

1 Symbiosis between river and dry lands: phycobiont dynamics 2 on river gravel bars

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30 Abstract

31 River gravel bars are dynamic and heterogeneous habitats standing on transition between
32 aquatic and terrestrial environment. Periodical flooding, low nutrient content, frost, missing safe
33 sites, drought, and heat on the ground surface significantly influence life in these habitats.
34 Mutualistic symbiosis may be a successful strategy for organisms to survive and to proliferate under
35 harsh conditions. The lichen genus *Stereocaulon* was selected as a model symbiotic system among
36 the organisms living on river gravel bars. The aim of our work was to determine effect of this
37 dynamic environment on a phycobiont (i.e., green eukaryotic photobiont) community structure. We
38 analysed 147 *Stereocaulon* specimens collected in the Swiss Alps using Sanger sequencing (fungal ITS
39 rDNA, algal ITS rDNA, algal actin type I gene) and 8 selected thalli and 12 soil samples using Illumina
40 metabarcoding (ITS2 rDNA). We performed phytosociological sampling on each study plot (n=13).
41 Our analyses of communities of phycobionts, lichens, bryophytes, and vascular plants indicated an
42 ongoing colonisation by phycobionts and gradual change of phycobiont community along to the
43 successional gradient. We recovered great phycobiont diversity associated with *Stereocaulon*
44 mycobionts including algae reported as phycobionts for the first time. Each of two *Stereocaulon*
45 mycobiont OTUs has distinct pool of predominant phycobionts in the study area. Finally, all thalli
46 selected for Illumina metabarcoding contained a wide range of additional intrathalline algae, i.e.,
47 showed algal plurality. In general, succession process on newly emerged or recently disturbed
48 localities also takes place within a community of microscopic symbiotic organisms, such as
49 phycobionts.

50 Key words: selectivity, lichen phycobionts, succession, community composition, metabarcoding, algal
51 plurality

52 Introduction

53 River gravel bars are dynamic and heterogeneous habitats ranging from glacial floodplains and
54 alpine wide river valleys to piedmont (Montgomery and Buffington 1998; Malard et al. 2006; Tockner
55 et al. 2006; Hohensinner et al. 2018). Periodical floods together with variation of speed and intensity
56 of the water current, model richly braided channels with a mosaic of channels, pools, bars and
57 islands (Junk et al. 1989; Tockner et al. 2000; Ward et al. 2002). Destruction and reformation of the
58 river gravel bars by floods results in a wide structural change and a cyclic vegetation succession.
59 Gravel bar vegetation is characterized by early-successional pioneer vegetation and subsequent,
60 mainly shrub, vegetation stages in the colonization sequence (Pettit and Froend 2001; Prach et al.
61 2016). These vegetation types form a mosaic of units according to microhabitat environmental
62 conditions and disturbance level (Müller 1996; Tockner et al. 2000; Wellstein et al. 2003; Gilvear et

63 al. 2008). Gravel bars in alpine zones contribute significantly to the regional diversity of an otherwise
64 harsh alpine environment (Tockner and Malard 2003). Early- to mid-successional stands are often
65 occupied by relatively species rich communities of vascular plants of high evenness and relatively low
66 vegetation cover. In later successional stages, the evenness and species richness decrease as organic
67 matter and nutrients accumulate and the competition from established dominant species increase.
68 This pattern was shown gravel bars, for example, by Corenblit et al. (2009), Prach et al. (2014),
69 Kalníková et al. (2018).

70 Alpine rivers with glacier source are highly influenced by daily flooding, due to melting of
71 glaciers which makes the environment even more extreme (e.g. Milner and Petts 1994; Tockner et al.
72 2000; Malard et al. 2006). Low nutrient content in a substrate surface represents the most limiting
73 environmental factor in the glacier floodplains. Especially the recently unglaciated terrain is
74 characterized by bare soils which do not contain any organic matter and the soil seed bank is initially
75 not present. In addition, frost, missing safe sites, drought, and heat on the ground surface
76 significantly influence life in these habitats (Stöcklin and Bäumler 1996; Tockner et al. 2006;
77 Marcante et al. 2014). Common strategies of the gravel bar species are usually high diasporas
78 dispersability, fast growth, disturbance tolerance, clonal growth and ability to grow on poor soils
79 (Jeník 1955; Vitt et al. 1986; Muotka and Virtanen 1995; Stöcklin 1999; Karrenberg et al. 2003;
80 Ellenberg and Leuschner 2010).

81 Under harsh conditions of river gravel bars, mutualistic symbiosis may be a successful strategy
82 for the organisms to survive and to proliferate (Doty et al. 2016). Since lichens represent one of the
83 oldest known and recognizable examples of mutualistic symbiosis living under stressful conditions
84 (Seckbach and Grube 2010), we selected the *Stereocaulon* lichens, among the organisms living on
85 river gravel bars, as a model symbiotic system. The *Stereocaulon* genus is widespread and
86 ecologically successful and is a pioneer lichen growing in harsh conditions on newly formed
87 substrates (Stretch and Viles 2002; Meunier et al. 2014). Moreover, previous studies confirmed its
88 ability to survive episodic submersion (Sadowsky et al. 2012), even though it is not natively aquatic.
89 Recently, an exceptional diversity of phycobionts was discovered to be associated with *Stereocaulon*,
90 including three ecologically diversified trebouxiophycean genera *Asterochloris*, *Vulcanochloris* and
91 *Chloroidium* (Vančurová et al. 2015, 2018).

92 The lichen host ecological amplitude may be greatly influenced by its specificity towards the
93 photobionts (Rolshausen et al. 2017; Vančurová et al. 2018). Symbiotic interactions vary along the
94 environmental gradients (Godschalx et al. 2019) and could be affected by stressful environments
95 (Romeike et al. 2002; Engelen et al. 2010). Therefore, the aim of our work was to determine the

96 phycobiont diversity pattern of *Stereocaulon alpinum* in the gradient of the vegetation succession.
97 We applied both Sanger sequencing (n=147) and Illumina metabarcoding (n=8) to *Stereocaulon*
98 *alpinum* specimens collected in 13 study plots to address the following questions: 1) Is the
99 phycobiont diversity influenced by succession, water regime, or alternatively other biotic and abiotic
100 conditions?; 2) Do *Stereocaulon* lichens use their ability to co-operate with various phycobionts to
101 cope with extreme conditions on river gravel bars of glacial floodplains?; 3) Do *Stereocaulon* lichens
102 show algal plurality in this habitat?

103 Material and Methods

104 Study area and field sampling

105 The sampling was carried out in August 2017. The study area covered three glacial valleys (1)
106 Morteratsch valley and 2) Roseg valley in the Bernina range and 3) Lötschental valley of the Lonza
107 River in the Bernese Alps in the Swiss Alps. Four localities (Morteratsch, Roseg I, Roseg II and Lonza)
108 situated on the river gravel bars of glacial floodplains (1995–2070 m a. s. l.) were sampled. We
109 carried out 13 vegetation plots of 4 × 4 m. A detailed description of the location of each plot is
110 provided in Online Resource 1/Table S1. Study plots in the localities belong to three succession
111 stages: first (early-successional), second (defined by shrub layer), and third (the most developed
112 usually with tree layer). In the locality Roseg II, only first and second stages were present. Examples
113 of study plots belonging to different succession stages are given in Online Resource 2/Fig. S1.

114 Coordinates of each plot were recorded using a portable GPS device (WGS-84 coordination
115 system). We recorded all lichen, bryophyte and vascular plant species within the vegetation plots.
116 Lichens and bryophytes were collected from the ground and stones. In each plot we estimated
117 visually: a) the cover of each species according to the extended Braun-Blanquet cover scale
118 (Westhoff and Van Der Maarel 1978), b) the total vegetation cover and the cover of each layer (tree,
119 shrub, herb, moss and lichen). We measured the elevation of the gravel bar (as a distance from its
120 highest point to the actual water level with a tape) as well as distance from the river. One soil sample
121 per plot was taken. Data on vegetation structure for each plot are listed in the Online Resource
122 1/Table S2. The substrate of all localities is rather acidic (in several cases with basic fractions), since
123 the *Stereocaulon* lichens do not occur on calcareous substrata (Lamb 1951).

124 On each plot, at minimum 10 *Stereocaulon* samples were collected. For each *Stereocaulon*
125 sample the type of substrate and affiliation to the plot was noted. Lichen morphospecies were
126 identified in the field as well as in the laboratory using standard microscopic and chemical methods,
127 including spot tests and thin-layer chromatography (TLC). *Stereocaulon* vouchers were deposited in
128 Herbarium collection of the Charles University in Prague (PRC). Vouchers of accompanying lichens

129 were deposited in the personal herbarium of J. Malíček. Vascular plants and bryophytes which were
130 not identified in the field (unambiguous cases) were collected and identified in the lab. All records on
131 vegetation plots were stored in the Gravel bar vegetation database – ID: EU-00-025 (Kalníková and
132 Kudrnovsky 2017), which is included in the European Vegetation Archive (Chytrý et al. 2016).
133 Nomenclature follows Euro+Med PlantBase (2006–2019) for vascular plants, Hill et al. (2006) for
134 mosses, Grolle & Long (2000) for liverworts, and Nimis et al. (2018) for lichens.

