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Non-developing ascospores in apothecia of asexually reproducing lichen-forming fungi

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Summary. The presence of apothecia in mixed species (vegetatively reproducing lichens, occasionally producing ascomata) has been interpreted as a mechanism to increase genetic variability in mostly clonal populations. However, spore viability from these apothecia has not been studied. We asked whether ascospores of the mixed species *Physconia grisea* are viable and thereby contribute to increasing the genetic diversity within populations of this species. An ontogenetic study of spores in cultures of *P. grisea* and a related sexual species (*P. distorta*), showed that although mature apothecia from both species produced and discharged meiospores capable of germination, spores from *P. grisea* were only rarely (0.43 %) able to continue development whereas those from *P. distorta* germinated and developed successfully. The strongly reduced viability of *P. grisea* spores suggested that they do not have a strong reproductive function, at least in the two local populations analyzed. Additionally, we show that the segregation of *Physconia grisea* ssp. *lilacina* does not have molecular support. [**Int Microbiol** 2013; 16(3):145-155]

Keywords: *Physconia* spp. · apothecia · sexual reproduction · germination · ontogenetic development · mixed species

Introduction

Lichen fungi form obligate symbiotic relationships with their photobionts, either a green alga and/or a cyanobacterium. The largest number of lichenized fungi is found among the Ascomycota, in which over 40 % of the known species live in symbiosis with algae as lichens [26]. Lichen-forming fungi reproduce by sexual (e.g., apothecia, perithecia, or mazaediate ascomata) or asexual (vegetative) reproduction and develop the respective structures. When apothecia are sexually mature, meiospores are propelled upward from the asci and dispersed through several mechanisms, such as wind, water, and birds [2,41]. Sexual reproduction carries a high risk of failure since after spore germination the fungi, an obligate partner in the symbiosis, might not be able to find a compatible photobiont, thereby failing to re-lichenize. Printzen and Ekman [49] have concluded that dispersal by ascospores over long distances is

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rather ineffective in the fertile lichen *Cladonia subcervicornis* (Cladoniaceae). Lichens can potentially disperse vegetatively by symbiotic thallus fragments that have broken off [8]. Although these fragments may not necessarily regenerate a complete thallus, some lichens, such as *Thamnolia subuliformis*, use this mechanism exclusively [9]. Lichens can also spread through vegetative diaspores containing algal and fungal somatic cells (e.g., isidia and soredia) or with pycnidia, asexual organs that produce mitospores (conidia). Isidia are elongated, corticated outgrowths from the thallus surface that detach for mechanical dispersal. Soredia are powdery areas on the upper surface of the thallus that contain small groups of algal cells surrounded by fungal filaments. Thallus fragmentations, isidia, and soredia reduce the risk of reproductive failure since the two symbionts are dispersed together. However, the viability of vegetative diaspores after dispersion can be affected by environmental conditions, such as pollutants [21]. When the reproductive mode is vegetative, lichen species usually present either isidia or soredia, very rarely both at once. In a recent molecular study of a population of *Pseudoevernia furfuracea* (Parmeliaceae), a species that typically uses isidia as its dispersal mechanism, Ferencova et al. [18] have concluded that specimens bearing isidia and soredia at the same time should not be considered as separate species, but as a morphological variants within a polymorphic species that is simultaneously capable of executing different reproductive strategies.

It was long thought that closely related species in many groups of lichenized fungi might differ in their reproductive modes. In these "species pairs" there is a "primary" or "mother" taxon that only reproduces sexually, and a "derived" or "secondary" taxon that only reproduces vegetatively [46,55]. Sometimes, vegetative species develop apothecia together with asexual reproduction structures; in such cases, the terms "mixed reproduction" or "mixed taxa" have been used. However, molecular phylogenetic studies of species pairs have suggested that in many cases neither the sexual nor the vegetative taxa (species) form monophyletic sister clades. Rather, phylogenetic trees usually show individuals from sexual and asexual taxa to be intermixed and neither taxon forms monophyletic clades (see reviews by Crespo and Pérez-Ortega [12], Lumbsch and Leavitt [32]). Accordingly, Tehler et al. [56] have suggested that the species pair concept should be abandoned, evolutionarily and taxonomically. However, the term "mixed species" is still used to refer to populations that mostly undergo asexual reproduction but rarely bear apothecia.

The various hypotheses on the evolution of reproductive strategies and the evidence supporting them have been contradictory. While some scientists have demonstrated that sorediate specimens can arise independently from sexual lineages at different geographical locations [30,42], others have shown the development of apotheciate (sexual) clades from rarely sexually reproducing, mostly isidiate morphotypes, i.e., mixed species [11]. Hestmark et al. [22] suggested that independent reversal to sexual reproduction from asexual stages could explain the evolution of two types of apothecia from a vegetatively reproducing lineage, which implies that the genes for sexual reproduction do not become permanently lost but only temporarily silenced. Tehler et al. [56], in their molecular phylogenetic study of *Roccella* from the Galapagos Islands, have emphasized that either a sorediate or a sexual ancestor of the *Roccella galapagoensis* aggregata species complex (Roccellaceae) can be postulated. In that particular case, either evolutionary direction, from sexual to asexual reproduction or vice versa, seems equally plausible.

Lichen-forming fungi rarely invest in both sexual and asexual reproduction on the same thallus, i.e., within the same individual, which suggests that such an energetic investment is too costly to maintain under conditions of limited resources [5]. It is unclear why certain lichen species produce ascomata infrequently. In these mixed taxa, heterothallism (obligate cross-fertilization), lack of mating partners, mutations in genes involved in gamete, gametangia or ascomata formation, and environmental factors have been invoked [23] but none has been proven. Martínez et al. [34] reported that in *Lobaria pulmonaria*, a lichen with a mixed reproductive strategy, the largest individuals are more likely to develop reproductive structures, initially carrying out asexual reproduction that later is replaced by sexual reproduction.

