The Lichenologist **51**(2): 175–186 (2019) doi:10.1017/S0024282918000580

Differences in the sexual aposymbiotic phase of the reproductive cycles of *Parmelina carporrhizans* and *P. quercina*. Possible implications for their reproductive biology

D. ALORS, Y. CENDÓN-FLÓREZ, P. K. DIVAKAR, A. CRESPO, N. GONZÁLEZ.BENÍTEZ and M. C. MOLINA

Abstract: Our knowledge of ontogenetic development and reproductive biology in lichen-forming fungi is rather poor. Here, we aim to advance our understanding of the reproductive biology of *Parmelina carporrhizans* and *P. quercina* for which mycobiont fungi of both species were cultured in aposymbiotic conditions from ascospores. For *P. carporrhizans* 48 hours were necessary for 98·6% of apothecia to eject spores, while for *P. quercina* 100% of apothecia ejected spores in the first 24 hours. In *P. quercina*, large apothecia ejected more spores than smaller ones. In both species the percentage of spores germinating seemed independent of apothecium size. The percentage germination was higher in *P. carporrhizans* (72·4%) than in *P. quercina* (14·3%). Moreover, *P. carporrhizans* was grown more successfully on culture media than *P. quercina*. These results suggest that these species have different reproductive strategies, given that *P. carporrhizans* expels larger spores and in greater numbers than *P. quercina* as well as having different nutritional requirements (since *P. carporrhizans* grew successfully in the selected media but *P. quercina* did not). These characteristics may explain the sympatric speciation of these species.

Key words: axenic culture, mycobiont, ontogeny, reproductive success, size and number theory

Accepted for publication 12 *July* 2018

Introduction

Lichens reproduce by vegetative dispersal of both symbionts together and/or re-establish by fungal spores (ascospores and/or conidia) that disperse aposymbiotically and subsequently find a compatible photobiont (e.g. Sanders & Lücking 2002). Sanders (2014) showed that in *Calopadia puiggari* macroconidia and photobiont cells were co-dispersed but that upon germination the fungus might grow away from the algal cells leaving them to form a free-living population. Apart from important but limited field observations, knowledge of lichen-forming fungal reproductive biology is still scarce for two reasons: their inability to successfully execute the complete reproductive cycle under in vitro conditions and the difficulty in tracing the reproductive propagules of most lichen species in nature (Schuster *et al.* 1985; Ott 1987; Sanders 2014; Morando *et al.* 2017). However, traceability of lichen spores could now be much easier thanks to the increased sensitivity of new methods developed by Eaton *et al.* (2018) combining aerobiological traps with nested PCR protocols.

The aposymbiotic culture of lichens and their symbionts using sexual and asexual propagules has led to a new understanding of the biology of these organisms, including the genetic variability of populations (Dal Grande *et al.* 2014; Degtjarenko *et al.* 2016) and horizontal gene transfers (e.g. McDonald *et al.* 2012; Beck *et al.* 2015). Similarly, Molina *et al.* (2015) were able to obtain valuable direct information about sexual reproduction such as spore discharge (productivity), germination and ontogenetic development before association with an algal

D. Alors, P. K. Divakar and A. Crespo: Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain. Email: d.alors@gmail.com
Y. Cendón, N. González and M. C. Molina: Departamento de Biología y Geología (Área de Biodiversidad y Conservación), ESCET, Universidad Rey Juan Carlos, Móstoles, 28933 Madrid, Spain.

partner (the sexual aposymbiotic phase of the reproduction cycle). Molina *et al.* (1997) suggested a co-relationship between sexual maturation and size of apothecia on the basis of studies on aposymbiotic cultures. Ascertaining a relationship (should there be one) between size of apothecium and productivity and germination of spores, could provide baseline information for research on reproductive biology in these organisms.

Öckinger *et al.* (2005) demonstrated a relationship between propagule dispersion capacity and the distribution of *Lobaria pulmonaria* (L.) Hoffm., a relationship that is modulated by environmental factors. Furthermore, Tibell (1994) noted that species with small spores have a wider global distribution than those with larger spores. Johansson *et al.* (2007) established a relationship between the age of trees and the size of spores of epiphytic lichens. Morando *et al.* (2017) investigated the relationship between size and number of pores and spatial structure; they noted that discharge of fewer and larger spores could result in spore aggregation and reduced dispersion. As far as we know, no hypotheses in lichen-forming fungi have been proposed for the relationship between size and number of spores and germination success. However, in plants, relationships between seed size and number of seeds have been proposed to explain the ecological adaptative advantages of two different strategies: 1) having many small seeds; 2) having a small number of large seeds. In general, plants producing large seeds have a competitive advantage over those producing smaller seeds because of their higher germination rates and greater nutrient reserves (Easton & Kleindorfer 2008*a*). Nevertheless, small-seeded species also have great success, perhaps due to their dispersive advantage.

Parmelina carporrhizans (Taylor) Poelt & Vězda and *P. quercina* (Willd.) Hale were chosen as model organisms for *in vitro* culture studies. They are phenotypically similar but genetically different so that they are two different species and are phylogenetically unrelated (see e.g. Argüello *et al.* 2007; Núñez-Zapata *et al.* 2011, 2017). They are foliose epiphytic lichens that reproduce only sexually, and both are heterothallic (Honegger & Zippler 2007). *Parmelina quercina* has a mainly Mediterranean distribution in southern Europe and Morocco, although it is reported from a small number of other sites (Nimis 1993; Clerc & Truong 2008). *Parmelina carporrhizans* grows in more oceanic and humid sites, mainly in the Mediterranean but also in the Macaronesian region, and is even occasionally found in Great Britain (Schauer 1965; Argüello *et al.* 2007; Clerc & Truong 2008; Hawksworth *et al.* 2008; Alors *et al.* 2014). The two species rarely coexist. Recently, *P. carporrhizans* was confirmed as heterothallic and long-distance dispersal of its ascospores (up to 900 km) was demonstrated using molecular data (Alors *et al.* 2017).

In the current study, we aim to investigate some aspects of the reproductive behaviour of these two closely related species, with similar (but slightly different) morphologies, in the light of size and number theory. Specifically, we tested 1) different patterns of spore discharge and germination, 2) new mycelia development and growth *in vitro*, and 3) the importance of apothecia size in spore ejection and germination.

