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HOST MICROBE INTERACTIONS

Does the Reproductive Strategy Affect the Transmission and Genetic Diversity of Bionts in Cyanolichens? A Case Study Using Two Closely Related Species

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Abstract Observed levels of population genetic diversity are often associated with differences in species dispersal and reproductive strategies. In symbiotic organisms, the genetic diversity level of each biont should also be highly influenced by biont transmission. In this study, we evaluated the influence of the reproductive strategies of cyanolichen species on the current levels of population genetic diversity of bionts. To eliminate any phylogenetic noise, we selected two closely related species within the genus *Degelia*, which only differ in their reproductive systems. We sampled all known populations of both species in central Spain and genotyped the fungal and cyanobacterial components of lichen samples using DNA sequences as molecular markers. We applied population genetics approaches to evaluate the genetic diversity and population genetic structure of the symbiotic components of both lichen species. Our results indicate that fungal and cyanobiont genetic diversity is highly influenced by the reproductive systems of lichen fungus. We detected higher bionts genetic diversity values in the sexual species *Degelia plumbea*. By contrast, the levels of fungal and cyanobiont genetic diversity in the asexual species *Degelia atlantica* were extremely low (almost clonal), and the species shows a high specificity towards its cyanobiont. Our results indicate that reproduction by vegetative propagules, in species of the genus *Degelia*, favors vertical transmission and clonality, which affects the species' capacity for resources and competition, thereby limiting the species to restricted niches.

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Introduction

Current levels of population genetic diversity are often associated with dispersal and reproductive strategies of species. Thus, colonization, population persistence, and extinction probabilities are influenced by the reproductive and dispersal systems of species [6, 7, 55, 62]. Sexual reproduction is considered to be one of the most important processes for species survival because it produces new genotypes and tends to increase the likelihood of successful dissemination by long range dispersal [14]. Nevertheless, a large number of fungal organisms also exhibit asexual reproduction to produce genetically identical offspring and clones, which typically enhances population persistence by short range dispersal but reduces the opportunities for adaptive evolution [14]. However, the dispersal potential of sexual and asexual propagules is rather variable. In fact, in some fungal lineages the asexual propagules tend to be more effective for long range dispersal than sexual ones [21, 33, 62].

Among the wide range of fungal organisms, many species exhibit both sexual and asexual reproduction. Some lichen species are capable of only one form of reproduction, i.e., sexual or asexual [23]. Lichens are composed of a fungus in association with a photosynthetic symbiont (algae or cyanobacteria), which produces a stable thallus with a unique and complex structural association [2]. This symbiotic association allows the fungal and algal partners to colonize a range of habitats that would otherwise be uninhabitable by either of them [2]. Because they are symbiotic organisms, lichen reproduction is quite complex [23]. While photobionts reproduce mainly by cell division within the

lichen [22], mycobionts can reproduce either sexually or asexually in the lichen thallus. Lichen fungi can reproduce sexually by producing individual microscopic spores of the mycobiont, which must associate with a new algal or cyanobacterial partner, though a few exceptions exist where spores can be dispersed together with the photobiont [23]. Asexually, lichen fungi disperse either by means of asexual spores (conidia) or vegetative propagules (containing both symbionts), such as isidia or soredia [23, 59]. Vegetative propagules are often better suited to the establishment of new individuals [9]. The establishment of new lichens via a sexual mode implies independent partner dispersal, mycobiont germination in a suitable microhabitat, and making rapid contact with an appropriate photobiont [23, 54]. This form of reproduction favors horizontal photobiont transmission, where the photobiont cell does not derive from the parental thallus, therefore tends to increase the genetic diversity of both symbiotic components [8, 15, 59, 63]. Asexual reproduction often does not require re-lichenization, so vertical photobiont transmission is expected (photobiont cell derives from the parental thallus), with a strong biont population structure reducing the genetic diversity levels of both mycobiont and photobiont. However, this appears not to be the rule because recent reports have demonstrated that some lichen species with asexual reproductive strategies can also exhibit horizontal transmission of bionts with high levels of genetic diversity [21, 33, 37, 62].

Studying the patterns of genetic diversity in both bionts would allow infer the mode of biont transmission in lichens. Additionally, parallel studies of closely related species with different reproductive strategies (asexually via specialized vegetative propagules or sexually via spores) should provide insights into the importance of the reproductive mode for biont transmission. The link (cause and effect) between the reproductive mode and symbiont transmission was shown to be nonexistent when it was tested in green algal-lichen species [62]. A previous study using green algal-lichen showed that the reproductive mode affected mycobiont diversity but not algal diversity, suggesting successful horizontal photobiont transmission by asexual reproductive species. These observations suggest that dispersal via vegetative propagules does not involve the maintenance of partnerships in green algal-lichens [62]. However, similar studies have not been conducted using cyanolichens. A recent study [37] used a phylogenetic approach to infer that vertical biont transmission in cyanolichens was highly correlated with sterile species found in narrow ecological niches, but population genetic studies have not been conducted to compare fungal and cyanobiont diversity.

In this study, we evaluated the differences in the current levels of bionts genetic diversity within and among populations for two cyanolichen species with different dispersal and reproductive strategies, i.e., *Degelia atlantica* and *D. plumbea*, which co-occur and are recognized as vulnerable species in Spain [32]. Our goals were to test the importance of reproductive strategies for the genetic diversity of two threatened cyanolichens and to infer the importance of reproductive strategies for biont transmission. We hypothesized that species with sexual reproductive structures involving horizontal transmission should exhibit higher levels of biont diversity compared with asexual species that use vertical biont transmission. To address these objectives, we used DNA sequences as molecular markers and we applied population genetics approaches to evaluate the genetic diversity and population genetic structure of both symbiotic components.

Degelia atlantica and *D. plumbea* (*Pannariaceae*) are morphologically very similar cyanolichens with *Nostoc* species as photobiont. These species have foliose thalli with blue-grey lobes that form rosettes reaching 7 cm in diameter. *D. plumbea* typically produces numerous sexual structures (apothecia) and no specialized vegetative propagules (isidia). By contrast, *D. atlantica* produces marginal and laminal isidia whereas its production of apothecia is very rare or practically unknown in central Spain [11]. *D. plumbea* populations are distributed primarily in Mediterranean-Atlantic regions of Africa, Europe (Portugal to Norway and the Crimea), and the Macaronesian islands. *D. atlantica* has a narrower distribution and is more common in regions with a high oceanic influence [24–26]. In central Spain, both species occur in a very limited number of localities, specifically in humid valleys and ravines, and within well-preserved forests ranging from the meso- to supramediterranean belts (750–1,280 m altitude) [11, 32]. Both species usually occur together within the same forest and even on the same tree, so they were often intermixed within the studied area. However, *D. atlantica* is scarcer, being found in fewer localities than *D. plumbea.* Because of the limited number of populations in central Spain, both species are classified as regionally vulnerable [32].

