemental material is available for this article, accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2014.945190

Fig. S1. Alignment of the *rbc*L sequences.

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