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Lessons from culturing lichen soredia

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Abstract
Vegetative propagules play various important roles in lichen biology. We cultured soredia of *Cladonia* lichens in vitro and obtained three noteworthy results. Firstly, soredia are a beneficial source for the isolation of lichen symbionts. The mycobiont was obtained from 66% and the photobiont from 67% of the cultured soredia that were not contaminated. Secondly, the development of soredia followed a previously recognized pattern, arachnoid stage – soredium field – primordium, but a thalline structure was not achieved. We suggest that thallus formation in vitro is a question of favourable environmental factors, not partners compatibility. Finally, we discovered that fungi, other than the mycobiont, as well as airborne contaminants are dispersed together with lichen soredia. This is the first-ever report of such a phenomenon. The possible ecological consequences are discussed. Cystobasidiomycete yeasts were found among these fungi. We isolated representatives of three different lineages from a single thallus suggesting a low specificity for this association.

1 Introduction

Vegetative dispersal propagules are an exclusive expression of the lichen symbiotic phenotype (Ahmadjian 1993b). Soredia are small (20–50 μm) spherical clumps of a few algal cells and short hyphae, and among the most common means of reproduction in many lichens (Büdel and Scheidegger 2008). Their role in lichen biology is quite well-understood.

Soredia provide a lichen the clear advantage of co-dispersal of both symbiotic partners, eliminating the need for recruitment of compatible algae, which are considered to be rare in the environment (Vančurová et al. 2020). As a result, however, sorediate lichen-forming *Cladonia* species have been shown to be more specific towards their photobionts, i. e. their potential range of compatible partners is lower (Steinová et al. 2019), which may limit their ecological niches and distribution ranges (Rolshausen et al. 2018; Vančurová et al. 2018). In addition to dispersal, soredia also serve as photobiont source for other lichens (Ahmadjian 1993a). This fact plays an important role in establishment of whole lichen communities. According to the core-fringe species hypothesis (Rikkinen et al. 2002), sexual lichen species (fringe) depend on the dispersal of suitable photobionts by asexual species (core). This hypothesis has been supported by recent studies (Belinchón et al. 2015; Cardós et al. 2019).

Soredia are dispersed continuously in large amounts, often landing near the parent lichen thallus (Armstrong 1987). However, they are also carried by the wind up to distances of tens of meters (Armstrong 1987; Werth et al. 2006), and exceptionally hundreds or thousands of kilometers (Harmata and Olech 1991). Soredia are also effectively dispersed by invertebrates, such as mites, ants or snails (Stubbs 1995; Lorentsson and Mattsson 1999; Boch et al. 2011). Success of reestablishment of lichen thalli from soredia has been demonstrated in various transplantation experiments (Armstrong 1990; Scheidegger 1995; Kon and Ohmura 2010).

Soredia have been studied experimentally mainly with the purpose of lichen synthesis in vitro (e.g. Stocker-Wörgötter 1995; Valarmathi and Hariharan 2007) or viability testing (e.g. Hauck and Zöller 2003; Buldakov 2010). Successful syntheses of lichens in vitro are infrequent and factors that determine the underlying processes are still only partially understood (as reviewed e. g. by Stocker-Wörgötter 2001; Joneson and Lutzoni 2009). Thus, procedures for such experiments are not standardized and need to be established for each experimental series anew. Additionally, although the contamination rate associated with culturing lichen material is generally high (Crittenden et al. 1995), studies about culturing soredia did not provide information on contaminating fungi (see e.g. Stocker-Wörgötter and Türk 1988, Stocker-Wörgötter 1995, Zorer et al. 1997, Trembley et al. 2002 and references above). It might, however, be expected that besides airborne and laboratory contaminants, fungi associated with

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lichen thalli (Hawksworth and Grube 2020) are co-dispersed with soredia, as has already been shown for bacteria (Aschenbrenner et al. 2014).

We cultured soredia to observe their development with the objective of setting a reference frame for future in vitro compatibility testing. Specifically, our aims were to 1) evaluate suitability of soredia for the isolation of symbiont cultures, 2) inspect their development in vitro, and 3) have a first-ever look into diversity of fungi spread with soredia.

2 Material and Methods

2.1 Materials

Cladonia rei was collected from soil on silicate rock outcrops in Svatá, Czech Republic, N49.9399972 E13.9607781, 480 m a. s. l. on 5 March 2019 and *C. fimbriata* from soil on the lower edge of an oak-pine forest in Černošice, Czech Republic, N49.9470725 E14.3388042, 200 m a. s. l. on 11 September 2019. The specimens were deposited in PRC (PRC 4638 and PRC 4639, respectively). *C. rei* was processed the day after collection, *C. fimbriata* after two days and then again three weeks after collection. Both were used for evaluation of isolation success of the mycobiont and photobiont. Only soredia from *C. fimbriata* were used to study development and to collect information on the associated fungi.

2.2 Isolation

The thalli were used unwashed. In addition to soredia development, we were also interested in fungi co-dispersed with soredia, both within the soredia and on their surface (see Discussion). We are aware of disadvantages of not washing, but we believe molecular methods give us a powerful tool for distinguishing airborne fungi and laboratory contaminants. Also, thorough washing could result in detachment of the most mature soredia that might be expected to germinate most readily.

Under a binocular microscope, the soredia were separated directly from the thalli using a sterile needle and placed onto cultivation media. Sterile 12 well cell culture plates (Cellstar, USA) were used, each well 22 mm in diameter. Care was taken to separate as little lichen material as possible, resulting in removal of individual soredia or clusters of a few. Media recipes are to be found in Stocker-Wörgötter and Hager (2008). The media used were Bold's Basal medium (BBM) and Malt-yeast extract medium (MYA) with no sugars added, or BBM, Trebouxia organic medium (TOM) and Sabouraud agar (SAB) with the addition 1% or 2% of ribitol, mannitol or glucose. Ribitol and mannitol were used as alternatives to glucose because they have been reported to stimulate the growth of lichen-forming fungi (Guzow-Krzemińska and Stocker-Wörgötter 2013, Meeβen et al. 2013). The inoculated plates were kept in an incubator (Electrolux, ERC2543, 250CI, with thermostat TS-3, FK technics and fluorescent bulb controlled by a digital time switch TR610, Theben) at 16.5 °C and 12 h light regime (18 μmol photons m^2 s^1).