Those authors have also found indications that macro- and microclimatic variables influence the type of reproductive structures that are formed. Lawrey [28] correlates the mixed reproductive mode in lichens with temperate ecosystems, which climate-wise are seasonally more variable and unpredictable than tropical climates. It is possible that mixed taxa can "turn on" their sexual reproductive strategy as a response to environmental stress and high selection pressure (limited resources, desiccation, low relative humidity, high solar radiation, low diversity of compatible photobionts, forest fragmentation, air pollution, etc.), as an alternative means to reproduce. Populations that had some degree of sexual reproduction in the past might have been favored, through natural selection, during stressful events, since sexual reproduction increases genetic variability and therefore also the likelihood of adaptation and survival in new environments. By contrast, if conditions are stable then asexual reproduction allows population maintenance with fewer resources such that these

populations would be favored by natural selection [5]. Buschbom and Mueller [6] have also suggested that changes in the reproductive strategy of lichens are governed by trade-offs in the success of the symbiosis, with the two modes of reproduction alternating based on selective pressures. These hypotheses presume that occasional apothecia from mixed species are functional and generate viable meiotic spores. However, until now, there has been no effort to determine the viability of spores from such rare apothecia in mixed species. As far as we know, ours is the first study evaluating this hypothesis.

The mixed species *Physconia grisea* has sorediate margins and simple white rhizines on the lower thallus side and only rarely does it produce apothecia (although when the apothecia are present, they can be abundant) [45]. It occurs on the nutrient-rich or eutrophicated bark of deciduous trees, often along roads, and on calcareous as well as eutrophicated non-calcareous stones. Its distribution is restricted to Europe, the Atlas Mountains of northern Africa, and the Near East [45]. Some authors have proposed that the vegetative-only morphotype and the rare apothecia morphotype in *P. grisea* populations should be distinguished as different subspecies (*P. grisea* ssp. *lilacina*, Poelt 1966), i.e., forming a subspecies pair, although this subspecies segregation has yet to be tested at the molecular level. Other authors (e.g., Lawrey [28]) have interpreted such morphotypes as morphological variations within a single species with mixed reproduction. Wornik and Grube [57] have proposed that *Physconia distorta* is the sexually reproducing species pair partner of the mixed species *P. grisea*. However, phylogenetic studies have shown that the two species are not sister species or close-clade species [15], even though both belong to the monophyletic *Physconia* group [16].

The mixed species *Physconia grisea*, a primarily vegetatively reproducing species, was chosen here as a study model to determine whether the occasionally occurring apothecia produce viable sexual spores. Our hypothesis was that viable spores from these rare apothecia provide an adaptive advantage that can increase genetic variability in later generations and populations. Accordingly, we compared sporulation and germination rates as well as the growth of ascospores in various axenic cultures of *P. grisea* and *P. distorta*. The latter was selected as a comparison species since it only undergoes sexual reproduction and has already been successfully cultured in vitro [35,37]. Moreover, *P. distorta* and *P. grisea* are sympatric species that can compete for the same *Trebouxia* species as their photobiont [57]. Additionally, DNA sequencing of the field-collected apotheciated morphotypes of *P. grisea* has confirmed them as belonging to the *P. grisea* s. str. clade [15].

Materials and methods

Lichen material. *Physconia grisea* specimens were collected from Casa de Campo on *Quercus ilex* (N 41º 24′ 32.88″, W 03º 45′ 10.52″, alt. 630 m, Madrid, Spain) by P. Divakar. *Physconia distorta* was collected from Montejo de la Sierra on *Quercus pyrenaica* (N 41º 04′ 63″, W 03º 29′ 65″, alt. 1293 m, Madrid, Spain) also by P. Divakar. One thallus from two local populations was used from each species, selected among 15–20 apothecia per thallus. According to Fedrowitz et al. [17], a "local population" is defined as all single thalli of the same species on a tree, and the metapopulation consists of all local populations within a certain forest landscape. The selected apothecia were of intermediate sizes (about 5 mm in diameter) to ensure sexual maturity [39]. Both localities have acceptable air quality, with pollutant concentrations lower than those that usually affect medium-sensitive lichens. Apothecia density was calculated as the number of apothecia per cm^2 and three random apothecia were used. Samples were processed for spore discharge immediately after field collection (see below).

Isolation and culture. Plurisporic isolates were obtained from apothecia of both lichen species. The fungi were cultured from discharged spores following the methods of Ahmadjian [1]. The apothecia were washed for 20 min in distilled water followed by 30 min in phosphate-buffered saline (PBS) containing 0.01 % Tween 80 (v/v). Ascomata were then soaked in sterile double-distilled water for 2 h, with the water changed several times during that period. Finally, apothecia were carefully cleaned and lightly dried with absorbent paper under a magnifier glass.

Clean ascomata were attached to the inner side of Petri dish lids with petroleum jelly. The bottom halves of the Petri dishes contained either Basal Bold medium (BBM) or 1 % glucose BBM (1G-BBM; [3]) and were inverted over the lids, allowing the ascospores to discharge upwards onto the medium. The lids with apothecia were removed after spore discharge was complete and replaced by new lids. The Petri dishes were then inverted to the normal culture position with medium at the bottom of each dish.