Materials and Methods

Lichen material

Two subsets of samples were considered. Initially we analyzed three samples of each species, collected in 2012. However, the statistical analyses based on Akaike criterion showed the need to increase the sampling. We therefore also collected further specimens in 2016 and analyzed the number of spores ejected by apothecia and percentage of germination by apothecia from ten thalli of each species. Only the percentage of apothecia able to eject spores, ontogenetic observations and mycelia development are based on the initial samples collected in 2012.

The first subset of samples corresponds to three fresh thalli of *Parmelina carporrhizans* growing on *Castanea sativa* Miller collected from Cuevas del Valle, Spain (40°18′28·4″N, 5°00′39·0″W) on 11 October 2012 at 1007 m (Maf-Lich 19190, Maf-Lich 19191), and three thalli of *P. quercina* collected from *Quercus ilex* L. in Monfragüe, Spain (39°50′17·47″N, 5°59′36·41″W) on 29 October 2013 at 200 m. Specimen identifications were confirmed using ITS sequences. We used five large and five small apothecia per thallus.

The second subset of samples was collected in October 2016 and consisted of ten thalli of each species; we selected six apothecia from each thallus. Collections were carried out in the same localities as the first subset.

We assessed the possible positive correlation between size of the apothecia (apothecia size range) and the reproductive parameters. Sizes ranged between 3·8–7·0 mm for *P. carporrhizans* and 1·9–5·9 mm for *P. quercina*. Apothecia were classified into two size categories: 3·8–4·9 mm (small) and 5–7 mm (large) diam. for *P. carporrhizans*; and 1·9–3·5 (small) and 3·6–5·9 mm (large) diam. for *P. quercina*. For each species the two categories were selected such that one was less than and the other greater than the median value of apothecium diameter, discarding very small apothecia without a red hymenial disc, which were considered immature. To test the relationship between the reproductive parameters and apothecium size, data were grouped in discrete intervals of 0·4 mm.

Isolation and culture

Parmelina carporrhizans and *P. quercina* mycobionts were isolated from discharged ascospores following the inverted Petri dish method of Ahmadjian (1993). Apothecia were mechanically cleaned and washed following the protocols established by Molina & Crespo (2000); the clean ascomata were attached to the inner side of inverted Petri dish lids with petroleum jelly. The plates contained Bold's Basal Medium (BBM, Deason & Bold 1960) which is inorganic and suitable for spore germination (Molina *et al.* 1997), and the ascospores were discharged upwards. The Petri dishes were kept at 18–20 °C in the dark (Molina & Crespo 2000).

After germination, 90 randomly selected, uncontaminated multispore agar pieces from *P. carporrhizans* and 35 from *P. quercina* were subcultured on different media: 3% glucose LBM (w/v) (3G-LBM) according to Lilly & Barnett (1951) as modified by Lallemant (1985); 0·2% glucose malt-yeast extract (0·2G-MY) according to Molina *et al.* (2013); and Corn Meal Agar (CMA) following the manufacturer's instructions (Difco Laboratories, Detroit, MI, USA). Cultures were incubated at 20 °C in the dark (Molina & Crespo 2000). Periodically, mycobionts were examined using a Nikon SMZ800 stereomicroscope and an Olympus CX40 microscope. Photographs were taken with an automatic ring flash system attached to a Canon 450-D camera. The observations were carried out using white light and Nomarski interference contrast.

Ontogenetic parameters measured

The parameters measured to assess the ontogenetic development of these lichen-forming fungi in culture were: spore morphology, volume; germination type; mycobiont aggregate growth over 160 days on different media; the capacity to produce pigments, which was qualitatively assessed.

In the 2012 samples, the fitness parameters studied were: percentage of apothecia able to eject spores (after 24 and 48 h); productivity (number of octet spore packs or plurisporic groups ejected by apothecia in 48 h) of large apothecia (LAP) and small apothecia (SAP); germination (percentage of spore packs with at least one germinated spore) of large apothecia (LAG) and small apothecia (SAG); growth capacity after germination on different media. Growth was analyzed with ImageJ (http://imagej.nih.gov/ij/) using images taken with an Olympus SZ30 binocular microscope. Relative productivity (rprod) and relative germination (rger) of spores were also estimated using the following formulae with values ranging between −1 and 1:

$$\text{rprod} = (\text{LAP} - \text{SAP})/(\text{LAP} + \text{SAP})$$

$$\text{rger} = (\text{LAG} - \text{SAG})/(\text{LAG} + \text{SAG})$$

In the second collection (2016), a total of 10 thalli and 60 apothecia were analyzed to assess rprod and rger.

To estimate the volume of spores of both species, the length and width of spores were measured, as described by Argüello *et al.* (2007), whereby at least 120 spores from six thalli were measured for each species. The mathematical formula for the volume of a spheroid, or ellipsoid of revolution, was used ($V = 4/3\ \pi a^2 b$), since this is the geometric figure that most closely resembles the shape of spores.

Statistical analysis

Generalized linear mixed models (GLMMs) were used to test the hypotheses that spore ejection and germination differ between the two species studied (fixed variable with two levels) and thalli (random variable with three levels). GLMMs were fitted assuming a Gaussian distribution error and, for each response variable, three alternative models were compared: 1) null (or intercept only) model ($y = \beta_0$); 2) fixed-effect model (y = species); 3) mixed-effect model (y = species + (thallus (species))). The mixed-effect model contained thalli as a random factor nested within species type. In a nested design, each level of the nested predictor is uniquely associated with only one level of the higher-level predictor. With nested data structures, the interaction variance is pooled with the main effect variance of the nested factor (Schielzeth & Nakagawa 2012).

An information theory approach (Burnham & Anderson 2002) was used as an alternative to the traditional hypothesis testing approach (Johnson & Omland 2004) to select the best-fitting models accounting for spore ejection and germination. Models were selected based on the Akaike Information Criterion corrected for small sample sizes (AICc). The AICc rewards goodness of fit (based on the likelihood function), but also penalizes over-fitting (based on a function of the number of estimated model parameters). For each model set, we calculated the difference in AICc between each candidate model and the model with the lowest AICc (ΔAICc). The best model was that with the smallest AICc (Burnham & Anderson 2002). All analyses were conducted using R (R Development Core Team 2013). We used the lmer function in the lme4 package and the gls function in the nlme package (Pinheiro *et al.* 2016) for fitting models; the MuMIn package was used for AICc-based model comparison (Barton 2013).

