The interplay between habitat fragmentation and life traits affects specialization in lichen symbioses

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Abstract

1. Interactions between organisms are determined by species traits and differ in specialization, from generalist to highly specialized relationships. Although we expect that the effect of habitat loss and fragmentation on the abundance and survival of species depends on their degree of specialization, few studies have deepened into the interplay between interaction specialization and habitat loss.

2. Here, we investigate the combined effect of habitat fragmentation and life traits (growth type and reproductive mode) on the specialization of lichen-forming fungi (mycobionts) towards its photosynthetic partners (photobionts) in lichen symbioses.

3. We studied mycobiont specialization in epiphytic lichen communities present in ten fragments of *Quercus rotundifolia* trees embedded in an agricultural matrix. Both mycobionts and photobionts were identified through DNA sequencing and mycobiont specialization was measured through interaction parameters calculating the relative number of interactions (normalized degree; ND) and the specialization of each species based on its discrimination from a random selection of partners (d'). Phylogenetic generalized linear mixed models were used to

analyze the effect of patch size as well as the life traits growth type (crustose, foliose, fruticose) and reproduction mode (sexual vs asexual) on mycobiont specialization.

4. Both mycobiont and photobiont richness along the patch size gradient followed a hump-back pattern, which was more pronounced in photobionts. Mycobionts forming crustose biotypes established the largest number of interactions. Mycobiont specialization (d') was larger for fruticose and foliose biotypes and for species with vegetative reproduction. Along the gradient of fragment size, the relative number of interactions decreased and the specialization of mycobionts with vegetative reproduction increased.

5. *Synthesis*: The analysis of mycobiont specialization towards their photobionts in communities of epiphytic lichens in a fragmented Mediterranean forest revealed that the interaction between species life traits and habitat loss on specialization cannot be neglected. The results also pointed to the ability of some species to modulate their interactions to certain extent, suggesting that species might have a greater resilience to abiotic changes than expected, presumably creating extinction debt or even avoiding extinction processes to some extent.

Keywords: Epiphytic, lichen-forming fungi, Mediterranean forest, photobionts, selectivity.

Resumen

1. Las interacciones entre organismos están determinadas por los rasgos funcionales de las especies y difieren en su grado de especialización, desde interacciones generalistas hasta las altamente especializadas. Aunque se espera que el efecto de la pérdida y fragmentación del hábitat sobre la abundancia y la supervivencia de las especies dependa de su grado de especialización, pocos estudios han profundizado en la interacción entre la especialización de las interacciones y la pérdida de hábitat.

2. En este artículo investigamos el efecto combinado de la fragmentación del hábitat y los rasgos funcionales de las especies (tipo de crecimiento y modo reproductivo) en la especialización de

los hongos liquenizados (micobiontes) hacia sus socios fotosintéticos (fotobiontes) en las simbiosis de líquenes.

3. Se estudió la especialización de micobiontes en comunidades de líquenes epífitos presentes en diez fragmentos de árboles de *Quercus rotundifolia* incrustados en una matriz agrícola. Tanto los micobiontes como los fotobiontes fueron identificados mediante la secuenciación del ADN y la especialización de los micobiontes se midió a través de parámetros de interacción calculando el número relativo de interacciones (grado normalizado; ND) y la especialización de cada especie en función de su discriminación de una selección aleatoria de simbiontes (d'). Se utilizaron modelos lineales mixtos generalizados filogenéticos para analizar el efecto del tamaño del parche, así como los rasgos de tipo de crecimiento (crustáceo, folioso, frutal) y el modo de reproducción (sexual frente a asexual) sobre la especialización de los micobiontes.

4. Tanto la riqueza de micobiontes como de fotobiontes a lo largo del gradiente de tamaño de los parches siguió un patrón de U inversa, que fue más pronunciado en los fotobiontes. Los micobiontes que forman biotipos crustáceos establecieron el mayor número de interacciones. La especialización de los micobiontes (d') fue mayor para los biotipos fruticulosos y foliosos y para las especies con reproducción vegetativa. A lo largo del gradiente del tamaño del fragmento, el número relativo de interacciones disminuyó y la especialización de los micobiontes con reproducción vegetativa aumentó.

5. Síntesis: El análisis de la especialización de los micobiontes hacia sus fotobiontes en comunidades de líquenes epífitos en un bosque mediterráneo fragmentado reveló que no se puede descartar la interacción entre los rasgos de vida de las especies y la pérdida de hábitat sobre la especialización. Los resultados también apuntaron a la capacidad de algunas especies para modular sus interacciones hasta cierto punto, sugiriendo que las especies podrían tener una resiliencia a los cambios abióticos mayor de lo esperado, presumiblemente creando una deuda de extinción o incluso evitando los procesos de extinción hasta cierto punto.

Palabras clave: Bosque mediterráneo, epífitos, fotobiontes, hongos liquenizados, selectividad.

Introduction

Habitat loss due to anthropogenic land use represents one of the main drivers of biodiversity loss under the current scenario of global change (Haddad et al., 2015). A common process following habitat loss is habitat fragmentation in which natural homogeneous habitat is transformed into smaller fragments embedded in a matrix different from the original one (Wilcove et al., 1986). Consequently, fragmentation leads to a reduction in the amount of available habitat for species as well as its connectivity, and to changes of abiotic and biotic conditions in the remaining habitat (Fahrig, 2003). This process usually entails the decrease of population sizes, increasing the risk of species extinctions, and subsequent reduction of ecosystem structure, functioning and services (Isbell et al., 2011; Millennium ecosystem assessment 2005).

Changes derived from habitat fragmentation affect species unevenly depending on life strategies, population sizes and reproductive and physiological traits (Bovo et al., 2018; Farneda et al., 2015; Henle et al., 2004). Ecological specialization towards abiotic and biotic conditions is a highly relevant trait that may determine species fate during habitat fragmentation, being specialists more prone to suffer fragmentation effects (Devictor et al., 2008; Nordén et al., 2013).

As species interact with other species in natural habitats, fragmentation and habitat loss jeopardize species interactions (Tylianakis et al., 2008). Whereas the loss of taxonomic diversity has received large attention during the last decades, knowledge on the effect of habitat loss on interactions is still scarce (Gonzalez et al., 2011). The loss of interactions often precedes the loss of species diversity in ecosystems and it is seen as an early signal of ecosystem decay (Aizen et al., 2012; Valiente-Banuet et al., 2015). Not all kinds of interactions are equally affected by fragmentation, which has been shown to produce more negative effects on mutualisms than on

antagonisms (Magrach et al., 2014). Most of the knowledge we have on the effect of fragmentation on mutualisms comes from non-intimate interactions (e.g., seed dispersal, plant pollination) whereas intimate mutualisms have been largely ignored.

Independently of the interaction type, species show variability regarding the degree of specialization towards their partners, which ranges from generalists, interacting with many partners, to specialists, which interact with a few or even a single partner (Solé and Montoya 2001; Thompson, 1988). These strategies involve different evolutionary and ecological advantages and constraints (Dennis et al., 2011; Ollerton, 2006). Furthermore, they have been highly relevant to predicting the loss of interactions and species in gradients of habitat availability (Aizen et al., 2012). In addition, specialization is correlated with morphological and reproductive traits (Maglianesi et al., 2014; Otálora et al., 2013; Reif et al., 2016; Santamaría and Rodríguez-Gironés, 2007) as well as with species´ evolutionary history (Webb et al., 2010). However, it is largely ignored whether species might modulate their specialization under changing environmental conditions in order to survive. Overall specialization tends to decrease together with habitat availability (Hadley et al., 2018; Jauker et al., 2019). This decrease may be due to the loss of specialists (Aizen et al., 2012; Bagchi et al., 2018) or due to the variation of specialization within species, which may show certain plasticity to cope with new habitat conditions.

Lichens, the symbiotic phenotype of lichen-forming fungi (hereafter, the mycobiont) associated with, at least, one photosynthetic partner (the photobiont) a cyanobacteria and/or a green alga (Grube and Hawksworth 2007) are paradigmatic examples of intimate mutualisms. Due to their high sensibility to subtle changes in abiotic conditions in the environment, they are renowned bioindicators of habitat conditions (Marmor et al., 2011; McCune 2000; Nascimbene and Marini 2015; Rivas Plata et al., 2008), including changes in land management and habitat fragmentation (Aragón et al., 2010; Brunialti et al., 2013; Matos et al., 2017; Svoboda et al., 2010).