135 DNA extraction, amplification, and Sanger sequencing

136 DNA was extracted from lichen thalli (total lichen DNA). Lichen thalli were examined under a
137 dissecting microscope and washed with water before DNA extraction to remove possible surface
138 contamination. Total genomic DNA was isolated from thallus fragments following the CTAB protocol
139 (Cubero et al. 1999). Both algal and fungal nuclear internal transcribed spacers (ITS rDNA) and the
140 algal actin type I gene (including one complete exon and two introns located at codon positions 206
141 and 248; Weber and Kabsch 1994) were PCR amplified using primers listed in Online Resource
142 1/Table S3. PCRs were performed as described in Vančurová et al. (2018). All PCR were performed in
143 20 µl using Red Taq Polymerase (Sigma) as described by Peksa and Škaloud (2011) or with My Taq
144 Polymerase. Negative controls, without DNA template, were included in every PCR run to eliminate
145 false-positive results caused by contaminants in the reagents. The PCR products were sequenced
146 using the same primers at Macrogen in Amsterdam, Netherlands. The newly obtained sequences
147 were deposited in GenBank under accession numbers xxx (Online Resource 1/Table S4).

148 Sequence alignment and DNA analyses

149 *Asterochloris* datasets were analyzed both as a single locus for the ITS rDNA (data not shown)
150 and as a concatenated dataset of ITS rDNA and actin type I loci. The *Asterochloris* ITS rDNA dataset
151 consisted of 202 sequences: 142 newly obtained sequences and 60 previously published sequences
152 from *Stereocaulon* and other lichens retrieved from GenBank. The actin type I dataset consisted of 68
153 sequences: 7 newly obtained sequences, and 60 previously published sequences. The alignment was
154 automatically performed by MAFFT v.7 software (Katoh and Standley 2013) under the Q-INS-I
155 strategy and manually edited according to the published secondary structures of ITS2 rDNA (Škaloud
156 and Peksa 2010) using MEGA v.6 (Tamura et al. 2013). The actin type I sequences were aligned using
157 MAFFT v.7 software (Katoh and Standley 2013) under the Q-INS-I strategy. After deleting identical
158 sequences, the resulting concatenated alignment comprised 64 samples represented by unique ITS
159 rDNA and actin type I sequences.

160 The *Stereocaulon* (mycobiont) ITS rDNA dataset comprised 171 sequences: 145 newly obtained
161 sequences and 26 representative sequences selected to cover all main clades 1–8 published by
162 Högnabba (2006). The alignment was automatically performed by MAFFT v.7 software (Katoh and

163 Standley 2013) under the Q-INS-I strategy. After removing identical sequences, the resulting
164 alignment comprised 48 sequences. All DNA alignments are freely available on Mendeley Data:
165 <http://dx.doi.org/10.17632/jchg5h3t5k.1>.

166 Phylogenetic relationships were inferred with Bayesian Inference (BI) carried out in MrBayes
167 v.3.2.2 (Huelsenbeck and Ronquist 2001), maximum likelihood (ML) analysis implemented in GARLI
168 v.2.0 (Zwickl 2006), and maximum parsimony (MP) analysis using PAUP v.4.0b10 (Swofford 2003). BI
169 and ML analyses were carried out on a partitioned dataset to differentiate among ITS1, 5.8 S and ITS2
170 rDNA, actin intron 206, actin intron 248, and actin exon regions. The best-fit substitution models
171 (Online Resource 1/Table S5) were selected using the Bayesian information criterion (BIC)
172 implemented in JModelTest2 (Guindon and Gascuel 2003; Darriba et al. 2012). ML analysis was
173 carried out using default settings, five search replicates, and the automatic termination set at 5
174 million generations. The MP analysis was performed using heuristic searches with 1000 random
175 sequence addition replicates and random addition of sequences (the number was limited to 10^4 per
176 replicate). ML and MP bootstrap support values were obtained from 100 and 1000 bootstrap
177 replicates, respectively. Only one search replicate was applied for ML bootstrapping.

178 Statistical analyses

179 From the total of 147 samples with successfully sequenced phycobiont, two were excluded
180 because of the absence of the mycobiont sequence. The phylogeny of *Stereocaulon alpinum* showed
181 two lineages (OTU35 and OTU2; see below). Since the mycobiont identity affects the phycobiont
182 diversity (Vančurová et al. 2018) four samples belonging to the minority species-level lineage (OTU2)
183 were also excluded. Thus, statistical analyses were carried out using 141 members of the prevailing
184 mycobiont species-level lineage (OTU35) and their photobionts.

185 The relationship between species richness of *Stereocaulon alpinum* (OTU35) phycobionts and
186 overall lichen species richness was inspected as a correlation between the number of phycobiont
187 species-level lineages and number of lichen species. Since the number of samples per plot varied, the
188 number of phycobiont species-level lineages were down-sampled to the smallest sample size in the
189 data set which was 5 samples (Online Resource 2/Fig. S2). After excluding the study plot number 11
190 (with sample size of 5 samples), the smallest sample size in the data set increased to 10 samples. The
191 rarefaction was performed using the *rarefy* function in *vegan* R package (Oksanen et al. 2019). The
192 linear regression was performed separately for dataset including all plots and for dataset restricted
193 to plots with sample size ≥ 10 . Since the parametric regression analyses can be significantly biased in
194 small sample sizes, we performed the Bayesian linear regression instead. We constructed a
195 regression model where we modelled the number of phycobionts (X_i) as $X_i \sim Normal(\mu_i, \sigma)$, where μ_i
196 was determined as $a + b * \text{number of lichen species}_i$ (a = intercept, b = slope of the regression line)

197 and σ as the variance of the residuals. The priors were set as follows: $a \sim \text{Normal}(0, 0.001)$, $b \sim$
198 $\text{Normal}(0, 0.001)$, $\sigma \sim \text{Uniform}(0, 100)$.

199 The gradual change of phycobiont community composition was inspected as a correlation
200 between the proportion of the two most abundant phycobiont species-level lineages ((number of
201 *Asterochloris phycobiontica* samples + number of StA5 lineage samples)/number of all samples) and
202 the succession stage (coded 1, 2, 3). We constructed a regression model where we modelled the
203 proportion of the most abundant phycobionts (X_i) as $X_i \sim \text{Normal}(\mu_i, \sigma)$, where μ_i was determined as
204 $a + b * \text{succession stage}_i$ (a = intercept, b = slope of the regression line) and σ as the variance of the
205 residuals. The priors were set as described above. We ran three chains of the model for 1,000,000
206 iterations, discarding the initial 100,000 as burnin. We fit the regression model in program JAGS v.
207 4.2.0 (Plummer 2003) through the *R2JAGS* package (Su and Yajima 2009) in R.

208 To inspect the dynamics of phycobiont communities in the context of the river gravel bar
209 habitats, two PCoA ordinations were created. We used two different proxies to explain the
210 vegetation succession on river gravel bars: the cover of shrub layer and the cover of crustose lichens.
211 For this purpose, successional status of study plots was classified into two groups (early successional
212 and developed). This classification provides two alternative views on successional processes. Both
213 ordination analyses were based on the square-rooted Bray-Curtis dissimilarity. The original Braun-
214 Blanquet cover-abundance values were transformed to percentage and square-root transformed in
215 order to calculate the dissimilarity index. The first ordination was computed based on vascular plant
216 communities and the second on bryophytes + lichens. In both ordination plots, Ellenberg-type
217 indicator values (Ellenberg et al. 1991) for light and temperature were passively superimposed. In the
218 plot based on bryophytes + lichens, indicator values for bryophytes were used (Hill et al. 2006). We
219 also compared if the variation of different taxa groups corresponds to similar environmental
220 conditions, by calculating procrustes distances between taxa groups. The output was then used as
221 input distance matrix for PCoA ordination, visualizing a relative dissimilarities of community
222 composition gradients of the taxa groups.

223 The vegetation plot data were stored in Turboveg for Windows v.2 database (Hennekens and
224 Schaminée 2001) and further managed with JUICE software (Tichý 2002) and in the R environment (R
225 Core Team 2017) with the help of the *vegan* R package (Oksanen et al. 2019).

226 Bipartite association network between study plots and phycobiont species-level lineages was
227 produced using *bipartite* R package (Dormann et al. 2008).

228 Illumina metabarcoding of algal communities in selected lichen thalli and soil samples

229 In order to describe algal plurality in *Stereocaulon* thalli, Illumina metabarcoding was
230 performed. Eight selected thalli (four assigned to mycobiont OTU35 and four to OTU2) were
231 rehydrated with Milli-Q sterile water one day before being processed and stored in a growth
232 chamber at 20°C under a 12h/12h light/dark cycle (15 $\mu\text{mol}/\text{m}^2/\text{s}$). Thalli were cleaned under a
233 stereomicroscope to remove soil particles and then superficially sterilized following Arnold et al.
234 (2009). Fragments from different parts of each thallus were randomly excised and pooled together
235 (0.1 mg). Total genomic DNA was isolated and purified using the DNeasy Plant Mini Kit (Qiagen,
236 Hilden, Germany).

237 Soil from study plots (one sample per plot) was sampled. Soil samples were sieved to remove
238 all possible contaminations. Total genomic DNA was isolated and purified using the Soil DNA Isolation
239 Plus Kit® (Norgen Biotek Corp.), following the manufacturer's instructions.

240 Chlorophyta algal communities associated with the eight thalli and twelve soil samples were
241 assayed using Illumina high-throughput sequencing of ITS2 of the rRNA operon, proposed as a
242 universal barcode across eukaryotic kingdoms (Coleman 2009). High-coverage PCR primers at
243 conserved sites were designed using a custom database for the algal phylum Chlorophyta (Online
244 Resource 1/Table S3).