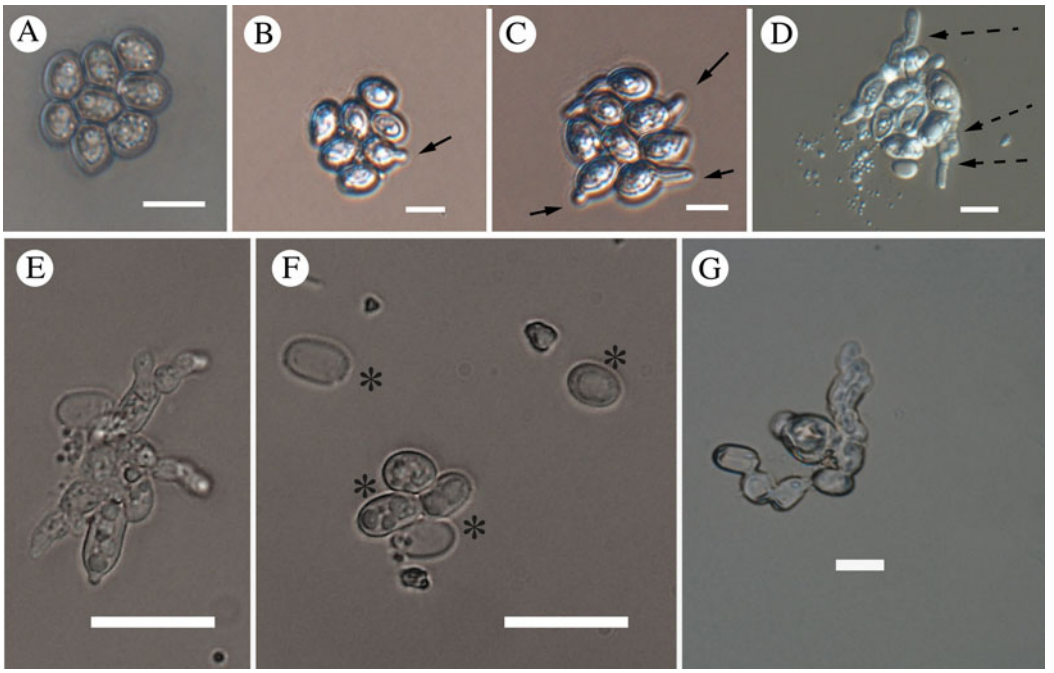


FIG. 1. A–D, *Parmelina carporrhizans*, spore groups. A, plurisporic aggregate 3 days after discharge; B & C, germination tubes (arrows) in spores 4 days after germination; D, septa (arrows) formed 7 days after germination. E–G, *P. quercina*, spore groups. E, germination of spores; F, ungerminated spores (asterisks) 10 days after spore ejection; G, degenerate culture dying at 30 days. Scales = 10 μm. In colour online.

Results

Maturity of apothecia

On the basis of the 2012 study, we found that the spores were ejected mainly in groups or packs of eight (Fig. 1A). Almost all the apothecia ejected spores after 48 hours, except some small apothecia of *Parmelina carporrhizans* (2·8%). For *P. carporrhizans*, in the first 24 hours, only 61·1% of large and 33·1% of small apothecia were sexually mature and had ejected spores, while apothecia of all sizes of *P. quercina* had ejected in that time.

Ontogeny

Based on measurements carried out in 2016 on 120 spores of each species, we found that *P. carporrhizans* spores had a volume of 1600·25 ± 0·20 μm^3, while those of *P. quercina* had a volume of 1418·69 ± 1·35 μm^3. The volume of *P. carporrhizans* spores was 18% greater than that of *P. quercina*. Spores began to germinate 3–4 days after sporulation in both species. *Parmelina carporrhizans* produced hyaline meiospores (Fig. 1A), which generated one or two germ tubes growing from the endosporium (Fig. 1B & C). A few days later, the germ tubes developed a clearly defined septum (Fig. 1D). Initially, the development in *P. quercina* was similar to that of *P. carporrhizans* but after the germ tube formed, they were often observed to degenerate (Fig. 1E), while many spores remained ungerminated (Fig. 1F), and a substantial number of aborted mycelia were observed after 30 days (Fig. 1G).

Spore productivity

On the basis of the 2012 study (n = 3) of the mean ejection of spore packs, *P. quercina* had greater productivity than *P. carporrhizans* (637 ± 347 vs. 221 ± 360 spores), especially in small apothecia (539 ± 179 vs. 95 ± 134

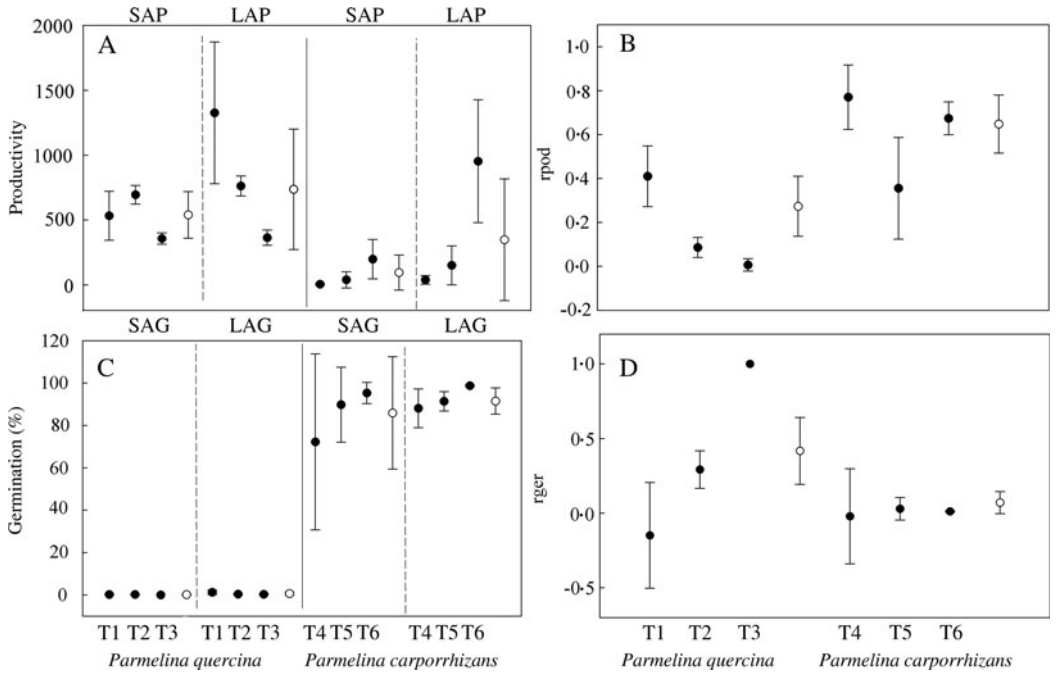


FIG. 2. Spore production and germination in *Parmelina quercina* and *P. carporrhizans*. A, productivity as absolute production of spores; B, relative productivity of spores (rprod); C, percentage germination; D, relative germination of spores (rger). Closed symbols represent values for each thallus sample (T1–T6). Mean values are plotted as open circles ($n = 3$). SAP = small apothecium production; LAP = large apothecium production; SAG = small apothecium germination; LAG = large apothecium germination. Error bars represent standard errors (SEM).