Cultures of lichen mycobiont (*Cladonia* spp.) and photobionts (*Asterochloris* spp.) were identified morphologically. Ten representative isolates of the mycobiont were chosen for molecular study and confirmation. We expressed isolation success in two ways; as isolation rate and viability rate. The isolation rate was calculated as the percentage of inoculantes from which the mycobiont/photobiont grew. The viability rate was calculated as the percentage of the number of obtained isolates from the number of inoculated wells minus the number of contaminated wells. A well was considered contaminated if it was overgrown by common airborne fungi, such as *Cladosporium*, *Penicillium* etc. We believe that the number of contaminated plates provides information about the state of laboratory equipment and skillfulness of the isolator rather than the quality of the studied material. Thus, the isolation rate provides information about how fruitful the effort was, and the viability rate allows us to compare our results with other methods of isolation. Contamination rates are high in all of them. Fungal isolates that were neither airborne and ubiquitous fungi, nor lichen mycobionts, were considered as soredia-associated fungi.

2.3 Soredia cultivation on natural substrates

Soil or pieces of sandstone, both collected from natural *Cladonia* habitats were autoclaved. They were placed in glass petri dishes (4 cm in diameter) and autoclaved again after one week. The material was fixed in the petri dish with water agar.

After three months of culturing, most soredia had developed into primordia (see Results and Discussion). Six of them were picked, divided into 22 smaller pieces and transferred onto sterile soil or sandstone. At first, they underwent four drying and re-wetting cycles. Drying was done in the following way: the agar surrounding the natural substrata was cut out from the petri dishes, open dishes were then placed in a running laminar flow box for four hours. During this time the air flow dried both the developing soredia and the substrata completely. The petri dishes were closed and sealed with parafilm. After four days, they were re-wetted as follows: hot water agar was carefully poured inside to surround the soil/sandstone. After the agar stiffened, the dishes were sealed with parafilm. This way 100% moisture was kept inside until the dishes were open again four days later. The developing soredia thus absorbed the humidity from the air surrounding them. They were inspected microscopically before each drying and photos were taken under a stereomicroscope. After the first month (four drying and re-wetting cycles), they were re-wetted monthly as described above. After each wetting the

petri dishes were covered with their lids but were not sealed with parafilm, so that slow continual drying was allowed. They were completely dry after about three weeks. The final evaluation was made after six months of culturing on the natural media (December 2019 – June 2020). The petri dishes were kept at circa 22 °C on a window sill to simulate natural light conditions.

2.4 Molecular methods

DNA from both cultures and the original thalli was isolated using the CTAB protocol (Cubero et al 1999) with minor modifications. ITS rDNA was amplified using the primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990). PCR began with denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C denaturation for 45 s, 54 °C annealing for 1 min and 72 °C elongation for 2 min and finished with extension at 72 °C for 10 min. For the cultures of yeast belonging to Cystobasidiomycetes SSU and LSU rDNA was amplified too. For SSU the primers SSU_symrho_2F and NS6 (Spribille et al. 2016) were used and the PCR consisted of 35 cycles of 95 °C denaturation for 30 s, 56 °C annealing for 30 s and 72 °C elongation for 45 s. The primers LR0R and LR6 (Vilgalys and Hester 1990) were used for LSU and the PCR consisted of 35 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 1 min. The PCR products were sequenced by Macrogen Europe, Amsterdam, the Netherlands. GenBank accession numbers of the newly obtained sequences are given in Table 1.

2.5 Identification of associated fungi

Because all the isolates were sterile we used ITS rDNA to designate their taxonomic position. The obtained chromatographs were examined and sequences were edited if needed. They were compared to GenBank using BLASTn. For each isolate the closest match was recorded. If the closest match was an unidentified fungus and/or from environmental sample, the closest reliably identified match, e. g. a sequence from type specimen or from a curated culture collection, was recorded as well (Table 1). Provisional names were given to the isolates based on sequence similarity; at the similarity of at least 98% the isolate was given a species names, at similarities between 90 and 97% the isolate was classified into an order and at lower similarities it was classified either to a subclass or class (Table 1).

2.6 Phylogeny of Cystobasidiomycetes

Cystobasidiomycetes yeasts were previously hypothesized to form specific symbiosis with lichens (Spribille et al. 2016). So, in order to position isolates SOR11c5, SOR11d6, SOR12b5 and SOR12d2 within the class a phylogeny based on the three ribosomal DNA markers was performed. Representatives of all main lineages of the class (Wang et al. 2015; Spribille et al. 2016; Černajová and Škaloud 2019) were included in the dataset taking into account the closet BLAST matches of our sequences (Table 2). *Sporidiobolus salmonicolor* (Microbotryomycetes) was used as the outgroup. Each marker was processed separately. Sequences downloaded from the GenBank were aligned with the newly obtained sequences using MAFFT v.7 (Katoh et al. 2017) using the Q-INS-I method. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana 2000) and removed. Final datasets consisted of 866 SSU, 314 ITS and 536 LSU positions. Substitution models were estimated with Bayesian Information Criterion using JModelTest v. 2.1.4 (Darriba et al. 2012) as follows: TrN + I + G for SSU (p-inv 0.588, gamma shape 0.71), JC + G for ITS1 (gamma shape 1.645), K80 for 5.8S, TVMef + G for ITS2 (gamma shape 0.648) and TIM2ef + I + G for LSU (p-inv 0.414, gamma shape 0.55).

The phylogenetic trees were inferred by Bayesian Inference in MrBayes v. 3.2.6 (Ronquist et al. 2012), initially for each locus separately. All three loci gave congruent topologies. So, the final analysis was performed on a concatenated dataset using the five partitions. Two parallel MCMC runs, with one cold and three heated chains, were run. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF), which was 0.001 after final 11 million generations. The first 25% of the trees were discarded as burn-in in each run. 50% majority-rule consensus trees were obtained using the sumt option. The analyses were run on the CIPRES Science Gateway v. 3.3 web portal (Miller et al. 2010).

3 Results

3.1 Mycobiont (*Cladonia* spp.) and photobiont (*Asterochloris* spp.) isolation success

Either the lichen symbionts or other fungi grew from all the soredia. Bacteria grew on only a few plates; the colonies were very small and did not perturb the symbionts. The isolation and viability rates are given in Table 3. Overall, isolation/viability rate was 46/66% for the mycobiont and 50/67% for the photobiont. Mycobiont viability rate was 75% from the soredia of *C. rei* and 62% from the soredia of *C. fimbriata*. Photobiont (*Asterochloris* sp.) viability rate was 83% and 59% from the soredia of *C. rei* and *C. fimbriata*, respectively. Viability of the soredia of *C. fimbriata* did not decrease after three weeks. The viability rate of the mycobiont was 62% in both cases. The viability rate of the photobiont was 57% two days and 62% three weeks after collection. Interestingly, for

Table 1 Identification based od ITS rDNA and best GenBank matches of the isolates obtained from soredia of *Cladonia fimbriata*