126 Some functional traits of lichens, mostly related to growth types and reproductive modes, are
127 commonly used in ecological and indicator studies as a response to many environmental or
128 anthropogenic changes (Ellis et al., 2021; Giordani et al., 2012; Hurtado et al., 2020; Koch et al.,
129 2019; Nelson et al., 2015). Another functional trait commonly used is the photobiont type, often
130 categorized as highly broad taxonomic categories (e.g. Giordani et al., 2012). The last two
131 decades have seen great advances in the understanding of photobiont diversity and the range
132 of fungal-algal interactions in lichen symbioses (Muggia et al., 2020), especially to know the
133 breadth of compatible photobionts at different systematic levels (e.g. (Dal Grande et al., 2012;
134 Fernández-Mendoza et al., 2011; Leavitt et al., 2015; Thüs et al., 2011)). During this time, it has
135 become clear that mycobiont specialization towards the photobiont varies among species and
136 it is likely a species-specific trait (Magain et al., 2016; Pérez-Ortega et al., 2012). However, the
137 knowledge about the influence of certain functional traits on photobiont specialization is scarce.

138 Lichens show a wide range of reproductive strategies (Tripp and Lendemer, 2018), but they can
139 be simply divided between asexual versus sexual reproduction. Most common asexual
140 reproductive strategies involve vegetative clonal propagules (soredia, isidia…) in which both
141 bionts are dispersed together ensuring the availability of compatible photobionts for the fungi
142 at the expense of suppressing genetic variability in both bionts (Buschbom and Mueller 2005;
143 Yahr et al., 2006). On the contrary, the dispersal of the mycobiont via ascospores implies the
144 propagule to find a compatible photobiont after its establishment on the new substrate
145 (Fernández-Mendoza et al., 2011; Yahr et al., 2006). The scarce studies in which specialization
146 towards the photobiont has been explored within a reproductive framework offer contradictory
147 results (Otálora et al., 2013; Wornik and Grube, 2010), likely due to the possibility of photobiont
148 switches after the vegetative propagule is settled (Nelsen and Gargas, 2008)

149 Lichen thalli also show a number of growth forms named biotypes (Honegger, 2001), which have
150 numerous implications for associated photobionts since different architectures and topologies

151 imply the existence of different microniches (Hawksworth and Grube, 2020). The most common
152 biotypes, which are the focus of this study, are: 1) crustose, in which the thallus lacks a lower
153 cortex and is attached to the substrate through the medulla, 2) foliose, in which the thallus has
154 a laminar form, partially adhered to the substrate by certain fixation structures, 3) fruticose, in
155 which thallus shows a shrub-like form attached to the substrate usually by a single point. The
156 role of thallus morphologies on photobiont specialization has not been formally tested, although
157 it is known that, for instance, crustose species usually display a high phylogenetic breadth of
158 photobiont partners (Blaha et al., 2006; Guzow-Krzeminska, 2006; Muggia et al., 2014).

159 In this study, we explore the effect of habitat fragmentation on mycobiont specialization toward
160 their photobionts as well as the effect of growth and reproduction traits on the specialization
161 along the fragmented habitat. We study the richness and specialization of epiphytic lichen-
162 forming fungi and their associated photobionts in habitat fragments of Mediterranean forest of
163 increasing size. We hypothesize that habitat loss reduces both biont richness and specialization.
164 Finally, we explore whether species specialization may be affected by species-specific functional
165 traits such as reproductive mode and growth form.

166 **Material and Methods**

167 **Study area**

168 The area of study was located in Central Spain (42° 5-8’ N; 3º 36-44’ W; 923-1093 m a.s.l.). The
169 area harbors remnants of forest embedded in an agricultural matrix (cereal crops) (Figure 1).
170 Fragments are either dominated by one of the main dominating tree species in the area (*Quercus*
171 *rotundifolia, Q. pyrenaica* and *Q. faginea*) or show mixed formations in which *J. oxycedrus* also
172 occasionally occur.

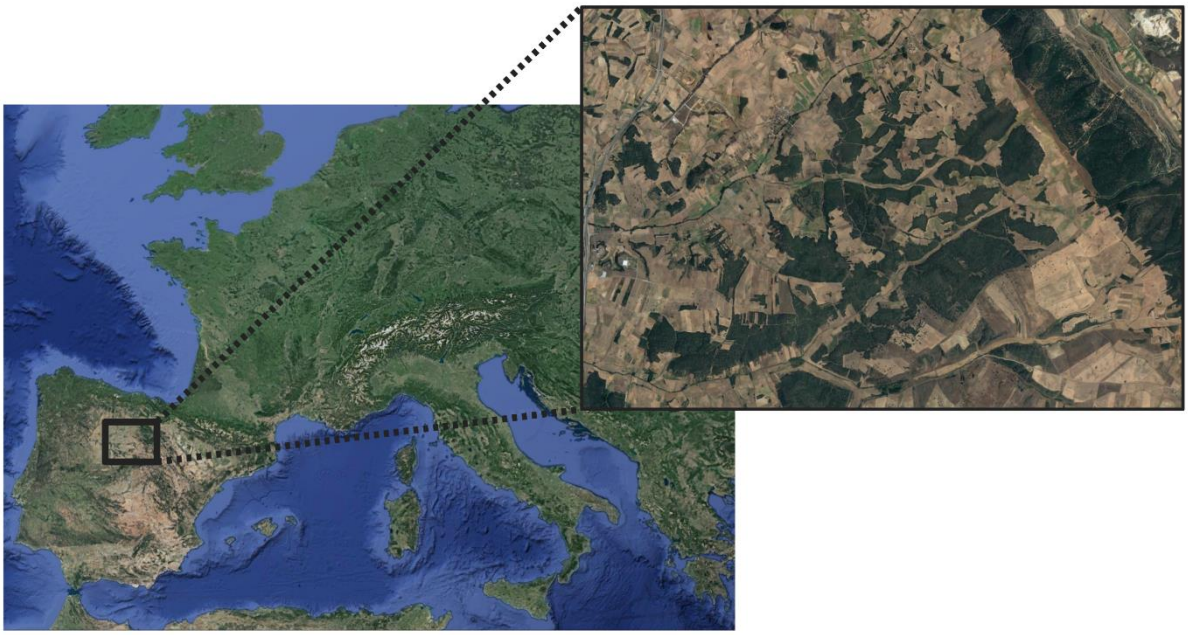


Figure 1. Map detailing the area where the study was carried out. Images obtained from Google Earth (14/12/2015) Europe. (Landsat/Copernicus Data SIO, NOAA, U.S. Navy, NIGA, GEBCO, GeoBasis-DE/BKG (©2009) Map GISrael Instituto Geográfico Nacional; 45º09'57"N 11º06'33"E. https://earth.google.com/web/@45.16589093,11.10934345,1020.81870028a,3304630.75835109d,35y,0h,0t,0r) The second map (smaller one on the right) is also obtained from Google Earth (20/08/2018). Madrigal del Monte. Instituto Geográfico Nacional. 42º07'17"N 3º38'41"W. https://earth.google.com/web/@42.12151496,3.64472552,955.37218271a,9380.0627212d,35y,0h,0t,0r

Sampling

The selection of the habitat fragments was made as follows: Orthorectified photographs were downloaded from the National Centre for Geographic Information (http://centrodedescargas.cnig.es/CentroDescargas/-index.jsp) corresponding to the year 2017,

186 and by means of the Google Earth Pro software (available at
187 https://www.google.com/earth/download/gep/agree.html) all forest fragments present in the
188 area were identified. Perimeter and area were measured for each fragment. Fragments with
189 highly irregular geometries (low area/perimeter ratio) were discarded to avoid disproportionate
190 edge effects. The remaining fragments were inspected during the fall of 2017 in order to check
191 the tree composition homogeneity. Considering the available diversity of fragment sizes, we
192 chose fragments in which *Quercus rotundifolia* was the dominant species (> 75 %). Finally, a total
193 of 10 fragments were selected in a size gradient ranging from 0.002 ha (a single isolated tree) to
194 250ha (Table 1).