spores) (Fig. 2A). Both species had positive rprod values, indicating that large apothecia eject more spores than smaller ones, and the mean rprod value was higher in *P. carporrhizans* (Fig. 2B). The mixed-effect model (full model including thalli effect) was best at explaining the patterns of spore ejection (LAP, SAP and rprod) because it showed the lowest values of AICc. These results highlight the need to increase the number of thalli analyzed (Table 1).

When ten thalli were analyzed, it was confirmed that that the average productivity per apothecium was higher for *P. quercina* than for *P. carporrhizans* (807·64 ± 76·56 vs. 686·13 ± 67·72). In addition, a positive correlation between class size of apothecium and ability to produce and eject spores was observed in *P. quercina* (Fig. 3A). However, in *P. carporrhizans* productivity remained constant and independent of apothecium size (Fig. 3C).

Spore germination

In the preliminary results ($n = 3$), the total percentage germination (disregarding apothecium size class) was much higher in *P. carporrhizans* (89·0%) than in *P. quercina* (0·4%) (Fig. 2C). The best model to explain patterns of spore germination (LAG, SAG and rger) was the mixed-effect model (Table 1). The mean rger showed higher positive values for *P. quercina*, and positive but near-zero values for *P. carporrhizans* (Fig. 2D). Differences in apothecium size had no effect on percentage germination (Table 1, Fig. 3D).

When the numbers of thalli and apothecia were increased, the percent germination was significantly higher in *P. carporrhizans* than

TABLE 1. *Generalized Linear Model comparing ejection of spores (production) and germination of spores between species (fixed-effect), between thalli nested within species (mixed-effect) and null effect as an alternative hypothesis, using the Akaike Information Criterion (AIC).*

Model	k	rprod		Productivity				rger		Germination			
				LAP		SAP				LAG		SAG	
		AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc	AICc	ΔAICc
Mixed-effect	4	**−9**	0	**335**	0	**320**	0	**40**	0	**320**	0	**320**	0
Fixed-effect	3	7	16	347	12	332	12	54	14	332	12	332	11
Null	2	26	35	361	26	347	27	51	11	347	27	347	27

k, degrees of freedom; rprod, relative productivity; productivity (number of octet spore packs or plurisporic groups ejected by apothecia in 48 h) of large apothecia (LAP) and small apothecia (SAP); rger, relative germination; germination (percentage of spore packs with at least one germinated spore) of large apothecia (LAG) and small apothecia (SAG); AICc, Akaike information criterion corrected for small sample size; ΔAICc, difference in AICc.

in *P. quercina* (72·40 ± 3·49 vs. 14·30 ± 2·87). With increased sampling, the percentage germination was also shown to be independent of the apothecium size in both species (Fig. 3B & D).

Growth in different culture media

The subcultures were transferred to three different culture media to ensure growth until the formation of mycobiont aggregates in both species. The growth success in *P. carporrhizans* was 100% ($n = 90$), but was very low in *P. quercina* at only 7% ($n = 35$). For this reason, the growth kinetics of *P. carporrhizans* only were studied. *Parmelina carporrhizans* grew faster when subcultures were transferred to 0·2G-MY and CMA culture media (mean growth rates of 2·5 ± 0·02 and 1·5 ± 0·01 µm d^{-1} ($n = 30$), respectively),

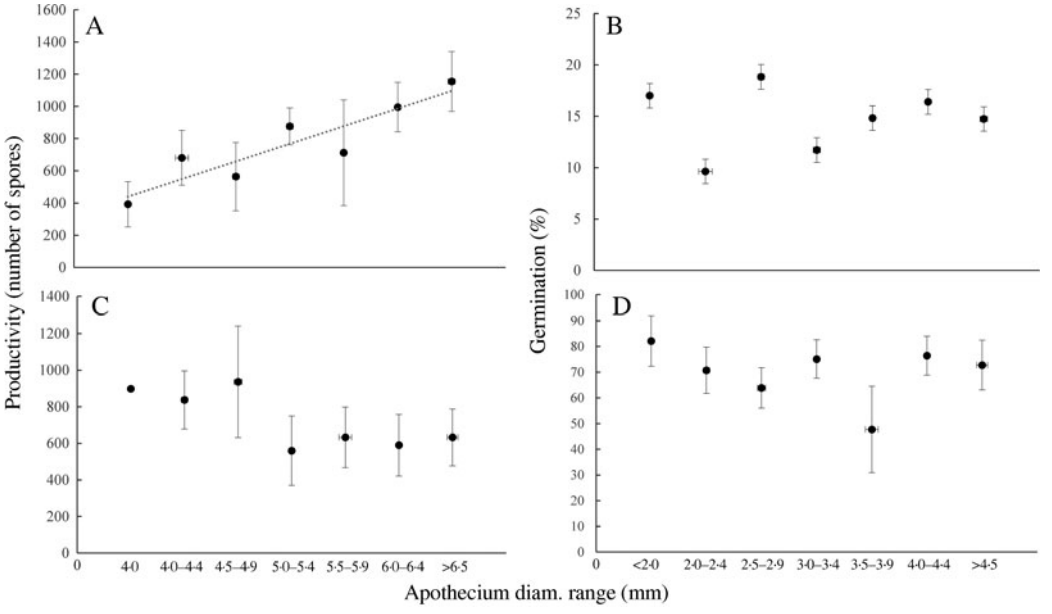


FIG. 3. Relationships between spore production, germination and apothecium size class in *Parmelina quercina* and *P. carporrhizans*. A & B, *P. quercina*; A, productivity, with regression line, $r^2 = 0.82$; B, percentage germination. C & D, *P. carporrhizans*; C, productivity; D, percentage germination. Mean values are plotted ±1 SEM ($n = 10$).