245 Amplicons for Illumina MiSeq sequencing were generated from nested PCR: in the first PCR the
246 forward 1378-Chlorophyta (newly designed; Online Resource 1/Table S3) and the reverse ITS4
247 primers (White et al. 1990) were used (27 amplification cycles were run), in the second PCR three
248 replicates were amplified using the primers 5.8F-Chlorophyta (newly designed; Online Resource
249 1/Table S3) and ITS4 modified with Illumina overhang adaptors (forward overhang: 5'-TCG TCG GCA
250 GCG TCA GAT GTG TAT AAG AGA CAG-3'; reverse overhang: 5'-GTC TCG TGG GCT CGG AGA TGT GTA
251 TAA GAG ACA G-3'; 22 cycles). These three replicates were then pooled together. PCR reactions were
252 performed as described in Moya et al. (2017).

253 PCR products were purified using AMPure XP beads (Beckman Coulter). Indexing PCR and
254 addition of Nextera sequence adapters were performed using Nextera XT Index kit (Illumina Inc., San
255 Diego, CA, United States) following the protocol for Illumina L library preparation. Finally, a second
256 purification round was carried out using AMPure XP beads. Libraries were then quantified and
257 pooled together. The libraries were sequenced on Illumina MiSeq platform using the MiSeq Reagent
258 Kit v3 (paired end 2x 300 bp), at STAB Vida, Lisbon, Portugal and Genomics Core Facility at the
259 University of Valencia, Spain.

260 Bioinformatic analyses

261 Quality control analysis of the Illumina MiSeq paired-end reads was performed using the
262 FastQC v.0.11.8. Raw reads were processed using Quantitative Insights Into Microbial Ecology 2
263 (QIIME2 v.2018.11; Bolyen et al. 2018). Demultiplexed paired-end sequence reads were
264 preprocessed using DADA2 (Callahan et al. 2016), a package integrated into Qiime2 that accounts for
265 quality filtering, denoising, joining paired ends, and removal of chimeric sequences. The first 20 bp
266 were trimmed from forward and reverse reads before merging to remove adaptors. In order to
267 remove lower quality bases, amplicon sequence variants (ASVs) were truncated at position 210
268 based on the FastQC reports during this step.

269 Subsequent analyses were based on the ASV table, which contained the count for each unique
270 sequence in each sample. Only ASVs with frequency ≥ 100 were further analyzed. BLAST searches
271 were used to confirm the sequence identity. Solely algal sequences were further analyzed.
272 Phylogenetic tree (Online Resource 2/Fig. S3) was inferred with Bayesian Inference (BI) using
273 MrBayes v.3.2.2 (Huelsenbeck and Ronquist 2001) as described above. Euler diagrams were
274 produced using *eulerr* R package (Larsson 2019).

275 Results

276 Vegetation on study plots

277 In total, we noted 88 taxa of vascular plants, 19 taxa of bryophytes and 45 taxa of lichens
278 within 13 study plots. Online Resource 1/Table S1 contains summary of species richness for particular
279 plots.

280 Phycobiont and mycobiont phylogenetic analysis

281 The predominant phycobiont detected in *Stereocaulon* thalli by Sanger sequencing of ITS
282 rDNA, belong in a 97% of samples to the genus *Asterochloris* and only five specimens represent other
283 trebouxiophycean algae. In the case of these five specimens, coded as A574, A574.1, A633, A634 and
284 A634V the identity of the phycobiont was confirmed by Blast search against the GenBank database.
285 Significant matches of 99% to 87% were obtained with *Coccomyxa viridis* HG973000 for A574,
286 *Elliptochloris reniformis* LT560354 for A574.1 and uncultured Trebouxiophyceae FJ554399 for A633,
287 A634 and A634V. These three sequences formed a well-supported clade with more distantly related
288 sequence KF907701 (86% sequence similarity; Online Resource 2/Fig. S3) previously assigned to clade
289 URa28 (Ruprecht et al. 2014). We follow this nomenclature hereafter for these sequences.

290 The phylogenetic hypothesis resulting from Bayesian analysis of the ITS rDNA and actin type I
291 sequences of *Asterochloris* (Fig. 1) is congruent with previous studies (Peksa and Škaloud 2011;
292 Gauslaa et al. 2013; Škaloud et al. 2015; Moya et al. 2015; Vančurová et al. 2018). The species

293 boundaries delimited in Vančurová et al. 2018 and nomenclature used *ibidem* were maintained. We
294 recovered a total of 14 lineages, including one novel lineage here referred to as StA9. Eleven of these
295 lineages were previously found as phycobionts of *Stereocaulon* (Vančurová et al. 2018 and references
296 therein), namely, *Asterochloris glomerata*, *A. irregularis*, *A. italiana*, *A. lobophora*, *A. phycobiontica*,
297 *A. aff. italiana*, *Asterochloris* clade 8, *Asterochloris* clade 12, *Asterochloris* StA3, *Asterochloris* StA4
298 and *Asterochloris* StA5. Two (*Asterochloris echinata* and *A. leprarii*) were found in association with
299 *Stereocaulon* mycobiont for the first time here. The most frequently occurring phycobionts linked
300 with the lineages *Asterochloris* StA5 and *A. phycobiontica*.

301 A phylogram resulting from Bayesian analysis of ITS rDNA sequences of *Stereocaulon*
302 mycobiont is shown in Fig. 2. The majority of the recovered mycobiont sequences formed a well-
303 supported lineage delimited as OTU35 by Vančurová et al. (2018). Five sequences matched with a
304 distant related OTU2 (sister to DQ396973 and DQ396974), despite the morphological similarity of all
305 studied samples. Both OTU35 and OTU2 fall into Group 8b *sensu* Högnabba (2006).

306 Phycobiont community structure and its changes

307 *Stereocaulon alpinum* OTU2 was sampled only in two study plots (numbers 8 and 11) and its
308 phycobionts were successfully sequenced in four cases. One sequence was assigned to *Asterochloris*
309 *italiana* and three sequences to trebouxiophycean lineage termed URa28. None of them was shared
310 with the predominant *Stereocaulon* mycobiont (OTU35) in the study area.

311 *Stereocaulon alpinum* OTU35 (n=141) associated with 15 species-level lineages of phycobionts
312 in the study area: 13 lineages of *Asterochloris*, one *Coccomyxa* and one *Elliptochloris*. Phycobionts
313 from two to six species level lineages were recorded in each of 13 study plots (Fig. 3). When the
314 sample size was reduced to five samples, 2-3.7 phycobiont species per plot were estimated. For the
315 sample size of 10 (plot 11 with only five samples was excluded), 2.8-5.3 phycobiont species per plot
316 were estimated.

317 Species richness of *Stereocaulon alpinum* OTU35 phycobionts increased significantly with the
318 species richness of all lichens recorded at a study plot (Fig. 4). Moreover, gradual change of
319 phycobiont community composition was proved using Bayesian linear regression (Fig. 5). The
320 proportion of two most abundant phycobiont species-level lineages (*Asterochloris phycobiontica* and
321 *Asterochloris* StA5) decreased significantly with the succession stage in favor of other species
322 recovered in lower frequency.

323 The gradient of succession in both ordinations (vascular-plants-based and non-vascular-plant-
324 based; Fig. 6) positively correlated with the diversity of lichens and phycobionts and the number of
325 locally rare phycobionts (defined as all species with the exception of the species *Asterochloris* StA5

326 and *A. phycobiontica* highly prevalent in the study area; Fig. 3). Phycobiont communities of early-
327 successional stages were relatively species poor and mostly consisted of species generally abundant
328 in the study area (thus, the species composition was similar within study sites). With ongoing
329 succession, the number of locally rare phycobiont species increased altogether with the total number
330 of phycobiont species and the number of lichen species. The diversity of vascular plants and
331 bryophytes was not associated with this gradient. Higher moss layer cover and higher amount of light
332 demanding species of both bryophytes and vascular plants were typical of the early-successional
333 stages.

334 As indicated by the PCoA based on procrustes distances of taxa groups (Fig. 7), the variation of
335 species composition of photobiont communities responded to the environment similarly as that of
336 bryophyte communities, while clearly differing from vascular plants and lichens (Online Resource
337 1/Table S2). Note that the gradient of species diversity of taxa groups did not correspond to that of
338 their community composition.

339 Microalgal metabarcoding

340 Phycobiont diversity within particular lichen thalli (n=8) was inspected using Illumina
341 metabarcoding. A total of 1,945,186 raw reads were generated, of which 1,240,063 passed the
342 demultiplexing step and quality filter. This represented a mean of 155,007 (median 179,415) algal
343 reads per sample, with a minimum of 38,329 and a maximum of 246,121 reads per sample. Filtered
344 metabarcoding dataset consisted of 116 hits (4 to 44 per sample).

345 Abundances of recovered algal clades by sample are shown in Fig. 8. The predominant
346 phycobiont covered from 52.4 to 98.8% reads. Thalli A523M, A554M, A563M, and A570M (assigned
347 to the mycobiont OTU35) contained various *Asterochloris* species as the predominant phycobiont:
348 lineage StA9 (sample A523M), *A. irregularis* (A563M) and *A. phycobiontica*/StA4/StA5 (A554M and
349 A570M). The species-level lineages StA4, StA5 and *A. phycobiontica* are undistinguishable using ITS2
350 rDNA marker. Relative frequency of ASVs linked to *Asterochloris* spp. by sample is shown in Online
351 Resource 2/Fig. S4. Thalli A597M, A598M, A633M, and A634M (assigned to mycobiont OTU2)
352 contained predominant phycobiont from the trebouxiophycean lineage URa28.