After germination, 36 uncontaminated multispores isolated (6 isolated on each plate and 6 plates per medium) from *P. distorta* and *P. grisea* were subcultured on 11 types of different media: 2 % glucose LBM (2G-LBM); 3 % glucose LBM (3G-LBM); 3 % sucrose LBM (3S-LBM) according to Lilly and Barnett [29]; 1 % glucose BBM (1G-BBM); 2 % glucose BBM (2G-BBM); 8 % sucrose BBM (8S-BBM); malt-yeast extract (MY), in accordance with Behera and Makhija [3]; 0.2 % glucose malt-yeast extract (0.2G-MY), modified by us from Ahmadjian [1] as follows: 5 g of malt extract, 2 g of glucose, 0.25 g of yeast extract, and 15 g of agar in 1 liter of twice-distilled water; potato dextrose agar (PDA); corn meal agar (CMA); and charcoal agar (CA), following the manufacturer's instructions (Difco).

Cultures were incubated at 18–20 °C in the dark. Periodically, mycobionts were examined using an Axioskop (Zeiss Germany) and a Nikon Eclipse 80 microscope with a magnifier glass. For photography, an automatic ring flash system was attached to the camera lens (SPOT Insight Wide-field 2MP). Photographs used white light and Nomarsky interphase contrast. When necessary, the colonies were slightly stained with lactophenol cotton blue, especially when colony growth was compact.

The variables used to evaluate sexual reproductive fitness were: percentage of apothecia that propelled spores upward (=sexually mature); number of spores ejected by each apothecium, percentage of spores that germinated from each apothecium, globular colony growth (defined as the diameter of the central part of the colonies, where the cells are globose and very compact) at 300 days. The lengths and widths of the spores were calculated based on at least 10 randomly selected spores and using the software NIS-Elements D 3.0 included with the Nikon Eclipse 80 microscope.

Statistical analysis. To determine whether the germination percentages on the two types of media (BBM and 1G-BBM) and obtained with the two species (*Physconia distorta* and *P. grisea*) were statistically different, a bifactorial analysis of variance (ANOVA) was performed. Variances were checked for homogeneity using Cochran's test. The Student-Newman-Keuls (SNK) test was used to discriminate among different treatments after a significant F-test. Data were considered significant when $P < 0.05$. The data of *P. distorta* growth on the different enriched media were not strictly parametric and thus were analalyzed with the Kruskal-Wallis test. All tests were done with the software Statistica 6.0 for Windows (Statsoft, Tulsa, OK, USA).

Molecular identity. Because of the frequent occurrence of cryptic species in lichenized fungi and especially in the *Physconia* group (e.g., *P. distorta* vs. *P. thorstenii*, [16]), we confirmed species identities using molecular sequencing of the internal transcribed spacer (nuITS). Samples derived from field-collected specimens of *P. grisea* (frozen specimens) and *P. distorta* (mycobiont cultures) were ground into powder with sterile plastic pestles. Total genomic DNA was extracted using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions, but with modifications as described in Crespo et al. [13], and then used for PCR amplification of fungal nuITS rDNA using the primers ITS1F and ITS4 (see [15]). PCR and sequencing were carried out following Crespo et al. [13]. Sequence and species identities were confirmed using the "megaBLAST" search function in GenBank [54].

Phylogenetic analysis. New ITS sequences were added to the alignment published in Divakar et al. [16]. The sequences generated for this study were deposited in GenBank under accession numbers KC559094 (*P. grisea*, MAF-Lich 17760) and KC559093 (*P. distorta*, MAF-Lich 17761).

Sequences were aligned using the program MAFFT ver. 6 [25] and the G-INS-I alignment algorithm, "1PAM/K = 2" scoring matrix, and offset value = 0.1; the remaining parameters were set to default values. The alignment was analyzed using Bayesian (B/MCMC) and maximum likelihood (ML) approaches. The program MrBAYES 3.1.2 [24] was used to analyze trees following a MCMC method. Two species of *Anaptychia* were selected as the outgroup, as this genus was supported as the sister group to *Physconia* in Cubero et al. [15]. The analyses were performed based on the general time reversible model of nucleotide substitution [50], including estimation of invariant sites, assuming a discrete gamma distribution with six rate categories and allowing site-specific rates (GTR+I+G). The nucleotide-substitution model and parameters were selected using the Akaike information criterion as implemented in jModelTest [48]. No molecular clock was assumed. Initial runs were conducted starting with random or neighbor-joining trees to check the number of simultaneous MCMC chains necessary to avoid trapping on local optima. Subsequently, a run with 2 million generations, starting with a random tree and employing 12 simultaneous chains each, was executed. Every 100th tree was saved into a file. The first 200,000 generations (i.e., 2000 trees) were deleted as the "burn in" of the chains. We plotted the log-likelihood scores of sample points against generation time using TRACER 1.0 to ensure that stationary phase was achieved after the first 200,000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value [24]. Of the remaining 18,000 trees, a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes.

A ML analysis was performed using the program RAxML v7.2.7, as implemented on the CIPRES Web Portal, with the GTRGAMMA model [52,53]. Support values were assessed using the "rapid bootstrapping" option with 1000 replicates. Branches with posterior probabilities (pp) ≥95 % and ML bootstrap values ≥70 % were considered strongly supported.