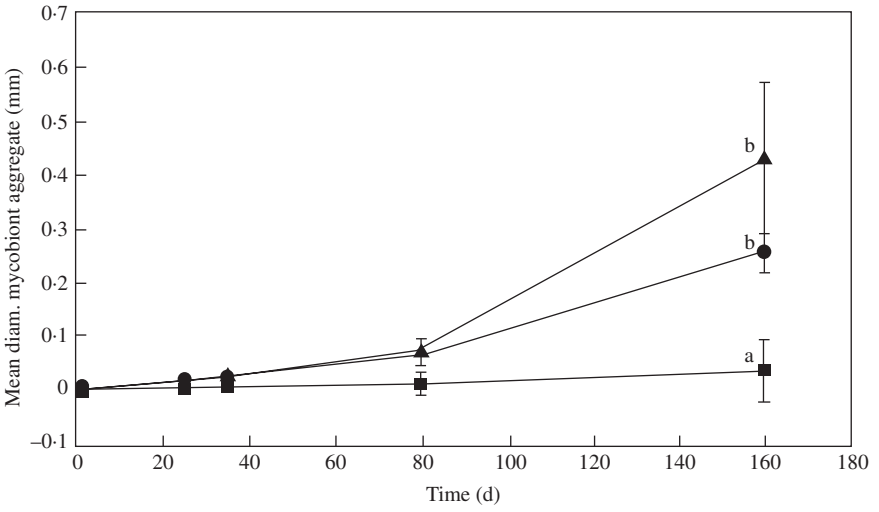


FIG. 4. Growth kinetics of *Parmelina carporrhizans* mycobiont aggregates over 160 days in different culture media. Values at 160 days with the same letter are not significantly different ($P < 0{\cdot}05$). ▲ = Corn Meal Agar; ● = 0·2% glucose malt-yeast extract; ■ = 3% glucose Lilly and Barnett's Medium. Mean values are plotted ± 1SEM ($n = 30$).

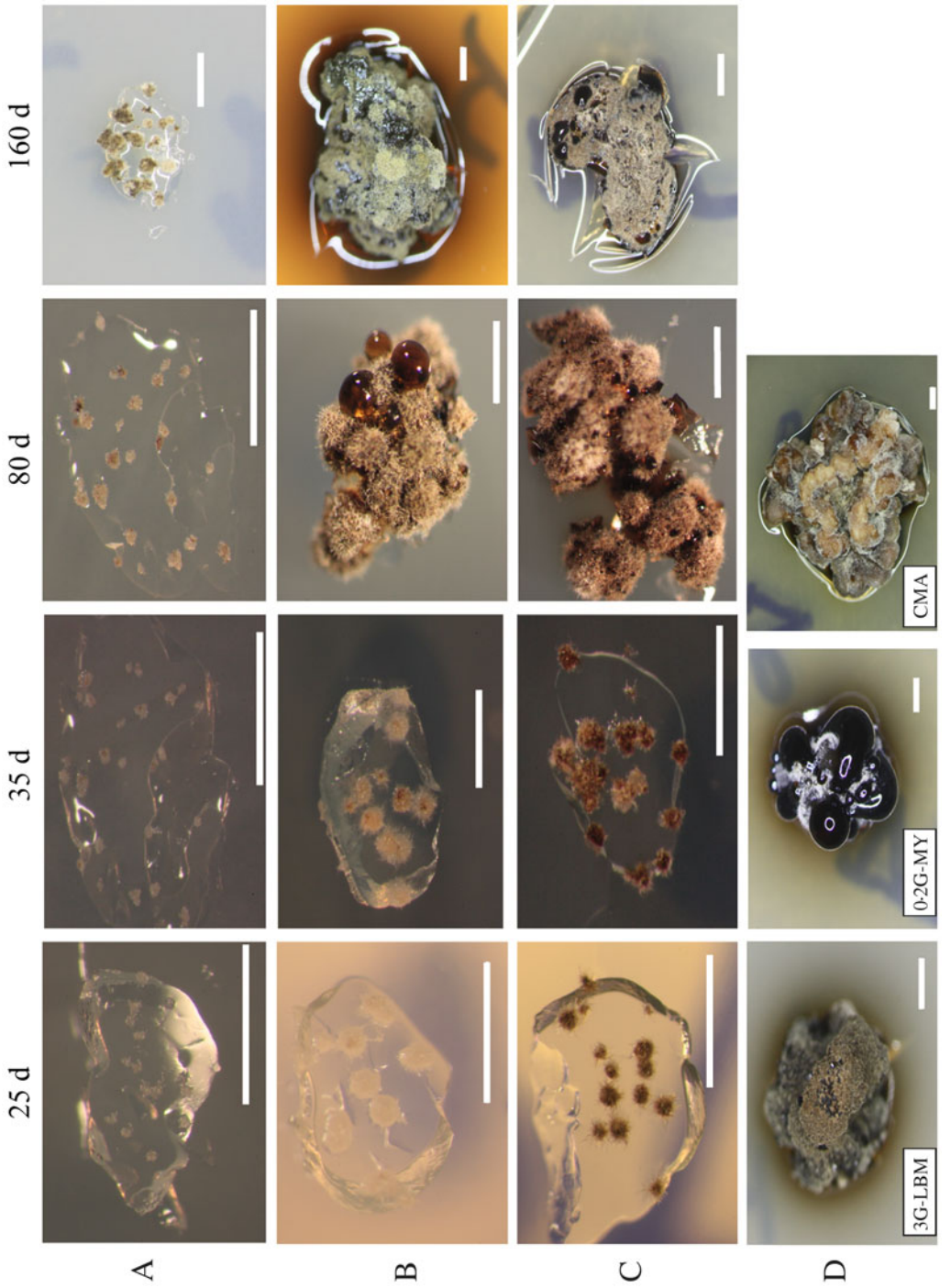
although its development was limited in 3G-LBM (Fig. 4).

The mycelium morphology of *P. carporrhizans* was similar in all media but the appearance of hyphal pigmentation was not. In 3G-LBM the pigmentation appeared after 80 days, whereas in 0·2G-MY media it appeared on day 25 and in CMA full pigmentation had occurred by this point (Fig. 5A–C). The cultures exhibited aerial as well as darkly pigmented hyphae forming the aggregates. After 20–30 days, they also started secreting dark brown liquid that crystallized on the surface. The cultures formed hollow aggregates which produced a halo of enhanced growth restricted to the area in contact with the culture medium. The morphology of *P. quercina* cultures was similar to those of *P. carporrhizans* but the growth of *P. quercina* cultures differed by being comparable in all three culture media (Fig. 5D).

