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Joint Dispersal Does Not Imply Maintenance of Partnerships in Lichen Symbioses

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Abstract Dispersal of symbiotic partners by joint propagules is considered as an efficient strategy to maintain successful associations and to circumvent low symbiont availability. Joint dispersal is widespread in diverse symbioses and a particularly common reproductive mode in lichens. We were interested in the implications of joint symbiont dispersal on population genetic structure and investigated patterns of symbiont association in populations of two closely related lichen species in the genus *Physconia*, with similar range of compatible algal partners. One of the lichen species is characterized by joint dispersal of both symbionts, whereas the other species propagates by meiotic fungal spores alone. The latter species must re-establish the symbiotic stage with appropriate algae sampled from the environment. Both fungal species have specialized on photobionts representing a monophyletic lineage of the algal genus *Trebouxia*. The results indicate no correlated association of symbiont genotypes in the species with joint symbiont dispersal. We rather show that algal gene diversity in populations of lichenized fungi with different propagation strategies is not necessarily different. The association with algae that differ from the co-dispersed genotypes during the vegetative development of the thalli is the most likely explanation for the observed pattern. Maintenance of symbiotic associations is an option but not a strict consequence of joint symbiont dispersal in lichens.

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Introduction

In many symbioses, the partners propagate independently, and the symbiotic life-cycle starts with de novo establishment of a symbiosis, after the independently distributed partners have encountered and recognized each other. The timely association with an appropriate partner species is therefore essential for establishing successful symbiotic phenotypes. Thus, the degree of specialization is limited by the availability of suitable partners in the environment. Problems of low partner availability are avoided by those symbioses which evolved mechanisms to disperse symbiotic partners jointly. Such partnerships can rapidly establish symbiotic phenotypes with the co-dispersed partner, which can be particularly advantageous for rapid colonization of newly available habitats. The transmission from parent to offspring, which captures an effective symbiont lineage, could further lead to co-evolution [13]. On the other hand, the joint dispersal of ecologically specialized partners can constrain the establishment of symbioses to narrow ecological niches, or high specialization in an ecosystem. Joint propagation of symbiotic partners is known from diverse symbioses, including bivalve-bacteria, ant-fungi, sponge-bacteria, and insect-bacteria associations (e.g., [25, 35, 38, 45]).

Lichens are the algal-associated life strategy of c. 18500 fungi. They represent symbioses that are at least obligate for reproduction of the fungal partner. General patterns of photobiont association were summarized for major evo-

lutionary radiations of lichens [29, 39, 47]. Meanwhile, also the knowledge about partner associations in selected species of lichens has substantially increased, predominantly with algae placed in the class Trebouxiophyceae that includes widespread and common aerial/terrestrial algae (e.g., [4, 5, 11, 22, 30–32, 36, 37, 50]). Recent research has found varying degrees of specificity for algal symbionts by lichen mycobionts. Some lichen mycobiont species can associate with a wide range of *Trebouxia* species. These less specific associations are known from lichens which also have a large ecological amplitude [6]. Other species are highly specific and accept only partners of a single algal lineage in *Trebouxia* [20].

Until recently, it was repeatedly put forward that species of the most common lichen-forming algal genus *Trebouxia* do not occur in free-living stages [1]. As a consequence of this view, fungal spores need to capture algae from either pre-established lichens or from lichen propagules that contain both symbionts. Free-living trebouxioid lichen algae are perhaps inconspicuous because macroscopically observable colonies are not developed. Instead, they are likely short-living in form of single cells or arranged in few-celled aggregates and present in mixed algal consortia. Initial colonization by lichens of abandoned glass pieces in Antarctica suggest that mycobionts acquired their photobionts from free-living stages [46]. Further, free-living lichen algae were detected on marble monuments using culture-independent molecular approaches [9]. Nevertheless, available lichen-forming *Trebouxia* species seem to be scattered and locally rare. To account for this situation, many lichens developed means for joint dispersal of symbionts.