353 Diversity of soil algae

354 Phycobiont diversity in twelve soil samples was analyzed using Illumina metabarcoding. A total
355 of 1,524,198 raw reads were generated, 876,596 of which passed the demultiplexing step and quality
356 filter. This represented a mean of 73,049 (median 63,827) algal reads per sample, with a minimum of
357 18,255 and a maximum of 164,643 reads per sample. Filtered metabarcoding dataset consisted of
358 427 hits (4 to 89 per sample). The phylogenetic hypothesis resulting from Bayesian analysis of the
359 ITS2 rDNA sequences obtained by Illumina metabarcoding of soil samples, selected lichen samples

360 and reference sequences from GenBank is shown in Online Resource 2/Fig. S3. We recovered
361 sequences in 44 well-supported clades, 27 of which contain exclusively soil algae, two contain
362 exclusively phycobionts and 15 are shared by these two groups. Occurrence of particular clades of
363 soil algae on each study plot is depicted in Online Resource 2/Fig. S5.

364 We also analyzed the occurrence of particular algal ITS2 haplotypes, including whole dataset
365 obtained by Sanger sequencing (probably predominant phycobionts; Fig. 9a). Nine haplotypes were
366 obtained by Sanger sequencing, in common with Illumina sequencing of soil and lichens. Vast
367 majority of haplotypes was unique for soil (n=256) or Illumina lichen (n=79) datasets. On the
368 contrary, 27 haplotypes were shared by soil and lichens but were not detected by Sanger sequencing
369 of *Stereocaulon* in the study area. Same analysis restricted to haplotypes with frequency ≥ 1000 (in
370 order to eliminate possible bias produced by errors from PCR and sequencing; Huse et al. 2010)
371 showed a similar pattern (Fig. 9b).

372 Discussion

373 Change of community structure along a succession gradient

374 The succession on river gravel bars is an important driver of both species' composition and
375 diversity. It is well documented in case of vascular plants (e. g., Gilvear et al. 2008; Prach et al. 2014),
376 but also applies to the microorganisms, such as soil bacteria or mycorrhizal fungi (Li et al. 2014;
377 Sheng et al. 2017). However, different taxa groups response differently to this gradient. For example,
378 vascular plants are known to follow a nested-community pattern when the highest species diversity
379 is connected to the early- to mid-successional stages and the community is being impoverished with
380 the ongoing succession (e. g., Walker and del Moral 2003; Corenblit et al. 2009; Chytrý et al. 2015).
381 This corresponds to the pattern of vascular-plant-species-richness observed within this study.

382 On the other hand, the pattern observed for the phycobiont communities of *Stereocaulon*
383 lichens differed. The phycobiont communities of early-vegetation stages were composed of relatively
384 few species lineages such as *Asterochloris phycobiontica* or *Asterochloris* StA5 (Fig. 3), which are
385 alpine and psychrophilic (Peksa and Škaloud 2011; Vančurová et al. 2018). As locally adapted, they
386 are probably frequent also in source populations in the surroundings of the study plots. Therefore,
387 newly emerged river gravel bars are easily colonized by *A. phycobiontica* and/or *Asterochloris* StA5
388 lineage. In subsequent stages, the observed species richness of phycobiont algae was mostly higher
389 and these two species-level lineages were gradually substituted by others (Fig. 5). We hypothesize
390 that some of them could be specialized to slightly different microhabitat conditions within particular
391 plots. For example, clades 8, 12 and StA3 tolerate higher pH (Piercey-Normore and DePriest 2001;
392 Bačkor et al. 2010; Vančurová et al. 2018; Steinová et al. 2019). On the river gravel bars of glacial

393 floodplains, organisms with various substrate optima could coexist together because of
394 heterogeneity of substrate transported by a river or glacier from more distant localities and various
395 substrate layers. In the study area, acidophilic vascular plants and bryophytes dominated. However,
396 occurrence of basophilic species such as *Didymodon fallax*, *Lophozia excisa*, *Syntrichia ruralis*, and
397 *Veronica fruticans* is considered as indication of basic fractions in substrate.

398 The species richness of terricolous lichens on glacier forelands in Alps were positively
399 correlated with time since deglaciation (Nascimbene et al. 2017) analogically to lichen species
400 richness on deglaciated plots in maritime Antarctica (Favero-Longo et al. 2012). In both cases, most
401 of species, once established, persisted to the oldest successional stages. On the river gravel bars the
402 species richness of phycobionts was positively correlated with that of lichens (Fig. 4) but less with
403 that of vascular plants and bryophytes (Fig. 6). This correlation probably indicates colonization of the
404 localities by phycobionts and mycobionts using the similar dispersal strategies. On the other hand,
405 we observed that the community composition variation (summarized by the procrustes tests; Fig. 7)
406 of lichens and phycobiont communities of *Stereocaulon* lichens differed.

407 We hypothesized ongoing colonization of river gravel bars by the phycobionts, or alternatively,
408 the acquisition of the phycobionts from soil *in situ* (Dal Grande et al. 2012; Fontaine and Beck 2012;
409 Ohmura et al. 2019). We found several algal clades both in soil and lichens (Online Resource 2/Fig.
410 S3), but the phycobiont pool seems to be independent of soil algae (Fig. 10). For example, the most
411 frequent ITS2 rDNA haplotype among Sanger sequences (*A. phycobiontica*/StA4/StA5 recovered in
412 69% of all samples) was present only in two soil samples in a very low amount (212 and 135 reads,
413 which is 2.4% and 3.1% of algal reads, respectively). This result supports our hypothesis that
414 phycobionts originating from the surroundings colonize the recently emerged plots without
415 substantial contribution of “soil seed bank”. Still, these results should be perceived as the basis for
416 future research. The number of soil samples is rather limited, and some taxa could be undetected
417 (Rippin et al. 2018). However, the taxonomic composition of algae occurring on river gravel bars is
418 comparable to the pool of soil algae detected in the forefield of Damma glacier in the Swiss Alps
419 (Frey et al. 2013). Notably, a significantly different algal community was found in an early-
420 successional stage, which is in their case represented by a bare soil near to a receding glacier. The
421 lichen phycobionts, including *Asterochloris*, were reported from soil in transitional and developed
422 stage. Besides others, they found the lineage StA9, firstly reported as lichen phycobiont in the
423 present study.

424 Beck et al. (2019) found two haplotypes *Stichococcus antarcticus*, phycobiont of *Placopsis*
425 lichen in maritime Antarctica, exclusively in areas deglaciated for a long time with more developed

426 soil and lichen community, unlike to other haplotypes occurring in whole area. The succession of
427 vegetation causes numerous physical and chemical changes in soil. Therefore, the correlation
428 between species richness of various organisms and successional stage (or age of the sample site) is
429 frequently connected with changing soil characteristics, such as nutrient concentrations (Li et al.
430 2014; Sheng et al. 2017). However, according the Ellenberg indication values (Online Resource
431 1/Table S1), we did not detect such gradients along successional gradient in the study area.
432 Contrarily, the gradients of distance from a river and the elevation above the water surface, are
433 detectable in our data. Since the succession is blocked by river on gravel bars; succession gradient
434 and distance from the river are naturally correlated. Nevertheless, no algae known from aquatic or
435 humid environment (e.g. *Chloroidium*; Vančurová et al. 2018) were found as predominant
436 phycobiont in samples growing in proximity to a river. Probably, the river disturbs the community
437 occasionally but does not favor individuals tolerating submersion.

438 Low specificity as an adaptive strategy

439 The specificity (i. e., the taxonomic range of acceptable partners; Rambold et al. 1998; Yahr
440 et al. 2004, 2006) of both mycobiont and phycobiont has been considered as crucial characteristic of
441 lichen interactions. The lower specificity towards symbiotic partners has been frequently reported as
442 advantageous strategy in harsh environments (Romeike et al. 2002; Engelen et al. 2010), because it
443 enables association with phycobionts adapted to a variety of conditions.

444 Two species-level lineages of *Stereocaulon* were recorded in the study area (Fig. 2). Both
445 lineages are morphologically identical, also indistinguishable in the field. Overwhelming majority of
446 samples belonged to OTU35, which has been reported to be low-specific towards its phycobionts
447 (Vančurová et al. 2018). Frequently, the mycobionts belonging to this lineage adopted algae known
448 generally as phycobionts of *Lepraria* lichens (*A. phycobiontica*, *A. echinata*, *A. leprarii*; Škaloud et al.
449 2015), in the study area. We hypothesize that such a low specificity (OTU35 associated with 13
450 species-level lineages of *Asterochloris* and in two cases also with representatives of other
451 Trebouxiophacean phycobionts in study area) could facilitate colonizing heterogeneous and harsh
452 habitat of river gravel bars of glacial floodplains.

453 On the other hand, none of the samples of *S. alpinum* OTU2 associated with the same
454 phycobionts as the mycobiont OTU35, despite growing in the same environment. The OTU2
455 mycobiont mostly associated with trebouxiophycean alga URa28 as a phycobiont. This alga was
456 previously detected in a soil sample from Canada either as a soil alga or possibly as a phycobiont of
457 *Stereocaulon* sp. which was recorded in the same place (Hartmann et al. 2009).