Discussion

In *Parmelina carporrhizans* and *P. quercina*, ascospores within an ascus are ejected simultaneously as a plurisporic pack or aggregate. This pattern is consistent with those noted in other groups of lichen-forming fungi (Molina & Crespo 2000; Sangvichien *et al.* 2011; Molina *et al.* 2013). However, the patterns of spore ejection differed between the two species including, over time, in the percentage of apothecia actively ejecting and the number of spores ejected by each apothecium. Regarding the time of spore discharge, all apothecia of *P. quercina* discharged in the first 24 hours while *P. carporrhizans* required an extra day (48 hours) to expel spores from almost all apothecia.

Spore productivity is a phenotypic trait that can vary yield from a small number of spores to thousands of plurisporic aggregates, depending on several environmental and genetic factors such as species and individual variability, hydration state and collection season (Yamamoto *et al.* 1998; Sangvichien *et al.* 2011; Molina *et al.* 2013). In the present study it was demonstrated that some apothecia of both *P. quercina* and *P. carporrhizans* can produce more than 1000 plurisporic aggregates. Together with *Usnea complanata* (Müll. Arg.) Motyka (Sangvichien *et al.* 2011) and *Myelochroa entotheiochroa* (Hue) Elix & Hale (Yamamoto *et al.* 1998), these are among the highest spore productivity values recorded



so far within *Parmeliaceae*. In terms of absolute productivity in the two species, *P. quercina* ejected more plurisporic packs than *P. carporrhizans*, with significant differences between species. *Parmelina quercina* showed a positive correlation between productivity and apothecium size, suggesting that the ability to generate and expel spores is acquired as the apothecium develops (Molina *et al.* 1997), perhaps because of the greater surface area of the hymenium. However, in the case of *P. carporrhizans* no significant differences were observed in the productivity of apothecia of different sizes. In the present study apothecia of *P. carporrhizans* with a red hymenium smaller than 4 mm in diameter were not found. However, it would be worth collecting new material of a size below this threshold to test the hypothesis of larger apothecia equating to higher productivity in *P. carporrhizans*.

Germination in *P. carporrhizans* and *P. quercina* occurred seven days earlier than that of *Parmelia saxatilis* (L.) Ach. (Molina & Crespo 2000). Furthermore, both species showed bipolar germination and radial centrifugal growth of plurisporic aggregates, similar to other parmelioid species (Molina & Crespo 2000; Armaleo 1991). Morphology of the mycelia was similar to that described for *Parmelina* species by Honegger & Zippler (2007). The percentage of germination was much higher in *P. carporrhizans* than in *P. quercina.* In both species, this reproductive parameter was independent of apothecium size and it did not show any recognizable pattern. This suggests that percentage germination does not depend on the development of the apothecium (excluding those apothecia which didn't develop a hymenial red disc) but could be taxon specific. This trait probably relies on the quantity of resources that each species expends on reproduction but little is known about this with regard to lichens. In plants, theories about the relationship between seed size and number have been proposed to explain the advantages of having a few large compared to many small seeds. Seedlings from large-seeded species with more nutrients should be able to establish under a range of environmental conditions that would not be tolerated by seedlings from small-seeded species. However, variation in seed size persists, despite the apparent advantages of being large-seeded (Westoby *et al.* 2002). Easton & Kleindorfer (2008*b*), using *Frankenia* L. species as a model, concluded that smaller-seeded species had lower germination success at medium and high temperatures but greater success at low temperatures. Furthermore, these species delay germination until they have experienced several days of soil-water contact. Our data suggest that *P. carporrhizans* and *P. quercina* could be used as models to test the "number-size theory" in lichens. Other studies have shown that species with spore sizes differing by one order of magnitude can have differences in ejection of two orders of magnitude, with those species with a smaller spore size ejecting more spores (Morando *et al.* 2017). In contrast to the study of Morando *et al.* (2017), we used two species of the same genus, which indicates that the reproductive strategies of genetically proximal species can differ substantially. *Parmelina carporrhizans* ejected upward fewer spores of greater size (and therefore with more reserve nutrients) and had a higher level of germination success in several culture media, while *P. quercina* ejected a greater number of smaller spores with lower germination success. The behaviour of *P. quercina* might be comparable to that of *Frankenia* species (Easton & Kleindorfer 2008*b*), which release many small seeds with low germination success but which may be delaying germination until appropriate environmental conditions prevail.

FIG. 5. Morphology and pigmentation of plurisporic aggregates of *Parmelina carporrhizans* and *P. quercina* mycobiont growing on Bold's Basal Medium after transferring to three different culture media. A–C, *P. carporrhizans* at four different times. A, 3% glucose Lilly and Barnett's Medium (3G-LBM); B, 0·2% glucose malt-yeast extract (0·2G-MY); C, Corn Meal Agar (CMA). D, morphology of *P. quercina* cultures after 160 days on the three culture media. Scales = 1 mm.

Despite presenting results from only one locality per species, differences in spore discharge and spore germination are not considered to be due to variation within populations. Spore discharge and spore germination were also measured in *P. quercina* samples from La Carlota (Cordoba) and Cuevas del Valle (Avila) collected in autumn 2012 (data not shown), and these yielded similar results of high discharge and very low germination.

The differences observed in spore volume, spore ejection and spore germination between *Parmelina* species might reflect adaptation to environmental conditions as seen in *Frankenia* species, since *P. carporrhizans* inhabits a more stable habitat than *P. quercina*, especially with respect to humidity. The germination of *P. quercina* spores could be delayed until triggered by environmental factors such as temperature or humidity.

Finally, *P. carporrhizans* grew and developed very well on several different media. It has been shown repeatedly that growth rates for lichen-forming fungi, in general, depend on culture media (Cordeiro *et al.* 2004; Brunauer & Stocker-Wörgötter 2005; Brunauer *et al.* 2007; Deduke & Piercey-Normore 2015; Shanmugam *et al.* 2016). Even closely related species such as *P. quercina* and *P. carporrhizans* as well as others (Molina *et al.* 2002) might have very different germination and developmental requirements. These results could explain, at least partially, why these closely related and partially sympatric species inhabit different ecological niches, since their nutritional requirements appear to be different. While *P. carporrhizans* grows very well in the culture media used, the success of development in these same media is lower for *P. quercina*. This has also been described in other species from the same genus (Molina *et al.* 2002). The conditions that are optimal for *P. carporrhizans* growth may be less favourable for *P. quercina*. Hence, in nature, it is possible that there are environmental filters that limit the aposymbiotic development of the latter species in favour of the former (or vice versa). After completing the aposymbiotic phase of the cycle, the mycobiont can find a compatible photobiont and establish a successful symbiosis (Marshall 1996; Fedrowitz *et al.* 2012). The low reproductive success of *P. quercina* under laboratory conditions contrasts with the distribution and abundance of this species in Europe (Clerc & Truong 2008), possibly because our laboratory conditions are not optimal for *P. quercina* (Deduke & Piercey-Normore 2015).