Lichenized algae can be distributed together with their fungal partners in mitotically produced organs, either produced directly on the vegetative thallus or in specialized regions thereof. Asexual propagules come in diverse types that can be morphologically distinguished. Types with a stratified anatomical structure resembling that of the mature thallus are called isidia. Others are called soredia and are more simply organized. They consist of few algal cells and hyphal elements. Some lichen mycobiont lineages, such as the sorediate *Lepraria*, have given up the production of meiotic fungal spores and still evolved a surprising diversity of asexual species that entirely rely on joint mitotic symbiont transmission. Other lineages have closely related sexual and asexual species, of which the latter reproduce solely by joint transmission of symbionts. Provided that these species share direct common ancestry, they are termed species pairs in lichenology ([14]; for a somewhat different meaning of this term in ichthyology, see [46]). Closely related sexual and asexual species, in particular, species pairs with complex morphology, often differ in their distributional ranges. The usually wider distribution of the sterile species suggests the colonizing success with joint symbiont transmission.

Joint symbiont transmission, on the other hand, should have clear consequences on the population genetic structure of symbiotic populations. We hypothesized that the sterile species should display a more distinct linkage of symbionts than the sexual species, which rather may randomly associate with locally available compatible photobiont strains to form symbiotic associations de novo. Consequently, algal diversity should be higher in populations of the sexual lichen species. To test this hypothesis, we studied symbiont association patterns in populations of two closely related lichen species with similar degree of specificity to algal lineages but differ in their propagation strategies. One species is reproducing with fungal ascospores, whereas the other species propagates both symbionts jointly by production of mitotic soredia. Since there are cases of doubtful taxonomic ranking of sterile and fertile morphs as species pairs (e.g., [26]), we selected the following species. The widely distributed bark-inhabiting foliose lichens *Physconia distorta* and *Physconia grisea* are closely related but distinct members of the family Physciaceae according to morphology and phylogenetic data [10]. *P. distorta* is fertile and propagates via ascospores (Fig. 1a, b), whereas *P. grisea* distributes both symbionts jointly in soredia (Fig. 1c, d; only very rarely ascomata were observed).

Materials and Methods

For this paper, sampling focused on the Italian peninsula (Fig. 2). A population was regarded as a number of co-occurring thalli of a lichen species in a given area of approximately 100×100 m. In each sampling plot, consisting of one or more neighboring trees, seven to 15 thalli were collected. Prior to DNA extraction, the lichens were checked for visible contaminations by other fungi. Species were determined according to standard references [49]. Special care was taken to avoid confusion with the recently introduced, closely related *Physconia thorstenii* [12]. A total DNA extraction of lichens, including fungal as well as algal DNA, followed a modified cetyltrimethylammonium bromide method [17]. For polymerase chain reaction (PCR) amplification using an ABI 2700 Cycler (Applera, Vienna), we used the fungal-specific primer pair ITS1F [16] and ITS4 [48] and, in a separate amplification, the algal-specific primers IT1T and ITS4T [24]. After initial denaturation at 95°C for 3 min, six touchdown cycles with annealing temperatures decreasing from 54°C for fungal template (56°C for algal template) to 48°C for fungal template (50°C for algal template) were carried out, followed by 35 cycles (94°C/30 s, 48°C/30 s, and 72°C/155 s) and terminating after a final elongation at 72°C for 7 min.