Materials and Methods

Study Area

The study area was located in central Spain and it comprised a vast and mountainous region covered with different types of Mediterranean forests and shrublands (Fig. 1; Table 1). All previously reported localities of *D. plumbea* and *D. atlantica* were visited to locate populations and were included in this study if confirmed. In central Spain, populations of both species are found in forests of *Quercus ilex* ssp. *ballota*, *Quercus faginea*, *Quercus pyrenaica*, and *Pinus pinaster*, and shrubs such as *Arbutus unedo*. *D. plumbea* and *D. atlantica* also occur on understory shrubs, including

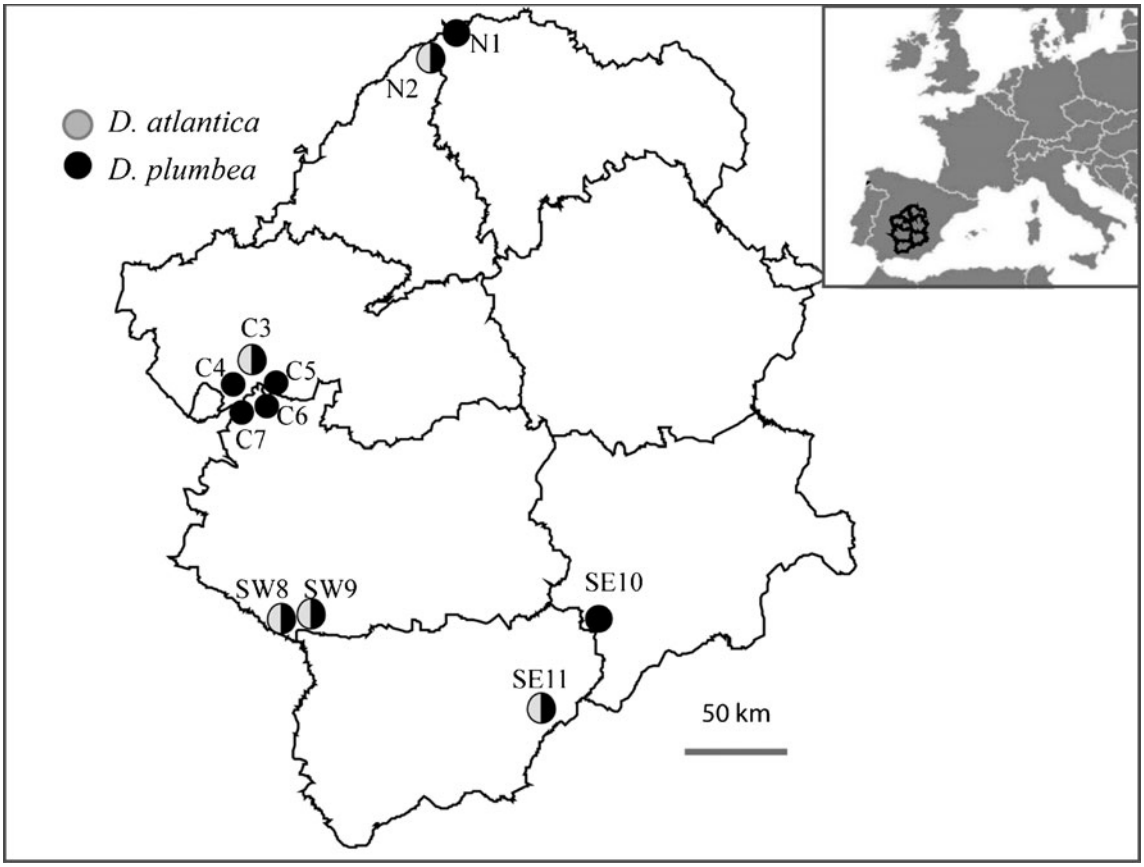

Fig. 1 Sampling locations and site identification codes for *D. plumbea* and *D. atlantica*. *Two-colored dots* indicate sites where both species were sampled

Table 1 Collecting localities for *D. plumbea* and *D. atlantica*

Pop. code	Locality	Host tree	Longitude	Latitude
North				
N1[b]	Guadalajara, S. Ayllón, S. Norte Guadalajar Natural Park	*Q. pyrenaica*	468113	4563754
N2[a]	Madrid, S. de Rincón, Hayedo de Montejo, N. Reserve[c]	*Q. pyrenaica*	458630	4551133
Central				
C3[a]	Toledo, Montes de Toledo, Cabañeros National Park	*Q. ilex* spp. *ballota*	363880	4380394
C4[b]	Ciudad Real, Montes de Toledo, Cabañeros National Park	*Q. ilex* spp. *ballota*	367232	4373563
C5[b]	Toledo, Montes de Toledo, Cabañeros National Park	*Q. ilex* spp. *ballota*	358583	4379545
C6[b]	Toledo, Montes de Toledo, Cabañeros National Park	*A. unedo*	363586	4367213
C7[b]	Toledo, Montes de Toledo, Cabañeros National Park	*A. unedo*	369865	4368818
Southwest				
SW8[a]	Ciudad Real, Sierra Madrona, Natural Park[d]	*Q. pyrenaica* + *Q. faginea*	389788	4256037
SW9[a]	Ciudad Real, Sierra Madrona, Natural Park[d]	*Q. pyrenaica*	382597	4255516
Southeast				
SE10[b]	Albacete, S. de Alcaraz, Calar del Mundo Natural park	*Q. ilex* spp. *ballota*	542801	4256898
SE11[a]	Jaén, S. de Segura, Cazorla Natural Park.	*A. unedo* + *I. aquifolium*	509507	4203051

Population code, locality (province), host tree, and geographic localization. Longitude and latitude coordinates correspond to the system WGS84

[a] Sites where *D. plumbea* and *D. atlantica* were sampled

[b] Sites where only *D. pumblea* individuals were sampled

[c] Sierra del Rincón biosphere reserve

[d] Valle de Alcudia y Sierra Madrona Natural Park

Phillyrea spp. and *Ilex aquifolium*. Most study localities are situated within protected areas including National Park, Natural Parks and habitat/species management areas. Individuals growing within a single forest were considered to constitute a population.

Collection Methods

A maximum of 20 individuals of each species were sampled per population. Small lichen fragments were collected from different trees to avoid sampling multiple ramets from individual clones. Five and 11 localities were sampled for *D. atlantica* and *D. plumbea*, respectively. A total of 67 *D. atlantica* and 174 *D. plumbea* individuals were sampled. Sampling was more extensive for *D. plumbea* because there is a higher number of populations of this species and the size of each population is greater than in *D. atlantica* (Table 1).