Isolate	GenBank Accession [1]	Class	Identification	Best GenBank matches				
				Similarity	e-value	Accession	Strain/Clone	Habitat
Lichen mycobiont								
SOR6a2[2]	MT981770-MT981779	Lecanoromycetes	*Cladonia fimbriata*	544/546(99%)	0.0	MK811629	Cladonia fimbriata O-L-200909	terricolous[3]
Ascomycota								
SOR6c3	MT981780	Dothideomycetes	Dothideomycetes sp.	543/546(99%)	0.0	MT236889	Uncultured fungus 4248_906	irrigation water from the pond[4]
				394/460(86%)	7E-101	NR_155853	*Saccharata eucalyptorum CPC 29222*	holotype culture, from *Eucalyptus bigalerita*[5]
SOR8a3	MT981784	Dothideomycetes	Dothideomycetes sp.	414/423(98%)	0.0	GU993541	Uncultured Capnodiales A11	energy transmission tower (corrosion dust)[6]
				443/496(89%)	2E-170	GU570527	*Devriesia pseudoamericana CPC:16,174*	fruit surface[7]
SOR11b6	MT981786	Dothideomycetes	Pleosporomycetidae sp.	473/495(96%)	0.0	KC222749	Uncultured fungus Toohyp3	soil in eucalyptus forest[8]
				435/502(87%)	1E-147	NR_154080	*Hermatomyces thailandicus MFLUCC 14–1143*	holotpye culture, from *Tectonia grandis*[9]
SOR12b1	MT981790	Dothideomycetes	Pleosporales sp	421/435(97%)	0.0	JX457096	Uncultured fungus HIC6	forest soil10forest soil[10]
				411/435(94%)	0.0	MN421854	*Lophiostoma chamaecyparidis isolate 4*	culture from *Nectandra lineatifolia*[11]
SOR12c3	MT981792	Dothideomycetes	Venturiales sp.	238/238(100%)	4E-119	KX194025	Uncultured fungus 1604	soil[12]
				312/341(91%)	4E-094	NR_168748	*Parafusicladium amoenum CBS 254.95*	holotype, from leaf litter of *Eucalyptus grandis*[13]
SOR13b1	MT981795	Dothideomycetes	*Pseudocamaropycnis pini*	502/507(99%)	0.0	NR_153459	*Pseudocamaropycnis pini CBS:115,589*	holotype culture, from a *Pinus elliotii* leaf[14]
SOR6d2	MT981781	Leotiomycetes	Helotiales sp.	464/479(97%)	0.0	KX908215	Leotiomycetes sp. 780 JMUR-2016	endophyte culture, from a leaf of *Pinus strobus*[15]
				454/501(91%)	0.0	MH221525	*Ciliolarina ligniseda SBRH847*	dead pinus log on the ground[16]
SOR8a2	MT981783	Leotiomycetes	Helotiales sp.	447/455(98%)	0.0	EF619699	Uncultured Helotiales 3S2.16.F04	forest soil[17]
				457/483(95%)	0.0	NR_156207	Hyalodendriella betulae CBS 261.82	*Alnus glutinosa*[13]
SOR12d3	MT981794	Leotiomycetes	*Lachnellula pulverulenta*	469/476(99%)	0.0	AB481260	*Lachnellula pulverulenta FC-2025*	*L. pulvurentula* fruit body[18]
SOR8d2	MT981785	Eurotiomycetes	Chaetothyriomycetidae sp.	509/509(100%)	0.0	KX147893	Uncultured fungus PO.1.69	pine sapwood [19]
				466/552(84%)	3E-139	NR_153652	*Bacillicladium lobatum CCF 5200*	type culture (walls of metro station)[20]
Basidiomycota								
SOR7c1	MT981782	Spiculogloeomycetes	Pucciniomycotina sp.	459/525(87%)	1E-162	MT236898	Uncultured fungus clone 4248_1241	irrigation water from the pond[4]
				299/370(81%)	3E-069	NR_121215	*Phyllozyma producta MAFF 654,001*	holotype culture, from a leaf of *Citrus unshiu*[21]
SOR11c4	MT981787	Exoboasidiomycetes	*Microstroma bacarum*	632/633(99%)	0.0	NR_153481	*Microstroma bacarum CBS 6526*	type, from fruit[22]
SOR11c5 = SOR12b5	MT990521/MT981788/MT974387	Cystobasidiomycetes	Cystobasidiomycetes sp.	504/531(95%)	0.0	KT581825	Uncultured Rhodotorula clone MDW-OTU-12	*Quercus* deserticola litter[23]
	MT990522/MT981791/MT974388			393/439(90%)	7E-151	KY104259		holotype, from soil [22]

Table 1 (continued)

Isolate	GenBank Accession [1]	Class	Identification	Best GenBank matches				Habitat
				Similarity	e-value	Accession	Strain/Clone	
SOR11d6	MT990523/MT981789/MT974389	Cystobasidiomycetes	Cystobasidiomycetes sp.	470/529(89%)	2E-176	AB263120	*Microsporomyces orientalis culture CBS:8594* Sakaguchia lamellibrachiae strain: SY-298	on *Calyptogena* in deep sea[24]
SOR12d2	MT990524/MT981793/MT974390	Cystobasidiomycetes	*Cystobasidium pinicola*	519/520(99%)	0.0	MH380197	Cystobasidium pinicola strain ICMP 2924	*Prunus persica* leaf[25]

[1] Accession numbers of newly obtained ITS rDNA sequences are provided except for Cystobasidiomycetes where SSU/ITS/LSU rDNA are given

[2] also SOR6c2, SOR6d3, SOR7a2, SOR8e1, SOR10-1, SOR10-3, SOR11d4, SOR12c4, SOR12c6

[3 – 25] References: Marthinsen et al. 2019, [4] Marčiulynas et al. 2020, [5] Crous et al. 2016, [6] Sette et al. 2010, [7] Frank et al. 2010, [8] Greenlaw 2012 unpubl., [9] Doilom et al. 2017, [10] Liu and Qiu 2012, unpubl., [11] Nelson et al. 2019 unpubl., [12] Beck et al. 2016 unpubl., [13] Crous et al. 2007, [14] Crous and Groenewald 2016, [15] U'Ren and Arnold 2016, [16] Helleman 2018, unpubl., [17] Parrent and Vilgalys 2007, [18] Hosoya et al. 2010, [19] van Nieuwenhuijzen et al. 2017, [20] Réblová et al. 2016, [21] Furuya et al. 2012, [22] Vu et al. 2016, [23] Rosales-Castillo et al. 2018, [24] Sampaio et al. 2006 unpubl., [25] Weir and Park 2018 unpubl.

both species the isolation and viability rates were the highest on BBM, i.e. mineral medium with no source of carbohydrates. The mycobiont viability rate was 83% for *C. rei* and 80% for *C. fimbriata*. On the other hand, it was 68% for *C. rei* and 60% for *C. fimbriata* on media with sugars added. The photobiont viability rate of both species was 100% on BBM and 76% for *C. rei* and 57% for *C. fimbriata* on media with sugars added (Table 3).