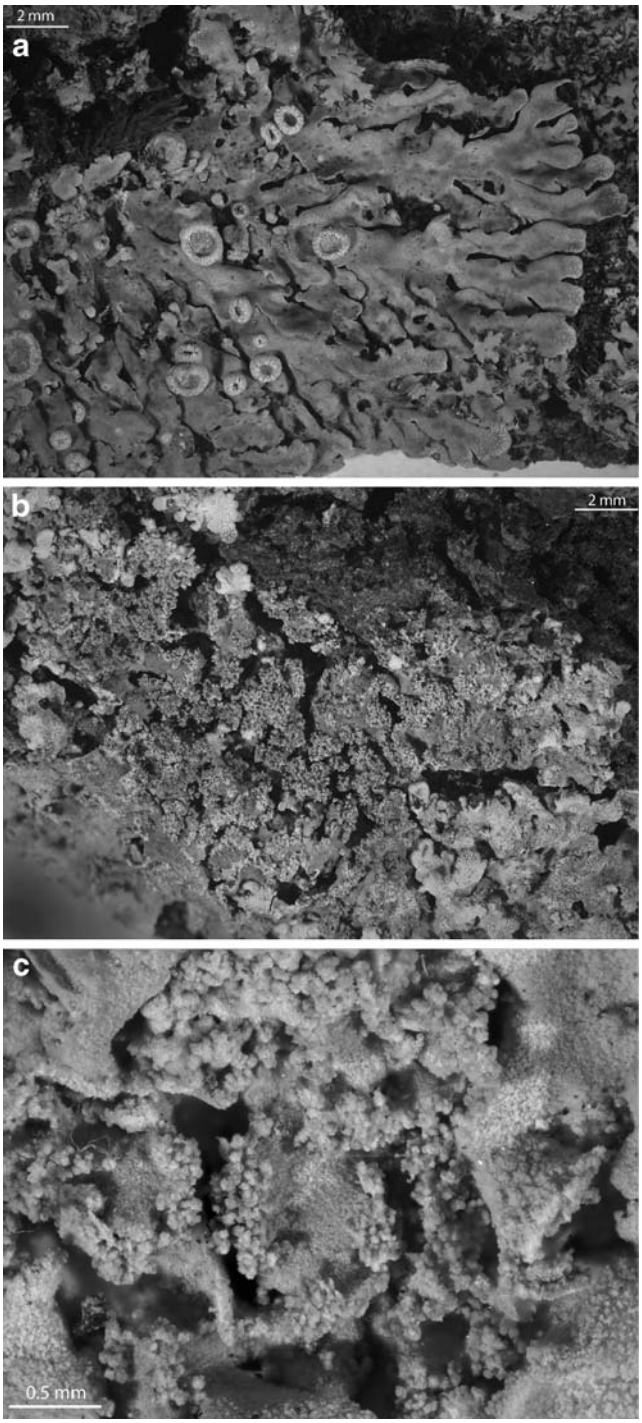

Figure 1 The studied species: (**a**) habit of *Physconia distorta*, (**b**) habit of *Physconia grisea*, and (**c**) soredia of *P. grisea*

Fifty microliters of PCR cocktail for the amplifications of the fungal internal transcribed spacer (ITS) region consisted of 5 µl/10× PCR Buffer (Qiagen, Vienna), 1.25 units Taq DNA polymerase (Qiagen, Vienna),10 µl/5× Q Solution (Qiagen, Vienna), 0.2 mM of each of the four dNTPs, 0.5 µl of each primer, and ca. 10–50 ng DNA. For the amplifications of the algal ITS region, we used the same amount of Taq DNA polymerase from another supplier (Amersham Pharmacia Biotech Inc.) and appropriate buffer conditions. Products were cleaned using the QIAquick PCR Purification Kit (Qiagen, Vienna). Both complementary strands were sequenced using the ABI Big Dye Terminator kit (Applera, Vienna). Sequencing reactions were separated on an ABI 3730xl sequencer and assembled using AutoAssembler (Applera, Vienna). Sequencing of the fungal ITS region was done using the primer pair ITS1-LM [28] and ITS2-KL [26]. The sequences were initially aligned using the Clustal algorithm as implemented in BioEdit [19] and optimized by eye. Gene diversities were calculated with Arlequin 3.01 [15].