DNA Sequencing

Small samples were prepared from the fresh material by grinding in liquid nitrogen. The total genomic DNA was extracted using a DNeasy kit (Qiagen), according to the manufacturer's instructions. In order to select suitable fungal molecular markers, the sequence variability of seven widely used genes was first tested using three specimens per population per species. The genes tested were: ITS rDNA (internal transcribed spacer region), IGS rDNA (intergenic spacer), RPB1 (RNA polymerase II subunit 1), RPB2 (RNA polymerase II subunit 2), EF1-alfa (translation elongation factor 1), GPD (glyceraldehyde-3-phosphate dehydrogenase) and beta-tubulin. Because, sequence variability was only detected in ITS rDNA, RPB1, and RPB2, only these three genes were selected as molecular markers for the population genetic study.

The total DNA was used to PCR amplify the fungal nuclear ITS rDNA region (including the 5.8S gene), the partial fungal sequence of the protein coding the loci RPB1 (spanning region B–F) and RPB2 (regions 5–7), and the photobiont (*Nostoc*) *rbc*LX gene cluster (Rubisco large and small subunits, and chaperone gene). ITS rDNA was amplified using the primer pair ITS5-ITS4 [60], according to a published method [38]. The protein coding RPB1 was amplified using two new specifically designed primers, RPB1-891F (5′-GGGTAAGAGAGTTGACTTCTCG-3′)-RPB1-2450R (5′-TATCGTGCARTTCGTGTATGGAGAG-3′), with the following cycling conditions: initial denaturation at 95 °C for 5 min; 35×(95 °C for 1 min, 48 °C ramping up to the extension temperature at 0.2 °C/s, and 72 °C for 2 min); 72 °C for 10 min. The RPB2 fragment was amplified with the primers fRPB2-5F and fRPB2-7R [29] using the same conditions described for RPB1. For RPB2, internally nested PCR reactions were performed using 1 μL of the PCR product from the first reaction and the newly-designed internal specific primer RPB2-957R (5′-CTTGTCGAGAGATATCTAAAC-3′), with the same cycling protocol used for the first PCR. The *Nostoc rbc*LX partial region was amplified using the primer pair CW and CX [51], according to a published method [36]. Amplifications were performed using a Peltier thermal cycler (PTC-100). PCR products were subsequently purified using the enzymatic method Exo-sap-IT (USB Corporation, Cleveland, OH). The purified PCR products were submitted for sequencing to Macrogen Inc. (Korea). DNA sequences were compared with sequences in the GenBank database using the BLAST algorithm [3] to verify the amplification of the correct regions. The sequences were aligned manually using MacClade 4.01 [30].

Analytical Methods

Sequences were collapsed into haplotypes using the tool COLLAPSE in the program ALTER [20]. One sequence from each haplotype was submitted to Genbank (Tables 2 and 3). Evidence for linkage disequilibrium between pairs of loci was assessed using the Recombination Detection Program v. 3.44 (RDP3) [31]. The genetic diversity among populations was compared using the sequence polymorphism statistics as nucleotide diversity (π, the average nucleotide substitutions per site among sequences; [34]) and haplotype diversity (*Hd* [34]), the probability of two randomly chosen haplotypes being different in a sample), which were calculated using DnaSP 5.10 [50]. The relationship between photobiont and fungal diversity was tested by building a simple linear model. The influence of lichen species (= reproductive system) on the genetic diversity was tested using a nonparametric Kruskal-Wallis test in the R package. Due to our unbalanced sample size, we applied the significance level of p value=0.1. Analysis of molecular variance (AMOVA) [16] for each locus was used to assess the population differentiation, which was conducted in ARLEQUIN [17]. Populations were grouped into four protected area units to assess the population genetic structure. The units of protected areas were defined according to the unit proximity and connectivity (Fig. 1). Thus, we defined four geographic units, which are referred to as N, C, SW, and SE (north, central, southwest and southeast). To understand phylogenetic relationships among haplotypes, a median-joining network was constructed [5] based on parsimony criteria as implemented in the software Network 4.2.1 [5]. All the analyses were performed for each locus and symbiont.

Results

The three fungal loci and the photobiont locus were polymorphic. The program RDP3 detected no recombination

Table 2 Distribution of haplotypes and base substitutions in the internal transcribed spacer region (rDNA ITS), DNA-directed RNA polymerase II subunit 1 and 2 (RPB1 and RPB2) of the mycobiot of *Degelia plumbea* and *D. atlantica*

Locus	ITS						RPB1						RPB2			
Position	1	1	2	3	4	5	1	1	2	6	7		1	1	2	
	6	6	0	0	6	1	9	2	8	3	5	5	4	7	2	
	5	6	9	5	5	9	4	9	4	7	8	9	7	7	5	
Site number	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	
Consensus	C	T	A	A	G	C	C	G	A	C	A	T	T	T	C	
D. plumbea																
Haplotype							Accession[b]	Haplotype					Accession[b]	Haplotype			Accession[b]
ITS-1(164)[a]	.	.	.	.	.	.	JX126704	RPB1-1 (103)	.	.	.	.	JX126695	RPB2-1 (99)	.	.	JX126702
ITS-2 (3)	.	.	T	.	.	.	JX126705	RPB1-2 (5)	.	.	.	C	JX126696	RPB2-2 (53)	C	C	T	JX126703
ITS-3 (3)	.	.	.	G	.	.	JX126706	RPB1-3 (29)	.	A	.	T	JX126697				
ITS-4 (1)	.	.	.	.	A	.	JX126707	RPB1-4 (12)	.	A	G	T	JX126698				
ITS-5 (3)	T	.	.	.	.	.	JX126708	RPB1-5 (1)	.	A	.	T	JX126699				
D. atlantica																
ITS-6 (67)	.	C	.	.	.	G	JX126709	RPB1-6 (64)	A	A	.	.	JX126700	RPB2-2 (27)	C	C	T	JX126703
														RPB2-3 (36)	.	C	T	JX126701

[a] Haplotype (frequency).

[b] GenBank accession number

events among the three fungal loci. Tables 2 and 3 show the fungal ITS, RPB1, RPB2, and photobiont *rbc*LX nucleotide polymorphism distributions. Not all three fungal loci could be obtained for each of the samples. Hence the total number of sequences varies for ITS, RPB1, and RPB2. Five ITS and RPB1 haplotypes and two RPB2 haplotypes were detected in *D. plumbea* samples (Table 2). Two RPB2 haplotypes were identified in *D. atlantica*, although sequence variation was not present in the ITS and RPB1 loci (only one haplotype per locus). *D. atlantica* and *D. plumbea* did not share ITS and RPB1 haplotypes, although they shared one RPB2 haplotype (RPB2-2; Tables 2 and 4).