3.2 Diversity of associated fungi

Alltogether 73 fungal isolates were obtained from the soredia of *C. fimbriata*. 47 of them were isolates of the lichen mycobiont, i. e. *C. fimbriata* and 26 were different from the lichen mycobiont. Ten of the former and 16 of the latter were successfully sequenced. The closest BLAST matches together with the closest reliably identified matches are shown in Table 1. Ten Ascomycota and six Basidiomycota isolates were obtained. Among them, Dothideomycetes (six isolates) and Cystobasidiomycetes (four isolates) prevailed respectively (Table 1). Majority of the isolates could not be assigned to a species or genus based on ITS rDNA. Sequences of only four isolates gave matches of 99% similarity: SOR11c4 matched *Microstoma baccarum* (632/633 bp), SOR12d2 *Cystobasidium pinicola* (519/520 bp), SOR12d3 *Lachnellula pulverulenta* (469/476 bp) and SOR13b1 *Pseudocamaropycnis pini* (502/507). Sequences from other cultures gave high matches (96–100%) with sequences of unidentified mostly uncultured fungi (Table 1) found in soil (SOR8a2, SOR11b6, SOR12b1, SOR12c3), pine needles and wood (SOR6d2 and SOR8d2) or even in corrosion dust (SOR8a3) and water (SOR6c3).

We obtained four isolates belonging to three distinct genotypes of Cystobasidiomycetes. Their position within the class as inferred by Bayesian Inference is shown in Fig. 1. SOR11c5 and SOR12b5 represent an unrecognized species related to *Microsporomyces pini* with full bootstrap support. SOR11d6 formed a unique lineage of uncertain position within the class. And SOR12d2 belongs to the genus *Cystobasidium* with full bootstrap support. It is conspecific with *Cystobasidium pinicola* based on the similarity of ITS sequence, but it appears as a separate species in the phylogram probably because of the fact that the similarity in both SSU and LSU is very high among various species within the genus.

3.3 Soredia development

The structure of soredia disintegrated after germination on all media. The symbionts grew in close association with one another but separately, each in its own way; the photobiont grew in an elevated globular form, typical of *Asterochloris* spp. and the mycobiont formed a loose arachnoid radial mycelium tightly fixed to the substrate (Fig. 2a). After five weeks, the

Table 2 List of GenBank sequences used for the phylogeny of Cystobasidiomycetes

Taxon	Strain/clone	ITS	LSU	SSU
Bannoa hahjimensis	JCM 10,336	AB035897	–	AB035897
Bannoa ogasawarensis	JCM 10,326	AB035713	AB082570	AB035713
Bannoa syzygii	JCM 10,337	AB035720	AB082573	AB035720
Buckleyzyma armeniaca	JCM 8977	AF444523	AF189920	AB126644
Buckleyzyma aurantiaca	JCM 3771	AF444538	AF189921	KJ708436
Buckleyzyma salicina	JCM 2959	AF444511	AF189995	AB021687
Cyphobasidium hypogymniicola	S-F264671	KU587700	KU587694	KU587705
Cyphobasidium usneicola	S-F264675	KU587704	KU587699	KU587706
Cystobasidium laryngis	JCM 10,953	AB078500	AB078500	AB126649
Cystobasidium pinicola	AS 2.2193	AF444292	AF444293	AB126652
Cystobasidium ritchiei	CBS 12,314	NR_154854	KY107445	NG_063085
Erythrobasidium elongatum	AS 2.1949	AF444561	AF189983	AB021669
Erythrobasidium hasegawianum	AS 2.1923	AF444522	AF189899	D12803
Lichenozyma pisutiana	CCF 6137	MK491195	MK491271	MK491263
Microsporomyces magnisporus	JCM 11,898	AB112078	AB111954	KJ708428
Microsporomyces pini	CBS 107,345	EU075190	EU075188	KJ708357
Occultifur externus	JCM 10,725	AF444567	AF189910	AB055193
Occultifur tropicalis	DMKU SE59	NR_148062	–	–
Sakaguchia dacryoidea	JCM 3795	AF444597	AF189972	D13459
Sakaguchia lamellibrachii	CBS 9598	AB025999	AB025999	AB126646
Sakaguchia oryzae	AS2.2363	AY335160	AY335161	KJ708352
Sporidiobolus salmonicolor	CBS 490	NR_149325	NG_056268	NG_063452
Symmetrospora coprosmae	JCM 8772	AF444577	AF189980	D66880
Symmetrospora foliicola	AS 2.2527	AF444521	AF189984	AB021671
Symmetrospora gracilis	JCM 2963	AF444578	AF189985	KJ708433
Uncultured Cyphobasidiales	T1385	KU948738	KU948871	KU948820
Uncultured Cyphobasidiales	T1645	KU948778	KU948917	KU948855

diameter of these flat mycelia was about 2 mm, ranging between 1.4 mm and 2.4 mm, regardless of presence or absence of sugars in the medium (Wilcoxon sum rank test, $W = 37$, $p = 0.54$, $n = 20$, not shown). The exceptions were two particularly large mycelia (4.7 and 5.6 mm in diameter) that developed on media with glucose.

After three months, the development differed depending on the medium. On BBM, there was no progress from the small arachnoid radial mycelia. On TOM and SAB both with glucose ($n = 21$), the symbionts came together and developed further in association (see below). It is uncertain whether this development was a result of the presence of glucose or organic nitrogen compounds (peptone) in the medium, because on BBM with glucose and on SAB with ribitol/mannitol growth of both symbionts unfortunately was not achieved, with only one exception. On BBM with ribitol ($n = 6$) or mannitol ($n = 2$) and on SAB with mannitol ($n = 1$) both symbionts grew, the mycobiont formed a dense and compact mycelium and the photobiont a compact colony that did not seem to interact in five of the soredia (Fig. 2b). In two others a soredium field (see below) was observed, and from one of these a primordium (see below) developed. However, it is impossible to draw conclusions about the effect of carbohydrate type in the medium as the contamination rate was very uneven (Table 3) and there were only a few soredia developing to these stages.