Conclusions

Although both species showed similar development parameters (*in vitro*) to other species of *Parmeliaceae*, *Parmelina carporrhizans* and *P. quercina* showed significant differences in ontogenetic development during the aposymbiotic phase of their life cycles. Under laboratory conditions, *P. carporrhizans* took longer to release plurisporic groups and ejected significantly fewer spores upwards than *P. quercina*, although these spores were larger and had a much higher percentage germination. In *P. quercina*, the productivity was proportional to the size of the apothecium. However, the percentage germination seems independent of productivity in both species. In this regard, we propose that *P. carporrhizans* and *P. quercina* represent a good 'model system' to test "number-size theory" in lichens.

We thank Dr Marcos Méndez (URJC) for his insightful comments and Mrs. P. Mason for the language revision of the text. We also want to thank the Managing Editor for her invaluable help in improving this manuscript. This study was supported by the Ministerio de Ciencia e Innovacion, Spain (CGL2013-42498-P) and Santander-Universidad Complutense de Madrid (PR75/18-21605).

References

Ahmadjian, V. (1993) *The Lichen Symbiosis*. New York: John Wiley & Sons, Inc.

Alors, D., Dal Grande, F., Schmitt, I., Kraichak, E., Lumbsch, H. T., Crespo, A. & Divakar, P. K. (2014) Characterization of fungus-specific microsatellite markers in the lichen-forming fungus *Parmelina carporrhizans* (*Parmeliaceae*). *Applications in Plant Sciences* **2:** 1400081.

Alors, D., Dal Grande, F., Cubas, P., Crespo, A., Schmitt, I., Molina, M. C. & Divakar, P. K. (2017) Panmixia and dispersal from the Mediterranean Basin to Macaronesian Islands of a macrolichen species. *Scientific Reports* **7:** 40879.

Argüello, A., del Prado, R., Cubas, P. & Crespo, A. (2007) *Parmelina quercina* (*Parmeliaceae*, *Lecanorales*) includes four phylogenetically supported morphospecies. *Biological Journal of the Linnean Society* **91:** 445–467.

Armaleo, D. (1991) Experimental microbiology of lichens: mycelial fragmentation, a novel growth chamber, and the origins of thallus differentiation. *Symbiosis* **11:** 163–178.

Barton, K. (2013) *MuMin: Multi-Model inference model selection and model averaging based on information criteria (AICc and alike)*. R package version 1.15.6.

Beck, A., Divakar, P. K., Zhang, N., Molina, M. C. & Struwe, L. (2015) Evidence of ancient horizontal gene transfer between fungi and the terrestrial alga *Trebouxia*. *Organisms Diversity and Evolution* **15:** 235–248.

Brunauer, G. & Stocker-Wörgötter, E. (2005) Culture of lichen fungi for future production of biologically active compounds. *Symbiosis* **38:** 187–201.

Brunauer, G., Hager, A., Grube, M., Türk, R. & Stocker-Wörgötter, E. (2007) Alterations in secondary metabolism of aposymbiotically grown mycobionts of *Xanthoria elegans* and cultured resynthesis stages. *Plant Physiology and Biochemistry* **45:** 146–151.

Burnham, K. P. & Anderson, D. R. (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. New York: Springer-Verlag.

Clerc, P. & Truong, C. (2008) The non-sorediate and non-isidiate *Parmelina* species (lichenized ascomycetes, *Parmeliaceae*) in Switzerland – *Parmelina atricha* (Nyl.) P. Clerc reinstated in the European lichen flora. *Sauteria* **15:** 175–194.

Cordeiro, L. M. C., Iacomini, M. & Stocker-Wörgötter, E. (2004) Culture studies and secondary compounds of six *Ramalina* species. *Mycological Research* **108:** 489–497.

Dal Grande, F., Alors, D., Divakar, P. K., Bálint, M., Crespo, A. & Schmitt, I. (2014) Insights into intrathalline genetic diversity of the cosmopolitan lichen symbiotic green alga *Trebouxia decolorans* Ahmadjian using microsatellite markers. *Molecular Phylogenetics and Evolution* **72:** 54–60.

Deason, D. R. & Bold, H. C. (1960) Phycological studies. I. Exploratory studies of Texas soil algae. *University of Texas Publications* **6022:** 1–70.

Deduke, C. & Piercey-Normore, M. D. (2015) Substratum preference of two species of *Xanthoparmelia*. *Fungal Biology* **119:** 812–822.

Degtjarenko, P., Marmor, L., Torra, T., Lerch, M., Saag, A., Randlane, T. & Scheidegger, C. (2016) Impact of alkaline dust pollution on genetic variation of *Usnea subfloridana* populations. *Fungal Biology* **120:** 1165–1174.

Easton, L. C. & Kleindorfer, S. (2008*a*) Germination in two Australian species of *Frankenia* L., *F. serpyllifolia* Lindl. and *F. foliosa* J. M. Black (*Frankeniaceae*) – effect of seed age, seed mass, light, and temperature. *Transactions of the Royal Society of South Australia* **132:** 29–40.

Easton, L. C. & Kleindorfer, S. (2008*b*) Interaction effects of seed mass and temperature on germination in Australian species of *Frankenia* (*Frankeniaceae*). *Folia Geobotanica* **43:** 383–396.

Eaton, S., Zúñiga, C., Czyzewski, J., Ellis, C., Genney, D. R., Haydon, D., Mirzai, N. & Yahr, R. A. (2018) A method for the direct detection of airborne dispersal in lichens. *Molecular Ecology Resources* **18:** 240–250.

Fedrowitz, K., Kuusinen, M. & Snäll, T. (2012) Metapopulation dynamics and future persistence of epiphytic cyanolichens in a European boreal forest ecosystem. *Journal of Applied Ecology* **49:** 493–502.

Hawksworth, D. L., Blanco, O., Divakar, P. K., Ahti, T. & Crespo, A. (2008) A first checklist of parmelioid and similar lichens in Europe and some adjacent territories, adopting revised generic circumscriptions and with indications of species distributions. *Lichenologist* **40:** 1–21.