The genetic diversity values were variable among loci across populations of *D. plumbea* (Table 5 and Fig. 2). Most populations with single ITS haplotypes had more than one RPB1 or RPB2 haplotype (Table 4). For instance, five out of seven populations with single ITS haplotypes had two RPB2 haplotypes and three had several RPB1 haplotypes. In the case of *D. atlantica* all populations had similar genetic diversity (Table 5). To compare the genetic diversity among species, a concatenated dataset of the three loci (1,831 bp in length) was constructed using 146 *D. plumbea* samples and 63 *D. atlantica* samples (Table 5). For the sexual species (*D. plumbea*), a total of 12 multi-loci genotypes were identified; 6 were unique to individual populations, and 6 occurred in more than 1 population (results not shown). Only two multi-loci genotypes were identified in the asexual species (*D. atlantica*), both of which occurred in more than one population. The average genetic diversity (*Hd*) was 0.4388 for *D. plumbea* and 0.1428 for *D. atlantica*.

Forty-eight photobiont sequences were analyzed for *D. atlantica* and 125 for *D. plumbea*. A total of nine haplotypes were distinguished, two of which were shared by *D. atlantica* and *D. plumbea* individuals, and seven that were unique to *D. plumbea* (Table 4). In *D. plumbea* populations, no relationship was detected between photobiont and fungal diversity (R^2=0.097, P>0.1). A relationship between fungal species (*i.e.* reproductive strategy) and photobiont genetic diversity was identified (Fig. 3). All *D. atlantica* populations lacked photobiont genetic diversity (*Hd*=0), whereas the average *Nostoc* genetic diversity (*Hd*) in *D. plumbea* was 0.3690.

The extent of population differentiation in the two species was quantified by evaluating a geographic subdivision scenario (corresponding to the units of protected areas, i.e., N, C, SE, SE) using an AMOVA based on each locus and species. For *D. plumbea* the results for a single AMOVA for ITS, RPB2, and the concatenated datasets indicated the absence of significant differentiation among geographic units (Table 6). However, AMOVA detected significant differences for RPB1 among protected area units (Table 6). The *Fst* values for *D. plumbea* DNA coding regions (RPB1

Table 3 Distribution of haplotypes and base substitutions in the gene cluster RuBisCo large and small subunits, and chaperone

Locus	rbcLX (Nostoc)																																
Position						1	1	1	1	1	2	2	3	3	3	4	4	4	4	4	5	5	5	5	5	5	5	5	5	5	6		
		1	2	7	8	0	3	4	7	9	3	5	4	5	7	1	3	7	8	8	0	1	2	4	5	6	7	7	8	9	1		
	2	1	7	7	0	4	4	0	3	1	5	0	7	3	5	0	6	3	2	8	0	9	4	5	1	3	5	8	5	8	2		
site number										1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3		
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1		
Consensus	C	G	C	C	G	C	T	T	A	G	G	C	T	A	C	T	A	C	C	C	C	A	T	C	A	A	G	C	T	A	G		
D. plumbea																																	
Haplotype																																Accession[b]	
rbclx-1 (75) [a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.	JX126710	
rbclx-2 (7)	T	A	T	.	A	.	C	C	G	A	.	.	C	G	T	G	G	T	T	G	T	G	C	.	G	G	C	.	A	G	C	JX126711	
rbclx-3 (3)	.	.	.	.	.	T	C	C	.	A	.	T	C	.	T	.	G	.	.	.	.	G	C	.	G	G	C	.	A	G	C	JX126712	
rbclx-4 (7)	T	A	T	.	.	.	C	C	.	.	.	T	C	G	T	G	G	.	.	G	.	G	C	.	G	G	C	.	A	G	C	JX126713	
rbclx-5 (15)	T	A	T	.	.	.	C	C	.	.	.	T	C	.	T	.	G	.	.	.	.	G	C	T	.	G	.	.	.	.	C	JX126714	
rbclx-6 (2)	T	A	.	.	.	.	C	C	G	A	.	.	C	G	T	G	G	T	T	G	T	G	C	.	G	G	C	.	A	G	C	JX126715	
rbclx-7 (14)	T	A	T	.	.	.	C	C	.	A	.	T	C	.	T	.	G	.	.	.	.	G	C	T	.	G	.	.	.	.	C	JX126716	
rbclx-8 (1)	T	A	T	T	.	.	C	C	.	.	A	.	C	G	T	G	G	T	.	G	T	G	C	.	G	G	C	.	A	G	C	JX126717	
rbclx-9 (1)	T	A	T	.	.	.	C	C	.	.	.	.	C	G	T	G	G	T	.	G	T	G	C	T	G	G	C	T	A	G	C	JX126718	
D. atlantica																																	
rbclx-1 (38)	.	.	.	.	.	.	.	.	.	.		.	.	.	.		.	.	.	.		.	.	.	.	.	.	.	.	.	.	JX126710	
rbclx-4 (10)	T	A	T	.	.	.	C	C	.	.	.	T	C	G	T	G	G	.	.	G	.	G	C	.	G	G	C	.	A	G	C	JX126713	

Gene (*rbc*LX) of the photobiont of *Degelia plumbea* and *D. atlantica*

[a] Haplotype (frequency)

[b] GenBank accession number

Table 4 Haplotype occurrence of the three mycobiont genes and a photobiont gene of *Degelia plumbea* and *D. atlatica* on each of the localities sampled

	Population										
		North		Centro					South west		South East	
	Locality	N1	N2	C3	C4	C5	C6	C7	SW8	SW9	SE10	SE11
Locus	Haplotype											
D. plumbea												
Mycobiont												
ITS	ITS-1	17	6	14	16	17	19	20	17	9	11	18
	ITS-2	0	2	1	0	0	0	0	0	0	0	0
	ITS-3	0	0	0	0	0	0	0	3	0	0	0
	ITS-4	1	0	0	0	0	0	0	0	0	0	0
	ITS-5	0	3	0	0	0	0	0	0	0	0	0
RPB1	RPB1-1	0	9	11	11	8	10	19	19	9	0	7
	RPB1-2	0	0	3	0	2	0	0	0	0	0	0
	RPB1-3	0	2	1	0	2	7	0	0	0	8	9
	RPB1-4	12	0	0	0	0	0	0	0	0	0	0
	RPB1-5	0	0	0	0	0	0	0	0	0	0	1
RPB2	RPB2-1	0	3	6	12	10	17	18	19	4	1	9
	RPB2-2	11	8	10	0	3	0	1	0	5	8	7
Photobiont												
*rbc*LX	*rbc*LX-1	3	5	9	6	10	15	6	10	9	2	0
	*rbc*LX-2	0	0	0	3	0	0	4	0	0	0	0
	*rbc*LX-3	0	0	0	3	0	0	0	0	0	0	0
	*rbc*LX-4	0	0	0	0	2	0	0	0	0	0	5
	*rbc*LX-5	0	0	0	0	0	0	4	0	0	6	5
	*rbc*LX-6	0	0	0	0	0	0	2	0	0	0	0
	*rbc*LX-7	8	3	0	0	0	0	0	0	0	3	0
	*rbc*LX-8	0	0	0	0	0	0	0	0	0	0	1
	*rbc*LX-9	0	0	0	0	0	0	0	0	0	0	1
D. atlantica												
Mycobiont												
ITS	ITS-6	–	10	10	–	–	–	–	19	12	–	16
RPB1	RPB1-6	–	10	10	–	–	–	–	19	10	–	15
RPB2	RPB2-2	–	5	0	–	–	–	–	7	6	–	9
	RPB2-3	–	3	10	–	–	–	–	12	4	–	7
Photobiont												
*rbc*LX	*rbc*LX-1	–	8	10	–	–	–	–	10	10	–	0
	*rbc*LX-4	–	0	0	–	–	–	–	0	0	–	10