The mycobiont cultures formed numerous lateral branches (Fig. 2c) and encircled algal cells (Fig. 2d). A well-developed so-called soredium field (Schuster 1985; Stocker-Wörgötter and Türk 1988), was observed that consisted of a layer of undifferentiated algal-fungal tissue after two–three months of inoculation. At the beginning it consisted of a mass of predominantly algal cells with a few interwoven fungal hyphae (Fig. 2e). If there were more soredia sown on a plate, they usually fused into one mass/tissue at this stage. Gradually, the network of hyphae became denser and the mycobiont started to dominate either in parts of the soredium field (Fig. 2f) or over the whole tissue at once (Fig. 2g). In the next stage, a so-called primordium appeared; the mycobiont enclosed the algal cells inside the tissue, thus forming the basis of thallus stratification (Fig. 2h). Rather than distinct phases, the development was a continuum. The various stages

Table 3 Isolation success; numbers of obtained isolates, contamination, isolation and viability rates are given

	Obtained isolates					Contaminated	Contamination rate	Mycobiont isolation rate[3]	Mycobiont viability rate[4]	Photobiont isolation rate[3]	Photobiont viability rate[4]
	Inoculates	Mycobiont only	Photobiont only	Mycobiont + photobiont	Other fungi						
Cladonia rei											
BBM	12	0	1	10	0	1	**8%**	**83%**	**91%**	**92%**	**100%**
+ glucose [1]	10	0	2	5	2	1	**10%**	**50%**	**56%**	**70%**	**78%**
+ mannitol [1]	8	0	0	5	3	0	**0%**	**63%**	**63%**	**63%**	**63%**
+ ribitol [1]	9	0	0	7	1	1	**11%**	**78%**	**88%**	**78%**	**88%**
total	39	0	3	27	6	3	**8%**	**69%**	**75%**	**77%**	**83%**
Cladonia fimbriata											
BBM	10	0	2	8	0	0	**0%**	**80%**	**80%**	**100%**	**100%**
MYA	26	3	1	3	4	15	**58%**	**23%**	**55%**	**15%**	**36%**
+ glucose 1	52	0	3	21	14	15	**29%**	**40%**	**57%**	**46%**	**65%**
+ mannitol [1]	17	5	1	2	4	6	**35%**	**41%**	**64%**	**18%**	**27%**
+ ribitol 1	10	1	0	6	3	0	**0%**	**70%**	**70%**	**60%**	**60%**
sum 2 days [2]	45	17	6	4	12	8	**18%**	**51%**	**62%**	**47%**	**57%**
sum 3 weeks [2]	70	23	3	3	13	28	**40%**	**37%**	**62%**	**37%**	**62%**
total	115	40	9	7	25	36	**31%**	**43%**	**62%**	**41%**	**59%**
C. rei + *C. fimbriata*											
total	154	40	12	34	31	39	**25%**	**49%**	**66%**	**50%**	**67%**

[1] combined numbers of isolates for BBM/SAB/TOM with glucose/mannitol/ribitol

[2] sum of isolates on all media inoculated two days or three weeks after collection

[3] isolation rate was calculated as number of obtained isolates / number of inoculates

[4] viability rate was calculated as number of obtained isolates / (number of inoculates—number of contaminated inoculates)

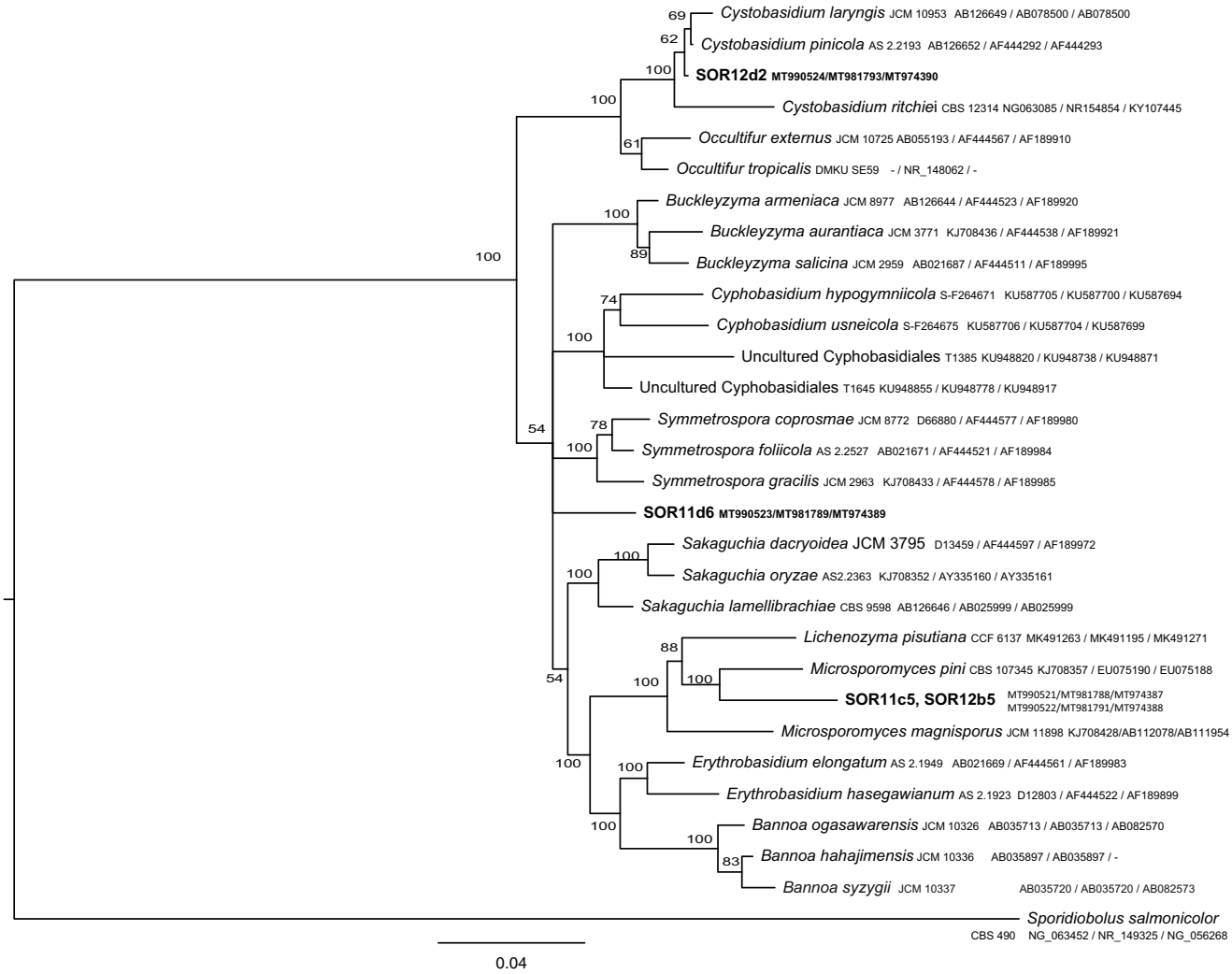

Fig. 1 Phylogeny of the *Cystobasidiomycetes* obtained by Bayesian inference of concatenated SSU, ITS and LSU rDNA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability. Newly obtained sequences are marked in bold. Scale bar represents the expected number of substitutions per site

could be observed simultaneously even within one well (Fig. 2i). Some of the soredia reached the primordium phase very quickly, the soredium field phase being very short. Others remained in the soredium field phase for a very long time and did not enter the primordium phase during the course of our experiment. This variation was observed even though all cultures were kept in the same conditions. No further development was observed on agar media even months later. After reaching the stage of primordium, no further development occurred. Although a layer of mycobiont tissue was formed on the surface it still consisted of loose hyphae with aerial hyphal strands sticking out (Fig. 3a) and thus the cultures did not form a cortical layer and no podetia or squamules were observed.