Results

The sequences obtained from one specimen from each species were identified as *Physconia grisea* and *P. distorta*, respectively, through Genbank comparisons, confirming our initial species identification. The ITS PCR products from these specimens are about 600 base pairs (bp) in length. In the molecular phylogenetic tree (Fig. 1), the sequence obtained from an axenic culture of *P. distorta* clustered with the sequences of other non-cultured samples of *P. distorta* in the "distorta clade." This relation was strongly supported; however, the monophyly of *P. distorta* was not. The *P. distorta* clade included other species, such as *P. detersa* and *P. subpulverulenta*. Whether *P. detersa* and *P. subpulverulenta* merit species-level ranking has been questioned in other studies [15,16]. Those authors have highlighted the species-pair problems and have suggested that both *P. detersa* and *P. subpulverulenta* belong to *P. distorta* and are not separate lineages. Since resolving this controversy was not the goal of our study we left this question open. The sequence obtained from apotheciated *P. grisea* sample grouped within the "grisea clade" (pp 1.00). This clade is formed by specimens from six different populations, two of which are fertile and four of which reproduce vegetatively. Phylogenetic clusters related to reproductive strategy (sexual vs. vegetative reproduction) were not distinguished in the "grisea clade".

The collected specimens of *P. grisea* and *P. distorta* showed abundant apothecia (respectively, 2.07 ± 0.33 and 3.10 ± 0.31 apothecia·cm^{-2}). All ascomata ejected spores from mature asci after 12 h and spore germination was observed after 6 days for both species. Contamination by bacteria or other fungi was observed near many spores or spore groups ejected from the apothecia. Contaminations are expected when working with field-collected specimens, although contamination levels were highly variable between apothecia (data not shown). The spores of both species were subglobular and uniseptated, with brown external ornamentation capsules (Fig. 2). The spore length and width (mean ± standard deviation) were 25.99 ± 8.66 µm × 15.32 ± 5.11 µm for *P. grisea*, and 28.62 ± 5.74 µm × 16.85 ± 3.75 µm for *P. distorta*. When a meiospore germinated, its capsule was broken open at two points (bipolar germination), allowing the development of germ tubes (Fig. 2A,B). The meiospores were attached to the media on the top plate as either isolated spores or groups of 2–3 or 6–8 spores (Fig. 2C). The number of ascospores ejected per apothecium varied considerably, with some ascomata releasing just a few dozens of spores and others releasing hun-

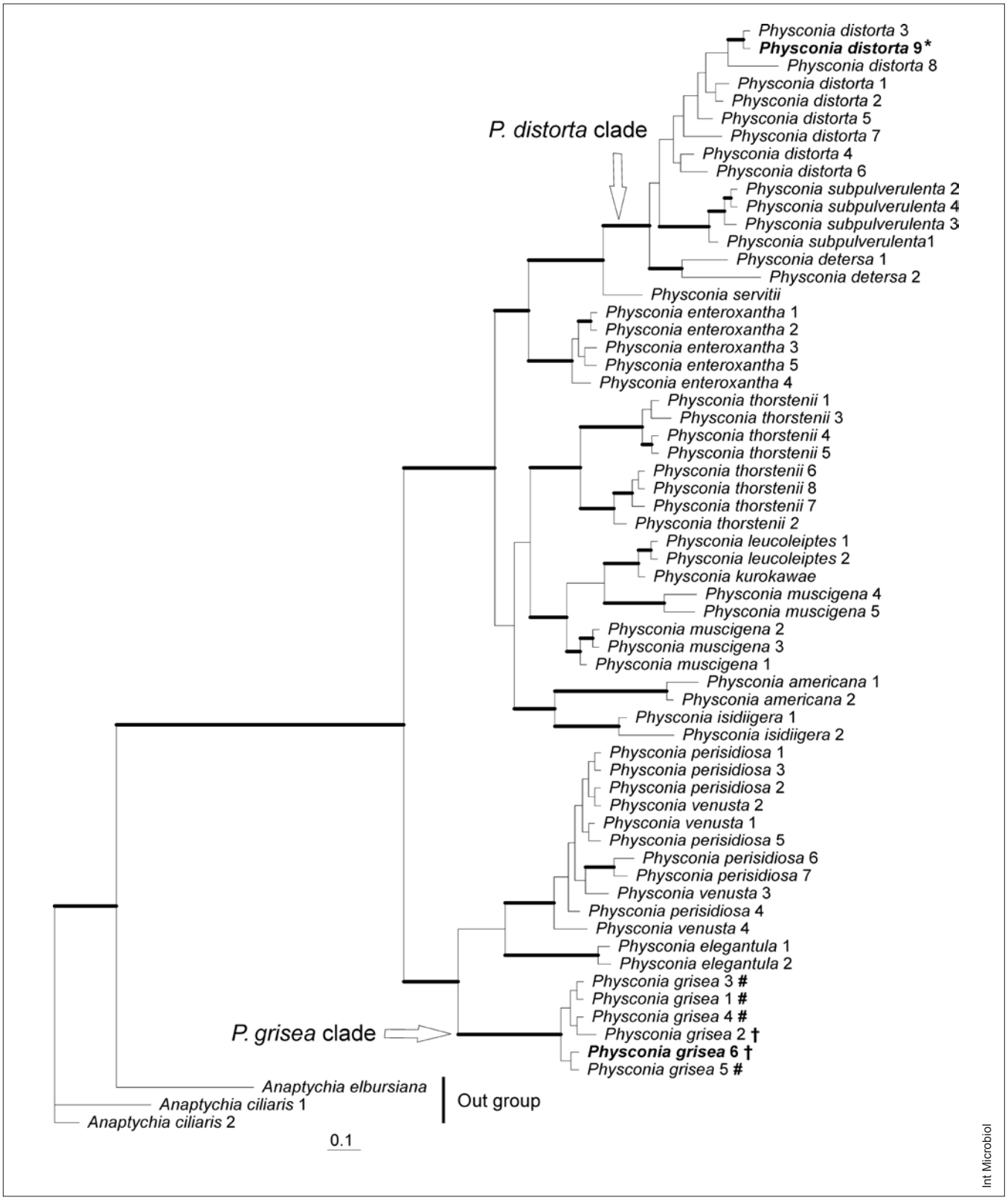

Fig. 1. The majority-rule consensus tree from the Bayesian analysis based on nuITS sequences of *Physconia*. Branches that received strong support in any of two analyses (pp ≥0.95 and RaxML bootstrap ≥70 %) are shown in bold. The phylogenetic position of the two new sequences of *P distorta* (obtained from culture) and *P. grisea* (field collected) are shown in bold and bold with an asterisk, respectively. Symbols: #, vegetative specimens; †, apotheciated specimens.