458 Algal plurality

459 The occurrence of more phycobiont species in a single lichen thallus (i.e., algal plurality) is a
460 common but overlooked phenomenon (e. g. Bačkor et al. 2010; Moya et al. 2017; Onuț-Brännström
461 et al. 2018) known also in *Stereocaulon* (Vančurová et al. 2018). However, various lichen species
462 differ in the prevalence of algal plurality. Dal Grande et al. (2017) proved an occurrence of more
463 phycobionts in 49.2% of *Lasallia hispanica* thalli but only in 1.7% of *L. pustulata* thalli. The algal
464 plurality is mostly undetectable by the Sanger sequencing. Using the Illumina metabarcoding, we
465 found more than one phycobiont in all selected samples from both mycobiont species-level lineages
466 (Fig. 8). However, these samples were selected on the ground of difficulties with Sanger sequencing
467 which could indicate the algal plurality (Paul et al. 2018). Illumina metabarcoding as well as the
468 Sanger sequencing uncovered URa28 as a predominant phycobiont of *Stereocaulon alpinum* OTU2. In
469 most cases, the phycobiont determined by the Sanger sequencing corresponds with a predominant
470 phycobiont according to the high-throughput sequencing (Molins et al. 2018; Paul et al. 2018).

471 Even though the two mycobiont species-level lineages OTU2 and OTU35 differ in
472 predominant phycobiont pool, they share the pool of other intrathalline algae, unlike two *Circinaria*
473 spp. collected at the same location shared the predominant phycobiont, but showed a completely
474 different pool of other intrathalline algae (Molins et al. 2018). The *Stereocaulon* OTUs differ
475 significantly in the frequency of intrathalline algae (Fig. 8). Above all, most of the OTU35 (with
476 *Asterochloris* as the predominant phycobiont) thalli contain also a small amount of URa28 algae and
477 *vice versa*. Several algal clades interacted exclusively with one mycobiont species-level lineage, but
478 their frequency was generally low. A comparable phycobiont pair *Trebouxia jamesii*/*Trebouxia* sp.
479 TR9 found in *Ramalina farinacea* lichen is assumed to bring physiological benefits to that symbiotic
480 system (Casano et al. 2011; Centeno et al. 2016). Alternatively, the minority phycobionts could occur
481 in thalli without direct effects to the lichen. They might be used as a source of algal symbionts for
482 other lichens in the locality.

483 Conclusions

484 The diversity of phycobionts shifts along the succession gradient on the river gravel bars. We
485 revealed positive correlation between the species richness of phycobionts and the species richness of
486 the accompanying lichens in the locality indicating ongoing colonization of this habitat by both
487 groups. The phycobiont communities of early-successional stages comprised relatively few species
488 lineages. In subsequent stages, the observed species richness of phycobionts was mostly higher and
489 the species-level lineages typical for early-successional stages were gradually substituted by others
490 probably adapted to heterogenous microhabitat conditions of the river gravel bars.

491 A substantial phycobiont diversity (including 14 *Asterochloris* species-level lineages and three
492 additional trebouxiophycean algae) recovered on the river gravel bars suggested low specificity of
493 *Stereocaulon* mycobionts. This range of phycobionts could help to cope with heterogenous and
494 dynamic conditions on river gravel bars of glacial floodplains.

495 We found more than one phycobiont in all selected samples belonging to both mycobiont
496 OTUs (OTU2 and OTU35). *Asterochloris* phycobionts were recovered as the predominant phycobionts
497 of OTU35 and trebouxiophycean lineage URa28 was the predominant phycobiont of OTU2. Although,
498 broad community of other intrathalline algae was shared by both mycobionts.

499 This study provides novel insights into community structure of symbiotic microorganisms
500 under harsh and dynamic conditions of river gravel bars. Additionally, it poses several challenging
501 questions concerning cryptic lichen species and specificity towards the photobionts, dispersal of
502 microscopic symbionts, an ecological function of additional intrathalline algae and an observed
503 discrepancy between communities of soil and lichen algae.

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748 Figures

749 **Fig. 1** Phylogenetic hypothesis of *Asterochloris* resulting from the Bayesian analysis of combined ITS

750 rDNA and actin type I sequences. Values at the nodes indicate the statistical supports of Bayesian

751 posterior probability (left), maximum-likelihood bootstrap (middle) and maximum parsimony

752 bootstrap (right). Fully supported branches (1.0/100/100) are marked with an asterisk. Scale bar

753 shows the estimated number of substitutions per site. Newly obtained sequences are in boxes. Clade

754 affiliations: clade 8, clade 9 *sensu* Škaloud and Peksa (2010), A4, A9, A11 *sensu* Peksa and Škaloud

755 (2011), URa14 *sensu* Ruprecht et al. (2014), I1, I2 *sensu* Řídká et al. (2014), S1, S3 *sensu* Nelsen and

756 Gargas (2006), *A. aff. irregularis*, *A. aff. italiana* and StA1 – StA8 *sensu* Vančurová et al. (2018). StA9

757 lineage was identified as new in present study. Online Resource 1/Table S6 contains accession

758 numbers of reference sequences retrieved from GenBank

759 **Fig. 2** Phylogenetic hypothesis of *Stereocaulon* resulting from the Bayesian analysis of ITS rDNA.

760 Values at the nodes indicate the statistical supports of Bayesian posterior probability (left),

761 maximum-likelihood bootstrap (middle) and maximum parsimony bootstrap (right). Scale bar shows

762 the estimated number of substitutions per site. Newly obtained sequences are in boxes. All new

763 sequences are belonging to Group 8b *sensu* Högnabba (2006), as marked

764 **Fig. 3** Bipartite association network between study plots and phycobiont species-level lineages. The

765 width of the links is proportional to the number of specimens forming the association

766 **Fig. 4** Bayesian linear regression of number of accompanying lichen species as a predictor of the

767 number of phycobionts associated with mycobiont OTU35 down-sampled to **a** sample size of 5, **b**

768 sample size of 10 per plot. Dashed lines show the 95% CRI around the regression line

769 **Fig. 5** Bayesian linear regression of number of a succession stage as a predictor of the proportion of

770 the most abundant phycobiont species-level lineages ((number of *Asterochloris phycobiontica*

771 samples + number of StA5 lineage samples)/number of all samples). Dashed lines show the 95% CRI

772 around the regression line

773 **Fig 6** Ordination diagrams of principle coordinate analyses of community composition of vascular

774 plants (a, b, c) and bryophytes + lichens (d, e, f). Colors and symbols indicate the proxy of

775 successional development (in case of a and d it is the cover of shrubs being higher than 10%; in case

776 of b and e it is the cover of crustose lichens being higher than 10%). Plots c and f show the passively

777 superimposed environmental and biological variables. Ellenberg-type indicator values (EIVs) for light

778 and temperature are calculated for vascular plants in case of plot c and for bryophytes in case of plot
779 f. On axis labels the variation explained by particular ordination axis is given.

780 **Fig 7** Ordination diagram (principle coordinate analysis) of procrustes distances between individual
781 taxa groups. The diagram shows the difference in response of communities of individual taxa groups
782 to ecological gradients covered by the sampled dataset. On axis labels the variation explained by
783 particular ordination axis is given.

784 **Fig. 8** Eight *Stereocaulon* thalli selected for Illumina metabarcoding and abundances of recovered
785 algal clades. A523M, A554M, A563M, A570M belong to mycobiont OTU35. A597M, A598M, A633M,
786 A634M belong to mycobiont OTU2. Clade affiliations: URa25, URa28 *sensu* Ruprecht et al. (2014).
787 Clades VII and XVIII were identified as new in present study. Amplicon sequence variants (ASVs) were
788 sorted into these clades based on phylogenetic hypothesis presented in Online Resource 2/Fig. S3.
789 Online Resource 1/Table S7 describes relative abundance of each ASV in each sample

790 **Fig. 9** Euler diagrams depicting sets of algal ITS2 rDNA haplotypes recovered from selected
791 *Stereocaulon* thalli (n=8) using Illumina metabarcoding, from all *Stereocaulon* samples (n=147) using
792 Sanger sequencing and from soil samples (n=12) using Illumina metabarcoding. In case of Illumina
793 metabarcoding sets, only haplotypes with frequency **a** ≥ 100 and **b** ≥ 1000 were included

794 **Fig. 10** Relative abundances of algal clades within soil (first line) and *Stereocaulon* (second line)
795 samples. Solely plots 4, 5, 6, and 8 with soil samples generating >5000 algal reads and with
796 *Stereocaulon* samples analyzed using Illumina metabarcoding were displayed. Clade affiliations:
797 URa25, URa28 *sensu* Ruprecht et al. (2014). Clade XVIII was identified as new in present study.
798 Amplicon sequence variants (ASVs) were sorted into these clades based on phylogenetic hypothesis
799 presented in Online Resource 2/Fig. S3

800 **Supplementary material**

801 **Table S1** Location, environmental and vegetation characteristics of study plots

802 **Table S2** Species composition of gravel bar vegetation plots. Species are ranked by decreasing
803 frequency of occurrence. The abundance of species is given on the nine-degree Braun-Blanquet
804 scale. All relevés are stored in Gravel bar vegetation database (Kalníková and Kudrnovsky 2017)

805 **Table S3** Primers used in this study

806 **Table S4** GenBank accession numbers, phycobiont species-level lineage, mycobiont OTU, substrate
807 and affiliation to study plot of *Stereocaulon* samples

808 **Table S5** Substitution models selected for each partition of *Asterochloris* and *Stereocaulon*
809 (mycobiont) datasets and for algal ITS2 rDNA dataset using the Bayesian information criterion (BIC)
810 as implemented in JModelTest2 (Guindon & Gascuel 2003, Darriba et al. 2012)

811 **Table S6** Accession numbers of *Asterochloris* reference sequences retrieved from GenBank