After the soredium field phase was transferred onto soil or sandstone, the mycobiont started to take over. It quickly increased the network of hyphae within the tissue (Fig. 3b) and fixed it to the substrate with hyphal strands resembling rhizines (Fig. 3c). A layer of only mycobiont was formed on the substratum, enclosing the inner photobiont layer (Fig. 3d, e). In contrast to agar media, the primordium phase was reached on all plates with natural substrata within the first month after the transfer. However, as on agar media, the cultures still lacked cortical structure in the strict sense. After each rewetting the mycobiont formed strands of hyphae protruding in all directions (Fig. 3f), thus colonizing the surrounding substratum. In some of them, formation of a white loose medullary tissue could also be observed (Fig. 3g). However, we did not observe any podetia or squamules.

4 Discussion

4.1 Isolation success

Lichen vegetative propagules, such as soredia and isidia, have been shown to be highly viable in laboratory testing

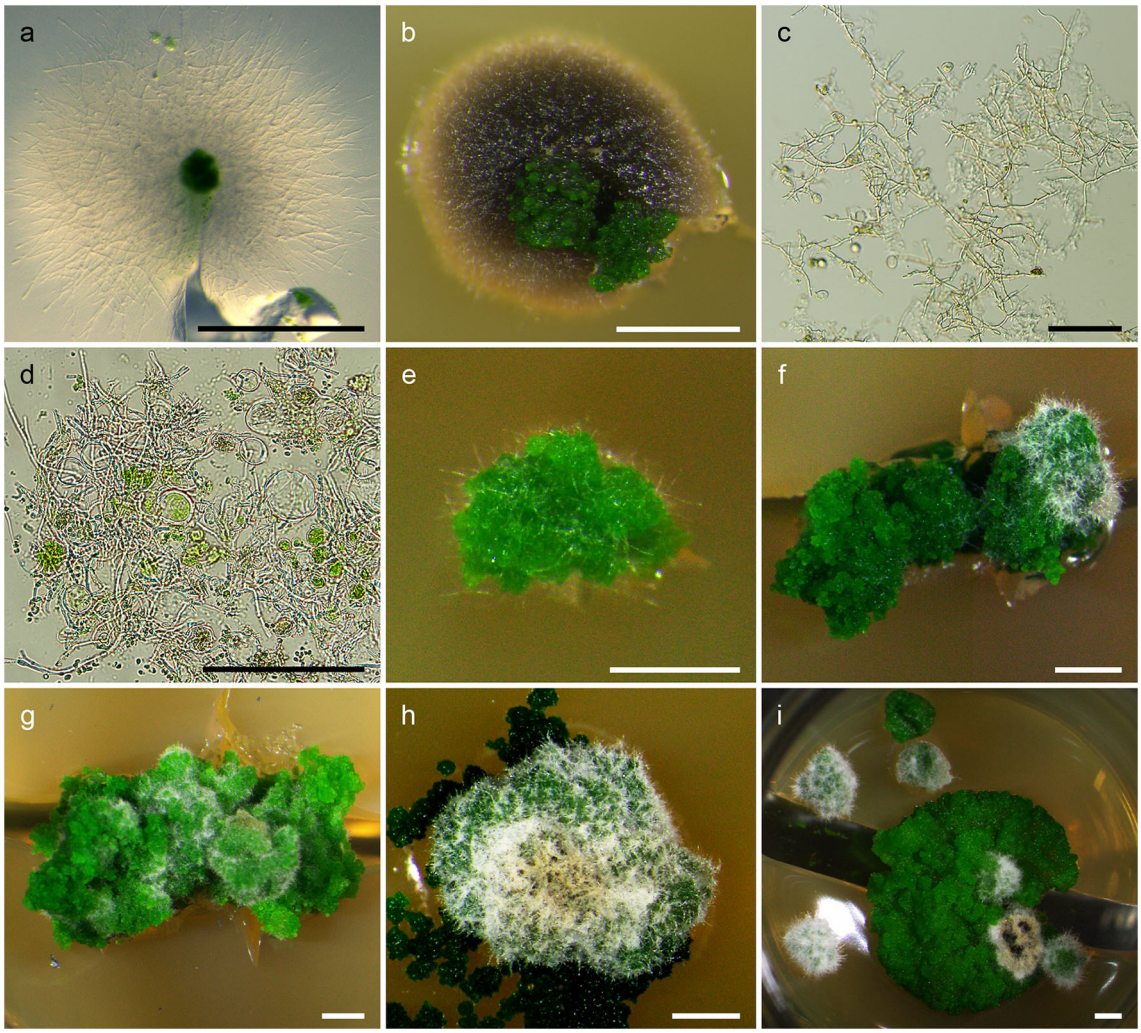

Fig. 2 Development of soredia in vitro: **a** Arachnoid stage, after one month of culturing; **b** – No interaction between the mycobiont and photobiont; **c** Frequent lateral branching with short internodes of the mycobiont hyphae; **d** Mycobiont hyphae encircling photobiont cells; **e** Soredium field, a mass of predominantly algal cells with a few interwoven fungal hyphae; **f** Soredium field, mycobiont dominates in a part; **g** Soredium field, mycobiont takes over; **h** Primordium, mycobiont forms a superficial layer enclosing the photobiont inside; **i** Different stages of soredium field and a primordium developing on a single plate. Scale bars represent 1 mm (**a**, **b**, **e**-**i**) or 100 μm (**c**, **d**)

(Buldakov 2010), resynthesis experiments (Stocker-Wörgötter and Türk 1988) and transplantation experiments (Ott 1987). Here we show that soredia are also a good source of mycobiont cultures. Mycobionts were obtained from 88% of the soredia that were not contaminated or overgrown by other fungi. From the remaining 12%, only photobionts grew. In contrast, isolation from spores and thallus fragments is, to a great extent, hindered by problems with obtaining spores discharge, inducing germination and the failure of thallus fragments to grow (Crittenden et al. 1995). In our experiments, both symbionts often grew together but their separation by subculturing was not difficult.

Contamination remains the biggest problem but we found for soredia that it is comparable to other isolation methods (Crittenden et al. 1995). Soredia culturing thus offers a straightforward and effective approach to the isolation of lichen mycobionts. Armaleo and May (2009) used soredia of *C. grayi* to obtain cultures that were the basis for genomes sizing. We believe that soredia culturing would facilitate studies, such as the recognition of signaling, secondary metabolites production, or whole genome sequencing.