and RPB2) were higher than the ITS loci *Fst* values. For *D. atlantica*, the *Fst* values calculated from RPB2 indicated little differentiation among populations and geographic units (Table 6). *D. atlantica* and *D. plumbea* shared photobiont haplotypes, so an AMOVA was performed for the entire *rbc*LX dataset (*D. plumbea* and *D. atlantica*), with protected area units and lichen species as the grouping criteria. The AMOVA results for the photobiont revealed significant differentiation among protected area units (Table 6). The phylogenetic relationship among the haplotypes of *D. atlantica* and *D. plumbea* are represented in the haplotype network in Fig. 4.

Discussion

Fungal Sequence Polymorphism and Diversity

High levels of genetic similarity were detected between the mycobionts of *D. atlantica* and *D. plumbea*. For the ITS

Table 5 Mycobiont and photobiont diversity statistics for 11 populations of *Degelia plumbea* and 5 populations of *D. atlantica* by individual locus and concatenated dataset

Mycobiont																			
	ITS				RBB1				RPB2				Concatenated				Photobiont *rbc*LX			
Population	n	h	Hd	π	n	h	Hd	π	n	h	Hd	π	N	h	Hd	π	n	h	Hd	π
Degelia plumbea																				
North																				
N1	18	2	0.1100	0.0002	12	1	0.0000	0.0000	11	1	0.0000	0.0000	11	1	0.0000	0.0000	11	2	0.4360	0.0100
N2	11	3	0.6546	0.0014	11	2	0.3270	0.0008	11	2	0.4363	0.0027	10	4	0.8000	0.0014	8	2	0.5350	0.0131
Central																				
C3	15	2	0.1333	0.0003	15	3	0.4470	0.0008	16	2	0.5000	0.0031	15	5	0.7810	0.0019	9	1	0.0000	0.0000
C4	16	1	0.0000	0.0000	11	1	0.0000	0.0000	12	1	0.0000	0.0000	11	1	0.0000	0.0000	12	3	0.6810	0.0199
C5	17	1	0.0000	0.0000	12	3	0.5454	0.0011	13	2	0.3846	0.0024	12	4	0.7273	0.0010	12	2	0.3000	0.0098
C6	19	1	0.0000	0.0000	17	2	0.5147	0.0013	17	1	0.0000	0.0000	17	2	0.5140	0.0006	15	1	0.0000	0.0000
C7	20	1	0.0000	0.0000	19	1	0.0000	0.0000	19	2	0.1053	0.0007	19	2	0.1052	0.0002	16	4	0.7660	0.0210
Southwest																				
SW8	20	2	0.2684	0.0005	19	1	0.0000	0.0000	19	1	0.0000	0.0000	19	2	0.2807	0.0002	10	1	0.0000	0.0000
SW9	9	1	0.0000	0.0000	9	1	0.0000	0.0000	9	2	0.5555	0.0035	9	2	0.5500	0.0009	9	1	0.0000	0.0000
Southeast																				
SE10	11	1	0.0000	0.0000	8	1	0.0000	0.0000	9	2	0.2200	0.0014	8	2	0.2500	0.0004	11	3	0.6454	0.0080
SE11	18	1	0.0000	0.0000	17	3	0.5820	0.0014	16	2	0.5250	0.0033	15	5	0.8190	0.0015	12	4	0.6960	0.0092
Total	174	5	0.1113	0.0002	150	5	0.4868	0.0013	152	2	0.4572	0.0028	146	12	0.6880	0.0014	125	9	0.3431	0.0075
Average			0.1060	0.0002			0.2196	0.0004			0.2478	0.0015			0.4388	0.0007			0.3690	0.0082
Degelia atlantica																				
North																				
N2	10	1	0.0000	0.0000	10	1	0.0000	0.0000	8	2	0.5351	0.0011	8	2	0.1781	0.0004	8	1	0.0000	0.0000
Central																				
C3	10	1	0.0000	0.0000	10	1	0.0000	0.0000	10	1	0.0000	0.0000	10	1	0.0000	0.0000	10	1	0.0000	0.0000
Southwest																				
SW8	19	1	0.0000	0.0000	19	1	0.0000	0.0000	19	2	0.4910	0.0010	19	2	0.1642	0.0004	10	1	0.0000	0.0000
SW9	12	1	0.0000	0.0000	10	1	0.0000	0.0000	10	2	0.5330	0.0011	10	2	0.1781	0.0004	10	1	0.0000	0.0000
Southeast																				
SE11	16	1	0.0000	0.0000	15	1	0.0000	0.0000	16	2	0.5820	0.0011	16	2	0.1940	0.0004	10	1	0.0000	0.0000
Totall	67	1	0.0000	0.0000	64	1	0.0000	0.0000	63	2	0.4987	0.0011	63	2	0.1662	0.0004	48	2	0.3316	0.1070
Average			0.0000	0.0000			0.0000	0.0000			0.4282	0.0009			0.1428	0.0003			0.0000	0.0000

n sample size, *h* number of haplotypes, *Hd* haplotype diversity, π nucleotide diversity