The phylogenetic hypothesis was established using a Bayesian approach as implemented in the program MrBayes 3.1.2 [23, 41]. The General Time Reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories (GTR+I+G) was used for likelihood calculations. The optimal nucleotide substitution model was found before with the program MrModeltest 3.7 (written by J.A.A. Nylander and available at http://morphobank.ebc.uu.se/mrbayes/) using the Akaike information criterion. For other parameters, the default settings were used. The Markov Chain Monte Carlo algorithm was run for two million generations, with six

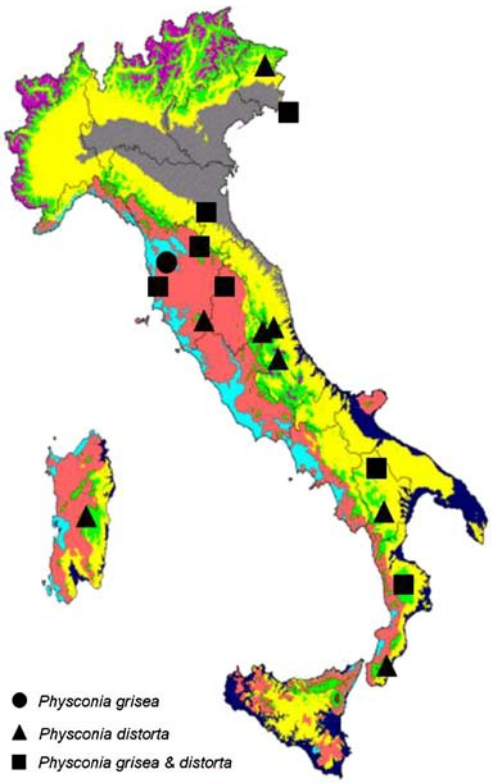

Figure 2 Map of Italy showing the distribution of sampling sites for this study (some of the symbols may indicate two adjacent populations of the same species)

chains starting from a random tree and using the default temperature of 0.2. Every tenth trees were sampled and the first 1,000 trees were discarded as burn-in. The consensus phylograms based on the mean branch lengths were calculated with the command *sumt* in MrBayes (see MrBayes 3.1 manual, [41]). The phylogenetic trees were drawn with the program TreeView [34].

Results

Thirty-nine populations were investigated for this study (27 of *P. distorta* and 12 of *P. grisea*). From seven to 15 thalli of the respective species from each population, we sequenced both mycobiont and photobiont ITS regions. A total of 1,066 new sequences were generated (539 of the photobiont and 527 of the mycobiont ITS). Representative sequences have been submitted to Genbank under the numbers EU795052–EU795082. The majority-rule consensus tree of the algal ITS dataset (Fig. 3) was calculated from 398,000 trees. The likelihood parameters in the sample had the following average values (variance) for the ITS partition: rate matrix r(GT) = 0.067 (±0), r(CT) = 0.417 (±0.002), r(CG) = 0.042 (±0), r(AT) = 0.162 (±0), r(AG) = 0.204 (±0.001), r(AC) = 0.105 (±0), gamma shape parameter α=0.206 (±0.001).

First, we analyzed the diversity of photobionts in both species to assess whether the species share symbiont