195 **Table 1**. Mycobiont and photobiont richness and mean mycobiont specialization in each studied
196 forest patch. A= Area of each forest patch, in hectares; M_d= Number of mycobiont species; M_e=
197 Number of exclusive mycobiont species in a forest patch; P_d= Number of photobiont ASVs; P_e=
198 Number of exclusive ASV species in a forest patch; d= mean degree; ND: mean normalized
199 degree.

A	M_d/M_e	P_d/P_e	d	ND
0.002	9/1	25/3	9	0.33
0.1	17/1	34/8	7.64	0.22
0.3	22/1	42/6	9.13	0.217
1.02	23/1	63/12	10.83	0.17
4.23	27/1	51/13	8.37	0.17
12.21	28/1	75/20	9.54	0.13
16.13	23/0	57/8	11.87	0.21
35.85	23/1	52/9	8.39	0.16
48.01	27/0	61/12	8.89	0.15
250	20/2	46/7	2.9	0.06

200

201 In each fragment, epiphytic lichen communities were surveyed at the geographic center of the
202 fragments. Sampling was carried out according to Asta et al., (2002). In summary, we sampled
203 the lichen community in a total of ten tree trunks (diameter ≥ 12 cm) in both north and south
204 orientation, using a 10×50 cm grid divided into five 10×10 cm squares and annotated all species
205 present and their abundance which was measured as the number of squares where the species
206 occurs. Once the inventory was completed, we attempted to collect a total of 10 individuals of

207 all the species identified in each fragment. This search lasted up to a maximum of 4 hours per
208 plot (two people). Samples were dried at room temperature and stored until further processing.

209 **Molecular methods**

210 **Mycobiont barcoding**

211 Field identifications of mycobionts were subsequently checked using currently used methods in
212 lichen taxonomy and available literature. Further, we sequenced the nrITS region, the universal
213 fungal barcode for fungi (Schoch et al., 2012) to corroborate identifications in each of the
214 identified morphospecies. Briefly, DNA extractions were performed using cationic exchange
215 resin Chelex 100 (BioRad, Madrid) following Ferencova et al., (2017). PCRs were performed using
216 the primers ITS1F and ITS4-Kyo2 (Gardes and Bruns 1993; Toju et al., 2012). Reactions included
217 6.5 µl of MyTaq Red Mix (BioLine, UK), 2 µl of diluted (1:10) DNA template, 0.5 µl (10uM) of each
218 primer and 5.5 µl of sterile distilled H_2O. PCR conditions were set as follows: a denaturation step
219 of 94ºC for 5 min followed by 35 cycles of 95ºC for 30 s, 57ºC for 30 s, and 72 ºC for 1 min ending
220 with an elongation of 72ºC for 7 min. Amplicons were sequenced in Macrogen (Madrid, Spain)
221 using the same primers as in the amplification reaction.

222 **Mycobiont phylogeny**

223 We constructed the phylogenetic tree depicting the evolutionary relationships of mycobionts to
224 account for statistical non-independence derived from common ancestry in the models. DNA
225 extractions were either using extractions obtained in the previous section or using E.Z.N.A.®
226 Forensic Kit (Omega Bio-Tek, Norcross, Georgia, USA) according to the protocol defined by the
227 company. To determine phylogenetic affinities, we used DNA three nuclear genomic regions, *i.e.*
228 the ribosomal internal transcribed spacer (nrITS) obtained in the previous section, the nuclear
229 large subunit region (nrLSU), and the largest subunit of RNA polymerase II (RPB1), as well as the
230 mitochondrial small subunit locus (mtSSU). The primers used for the amplification of these
231 regions are provided in Supplementary Material Table 1.

232 PCR reactions were performed in a total volume of 15 µL, containing 2 µL of template DNA for
233 amplification of the nuLSU, mtSSU (or 4 µL for the amplification of the RPB1 region), 0.5 µL of
234 each primer (10 mM) and 6.5 µL of MyTaq Mix wich contains MyTaq DNA Polymerase (Bioline,
235 UK)) and dNTPs; distilled water was added to reach the final volume.

236 The PCR conditions used for amplification for RPB1, nuLSU and mtSSU were the same as
237 described in Perez-Ortega et al., (2016). PCR products were sequenced by Macrogen Inc.
238 (Madrid, Spain) using the same primers as in the amplification reaction.

239 The four regions were aligned through MAFT tool v 1.4.0 (Katoh and Standley 2013) using the
240 algorithm "auto" implemented in Geneious Prime v 2019.0.3
241 (https://www.geneious.com/prime/). To eliminate the ambiguously aligned regions of those
242 alignments, Gblocks v 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html)
243 has been used (Castresana, 2000) allowing the least stringent parameters smaller final blocks,
244 gap position within the blocks and less strict flanking positions. In order to select the best-fit
245 partitioning schemes and nucleotide evolution models, we used PartitionFinder v 2.1.1 (Lanfear
246 et al., 2017). Phylogenetic relationships among taxa were calculated using Bayesian inference in
247 BEAST2 v 2.6 (Bouckaert et al., 2014) as implemented in CIPRES Science Gateway v 3
248 (https://www.phylo.org/) (Miller et al., 2010). We used an uncorrelated relaxed model with a
249 log-normal prior for modelling clock and a Yule process to model tree prior (Drummond et al.,
250 2006). The MCMC chain length was run during $5x10^9$ generations and results were logged every
251 5000 generations. Trace plots and effective sample sizes (ESS) were examined by TRACER v 1.7
252 (Rambaut et al., 2018). Finally, after discarding the first 25% sampled trees (burning), the results
253 were summarized and annotated in a maximum clade credibility tree (MCC) through
254 TreeAnnotator v 1.8.4 (https://beast.community/treeannotator) (Drummond et al., 2007). For
255 the visualization of the resulting consensus tree and comparison of retrieved phylogenetic
256 relationships with previous literature, we used Figtree v 1.4.4 (Rambaut, 2012). Accession

257 numbers for the sequences generated and used during this study are available in Supplementary
258 Material Table 2.

259 **Photobiont**

260 A small fragment (1-2 mm^2) of the thallus was taken from each specimen. Special care was taken
261 to avoid thallus areas with clear signs of epiphytic fungi or algae as well as necrotic areas. Up to
262 ten fragments per species and forest fragment were pooled in a single microcentrifuge tube.
263 DNA extractions were performed using E.Z.N.A. Forensic DNA Kit (Omega Bio-Tek, Norcross,
264 Georgia, USA) following the manufacturer´s instructions. The second part of the internal
265 transcribed spacers (ITS2) of the photobiont was amplified using the primers FDGITS2-f y
266 FDGITS2-r (Dal Grande et al., 2018) with Illumina adapters CS1 y CS2 attached (Available in:
267 https://rtsf.natsci.msu.edu/-genomics/sequencing-services/sample-requirements-for-illumina-
268 sequencing/). PCR reactions were performed using a mix of 25 µl which contained 0.625 U
269 PrimerSTAR GXL DNA Polymerase (Takara BioInc, Japan), 5 µl of Buffer, 2 µl of dNTP Mixture (2.5
270 mM), 3 µl of DNA template, 0.5 µl of each primer at 10 mM and 13.5 µl of sterile distilled H_2O.
271 PCR conditions were set as follows: a 94ºC denaturation step for 1 min followed by 30 cycles of
272 95ºC for 15 s, 52ºC for 15 s and 72 ºC for 30 s ending with an elongation of 72ºC for 1 min. PCR
273 products were visualized in agarose gels and quantified by using a Qubit® Fluorometer (Life
274 Technologies, Darmstadt, Germany) and normalized. Samples were indexed and pooled in a
275 single Illumina MiSeq run (2 x 250bp paired-end sequencing, v2 Standard 500 cycle) at the
276 Research Technology Support Facility Genomics Core at Michigan State University (USA).