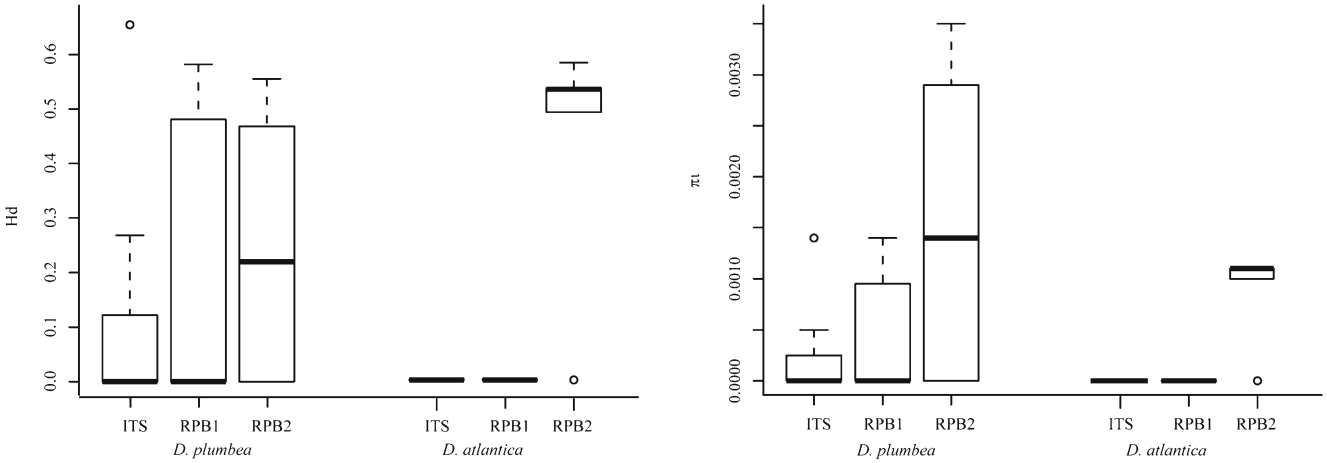

Fig. 2 Box plots of haplotype diversity (*Hd*) and nucleotide diversity (π) based on the locus of the mycobiont in the populations of *D. plumbea* and *D. atlantica*. The *thick central horizontal line in the box plot* represents the median. Each *box* encompasses 50 % of the samples, each *bracket* includes 95 % of the samples, and *individual points* represent outliers

region (544 bp) and RPB1 fragment (804 bp), only two pairwise differences were identified between the most common ITS and RPB1 haplotypes of *D. plumbea* and the unique ITS and RPB1 *D. atlantica* haplotypes (Table 2, Fig. 4). Previous studies, that assessed genetic diversity in lichens with the same three molecular markers used in this study, detected higher levels of polymorphism [13, 18, 27, 38, 48, 62]. The ITS gene usually has higher pairwise differences within and among species than that observed in this study. A pairwise difference among ITS haplotypes has frequently been attributed to differences at the species level [18, 27, 38, 48, 62]. Thus, our results may suggest that *D. atlantica* and *D. plumbea* do not correspond to two distinct species. However, there was no overall evidence of gene flow between the two species. The ITS and RPB1 haplotypes were not shared by *D. plumbea* and *D. atlantica* individuals (Fig. 4), although the species are sympatric (i.e., they were distributed in the same locality or even on the same tree). Therefore, our results show that reproductive isolation was present and that the current recognition of two distinct species should be maintained. Nevertheless, the species shared an RPB2 haplotype, which could indicate a recent speciation process where incomplete sorting has occurred. Although the species exhibited high levels of genetic similarity, *D. plumbea* and *D. atlantica* phenotypic characteristics are sufficient to diagnose each taxon (see introduction).

The differences in the levels of genetic diversity between the mycobionts of *D. plumbea* and *D. atlantica* (Fig. 2) indicate that the genetic diversity of these species is highly influenced by the reproductive system, which is consistent with a previous study of green algal-lichen species [62]. Our results indicated higher values of genetic diversity in *D.*

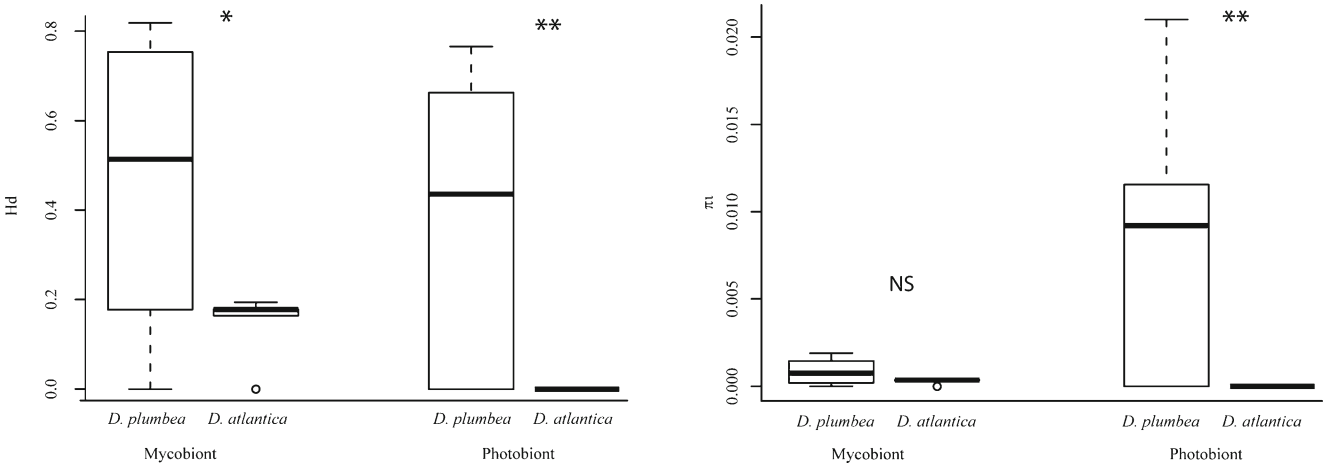

Fig. 3 Box plots of haplotype diversity (*Hd*) and nucleotide diversity (π) for the mycobiont and photobiont in populations of *D. plumbea* and *D. atlantica*. The mycobiont diversity values were obtained from a combined dataset of the three loci. The *thick central horizontal line in the box plot* represents the median. Each *box* encompasses 50 % of the samples, each *bracket* includes 95 % of the samples, and *individual points* represent outliers. Significance values $*P<0.1$, $**P<0.05$, and NS=$P>0.1$ (nonparametric Kruskal-Wallis test)

Table 6 Analysis of molecular variance (AMOVA) partitioned among regions (F_{CT}), among population within regions (F_{SC}) and within populations (F_{ST})

	D. plumbea			*D. atlantica*		
	F_{CT}	F_{SC}	F_{ST}	F_{CT}	F_{SC}	F_{ST}
Mycobiont						
ITS	0.0493	**0.1321**	**0.1280**	0.0000	0.0000	0.0000
RPB1	**0.2718**	**0.4701**	**0.1749**	0.0000	0.0000	0.0000
RPB2	0.2676	**0.3917**	**0.5545**	0.1268	**0.0447**	**0.1659**
Combined	0.1613	**0.4971**	**0.5783**	0.0401	**0.0109**	**0.05**
Photobiont						
*rbc*LX	**0.4083**	**0.2871**	**0.5782**			