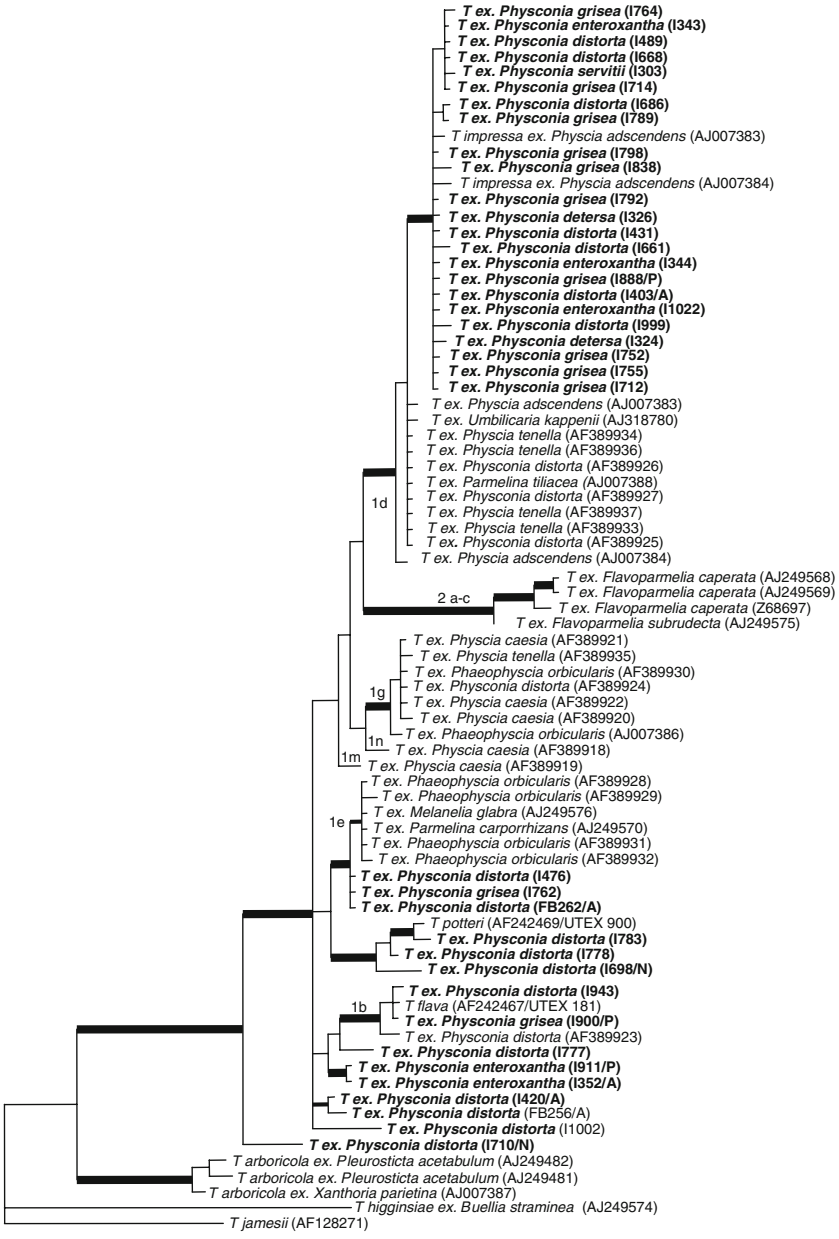

Figure 3 Phylogenetic tree of *Physconia* photobionts. Bayesian phylogenetic analyses. Posterior probabilities equal or more than 95% are indicated by thickened branches

specificity. Photobionts of *Physconia* belong to a monophyletic group, to which the described species *Trebouxia impressa*, *Trebouxia flava*, and *Trebouxia potteri* belong as well (Fig. 3). Within this complex, specific lineages are selected, i.e., Clade I1, as outlined by Helms [21].

The same photobiont genotypes were found in both lichen species, indicating that there is not a species-specific pattern. Moreover, there was no evidence for the preference of certain genotypes by either species. We also did not find differences in photobiont genotypes among northern and southern populations along our gradient.

The diversity of photobiont genotypes was variable in the populations under study. Algal gene diversity was highest in populations of *P. grisea* from Friuli-Venezia-Giulia, Italy (0.868), and in populations of *P. distorta* from Calabria, Italy (0.944). Moreover, we found that the photobiont genotypic variation within populations varies considerably.

Two geographically separate populations of the sterile *P. grisea* showed no variation in fungal genotypes (contrasting a diversity of algal genotypes of 0.571 and 0.868, respectively).

In comparison with photobiont gene diversity in populations of *P. distorta*, photobiont gene diversity was not significantly lower in *P. grisea*. The situation is clearly different when we compare the diversity of fungal genotypes in populations of these two species. Gene diversity in *P. grisea* was less than half the estimate for the sexual *P. distorta*. If there was a strict maintenance of partnerships after joint dispersal with the photobionts, the drop of gene diversity should be similar in mycobiont and photobiont. Despite the lower fungal diversity, *P. grisea* associates with almost as many photobiont genotypes as the fertile *P. distorta*. In *P. grisea*, we found two populations without variation in the mycobiont, but either population comprised more than one photobiont genotype.