277 **Bioinformatic analysis**

278 MiSeq reads were merged, demultiplexed and filtered using the package DADA2 v 1.8.0
279 (https://benjjneb.github.io/dada2/index.html) for R studio (v 3.4.3 https://cran.r-
280 project.org/bin/windows/base/old/3.4.3/). The first-left 22 bp corresponding to primer
281 sequences were trimmed using the *filterAndTrim* function. Based on quality plots forward and

282 reverse reads were trimmed to 200 and 170 bp respectively. Sequence filtering parameters was
283 set to maxN = 0, maxEE=c (1,4), trunQ =2. The remaining parameters were operated as default.
284 Finally, amplicon sequence variants (ASVs) were generated and a data matrix was built in which
285 the rows represented the species of mycobiont, columns the ASVs of the photobiont and each
286 cell contained the number of reads of each ASV inside the lichen, being the last a proxy for the
287 interaction strength. The table containing the data was rarefied using the function "*rrarefy*"
288 from the package "*vegan*".

289 We could not rule out the presence of contaminations in spite of the special care taken during
290 sample tissue collection, as thallus surface may dwell single algae cells not visible under the
291 stereoscope. Further, some algal species may occur inside the thallus but not taking part in the
292 symbiosis (Moya et al., 2017). In order to overcome this problem, we performed a double
293 filtering of the obtained results. First of all, we collected information available in the literature
294 about the photobionts associated with the species and/or genera found in our study (Blaha et
295 al., 2006; Muggia et al., 2014; Tschermak-Woess, 1988; Wornik and Grube, 2010). Secondly, we
296 established a limit of reads (100) below which we considered that the signal corresponded to
297 contaminations of transients or algae not primarily associated with the mycobiont. Thus,
298 sequences of taxa which based on the available literature do not represent the actual
299 photobiont of the species and/or those with less than 100 reads were eliminated from
300 subsequent analyses. All sequences obtained in this study will be available in the SRA (NCBI).

301 **Species specialization**

302 An interaction matrix between mycobionts and photobionts was built to account for interactions
303 in each forest patch. Mycobiont specialization towards their photobionts was explored by means
304 of two metrics, normalized degree and the parameter *d'*. Normalized degree is the number of
305 links (partners) per species normalized by the total number of possible partners. The parameter
306 *d'* measures the specialization of each species based on its discrimination from a random

307 selection of partners, ranging from 0 to 1 (a species interacts with only one other species, and
308 that species only interacts with the first one) (Blüthgen et al., 2006). Both metrics were
309 calculated for every species at all studied forest fragments using the function *specieslevel* from
310 the *bipartite* v. 2.16 R package (Dormann et al., 2008).

311 **Statistical analyses**

312 We first explored changes in ND and *d'* at the species level using Pearson correlation tests
313 between each of the two specialization parameters and the fragment size gradient in those
314 species present in at least five fragments. Analyses were carried out using the function *cor.test*
315 of the *stats* v. 3.6.2 R package. Then, we explored the independent and cross effect of fragment
316 size and two life traits, growth form and reproduction mode, on species specialization. All
317 species recorded during the inventories fitted well within the main three growth forms
318 recognized in lichens (Nash, 1996), i.e. crustose, whose thalli are strongly attached to the
319 substrate and cannot be separated from it; foliose, whose thalli have a more or less flat
320 appearance and are attached to the substratum by specialized structures in the lower cortex;
321 and fruticose, whose thalli have a three-dimensional architecture, similar to tiny bushes and are
322 attached to the substratum from a single point. Secondly, we categorized all species based on
323 their reproduction modes. Although several reproductive strategies may be found in lichens
324 (Tripp and Lendemer, 2018), species may be easily divided between those producing meiotic
325 propagules (ascospores) which once settled in a new substrate they must find a compatible
326 photobiont to reestablish the symbiosis, and those producing asexual propagules in which both
327 partners, fungus and alga, are dispersed together, and the new thalli correspond to clones of
328 the source thallus. We first explored the effect of area on the number of algae ASV and
329 mycobiont species using the function *lm* of the package *stats*.

330 We investigated the effect of both functional traits, growth form and reproduction type in the
331 mycobiont specialization towards their photosynthetic partners independently and in the

332 context of a gradient of habitat availability. We used generalized linear mixed models in a
333 Bayesian framework (Hadfield and Nakagawa, 2010) implemented in the package MCMCglmm
334 v. 2.29 (Hadfield, 2010) in order to control the phylogenetic effects on these relationships. The
335 uncertainty in the phylogenetic reconstructions was accounted for by running three
336 MCMCglmm´s, each one using a phylogenetic tree randomly chosen from the distribution of tree
337 topologies obtained in BEAST after discarding 25% of samples as burnin, and integrated over the
338 posterior samples by drawing 1000 random samples across models and using *HPDinterval*
339 function from the *coda* R package Plummer *et al.,* 2013). Models priors follow de Villemereuil
340 and Nakagawa (2014) using an inverse-Gamma distribution with shape and scale parameters
341 equal to 0.01 as priors for the random effects and for the residual variance.

342 All graphs for the analyses performed were created with the R packages *ggplot2* (Wickham
343 2016), *gridExtra* (https://CRAN.R-project.org/package=gridExtraand) and *wesanderson*
344 (https://CRAN.R-project.org/package=wesanderson).

345 **Interaction turnover**

346 In order to shed light on the origin of changes in mycobiont specialization along the fragment
347 size gradient, we analyzed the interaction beta diversity using the framework proposed by Poisot
348 et al. (2012). This approach decomposes the total beta diversity of interactions (β_{WN}) between
349 two sites into the interaction dissimilarity due to species turnover species (β_{ST}) and interaction
350 dissimilarity when species occur in both sites but interaction not (β_{OS}) (Poisot et al. 2012).