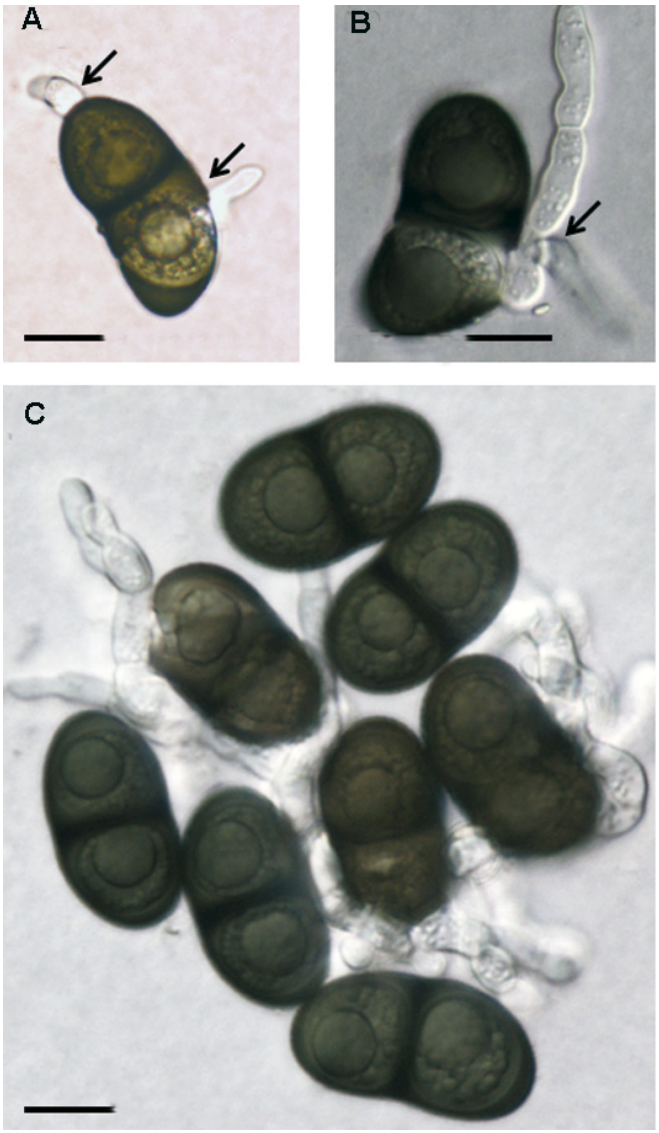

Fig. 2. Subglobular, bipolar, and uniseptate germinated ascospore from *Physconia grisea*. (**A,°B**). First germination stage. Arrows show the break points of the spore capsule with germ tubes. (**C**) Eight germinated spores group from asci. Scale bar = 10 µm.

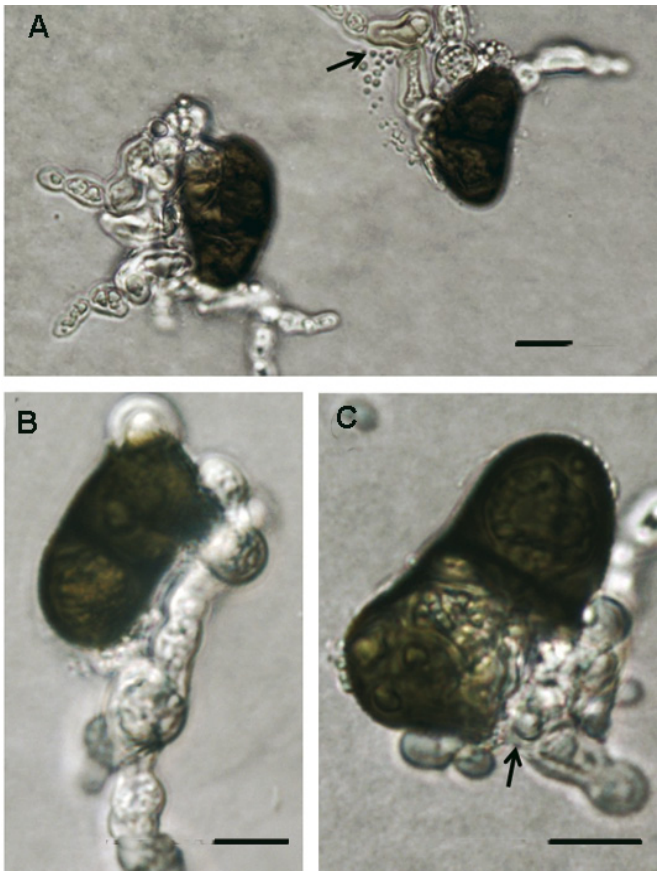

Fig. 3. Degeneration of *Physconia grisea* spores after a month on BBM (**A**), 0.2G-MY (**B**), and S-LBM (**C**). The arrow shows the collapsed cells and the cytoplasmic contents lost in the culture medium. Scale bar = 10 µm.

dreds of spores. *Physconia grisea* released an average of 62.3 ± 34.5 spores and *P. distorta* 73.4 ± 34.2 spores.