Fixation indices are indicated and values in bold indicates statistically significant ($P<0.05$)

plumbea (Figs. 2 and 3), which is a sexual species, suggesting the greater effectiveness of sexual reproduction in the generation of new genotypes compared with vegetative reproduction. However, two *D. plumbea* populations were monomorphic at three loci (Table 5 and Fig. 2). Overall, the levels of genetic diversity for the three loci in *D. plumbea* were markedly lower than levels reported for other threatened [27] or common lichen species [28, 48, 57, 62, 63]. If sexual reproduction facilitates genetic exchange and the generation of novel alleles, we expected an increased number of alleles per locus at the local and regional levels in *D. plumbea*. The low levels of genetic diversity detected in *D. plumbea* relative to other lichens may be a result of demographic (range contraction and habitat fragmentation) or genetic processes (genetic bottlenecks, founder effects, and drift) that were not tested in this study.

The fungal components of the five *D. atlantica* populations had a high level of clonality. Clonal reproduction typically enhances population persistence via short range dispersal, but it also reduces genetic variability and affects the species' capacity for resources and competition, which also increases the probability of local extinction [14]. The low number of *D. atlantica* populations and individuals could be a consequence of a bottleneck and demographic

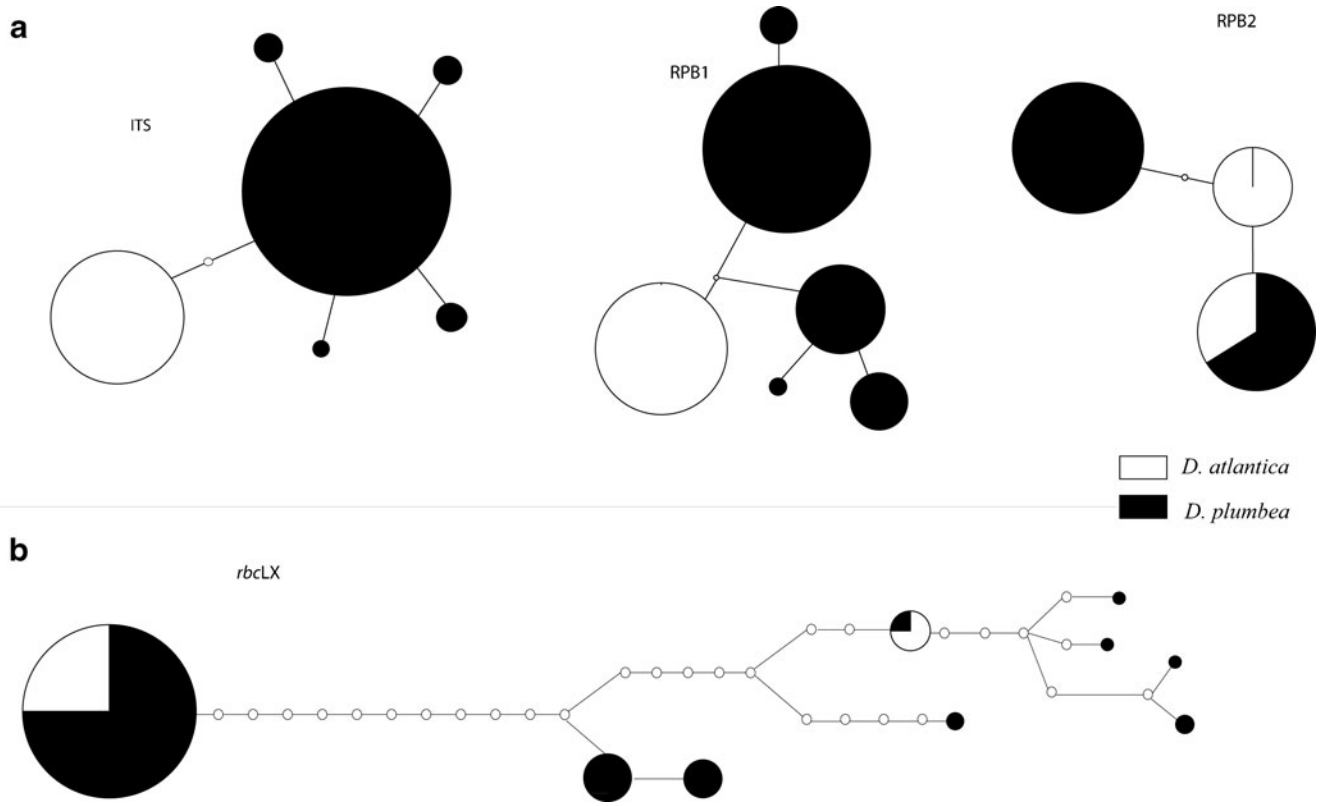

Fig. 4 Median-joining haplotype networks for (**a**) mycobiont ITS, RPB1 and RPB2 loci and (**b**) photobiont *rbc*LX locus within *D. plumbea* and *D. atlantica* in central Spain. Species are color coded in all networks

factors, which may have reduced its genetic diversity levels and its capacity to adapt to habitat modifications.

Photobiont Polymorphism and Diversity

In terms of the photobiont, our results showed that *D. plumbea* and *D. atlantica* shared *Nostoc* haplotypes, where *rbc*LX-1 was the most common haplotype detected in most localities (Table 4, Fig. 4). It is widely accepted that a single *Nostoc* haplotype can be associated with multiple species of lichen-forming fungi, sometimes from distantly related lineages [15, 36, 37, 46, 47]. In the case of *D. plumbea* (sexual species) the results showed that the mycobiont could associate with multiple *Nostoc* haplotypes, producing a generalist pattern and effective horizontal biont transmission. Generalist patterns should allow lichen species to extend their ecological range if the photobionts vary in their ecological requirements [35, 63]. Symbiont generalist patterns have been reported in cyanolichens [46, 61] and other symbiotic associations, including coral–algal symbioses [42, 49], mycorrhizae [43], and fungus-farming insects [1, 53].

Our results demonstrate that reproductive strategies significantly affect *Nostoc* genetic diversity in *D. plumbea* and *D. atlantica* (Fig. 3). Increased levels of photobiont (*Nostoc*) diversity were observed in *D. plumbea* compared with *D. atlantica*, indicating that sexual reproduction promotes horizontal transmission, whereas vegetative reproduction favors the clonal transmission of bionts in lichens of the genus *Degelia*. A generalist pattern was detected in *D. plumbea*, whereas *D. atlantica* expressed a relatively specific pattern where it associated with only two photobiont haplotypes (Table 4). In general, in species that lack vegetative propagules, such as *D. plumbea*, the bionts must be dispersed independently (horizontal photobiont transmission), and a re-lichenization process must occur, minimizing the specialization on one photobiont. In the case of *D. plumbea*, that necessary relichenization likely occurs with *Nostoc* dispersed by asexual species, which are recognized as important dispersers of the photobionts [46, 47]. This, therefore, explains why *D. plumbea* and *D. atlantica* share predominantly one *Nostoc* haplotype.