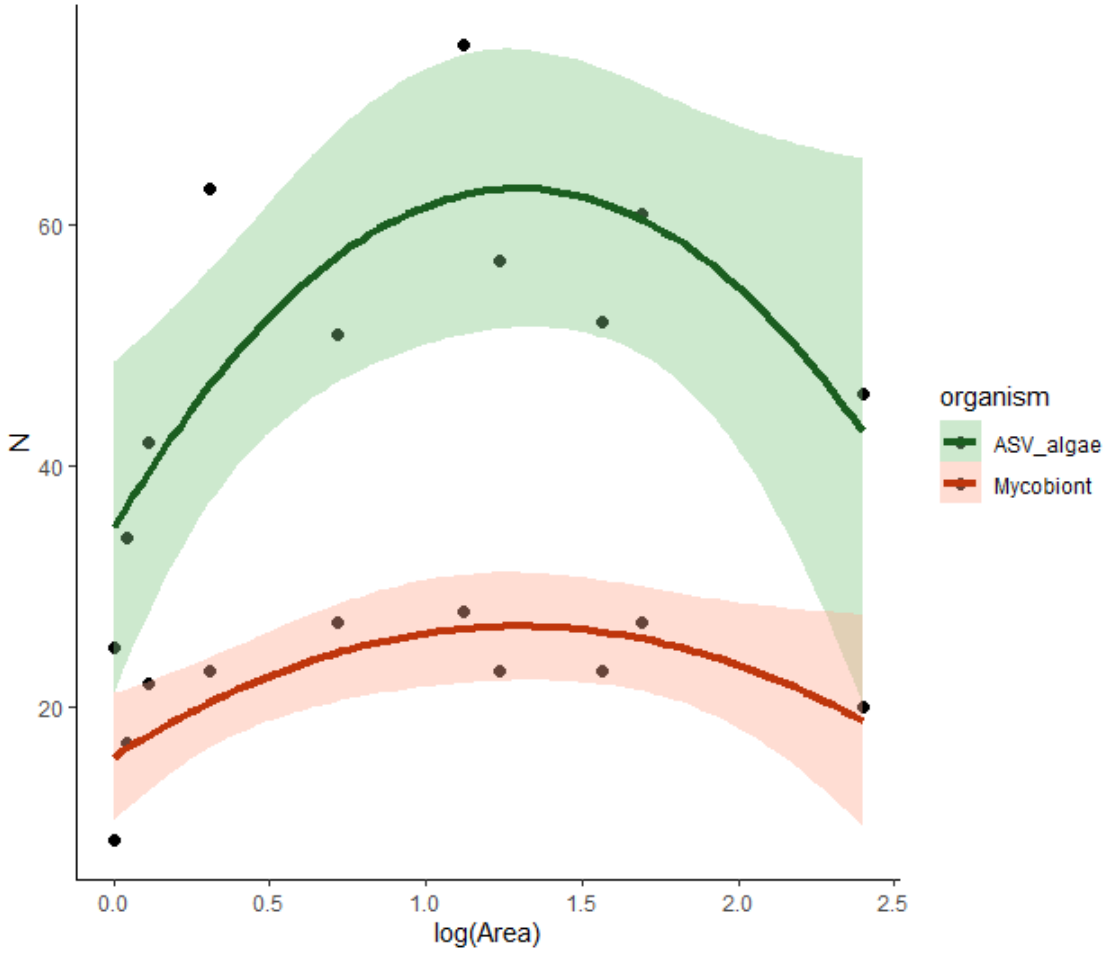
351 **RESULTS**

352 **Mycobionts**

353 We detected a total of 44 species of lichen-forming fungi in the study area (Sup. Mat. Table 2).
354 17 species were crustose, 19 foliose and 8 fruticose. Twenty species reproduced mainly sexually
355 whereas 24 showed vegetative reproduction ((Sup. Mat. Table 2). Number of mycobiont species

356 per forest fragment ranged from 9 at the 0.002 ha fragment to 28 at the 12.21 ha, with an
357 average of 22 species per fragment (Table 1), with a distribution along the area that was adjusted
358 to a quadratic model (R^2= 0.82; p<0.05; Figure 2). The number of exclusive taxa was low in all
359 fragments, occurring the highest, 2, at the largest forest fragment (Table 1).

360 The richness of crustose and foliose species did not vary along the area gradient while that of
361 fruticose species showed a quadratic relation (R^2= 0.571; F= 6.998, 2; d.f.=7; p-value<0.05), being
362 maximum in intermediate plots. Regarding reproduction mode, the richness of sexual species
363 showed a quadratic relationship with the log of area (R^2= 0.617; F= 8.237, 2; d.f.=7; p-
364 value<0.05), while the relationship for asexual species followed a positive linear trend (R^2=
365 0.346; F= 5.756, 1; d.f.=8; p-value<0.05).



366

367 **Figure 2**. Distribution of number (N) of mycobiont species and ASVs along the patch size gradient
368 (log(hectares)). Quadratic adjusted models (bold line) and confidence intervals (paler colored

369 area) are represented for the number of mycobiont species in red (R^2= 0.82; p<0.05) and green
370 for ASVs (R^2= 0.52; p <0.05).

371

372 **Photobionts**

373 A total of 2272 thalli were collected, corresponding to the 229 unique pools used for Illumina
374 MiSeq analysis. The average number of thalli per species and fragment was 9.92. A total of
375 8,478,480 paired-end reads were obtained from the photobiont ITS2 amplicons. After quality
376 filtering, a total of 4,956,878 reads remained. The processing of these reads resulted in 985
377 ASVs. After this processing we applied a manual filter removing the ASVs with less than 100
378 reads, obtaining a final number of 175 ASVs. Furthermore, the total number of reads remaining
379 after this manual filter was 4,903,976 with an average of 21,415 reads per sample. BLAST
380 searches of these remaining ASVs resulted in 158 corresponding to the genus *Trebouxia*, 13 to
381 *Dictyochloropsis* and 4 to *Asterochloris*. *Asterochloris* ASVs were restricted to *Cladonia*, while
382 the genus *Dictyochloropsis* was mostly found in the species *Phlyctis argena*.

383 The number of ASVs per forest fragment ranged from 25 at 0.002 ha to 75 at 12.21 ha (Table 1).
384 ASV diversity along the patch size gradient followed a quadratic relationship (R^2= 0.52; p <0.05;
385 Figure 2). The highest number of exclusive photobiont ASVs (20) occurred at the 12.21 ha forest
386 fragment (Table 1).

387 **Specialization**

388 The mean number of ASVs per mycobiont species, species degree, ranged from 2.9 at the 250
389 ha fragment to 11.87 at the 16.13 ha fragment (Table 1). The normalized degree ranged from
390 0.01 in *Evernia prunastri* at the 12.21 ha forest fragment to 0.54 in *Blastenia xerothermica* at the
391 0.3 ha forest fragment (Supplementary Material Table 3). The specialization parameter *d'* ranged
392 from 1 in *Cladonia fimbriata* at the 563.9 ha forest fragment to 0.16 in *Lecanora subcarpinea* at
393 the 1.02 ha forest fragment (Supplementary Material Table 3).

The correlation analyses between ND and area in species with occurrences in ≥ 5 forest patches showed negative Pearson's correlation coefficients for all species (Supplementary Material Table 4)., although they were only statistically significant in six of them, i.e. *Candelariella xanthostigma*, *Melanelixia subaurifera*, *Parmelina tiliacea*, *Physconia perisidiosa* and *Ramalina fastigiata*, all but the last one are vegetatively reproducing species. Regarding correlations between d' and area, they were only significant in *Physconia enteroxantha* and *Physconia perisidiosa,* for which d' increased along the forest fragment size gradient (Supplementary Material Table 4).

Phylogenetic generalized linear models showed differences in average normalized degree between crustose and fruticose species (Figure 3A), but not between reproduction modes (Figure 3B, Supplementary Material Table 4). Regarding the specialization parameter *d'* we found significant differences between crustose species and foliose and fruticose species (Figure 3C) as well as between reproduction modes, with vegetative species showing higher average d' (Figure 3D, Supplementary Material Table 4).