The germination rates of the two species did not significantly differ ($P > 0.05$). However, for both species the germination percentage on 1G-BBM was significantly lower than that on BBM ($F_{1,10} = 8,25$; $P = 0.016$). The averages and standard deviations were 79.73 ± 18.96 for *P. grisea* and 88.28 ± 15.2 for *P. distorta* on BBM and 40.1 ± 30.31 for *P. grisea* and 51.66 ± 34.01 for *P. distorta* on 1G-BBM. The standard deviations were high because of the large variations in the percentage of germination between apothecia. For example, for spores germinated in 1G-BBM, the range was 18–86 % for *P. distorta* and 12–72 % for *P. grisea*.

Germinated ascospores of *P. grisea* and *P. distorta* showed hyphae with short and septated cells, and the cytoplasmic content could be easily seen in cultures incubated in organic (1G-BBM) and inorganic (BBM) media. However, the behavior of the two species after germination was very different in each medium.

After one month, the hyphal cells of *P. grisea* cultured on BBM contained large vacuoles and the cells had begun to contract and shorten. Over the next 20 days, the hyphae became completely wrinkled and lost their cytoplasmic content so that they were no longer viable, excluding thus further growth (Fig. 3A). In a few rare cases, 10 % of the ascomata of *P. grisea* produced a small amount of ascospores (single or groups) that continued to grow after a month, but only on inorganic medium; however, they generated poor mycelia, with the hyphae growing only on the surface and at very slow rates (0.31 ± 0.1 µm per day; 100 µm in 315 days). These meiospores accounted

Table 1. Growth activity of sexual spores from *Physconia grisea* and *P. distorta* grown on inorganic media (BBM) and several enriched media. Activity is indicated by the following abbreviations: nt, not tested; –, without growth; –/+, only a 0.43 % of germinated meiospores grew, but very slowly and weakly. The number of + indicate intensity of growth according to Fig. 5

Media	*P. grisea*	*P. distorta*
Basal bold medium (BBM)	–/+	+
3 % Glucose LBM (3G-LBM)	–	++++
0.2 % Glucose malt-yeast extract (0.2 G-MY)	–	+++
Corn meal agar (CMA)	–	++
2 % Glucose BBM (2G-BBM)	–	++
1 % Glucose BBM (1G-BBM)	–	+
8 % Sucrose BBM (8S-BBM)	–	nt
Malt-yeast extract (MY)	–	nt
3 % Sucrose LBM (3G-LBM)	–	nt
2 % Glucose LBM (2G-LBM)	–	nt
Potato dextrose agar (PDA)	–	nt
Charcoal agar (CA)	–	nt

for 0.43 % of the total germinated spores from this species. When healthy and uncontaminated germinated spores of *P. grisea* were cultured on 1G-BBM, they rapidly degenerated without completing their development. To ensure that the poor development of *P. grisea* was not related to the choice of culture medium, eleven different media usually successful for lichen cultures were tested (Table 1); the result was always negative over the long term (Fig. 3B,C and Table 1).

After one month, all colonies of *P. distorta* in organic medium (1G-BBM) consisted of well-developed radial mycelia with globular cells in the center and more elongated, distally located cells. Fattened intersepta and small lipid drops were observed. During this time, pigment synthesis was considerable, leading to dark thallus-like colonies (Fig. 4A). The globular growth rate on 1G-BBM was 5.34 ± 0.26 µm per day (240 µm in 50 days). *P. distorta* mycelium also grew on basal media (BBM) but was more poorly developed, with long intersecta and a few lipid drops. Globular growth was slower in BBM (2.96 ± 0.57 µm per day; 133 µm in 50 days) and the hyphae were more filamentous, developing only on the surface and without generating colonies (Fig. 4B,C). The mycelia lacked pigmented areas. When germinated ascospores of *P. distorta* were transferred to enriched media (3G-LBM, 0.2G-MY, CMA, 2G-BBM, and 1G-BBM), after 15–20 days all

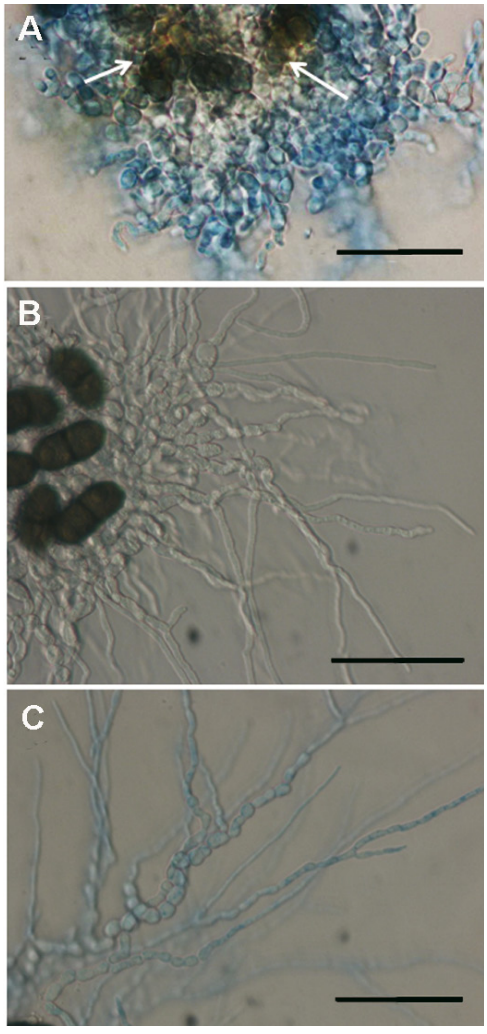

Fig. 4. Developmental stages of *Physconia distorta* spore germination. (**A**) Compacted growth with globular cells, observed on 1G-BBM media. (**B**) Poor and superficial growth with elongated cells, observed on BBM media. (**C**) Detail of hyphae stained with lactophenol. Scale bar = 100 µm.