812 **Table S7** Relative abundance of each ASV in each *Stereocaulon* sample

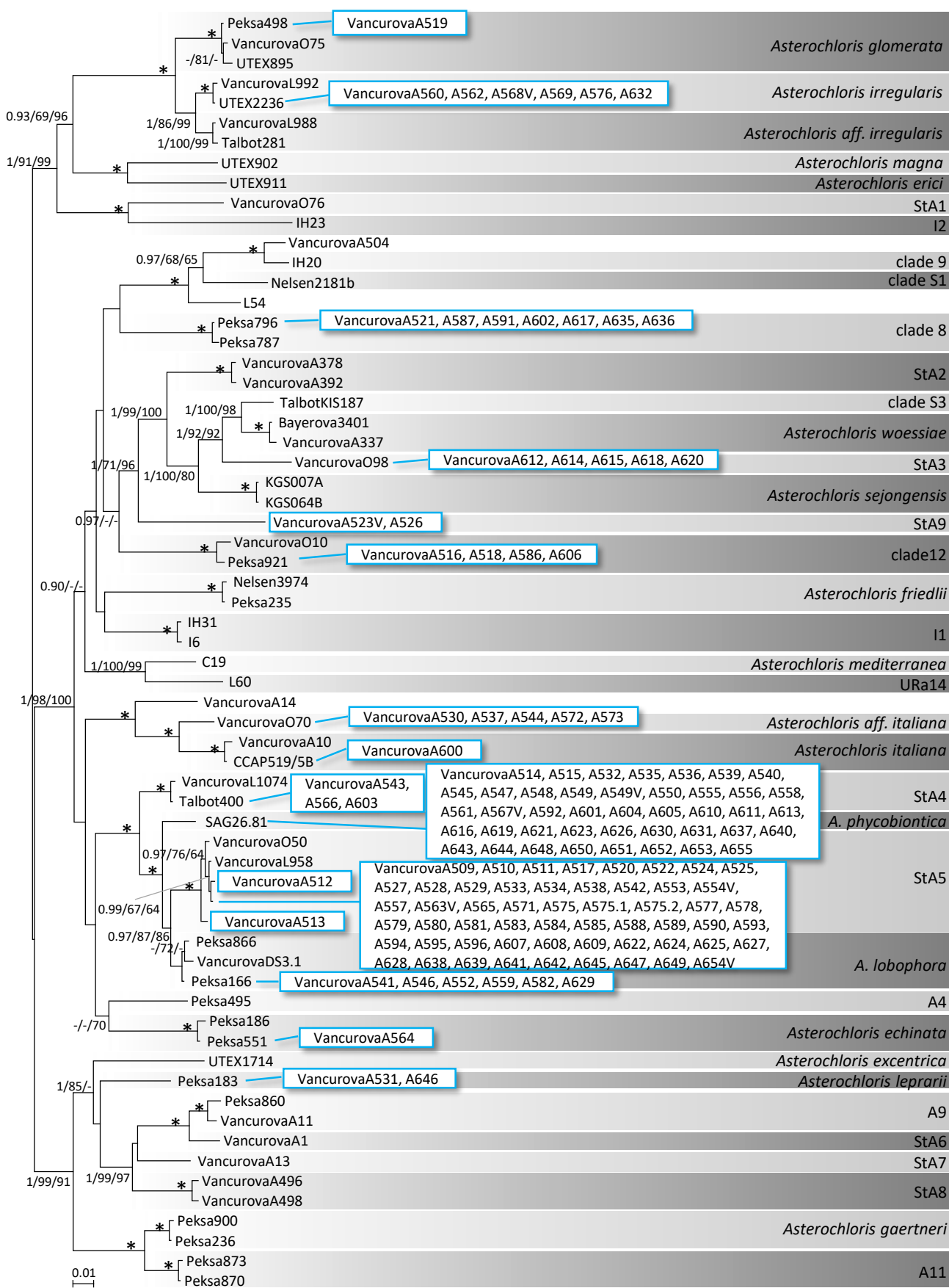
813 **Fig. S1** Examples of study plots belonging to different succession stages: **a**, **b** succession stage 1 (plots
814 5 and 9), **c** succession stage 2 (plot 2), **d** succession stage 3 (plot 3)

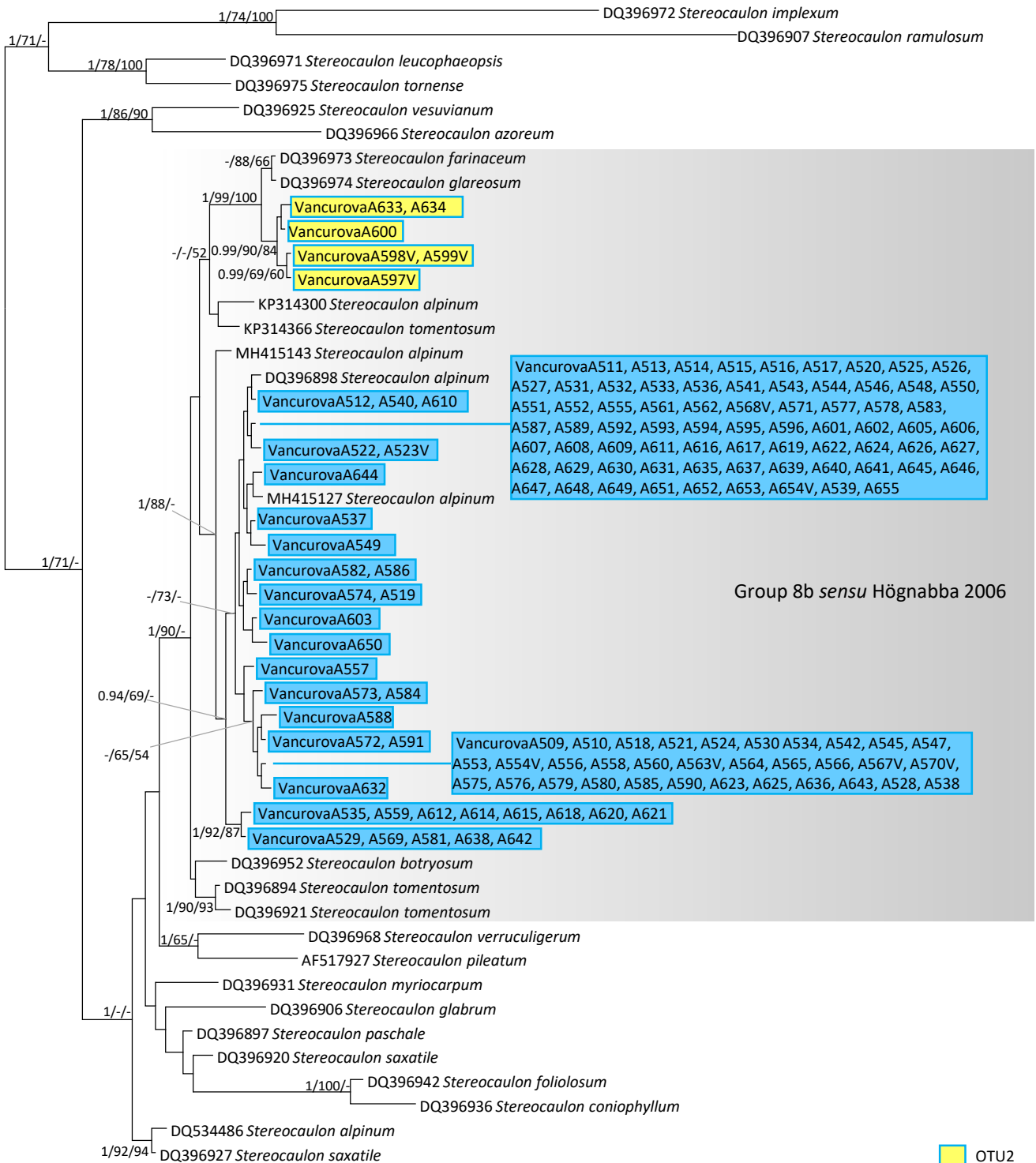
815 **Fig. S2** Rarefaction curves for 13 study plots. Vertical lines are drawn at sample size of n=5 and
816 sample size of n=10

817 **Fig. S3** Phylogenetic hypothesis resulting from Bayesian analysis of the algal ITS2 rDNA sequences
818 obtained by Illumina metabarcoding of soil samples, selected lichen samples and reference
819 sequences from GenBank. Clade affiliations: URa25, URa28 *sensu* Ruprecht et al. (2014). Clades I–
820 XVIII were identified as new in present study. Values at the nodes indicate the statistical supports of
821 Bayesian posterior probability