Discussion

The clade to which the *Physconia* photobionts belong comprises photobionts of diverse lichens of which different major groups belong (including photobionts of temperate species in other genera of Physciaceae such as *Heterodermia*, *Physcia*, and *Rinodina*).The photobiont genotypes can have extremely wide geographic ranges from Germany to Finland and Spain. Other species of the I-clade [21], such as *Trebouxia anticipata* and *Trebouxia gelatinosa* were not found as photobionts of *Physconia*. No apparent preferences of either *P. grisea* or *P. distorta* are observed within this clade.

A geographic component has been detected in photobiont selectivity of other lichens, though [6]. However, a shift in the frequencies of certain genotypes towards the warmer southern part of our gradient was not apparent. Temperate *Physconia* species share a rather narrow range of suitable photobionts, agreeing well with an overall trend of higher photobiont selectivity found in foliose and shrubby lichen species (e.g., [24, 32], but see an exception in *Umbilicaria* species from maritime Antarctica [40]). Especially when they exhibit wide ecological amplitudes, crustose lichens are less restricted in their choice of *Trebouxia* species ([6, 18], unpublished data). With narrower ecological demands, higher specificity is also known from crustose lichens (e.g., [20]), and at the edges of their ecological amplitude, lichens may be more restricted to particular photobionts (Beck, personal communication).

Our study shows considerable diversity of photobiont genotypes in populations of both studied lichens, and no obvious differences between the two lichens with different propagation strategies can be found. Individual populations may differ significantly in their photobiont gene diversity, but we did not observe a clear correlation with ecological conditions. A significant difference occurred in the gene diversities of the mycobionts of sterile and fertile species (Fig. 4). A lower mycobiont gene diversity in the asexual species compared to the sexual counterpart was observed in the species pair of *Cavernularia* (Printzen, personal communication). The original hypothesis that the sorediate species shows a more pronounced “coupling” of genotypes than the apotheciate species can be rejected. If this hypothesis would be valid, we would have observed a significant decrease of photobiont diversity in populations of the sterile lichen, similar to the decrease in mycobiont gene diversity. This is clearly not the case in our study, and the propagation strategy had no significant effect on the photobiont diversity. Possibly, rare sexuality in the sterile

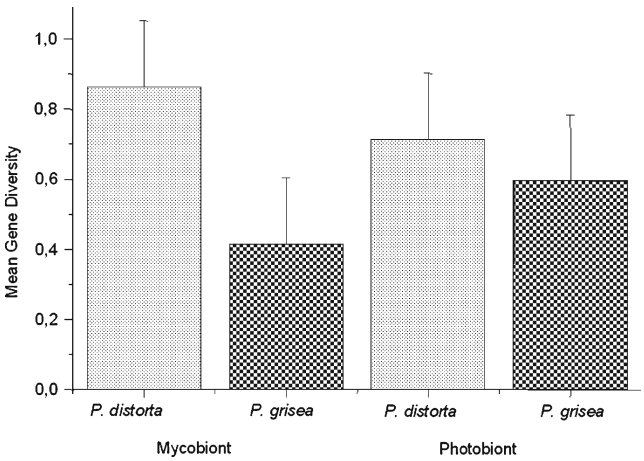

Figure 4 Comparison of mycobiont and photobiont diversity in populations of the two analyzed species (n=27 for *Physconia distorta*, n=11 for *Physconia grisea*)

species could have some influence on their photobiont diversity, but this could not explain the high variation found. We found two separate populations of *P. grisea* that showed no variation of the mycobiont, which probably distributed locally and is associated with six to eight algal haplotypes, respectively.