Honegger, R. & Zippler, U. (2007) Mating systems in representatives of *Parmeliaceae*, *Ramalinaceae* and *Physciaceae* (Lecanoromycetes, lichen-forming ascomycetes). *Mycological Research* **111:** 424–432.

Johansson, P., Rydin, H. & Thor, G. (2007) Tree age relationships with epiphytic lichen diversity and lichen life history traits on ash in southern Sweden. *Ecoscience* **14:** 81–91.

Johnson, J. B. & Omland, K. S. (2004) Model selection in ecology and evolution. *Trends in Ecology and Evolution* **19:** 101–108.

Lallemant, R. (1985) Le développement en cultures pures *in vitro* des mycosymbiotes des lichens. *Canadian Journal of Botany* **63:** 681–703.

Lilly, V. G. & Barnett, H. L. (1951) *Physiology of the Fungi*. New York: McGraw-Hill.

Marshall, W. A. (1996) Aerial dispersal of lichen soredia in the maritime Antarctic. *New Phytologist* **134:** 523–530.

McDonald, T. R., Dietrich, F. S. & Lutzoni, F. (2012) Multiple horizontal gene transfers of ammonium transporters/ammonia permeases from prokaryotes to eukaryotes: toward a new functional and evolutionary classification. *Molecular Biology and Evolution* **29:** 51–60.

Molina, M. C. & Crespo, A. (2000) Comparison of development of axenic cultures of five species of lichen-forming fungi. *Mycological Research* **103:** 595–602.

Molina, M. C., Stocker-Wörgötter, E., Türk, R. & Vicente, C. (1997) Axenic culture of the mycobiont of *Xanthoria parietina* in different nutritive media: effect of carbon source in spore germination. *Endocytobiosis and Cell Research* **12:** 103–109.

Molina, M. C., Crespo, A., Blanco, O. & Hawksworth, D. L. (2002) Molecular phylogeny and status of *Diploicia* and *Diplotomma*, with observations on *Diploicia subcanescens* and *Diplotomma rivas-martinezii*. *Lichenologist* **34:** 509–519.

Molina, M. C., Divakar, P. K., Zhang, N., González, N. & Struwe, L. (2013) Non-developing ascospores found in apothecia of asexually reproducing lichen-forming fungi. *International Microbiology* **16:** 145–155.

Molina, M. C., Divakar, P. K. & González, N. (2015) Success in the isolation and axenic culture of

Anaptychia ciliaris (*Physciaceae*, Lecanoromycetes) mycobiont. *Mycoscience* **56:** 351–358.

Morando, M., Favero-Longo, S. E., Carrer, M., Matteucci, E., Nascimbene, J., Sandrone, S., Appolonia, L. & Piervittori, R. (2017) Dispersal patterns of meiospores shape population spatial structure of saxicolous lichens. *Lichenologist* **49:** 397–413.

Nimis, P. L. (1993) *The Lichens of Italy: An Annotated Catalogue*. Torino: Museo Regionale di Scienze Naturali.

Núñez-Zapata, J., Divakar, P., Del-Prado, R., Cubas, P., Hawksworth, D. L. & Crespo, A. (2011) Conundrums in species concepts: the discovery of a new cryptic species segregated from *Parmelina tiliacea* (Ascomycota: *Parmeliaceae*). *Lichenologist* **43:** 603–616.

Núñez-Zapata, J., Alors, D., Cubas, P., Divakar, P. K., Leavitt, S. D., Lumbsch, H. T. & Crespo, A. (2017) Understanding disjunct distribution patterns in lichen-forming fungi: insights from *Parmelina* (*Parmeliaceae*, Ascomycota). *Botanical Journal of the Linnean Society* **184:** 238–253.

Öckinger, E., Niklasson, M. & Nilsson, S. G. (2005) Is local distribution of the epiphytic lichen *Lobaria pulmonaria* limited by dispersal capacity or habitat quality? *Biodiversity and Conservation* **14:** 759–773.

Ott, S. (1987) Sexual reproduction and developmental adaptations in *Xanthoria parietina*. *Nordic Journal of Botany* **7:** 219–228.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2016) *nlme: linear and nonlinear mixed effects models*. R package version 3.1–128.

R Development Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. URL: http:// www.R-project.org.

Sanders, W. B. (2014) Complete life cycle of the lichen fungus *Calopadia puiggari* (*Pilocarpaceae*, Ascomycetes) documented *in situ*: propagule dispersal, establishment of symbiosis, thallus development, and formation of sexual and asexual reproductive structures. *American Journal of Botany* **101:** 1836–1848.

Sanders, W. B. & Lücking, R. (2002) Reproductive strategies, relichenization and thallus development observed *in situ* in leaf-dwelling lichen communities. *New Phytologist* **155:** 425–435.

Sangvichien, E., Hawksworth, D. L. & Whalley, A. J. S. (2011) Ascospore discharge, germination and culture of fungal partners of tropical lichens, including the use of a novel culture technique. *IMA Fungus* **2:** 143–153.

Schauer, T. (1965) Ozeanische flechten in Nordalpenraum. *Portugaliae Acta Biologica* (*B*) **8:** 17–229.

Schielzeth, H. & Nakagawa, S. (2012) Nested by design: model fitting and interpretation in a mixed model era. *Methods in Ecology and Evolution* **4:** 14–24.

Schuster, G., Ott, S. & Jahns, H. M. (1985) Artificial cultures of lichens in the natural environment. *Lichenologist* **17:** 247–253.

Shanmugam, K., Srinivasan, M. & Hariharan, G. N. (2016) Developmental stages and secondary compound biosynthesis of mycobiont and whole thallus cultures of *Buellia subsororioides*. *Mycological Progress* **15:** 41.

Tibell, L. B. (1994) Distribution patterns and dispersal strategies of *Caliciales*. *Botanical Journal of the Linnean Society* **116:** 159–202.

Westoby, M., Falster, D., Moles, A., Vesk, P. & Wright, I. (2002) Plant ecological strategies: some leading dimensions of variation between species. *Annual Review of Ecology and Systematics* **33:** 125–159.

Yamamoto, Y., Kinoshita, Y., Takahagi, T., Kroken, S., Kurokawa, T. & Yoshimura, I. (1998) Factors affecting discharge and germination of lichen ascospores. *Journal of the Hattori Botanical Laboratory* **85:** 267–278.