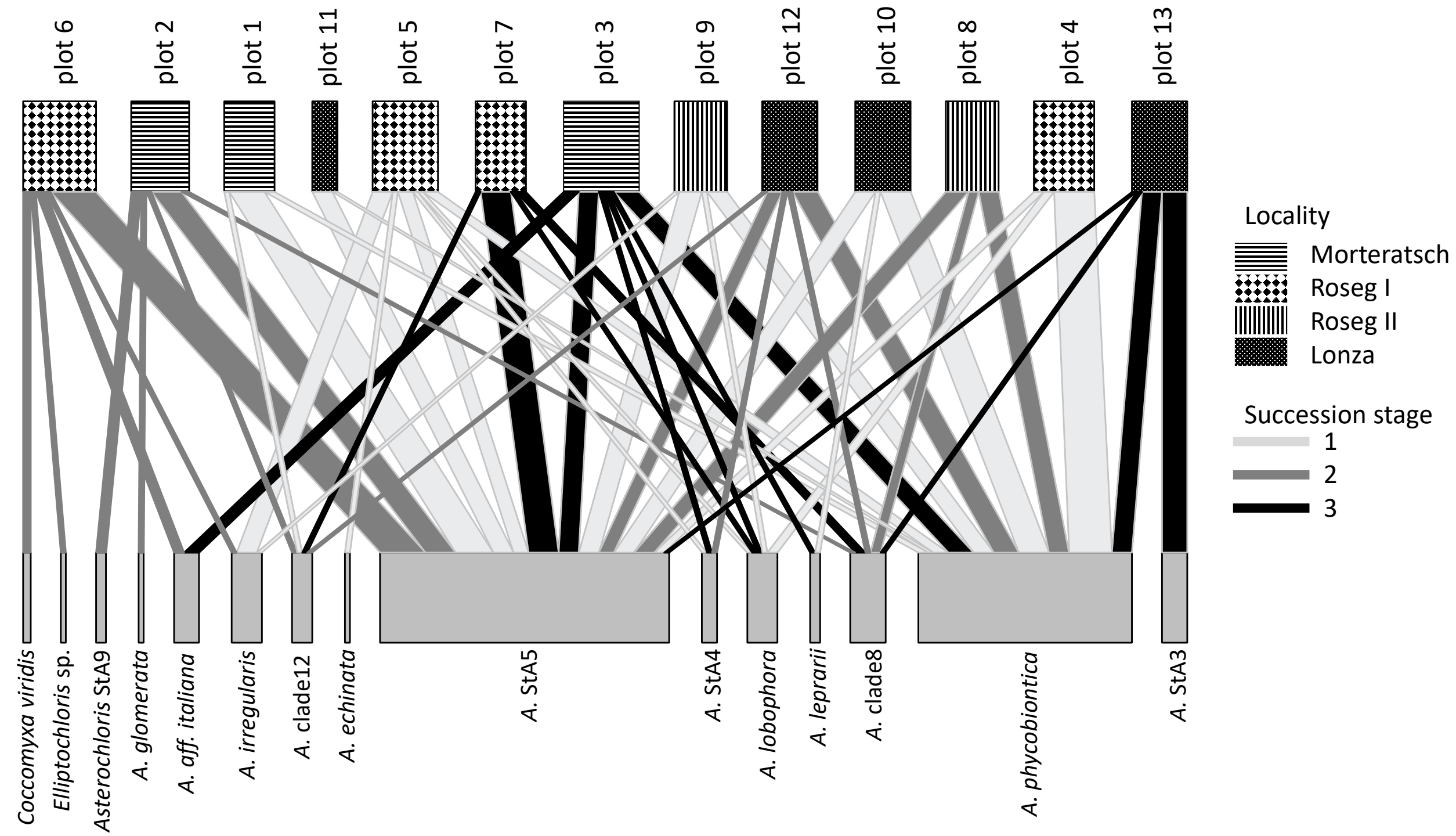
822 **Fig. S4** Relative frequency of ASVs linked to various *Asterochloris* species. Solely samples with
823 *Asterochloris* as a predominant phycobiont (>50% algal reads belonged to *Asterochloris*) were
824 displayed

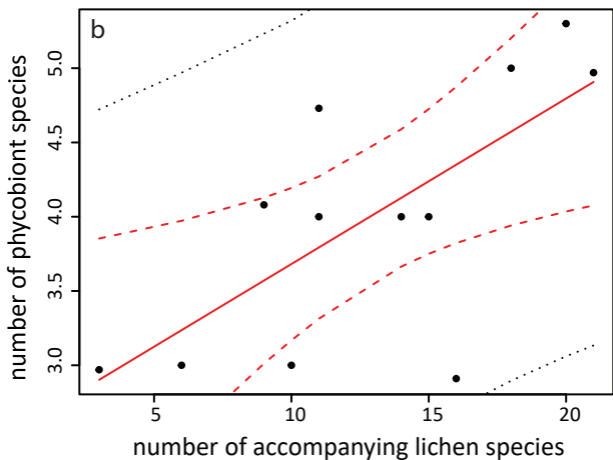
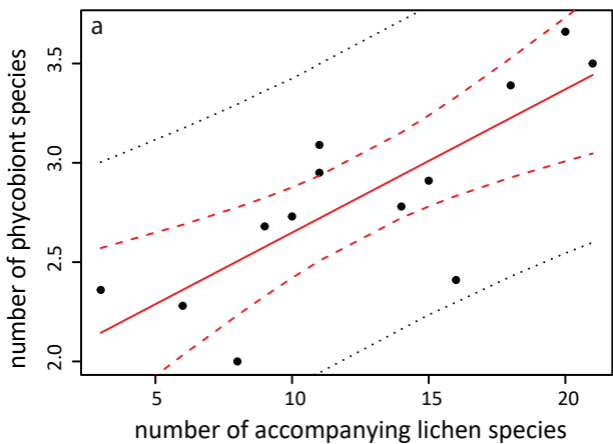
825 **Fig. S5** Frequency of ASVs linked to algal clades recovered from soil samples. Clade affiliations:
826 URa25, URa28 *sensu* Ruprecht et al. (2014). Clades I–XVIII were identified as new in present study.
827 Amplicon sequence variants (ASVs) were sorted into these clades based on phylogenetic hypothesis
828 presented in Online Resource 2/ Fig. S3

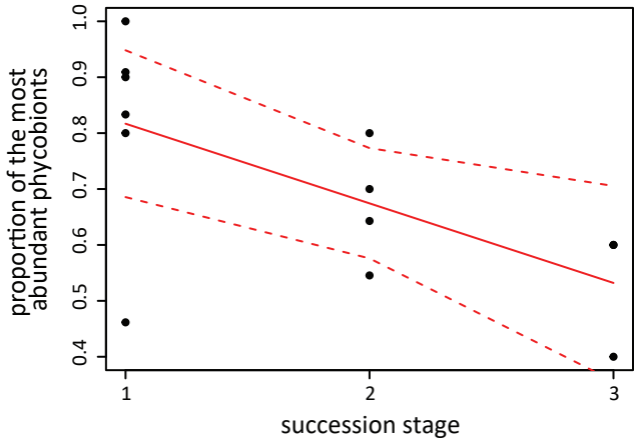


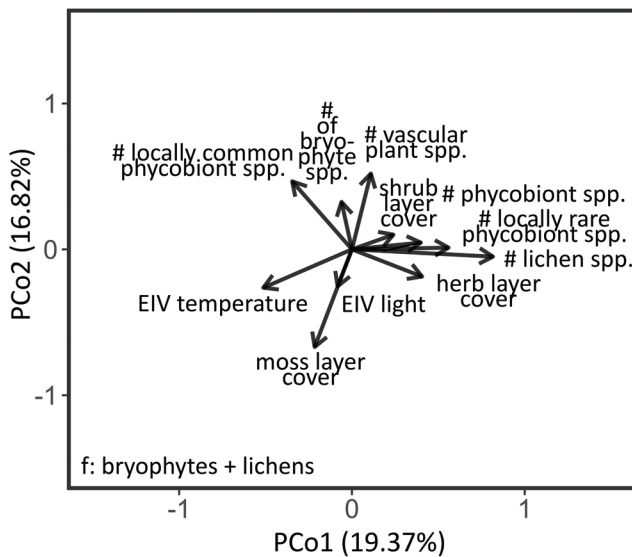
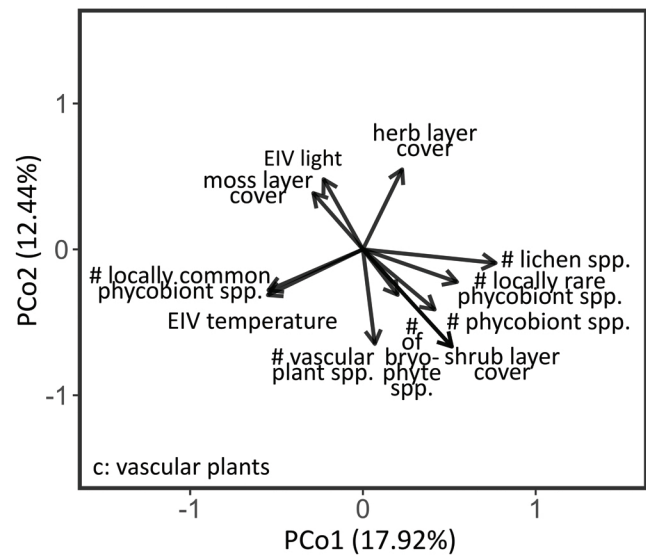
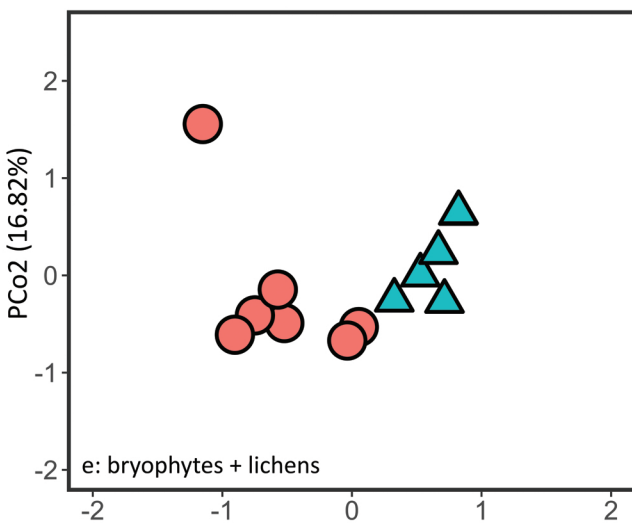
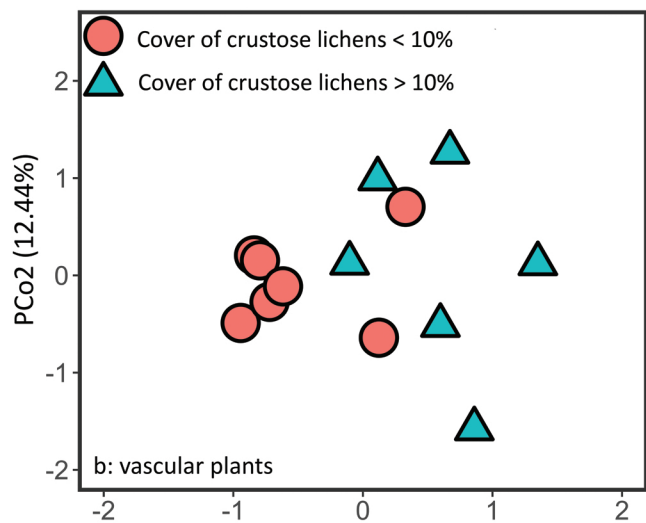
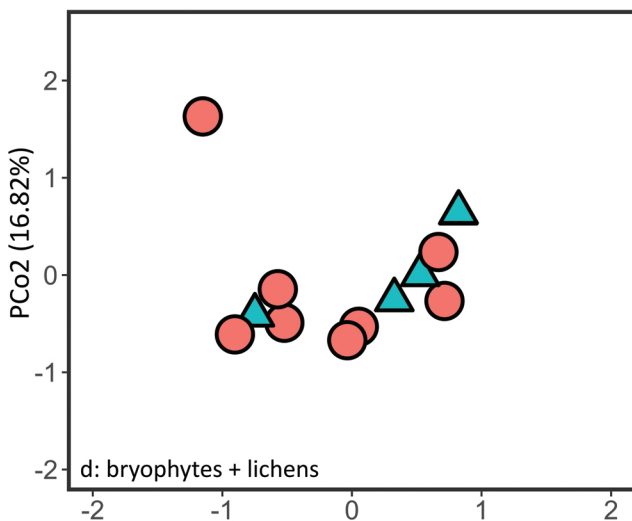
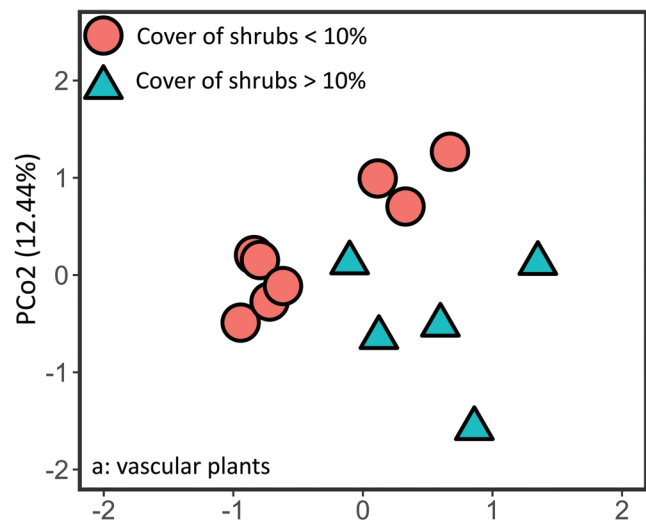