4.2 Soredia development

Development of soredia in culture was comparable to the development of soredia in the natural environment (Schuster et al. 1985; Stocker-Wörgötter and Türk 1988, 1989). The developmental series appears to be universal, including (1) arachnoidal stage, (2) soredium field, (3) primordium and (4) thalline stage (Schuster 1985; Stocker-Wörgötter and Türk 1989; Stocker-Wörgötter 1991, see below).

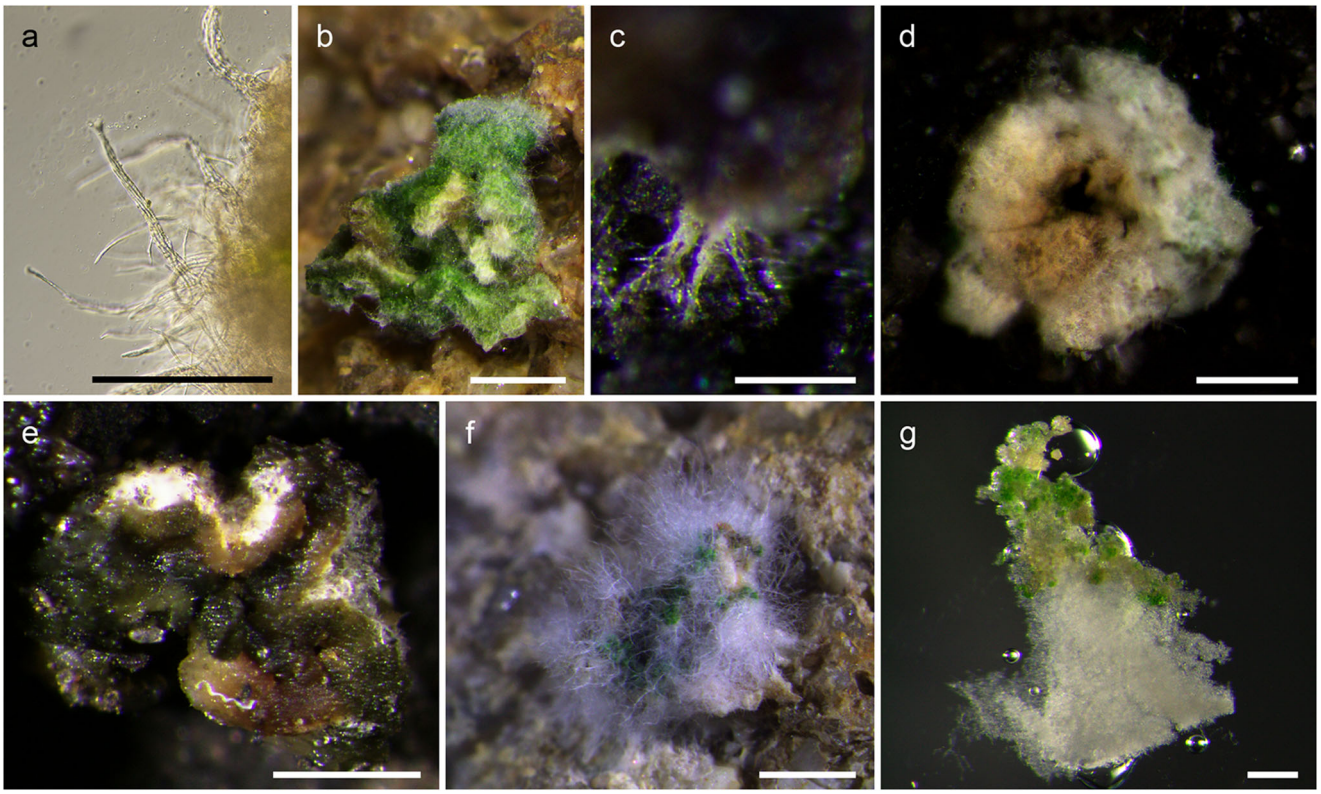

Fig. 3 Development of soredia in vitro: **a** Superficial mycobiont layer, strands of aerial hyphae stick out; **b-g** Development on soil: **b** Mycobiont quickly multiplied the network of hyphae within the tissue; **c** Rhizine-like structures fasten the developing soredium to its substrate; **d** Mycobiont layer completely enclosed the photobiont quickly, the figure shows primordium in Fig. 2h two weeks after transfer to soil; **e** Horizontal cut of primordium shown in Fig. 3d, photobiont layer enclosed by a mycobiont layer; **f** Strands of hyphae protruding in all directions after re-wetting; **g** Loose white medullary tissue formed below the mycobiont-photobiont interaction layer. Scale bars represent 1 mm (**b**, **d-f**) or 200 μm (**a**, **c**, **g**)

The soredium germinates into a loose arachnoid mycelium, the symbiotic partners thus come apart first (Fig. 2a, Stocker-Wörgötter and Türk 1988). Interestingly, isidia, which have stratified thalline structure, also disintegrate at the beginning of their development (Schuster 1985). This fact implies that after a vegetative propagule germinates, the partners need to recognize each other anew before further development. Thus the initial processes are analogous to reestablishment of the symbiosis de novo from mycobiont spores and photobiont cells (Athukorala et al. 2014). Although the molecular mechanisms of recognition are still largely unknown, it is clear that a complex pre-contact signaling is involved (Meeβen and Ott 2013). Initial steps of this signaling lead to release of specific polyols by the alga, i.e. ribitol in the case of *Asterochloris* (Richardson et al. 1968). Ribitol is not only the source of carbohydrates for the mycobiont but is probably the transformation signal that triggers lichenization (Ahmadjian 1993b; Meeβen et al. 2013). At the end of the pre-contact signaling, morphological changes in the mycobiont are induced; the hyphae grow forming numerous lateral branches with short internodes and encircle the photobiont (Fig. 2c and d; Athukorala et al. 2014; Joneson and Lutzoni 2009). However, this response has also been observed in co-cultures of certain incompatible partners (Ahmadjian and Jacobs 1981; Meeβen and Ott 2013), indicating low-specificity of the pre-contact signaling.

The next stage of the development is an undifferentiated mass of mingling symbionts that was termed a soredium field by Schuster (1985) (Fig. 2e-g). Soredium-like stages have also been reported from de-novo resyntheses (Galun and Garty 1988; 1995), even in incompatible partners (Ahmadjian et al. 1980; Galun and Garty 1988; Guzow-Krzemińska and Stocker-Wörgötter 2013). However, only under compatible combinations is the relative growth of the symbionts gradually balanced during this stage (Fig. 2f-i; Galun 1988; Stocker-Wörgötter and Türk 1989). This leads to turning the soredium field into a primordium.