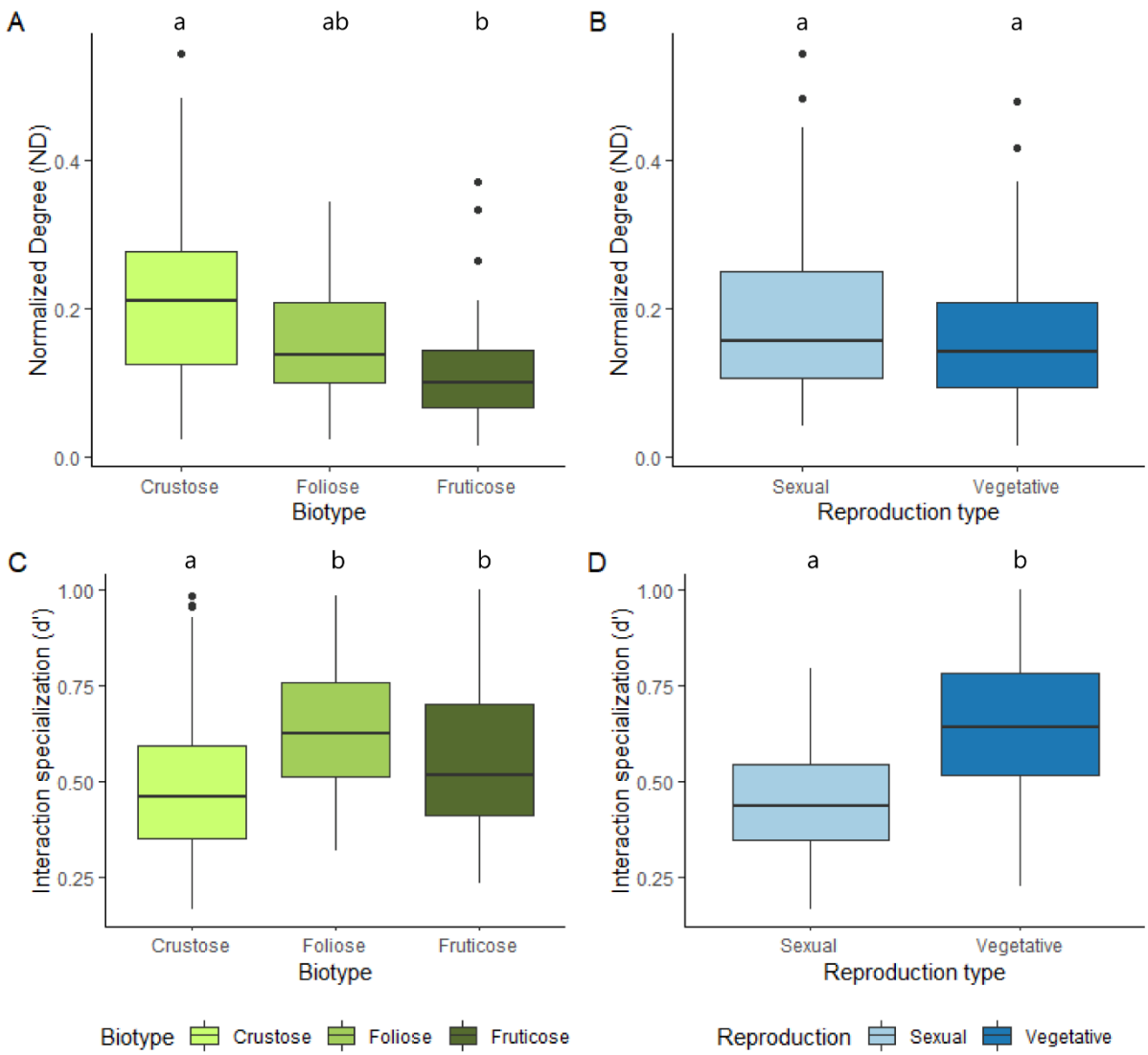


Figure 3. Boxplots show differences in specialization among species with different functional traits. A) Differences among biotypes in the specialization parameter *d'*; B) Differences between reproduction modes in the specialization parameter *d'*; C) Differences among biotypes in normalized degree (ND); D) Differences between reproduction modes in normalized degree (ND). The letters above boxplot depict significant differences among functional groups. Significance is based on phylogenetic controlled MCMCglm models (see. Mat. Supp. Table 3).

Phylogenetic generalized linear models also showed that area had a significant and negative effect on normalized degree (Figures 4A, B, Table 2). There were significant differences between crustose taxa and foliose and fruticose species and these differences were independent of the area (Table 2). No significant differences were found between reproduction modes regarding normalized degree along the area gradient (Table 2). Concerning the specialization parameter

422 d', the area had a significant effect, the larger the patch size the larger d' when considering
423 species biotypes (Figures 4C, D, Table 2). In addition, fruticose species had larger d' than crustose
424 species. Interestingly, there is a significant interaction between area and fruticose species, in
425 which the increase of the parameter d' along the area gradient is lower than in crustose species
426 (Figure 4C, Table 2). When considering the effect of area and reproductive modes on d' we
427 found differences between sexual and asexual species, with significant effects of interactions
428 (Figure 4D, Table 2). Vegetatively reproduced species increased their d' along the area gradient,
429 but the pattern was the contrary in sexual species.

430 **Table 2**. Results of Phylogenetic Generalized Linear Models explaining Normalized degree and
431 specialization (d´) as a function of biotype, reproductive mode and area. Variables with
432 significant effect, those in which the highest posterior density interval does not include zero, are
433 shown in bold. Area was implemented in all analyses as log(area+1).

	Highest Posterior Density intervals
*Normalized degree ~ biotype*area*	
Intercept	**[0.161 — 0.301]**
Area	**[-0.035 — -0.019]**
Biotype crustose	**[0.000 — 0.146]**
Biotype fruticose	[-0.134 — 0.033]
Area: Biotype crustose	[-0.015 — 0.012]
Area: Biotype fruticose	[-0.007 — 0.025]
*Normalized degree ~ reproductive mode*area*	
Intercept	**[0.203 — 0.353]**
Area	**[-0.037 — -0.019]**
Vegetative reproduction	[-0.092 — 0.016]
Area: Vegetative reproduction	[-0.002 — 0.023]
*d' ~ biotype*area*	
Intercept	**[0.434 — 0.815]**
Area	**[0.008 — 0.038]**
Biotype crustose	[-0.285 — 0.004]
Biotype fruticose	[-0.003 — 0.337]
Area: Biotype crustose	[-0.043 — 0.003]
Area: Biotype fruticose	**[-0.068 — -0.013]**
*d' ~ reproductive mode*area*	
Intercept	**[0.384 — 0.656]**
Area	[-0.024 — 0.009]
Vegetative reproduction	**[0.038 — 0.223]**
Area: Vegetative reproduction	**[0.001 — 0.044]**

434

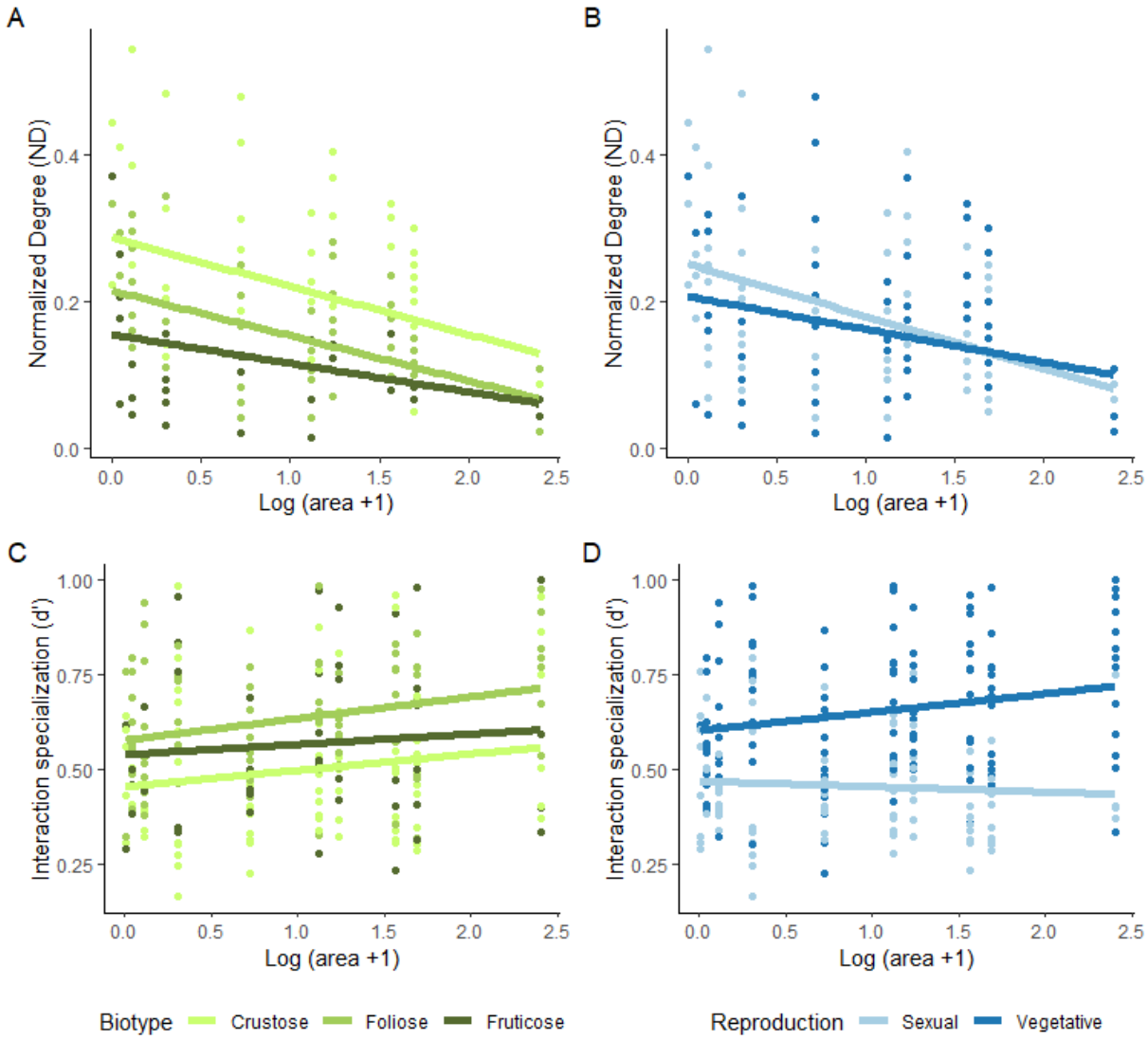


Figure 4. Representation of the interaction effect between area and reproduction type on ND (A) and d'(B), as well as the interaction effect between area and biotype on ND (C) and d'(D).