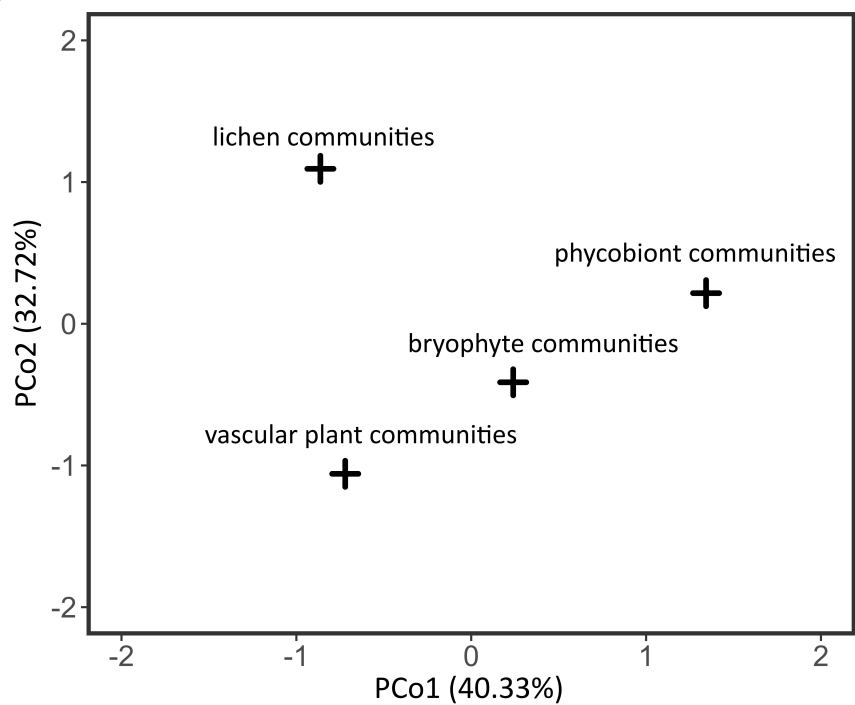
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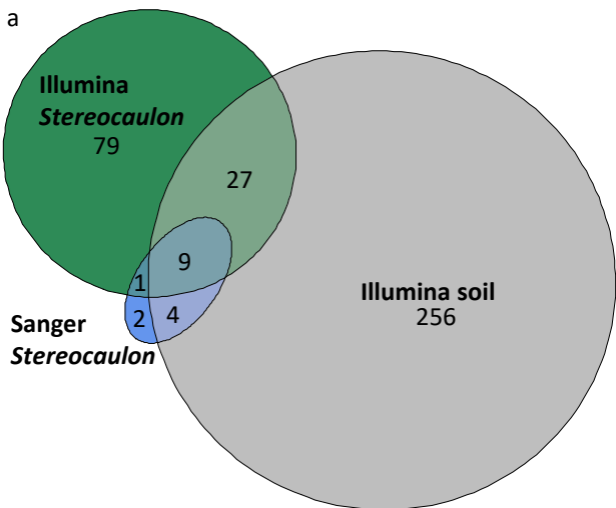




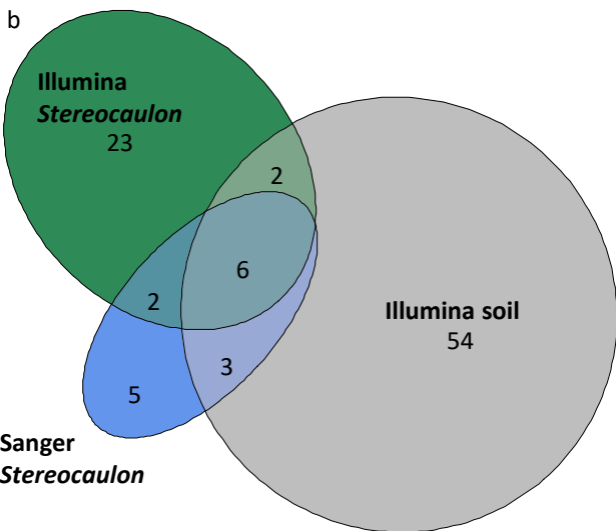




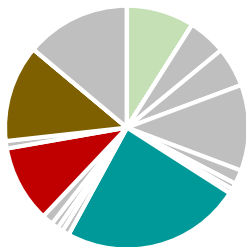
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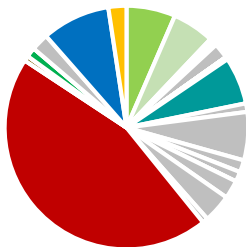
b



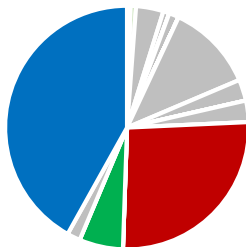
Plot 4
Soil 4



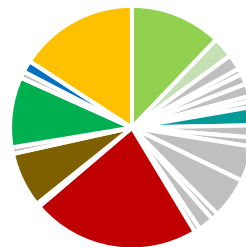
Plot 5
Soil 5



Plot 6
Soil 6

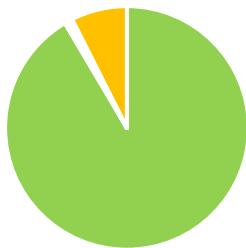


Plot 8
Soil 8

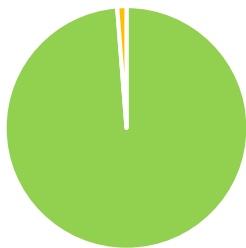


mycobiont OTU35

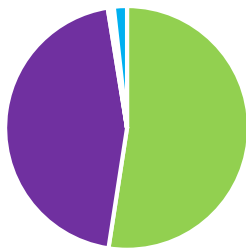
A554M



A563M

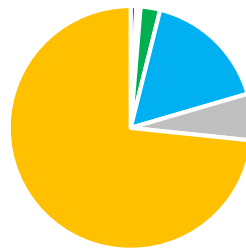


A570M

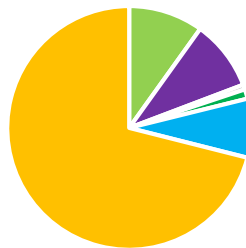


mycobiont OTU2

A597M



A598M




 *Asterochloris*


 *Coelastrella*

 *Parietochloris*


 *Sanguina*

 URa28

 others

 *Bracteacoccus*

 *Myrmecia*

 *Pseudochlorella*

 URa25

 Clade XVIII