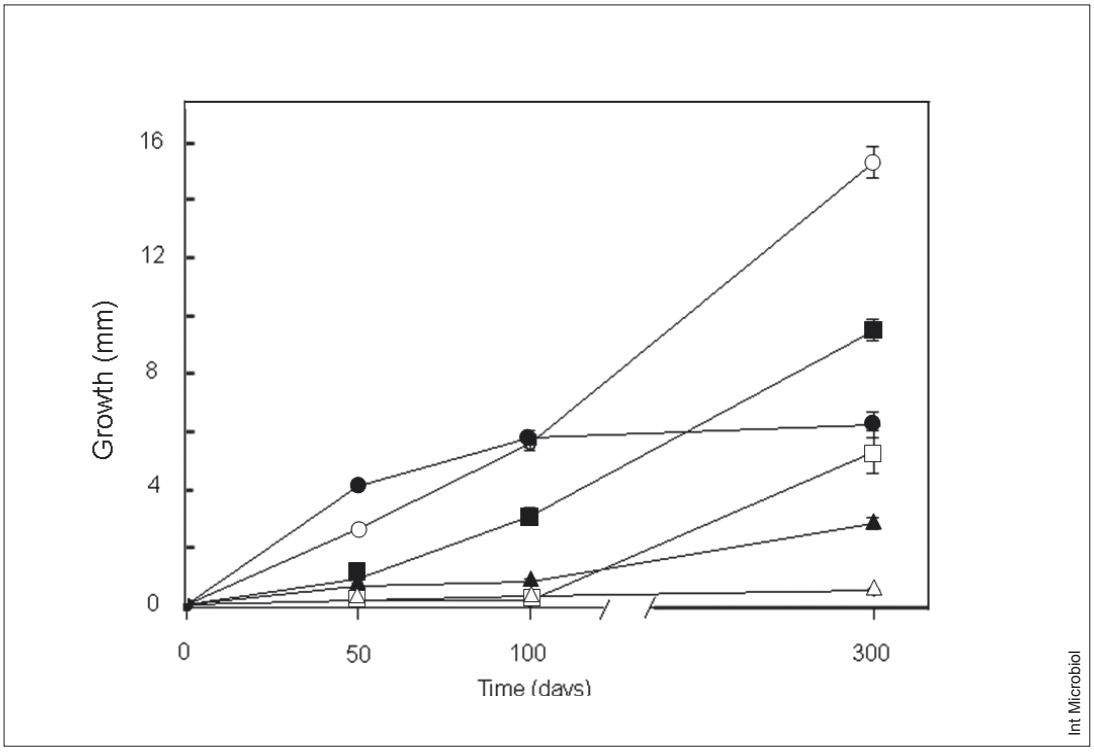

Fig. 5. Kinetics of *Physconia distorta* growth on different enriched media: CMA (closed circle), 3G-LBM (open circle), 0.2G-MY (closed square), 2G-BBM (open square), 1G-BBM (closed triangle), BBM (open triangle).

transferred spore groups grew, with the growth rate correlating with the type of medium (Fig. 5). After 50 days, the growth rates on the different media were statistically different ($\chi_5^2 = 52$; $P < 0.001$). Their ranking, in decreasing order, was as follows: CMA > 3G-LBM > 0.2G-MY = 2G-BBM = 1G-BBM = BBM, with CMA yielding statistically higher growth rates than the other media types for initial growth. However, there was a change in the rate of development starting after 100 days. Thus, at 300 days growth was also statistically different between cultures ($\chi_5^2 = 70.61$; $P < 0.001$), but the rank order of the growth rates on the tested media were: 3G-LBM > 0.2G-MY > CMA = 2G-BBM > 1G-BBM = BBM.

Discussion

The morphotypes of *P. grisea* ("mixed species") that have apothecia and undergo vegetative reproduction belong to the *P. grisea* clade s. str. described by Cubero et al. [15]. This allowed us to reject the hypothesis that these morphotypes should be considered as a separate subspecies (as suggested by Poelt [46]). All ascomata from the observed thalli of *P. grisea* and *P. distorta* species were sexually mature. The sexual-only and mixed species produced similar amounts of spores, which indicated that occasionally occurring apothecia of *P. grisea* could produce and eject spores in a similar manner to *P. distorta*, the sexually-only reproducing species. The amount of spore production varied widely in both species, suggesting that, although the selection of apothecia was standardized [39], the sexual age of the apothecia from different thalli was not homogeneous. This is not surprising considering the heterothallic life cycle of lichens (self-sterile), i.e., cross fertilization and genetic recombination among different individuals [23,55], as well as the slow growth rate of lichens in nature. Moreover, the ascospore germination rate was higher in inorganic medium (BBM) than in medium supplemented with glucose (1G-BBM). This inhibition or suppression of germination by glucose had been described previously by Molina et al. [39], who found that high concentrations of sugar control fungal germination. Since algal cells in the symbiosis are responsible for carbohydrate production, this behavior could be interpreted as an algal defense mechanism, according to the putative parasitism theory [40]. Belandria et al. [4] have attributed such results to the rapid proliferation of unwanted fungi or bacteria in

enriched media, which can promote allelochemical activities inhibiting the germination of the lichen ascospores.