In the case of asexual species that co-disperse both symbionts, a vertical photobiont transmission with narrower photobiont range might be expected. However, this has not always been observed. Previous reports have shown that horizontal biont transmission is also frequent in asexual lichen species, especially in those species with broad ecological ranges [37, 62]. Strict vertical transmission tends to reduce the genetic diversity of bionts, thereby affecting the capability of lichen species to colonize new habitats or niches [4, 37, 58, 61–63]. The low, almost null, diversity of the photobionts in populations of *D. atlantica* indicates that this species is highly specific in its selection of photobionts (narrow photobiont spectrum). Because narrow photobiont spectrum tends to limit the ecological range of a lichen species [58, 61–63], we hypothesize that the low photobiont diversity of *D. atlantica* might be one of the factors that affect the population number and population size of this species in central Spain.

Fungal Genetic Structure

A clonal population structure was detected in asexual *D. atlantica* populations from central Spain. Identical multiloci genotypes were observed in individuals that were geographically separated by more than 350 km (N2 and SW9). This could suggest effective long range dispersal propagules. However, vegetative propagules are better suited to the establishment of new individuals within short distances [9, 52]; therefore, it is not possible to assert that the observed clonality resulted from recent long distance dispersal. The genetic structure of *D. atlantica* populations is probably the result of demographic factors such as range contraction or habitat fragmentation. By contrast, the AMOVA detected a significant population differentiation in *D. plumbea* (Table 6) with some significant structure among the geographic units (only RPB1 data set). This suggested long distance dispersal limitations in *D. plumbea* (a fertile species with sexual spores). Similar results indicating a significant spatial structure and dispersal limitations have been reported for other lichen species [12, 56]. However, most studies have demonstrated that the propagules of lichen-forming fungi are widely and effectively dispersed over small and large spatial scales, and therefore populations do not exhibit spatial structures [9, 10, 27, 38, 39, 44, 45, 57].

Photobiont Genetic Structure

Contrasting patterns of genetic differentiation were found for the photobiont of *D. plumbea* and *D. atlantica*. Similar to the mycobiont, the photobiont associated with *D. atlantica* showed no population differentiation. With the exception of individuals in population SE11, most of the *D. atlantica* individuals (80 %) were associated with the common haplotype (*rbc*LX-1, Table 4). We hypothesize that it is likely that the common *Nostoc* haplotype (*rbc*LX-1) cannot inhabit the abiotic conditions in the SE11 locality. Indeed, the SE11 locality was the only site characterized by limestone substrate, whereas all others had siliceous and acid substrates. If both bionts were dispersed effectively in the past, *D. atlantica* may have needed to switch its photobiont to persist in the SE11 locality. A switch in favored photobionts by vegetative propagules during the colonization of new habitats has already been reported for green algal-lichens [40, 41, 58, 62]. However, previous studies demonstrated that the switching frequency was much higher than

that observed in *D. atlantica* [58, 63]. Switching the photobiont may allow an asexual lichen to colonize several niches and to extend its population number and population size [62]. However, a species' capability of colonizing new habitats or maintaining its population size is lower if the switch is a rare event, as we found with *D. atlantica*, therefore the species is forced to occupy very few niches.

High levels of differentiation among populations and geographic units were detected in *D. plumbea* photobionts (Table 6). Previous reports of green algal-lichens have shown that the photobiont genetic differentiation is higher than the mycobiont, being highly influenced by local abiotic factors [18, 58, 63]. The most common *Nostoc* haplotype (*rbc*LX-1) was present in almost all *D. plumbea* populations, indicating that this *Nostoc* haplotype is the most abundant. However, the fungal partner can also associate with other *Nostoc* haplotypes that are available in the habitat, which are locality-specific (i.e., rare haplotypes are specific to each locality, Table 4). These results and those for *D. atlantica* population SE11 suggest that the cyanobiont (*Nostoc*) has a weak habitat-substrate specialization. *Nostoc* is frequently found to be free-living on various substrates and in symbiotic associations with a diversity of hosts, including bryophytes, gymnosperms, and angiosperms [19]. A unique *Nostoc* haplotype can be free-living or associated with different cyanolichens and other hosts [37]. *D. plumbea* is dispersed by fungal spores, so it is highly likely that a spore might acquire a *Nostoc* haplotype that differs from the one in the parental thallus. It will associate with *Nostoc* from other symbiotic associations (including asexual lichen species) or with free-living *Nostoc* that grow in *D. plumbea* habitat [46, 47]. However, re-lichenization must occur, which involves integration of the appropriate photobiont and spore germination, and this process is controlled by unknown factors. These unknown factors probably explain why *D. plumbea*, which is a generalist species in its association with a photobiont, mainly selects a single *Nostoc* haplotype and why it experiences certain restrictions when colonizing new habitats. Although, *D. plumbea* is a more common species than *D. atlantica*, it is also reported to be vulnerable species in central Spain.

Conclusions

The genetic differences we detected between *D. plumbea* and *D. atlantica* (Fig. 2) suggest that fungal and photobiont genetic diversity were highly influenced by reproductive mode. Thus, the sexual mode of reproduction favored horizontal photobiont transmission from generation to generation. By contrast, asexual reproduction maintained vertical photobiont transmission, with reduced genetic diversity levels in both bionts. Therefore, vegetative reproduction favors the maintenance of partnerships in *Degelia* lichens. Our results agree partially with a previous study using species of the algal-lichen *Physconia* [62]. That study showed an effect of the reproductive strategy only on fungal diversity and not on photobiont diversity [62]. They concluded that biont horizontal transmission was effective for both reproductive strategies, i.e., sexual and asexual, in promoting the occurrence of asexual species in ample ecological situation. By contrast, we only observed horizontal transmission in a *Degelia* species with a sexual reproductive mode and wider distribution range, whereas strict vertical transmission was found in an asexual species with a narrower niche and distribution range. Probably one of the factors that explain the differences in symbionts transmission between *Physconia* and *Degelia* asexual species is the nature of vegetative propagules they produce. While *D. atlantica* produces isidia, which are small and corticated outgrowths of the thallus, *P. grisea* produces soredia, which are small powdery granules formed by few photobiont cells surrounded by fungal hyphae without cortex. Although comparative studies have not been done so far, it is highly likely that the compact nature of isidia makes them more difficult to switch photobiont in comparison with soredia.

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