A primordium (Fig. 2h and 3d) exhibits stratification; most importantly a layer of dense fungal network is formed on its surface enclosing the photobiont inside (Fig. 3e). In some cases, photobiont cells are continuously organized into a layer (Fig. 3e) and a loose white medullary tissue is formed below (Fig. 3g) from hyphae already in the primordium. In our experiments, the superficial mycobiont-only layer has a cottony appearance with long strands of aerial hyphae (Fig. 3a, also in Stocker-Wörgötter and Türk 1988). Although this layer is, for

sure, the basis for the cortex, we think it cannot be termed as such (*cf* Stocker-Wörgötter and Türk 1988.) until it develops the typical cortical tissue structure composed of tightly adhering hyphae (Büdel and Scheidegger 2008). Thus, the primordia in our experiments did not develop the cortex sensu stricto. On contrary, after each rewetting the superficial aerial hyphae expanded, enlarging the primordium and colonizing more substratum (Fig. 3f).

In contrast to previous studies (Ahmadjian 1966; Stocker-Wörgötter and Türk 1988; Stocker-Wörgötter 1995) no further structures, anatomical (cortex) or morphological (squamules or podetia), developed in our experiments, so the thalline stage was not reached. Obviously, the development of these structures, as well as reproductive structures, is not a question of compatibility but of environmental factors. The use of soil substrata and alternation of wetting and drying cycles are considered crucial in this aspect (Ahmadjian 1966; Jahns 1993; Stocker-Wörgötter 1995; Zorer et al. 1997). Both were tried in our experiments but still the development did not proceed. Thus, the conditions for further development remain poorly understood and might involve environmental stresses other than drying, for example night temperature drops, or air movement as a mechanical stimulus, could be important.

In conclusion, we have shown that the development of soredia of *C. fimbriata* follows the same pattern as described previously (Stocker-Wörgötter 1995), also for other species, e.g. *Peltigera didactyla*, *Hypogymnia physodes* and *Physcia tenella* (Schuster et al. 1985; Stocker-Wörgötter and Türk 1988, 1989). The same developmental stages as described here for soredia were observed in de-novo lichen resynthesis from spores (see Zorer et al. 1997 for *C. fimbriata* in vitro and Galun and Garty 1988 for *Xanthoria parietina* in situ). Thus, our observations can serve as a reference-frame for studies of compatibility of the mycobiont with diverse photobionts. Compatibility of the partners is not disproved by the lack of formation of advanced morphological structures, as long as the primordium stage is formed.

4.3 Soredia-associated fungi

Considering the limited number of isolates obtained we do not mean to give an exhaustive list of associated fungi but rather to look at the ecological groups they represent and indicate ecological consequences of such associations.

There are two possible causes of the association of other fungi with lichen soredia. First, they might be spores, conidia or other diaspores originating from fungi present in the surroundings of the lichen. This is probably the case of SOR12d3 matching *Lachnellula pulverulenta*, which forms fruit bodies on pine needles. Second, they might be derived from the interior of the lichen thallus. The hyphae that form soredia are of medullary origin (Darbishire 1927; Lallemant 1972). During the morphogenesis some of the numerous symptomless fungi present within the lichen thallus (e.g. Petrini et al. 1990; U'Ren et al. 2010; Honegger 2012) might be accidentally incorporated. The functional relationship of most of the endothallic fungi to their host is not known (U'Ren et al. 2010; Chagnon et al. 2016). Some are symptomless stages of strictly lichen-associated (lichenicolous) fungi (Oberwinkler 2017; Tuovinen et al. 2019) or other fungi with multiple ecological niches (Honegger 2012; Selosse et al. 2018). Others may be just inactive diaspores accidentally trapped within the thallus (Hawksworth and Grube 2020). For the latter two groups, vegetative propagules may be the only means of leaving the thallus and proceeding with their life cycle.

Majority of the fungi we found in association with soredia can be divided into three categories; firstly, fungi previously isolated from pine trees (SOR8d2, *Cystobasidium pinicola* SOR12d2 and *Pseudocamaropycnis pini* SOR13b1 known from pine sapwood, xylem and needles, respectively), secondly, fungi previously found in the soil (SOR8a2, SOR11b6, SOR12b1, SOR12c3) and thirdly, fungi that did not match any sequences deposited in GenBank (SOR7c1, SOR11c5, SOR11d6). Considering the current stage of knowledge, it is impossible to say for the former two groups whether they come from the surroundings or the interior of the thalli. The last group may be strictly lichen-associated, but we should avoid drawing conclusions before more is known about them.

Spribille et al. (2016) introduced cystobasidiomycetous yeasts as close and specific associates of lichens, even claiming them obligatory constituents of the lichen cortex. While mainly yeasts of Cyphobasidiales were found in Parmeliaceae lichens (Spribille et al. 2016) Microsporomycetaceae and another, yet undescribed, family-level lineage of Cystobasidiomycetes were found in a wide range of *Cladonia* species (Černajová and Škaloud 2019). This specificity was opposed by Mark et al. (2020) who found certain genotypes of Cystobasidiomycetes in several lichens and also single lichen species in association with various lineages of the yeast. They also anticipated yeast multiplicity in a thallus due to frequent mixed signal in Sanger sequencing chromatograms. In the present study we confirm this hypothesis; isolates of Cystobasidiomycetes yeast belonging to three distantly related lineages (Fig. 1) were obtained from a single thallus. This multiplicity indeed implies very limited specificity of the lichen-yeast association. It also suggests that lichens are commonly inhabited by diverse cystobasidiomycetous yeasts, contrarily to Lendemer et al. (2019) who found no evidence for such ubiquity. However, it is worth remembering that these yeast-like fungi occur on other hosts where they thrive on released carbohydrates and nutrients from tree leaves (Richardson et al. 1985; Richardson and Dowding 1988), and in the lichen context could be using carbohydrates released by dried lichens upon rewetting.

Regardless of their link to the host, whether they come from the surroundings and are attached at the surface, or come from inside of the lichen thallus and are incorporated within the soredium, it is apparent that other fungi are capable of co-dispersal with lichen soredia. Vertical transmission of endophytic fungi is also known in plants, for example grasses and forbs (White et al. 1993; Hodgson et al. 2014). It has also been shown that whole communities of bacteria are dispersed with lichen vegetative propagules (Aschenbrenner et al. 2014). At present, we can only speculate about consequences of such co-dispersal. Aschenbrenner et al. (2014) showed that bacterial communities on isidioid soredia of *Lobaria pulmonaria* are similar in composition to those on the original thalli and suggested that the newly developing thallus thus does not depend on de novo recruitment of bacteria, which may provide many essential functions to the lichen holobiont (Grube et al. 2015; Cernava et al. 2017). On the other hand, it has been suggested that lichen thalli host plant pathogens, including viruses and bacteria (Petrzik et al. 2014; Vilhelmsson et al. 2016). The vegetative propagules of lichens could be the way of their transmission. Here, we present evidence that other fungi are co-dispersed with lichen diaspores. The extent to which this co-dispersal influences life histories of both the lichen and the fungus remains to be explored.

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