Even though the processes of sporulation and germination were similar in the two species, later ontogenetic development was strikingly different. When grown on enriched media, sexual-only *P. distorta* formed well-developed thallus-like colonies. This behavior has been described in other lichen-forming fungi (e.g., [10,35]). The development of *P. distorta* varied significantly depending on the culture medium, with CMA being the most suitable during the first 50 days. However, after 300 days the best medium was 3G-LBM, likely because it is enriched with nutrients, vitamins, and asparagine. Colonies grown on basal medium (BBM) without a carbon source had poorly formed mycelium, with long intersepta and few lipid drops. Colonies on BBM also developed more slowly and only on the surface. Similar poor, superficial, and filamentous growth has been reported for lichenized [19,35] and phytoparasitic fungi (e.g., [38]) and is associated with fungal resource-searching in nutrient-poor media.

Germinated *P. grisea* spores (from the "mixed species") were unable to complete development in any of the enriched culture media, and only a very small fraction survived in non-enriched media. It is difficult to properly assess the finding that only 0.43 % of the total germinated spores of *P. grisea* showed extended growth in inorganic, non-enriched medium. Since only an extremely low percentage of *P. grisea* spores was able to grow and only at a remarkably slow growth rate (average of 100 μm growth in almost 1 year), we assumed that its sexual spores hardly contributed to the survival of mixed-species *P. grisea*. The probability is no doubt very low that these hyphae will find the needed compatible photobiont to establish a lichen association before the hyphae die [17,33].

Although the growing conditions of lichen-forming fungi are not genus-specific [10,36], it was surprising that none of the eleven culture media tested were adequate for the ontogenetic development of *P. grisea*. This suggests that 99.57 % of germinated meiospores of *P. grisea* would not be able to develop in any of the media, not because the spores were immature or in an unsuitable medium, but because in this species they might have been non-functional and/or non-viable over both the short and long term. Indeed, many of the media tested in this study have been used successfully in other lichen species, including Physciaceae [3,14,35].

Another reason for the lack of successful spore germination and development of *P. grisea* in the tested media could be that this species needs to find a suitable photobiont at a very early stage of its development and therefore does not thrive in laboratory cultures. If so, this poses an obstacle to successful lichen re-synthesis, especially when *P. grisea* shares its habitat with other species, such as *P. distorta* (sympatric species). Since these species also compete for photobionts [57], lichen re-synthesis in *P. distorta* could be more successful, favored by the initially strong ontogenetic development of this species to establish lichen symbiosis and by its fewer specific nutritional requirements. Regardless, the strong developmental differences between these two species are remarkable, with spores of *P. distorta* but not of *P. grisea* developing in all culture media tested.

Preliminary transplant experiments using the lichen *Icmadophila splachnirima* (Icmadophilaceae), another mixed species, have indicated that, in more exposed microhabitats, apothecial growth is reversibly arrested at an early developmental stage and always accompanied by the formation of marginal soralia. This observation suggests an environmentally triggered switch from sexual to asexual reproduction, possibly in response to adverse, more stressful growth conditions [31], in which there is a need to save resources. If the apothecia do not represent an adaptive advantage because the cost is greater than the benefit (as suggested by Ludwig et al. [31]), or if they are simply non-functional because the spores cannot complete their development, negative selection would be expected to discourage apothecia, which would then be observed only rarely or occasionally. This would account for the local populations of *P. grisea*. According to Honegger and Zippler [23], genetic defects in the cascade of ascomatal development are possible, although they cannot be explained without genetic studies of the mating-type pathway. Lichen forming fungi have yet to be studied in depth, but several asexual non-lichenized ascomycetes are known to fail to differentiate ascomata because of defects in genes of the complex mating-type pathway [25].

Harada [20] and Ohmura et al. [43] have considered that the high genetic variability observed in the mixed species *Parmotrema tinctorium* (Parmeliaceae) may be due in part to genetic recombination occurring in infrequent apothecia, but they have not tested this hypothesis empirically. Moreover, other mechanisms could explain such high genetic variability, such as somatic mutations, somatic recombination (parasexuality), historical genetic variability because of ancestral sexual states, or/and cryptic sex [9]. By contrast, Otalora et al. [44] have determined high genetic diversity values for bionts in *Degelia plumbea* (a sexual species) and extremely low values in *Degelia atlantica* (a mixed species). In our study, *P. grisea* meiospores produced from apothecia in in vitro conditions were almost always non-functional and therefore might not

have notably increased the genetic variability within the population. We suggest, following Honegger and Zipper [23], that in *P. grisea* the ascomatal developmental system would be defective. The defects might be isolated phenomena in a group of individuals or an established characteristic in the genetic structure of the population. To further test this hypothesis, an additional analysis of mating-type and related genes in populations of *P. grisea* is necessary.

It is possible that the non-viability of the spores of *P. grisea* grown in culture was due to seasonal or population factors [51]. Therefore, further studies are needed to clarify the presence and frequency of mating-type genes in the populations and/or to analyze the genetic variability in the population based on sequence or microsatellite data. Our work emphasizes the importance of testing spore viability at the population level before concluding that sexual spores are true sources of genetic variability in mixed system reproducing species, as has often been assumed [20,43]. This study of spore viability is of particular interest considering that lichens may have other sources of genetic variability, such as somatic mutations, somatic recombination (parasexuality), and historical genetic variability reflecting ancestral sexual states. The contribution of these sources to increasing genetic diversity in populations should be determined.

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