Either the photobiont in the mitotically produced joint dispersal unit is not the same as the one present in the subsequently establishing lichen thallus or the soredia may acquire different algal strains during their development on the parental thallus. Microscopic investigation rejects the latter hypothesis. We have not found any evidence that free-living algae attach to the developing soredia, which are tightly encaged by branching, hydrophobic hyphae. We also did not observe epithalline algae on other parts of the investigated lichen thalli (data not shown). On the other hand, we found rather dense occurrence of free-living algae among bark attached initial thalli and landed soredia in the vicinity of lichen thalli by calcofluor white staining and observation of bark substrates with an epifluorescence stereo microscope (Grube, unpublished data). It could also be argued that there is intrathalline variation of photobionts in a single thallus of lichens, but we would have had problems with direct sequencing if variation would be comparable to variation in the population. Even with soredia included, sequence data were unambiguous. This does not exclude occasional inhomogeneity in a thallus, but this kind of variation is differently patterned. In preliminary experiments, we found that regenerative outgrowths in the centers of large thalli can occasionally contain different algae in several, anatomically similar lichens. However, the algal partner is uniform within these outgrowths, in juvenile thalli, and in the growing margins. Thus, switching of symbiotic partners is possible by rejuvenation of ageing parts (Grube et al., preliminary results). For this reason, we always sampled young thalli or from the growing periphery thalli and confirmed uniqueness of algal strains in occasionally replicated extracts. The occasional photobiont switches during rejuvenating of thallus centers could actually promote longevity of the individual mycobionts.

We conclude that the algae in the dispersed soredia are not necessarily the same as in the developing lichens, which result from soredial propagation. In contrast to those in soralia, soredia on bark have a looser structure. In the latter, hyphae can branch out to contact either the bark substrate or neighboring algae. It may well be that algae in sheltered microsites of the bark are more vital than the few photobiont cells which were poorly protected while they joined the mycobiont during dispersal. We think that it is most likely that new photobiont-mycobiont combinations occur at this stage of development. We suggest that the main role of the photobiont in soredia is to prolong the survival of the co-propagated fungal hyphae. Depending on the viability of the soredial algae, the soredial fungus can choose between establishing a thallus with the rather few co-propagated alga or with adjacent, and possibly more vital free-living algae. We assume that the initial phase of thallus formation is most sensitive to local conditions and may sometimes vary at the scale of micrometers. Our hypothesis does not exclude the possibility of maintenance of lichen associations by joint distribution via soredia, but we suggest that symbiont switches are likely when fungi grow out from attached soredia. It remains to be addressed by future studies, whether photobiont switching may also be observed in lichens with more complex joint propagules such as isidia or phyllidia. In these types, stratification with an external fungal layer protects the co-propagated algae more efficiently against various environmental stress factors during phases of transport and initial establishment.

It has earlier been noticed [33] that lichen mycobionts may associate in nature loosely with other photobionts, until they find the appropriate alga to form a typical thallus. Whether such locally optimal algae are part of a pre-existing lichen symbiosis or represent free-living algae is still a matter of debate in lichens as no structures are developed for propagation of algal partners alone. Evidence for the existence of free-living *Trebouxia* increases [9, 43, 44], but they apparently do not form large colonies and are likely ephemeric. One source of free-living algae could be lichens which experience a shift to increased humidity during their life-time, e.g., by a closing vegetation in forests. Under such situations, algal colonies can grow out from the lichen thalli (Grube, unpublished observations). However, the dispersal of such algal colonies has yet to be studied in detail. Another source for algae is fecal pellets of orobatid mites, which frequently graze on lichens. Their droppings were shown to contain viable *Trebouxia* cells [27].

The mechanisms behind photobiont specificity are still poorly understood. Initial studies revealed a role of phycobiont-binding proteins [7, 8, 42], and likely, other factors are also involved. In any case, in vitro re-association experiments indicated that mycobionts can only form characteristic thalli with specific algae, whereas other associations resulted in undifferentiated associations or sorediate thalli [2, 3]. Formation of proper thalli can also be delayed, if the algae are related to the optimal lineages. Optional maintenance of jointly distributed symbionts likely increases the reproductive success of sterile lichens under a wider range of microclimatic conditions and prevents strict patterns of co-evolution. This aspect may be of significance also in other lichen lineages, such as *Lepraria*, where mycobiont species have diverged chemically and ecologically even in the absence of sexuality and with uniform co-dispersal by soredia [30].

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