Interaction turnover analyses revealed that most of the differences between forest patches were due to the establishment of new interactions even though partners were present rather than dissimilarity due to partner turnover (β_{ST}) (Figure. 5; t = 5.2741, df = 74.8, p-value<0.001).

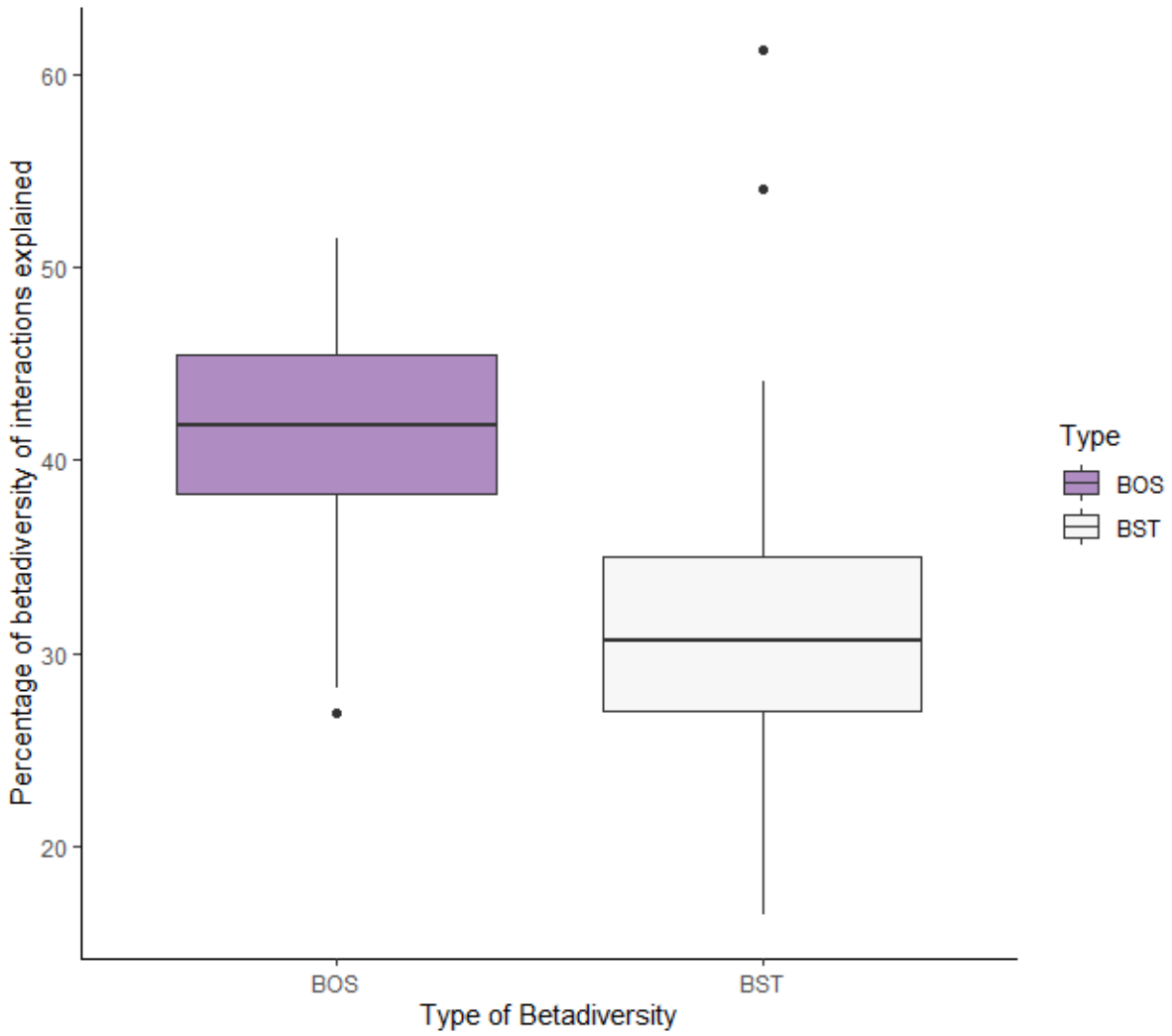


Figure 5. Percentage of beta diversity of interactions explained by the dissimilarity of interactions established between species common to both communities (BOS) and due to species turnover (BST) (t-test: t = 5.2741, df = 74.8, p-value<0.001).

Discussion

Our study represents the first attempt to disentangle the effect of habitat loss on interaction specialization in symbiotic organisms. The obtained results shed light on how habitat loss interplays with life traits to affect fungal-algal interactions in epiphytic lichen symbioses.

Diversity

Mycobionts and photobiont richness showed a humped-shaped distribution along the patch size gradient. This pattern is more pronounced for algae suggesting a higher sensitivity of algae to

454 abiotic conditions. Previous studies dealing with lichen diversity along gradients of habitat loss
455 in different forest ecosystems have reported a gradual loss of diversity as the available habitat
456 diminished (Svoboda et al., 2010), with changes in abiotic conditions associated to an increasing
457 fragment edge effect proposed as the main reason for such reduction (Asplund et al., 2014;
458 Belinchón et al., 2007; Boudreault et al., 2008; Esseen and Renhorn, 1998). Hump-shaped
459 diversity patterns have been reported for lichen-forming fungi along stand age (Asplund et al.,
460 2014; Miller et al., 2020) and elevational gradients (Nanda et al., 2021) so-far this is the first
461 report of a hump-shaped pattern of diversity along a fragmentation gradient. The intermediate
462 disturbance hypothesis (IDH) (Connell, 1978) predicts highest levels of biodiversity at
463 intermediate levels of frequency or intensity of disturbance (but see Mackey and Currie (2001)
464 and Fox (2013) for criticism). Although it has been proposed that the main driver for this peak
465 of diversity is the decrease of species densities, which entails reduced species competition (Fox
466 2013), it is not clear that this assumption may be applied to sessile organisms such as lichen-
467 forming fungi with high habitat specialization (Fritz et al., 2009). It could be that fragments of
468 intermediate size have intermediate abiotic conditions that allow the presence of habitat
469 specialist populations as well as species associated with the abiotic conditions existing in smaller
470 fragments. It is noteworthy that intermediate patch sizes dwell the largest number of exclusive
471 ASVs, a pattern not shared by mycobionts. Therefore, the hump-shaped pattern found in
472 mycobionts and photobionts may not respond to the same factors, or they may affect both
473 organisms differently. Intermediate abiotic conditions could be translated into a higher range of
474 available microhabitats, and higher spatial heterogeneity, promoting a larger amount of habitat
475 specialists and exclusive ASVs in photobionts (Rosenzweig, 1995). If mycobionts had broader
476 ecological requirements than photobionts, this would explain the difference in the observed
477 pattern between the two organisms.

478 Our results showed that a gradient of habitat availability influences the distribution of functional
479 traits in the studied lichen communities. Thus, except for crustose and foliose biotypes, the

480 number of fruticose, vegetative and sexually reproduced species changed along the gradient.
481 Changes in the distribution of functional traits in epiphytic lichen communities are directly
482 related to abiotic conditions such as solar radiation, water availability or air pollution (Giordani
483 et al., 2014; Koch et al., 2019; Matos et al. 2015; Paoli et al., 2017), and they change along
484 environmental gradients such as elevation (Bässler et al., 2016) or land use (Pinho et al., 2012).

485 *Functional traits and specialization*

486 Levels of specialization are affected by life traits in all known organisms (e.g. Armbruster, 2017;
487 Dehling et al., 2016). Our results showed differences in specialization among biotypes and
488 between reproductive modes, both in terms of ND and d'. Crustose species interacted on
489 average with a higher number of ASVs than fruticose and foliose species, although only the first
490 difference was significant. Regarding the parameter d', crustose species showed significantly
491 lower specialization than foliose and fruticose species. So far, no study had compared
492 differences in specialization among biotypes although most studies on photobionts associated
493 with crustose species have shown that they interact with a large number of species, in a wide
494 phylogenetic spectrum (Blaha et al., 2006; Guzow-Krzeminska, 2006; Muggia et al., 2014)
495 although examples of high specialization also occur (Hauck et al., 2007; Singh et al., 2015).

496 The interaction with a wider range of photobionts facilitates the settlement in a wider range of
497 substrates and ecological conditions (Muggia et al., 2014) since changes in photobiont allow
498 species to increase their ecological niche (Peksa and Škaloud, 2011; Fernández-Mendoza and
499 Printzen, 2013; Rolshausen et al., 2018). However, why most crustose lichens studied so far
500 show a higher number of associated photobionts still remains unclear. The constant contact with
501 the substrate during lichen growth mediated by prothallus ensures the constant contact with
502 new suitable free-living photobionts (Asmtrong and Bradwell, 2010; Sanders, 2005) that may be
503 incorporated into the lichen thallus in the course of development (Sanders and Lücking, 2002).
504 New photobionts might coexist with previous algal strains or replace them (Ott 1987). On the

505 contrary, chances to incorporate new algal strains in foliose and fruticose species are much more
506 reduced due to the scarce contact (single fixation points in fruticose species, rhizines in foliose
507 not adnate species) with substrate and global isolation due to cortical tissues. Molins et al.,
508 (2021) has recently shown that early developmental stages of *R. farinacea* show higher algal
509 diversity than older stages. This fact might indicate that, likewise it occurs in corals (Abrego et
510 al., 2009); it is in early stages of foliose or fruticose species when there is more intimate contact
511 with the substrate, therefore the mycobiont being able to establish contact with a wider array
512 of algal strains, although only one or a few of these will remain in the later stages.

513 The reproduction mode also showed a clear effect on mycobiont specialization. Sexual and
514 vegetatively reproduced species had on average a similar number of partners but the latter
515 showed considerably higher values of d'. Contrary to our findings, previous studies comparing
516 closely related species pairs had shown that species of lichen-forming fungi with clonal
517 reproduction via joint dispersal propagules tend to have lower associated algal diversity and are
518 more selective than sexual species (Cao et al., 2015; Otálora et al., 2013). The lower diversity
519 associated with species that reproduce by vegetative propagules has been attributed to the joint
520 dispersal of both bionts and the lack of need to search for new partners if the fitness of the
521 combination is optimal or suboptimal in the new environment. However, this interaction may
522 be broken and the mycobiont may interact with new photobionts in the new substrate, using
523 the original photobiont only for the settlement stage, thus avoiding the restriction to colonize
524 new environments. So far, no study had analyzed a high number of species to have a broad
525 overview. Thus, we showed that, on average, sexual and asexual species did not differ in terms
526 of the number of associated photosynthetic partners. Wornik and Grube (2010) showed that the
527 ease to switch photobiont in the vegetatively reproduced *Physconia grisea*, a Mediterranean
528 species dispersed via soredia and also found in our study area, was the only suitable explanation
529 for comparable photobiont diversity between a sexual and an asexual species. However,
530 regarding d' differences between both reproductive modes were significant and outstanding,

531 showing asexual species much higher specialization. Cao et al. (2015) suggested that the
532 decrease of selectivity in sexual species may be due to the need of finding a new photobiont
533 before the ascospores perish. In this case, the similar number of partners in both groups does
534 not point in that direction. The greater specialization found in vegetatively dispersing species
535 may reflect the higher fitness of a particular myco-photobiont combination at the local scale
536 which is favored by the clonal reproduction.

537 *Specialization along the gradient of area size*

538 Our results showed that average specialization at the species level changed along the gradient
539 of fragment size in the same way as occurs with other parameters that describe the structure of
540 interaction networks (Tylianakis and Morris, 2017). The variation of species specialization in such
541 gradients has only been scarcely studied (Aizen et al., 2012). Specialization is often considered
542 a species-level trait, conserved across the phylogeny (Gómez et al., 2010) and with relatively
543 little variation, although it is known to vary at geographical and phylogenetic scales (Poisot et
544 al., 2015; Trøjelsgaard et al., 2015). Carstensen et al. (2018) found that local and regional
545 specialization were strongly correlated in 14 plant-pollinator networks from two different
546 continents, considering species specialization consistent level across scales. On the contrary, we
547 found that average specialization may change due to habitat abiotic conditions, here derived
548 from habitat fragmentation. We observed a strong decrease in the average number of
549 photobionts associated with each fungal species as the fragment size decreased and an overall
550 higher specialization (*d'*) along the area gradient. The higher values of the normalized degree
551 found in smaller patch sizes regarding larger ones could be an artifact derived from the lower
552 number of photobionts available in small fragments (Fig. 2). However, the specialization
553 parameter *d'* did clearly point towards higher mycobiont specialization in larger fragments.
554 Although the number of available partners increased along most of the gradient, fungal species
555 become more selective. In addition, the analysis of the interaction beta diversity showed that

556 most of the turnover among forest patches was not due to changes in biont composition, that
557 is, the observed pattern of increasing specialization is not due to the absence of certain bionts,
558 but to an increase in the level of selectivity among the those available. Assuming a local
559 adaptation of the photobiont, the smaller fragments, with more homogeneous abiotic
560 conditions due to the greater edge effect (greater insolation, wind, nitrogen inputs, etc.) may
561 host only photobiont species that we could call weeds, adapted to the greater intensity of the
562 disturbance. New propagules arriving in the patch will have at their disposal only a pool of
563 equally suboptimal photobionts, perhaps interchangeable in terms of holobiont fitness, so the
564 degree of selectivity is low. On the contrary, in larger fragments, where a larger pool of
565 photobionts is available but also abiotic conditions are more heterogenous propagules of
566 different species will be able to establish more specialized interactions, thus increasing
567 holobiont fitness.

568 *Interplay between specialization, traits and fragmentation*

569 Changes in specialization along the area gradient were not even among morphological groups
570 and between the reproduction modes, showing that changes in specialization along the gradient
571 are also influenced by their life traits. For example, although the number of interacting
572 photobionts decreases along the area gradient in all biotypes, there was a significant difference
573 between how this number decreases in foliose and fruticose species and how it does in crustose
574 species. In addition, we found a significant interplay between the fruticose biotype and area,
575 regarding the specialization parameter d', showing that changes in habitat conditions and
576 availability do not affect similarly all biotypes in terms of discriminating among their
577 photosynthetic partners. It is known that different growth forms represent different strategies
578 of exploitation of water sources in lichens (Gauslaa, 2014). Thus, it is likely that the changing
579 abiotic conditions along the patch size gradient do not affect similarly photobionts occurring in

580 different growth forms since thallus biotypes may have different buffering potential of
581 environmental conditions.

582 Regarding reproductive modes, it is certainly striking that although the ND of both functional
583 groups similarly decreased along the area gradient, they showed contrasting trends regarding
584 the specialization parameter d' with sexual species decreasing in their specialization as the patch
585 area increases. The mode of reproduction has relevant implications with respect to constraints
586 on dispersal and colonization of new habitats (Ellis et al., 2021; Walser, 2004), thallus physiology
587 (Tretiach et al., 2005), and response to disturbance gradients such as air pollution (Koch et al.,
588 2019). The contrasting trends along the area gradient between reproductive modes in the
589 average d' may have their origin in differences in species composition and the distribution of
590 reproductive modes in the communities and also, in the different modulation of specialization
591 along the gradient at the individual level. Models indicated a significant change of reproduction
592 modes along the fragment size area. In addition, linear models for individual changes in
593 specialization along the gradient revealed five species in which species significantly modulated
594 their specialization in terms of ND (four of them asexually reproduced via soredia) and two cases
595 in which d' significantly increased along the gradient, both asexually reproduced via soredia.
596 Thus, the explanation of this contrasting pattern may be derived both from changes in species
597 composition and modulation of specialization in certain species. Modulation of specialization at
598 the species level is certainly a phenomenon that should be further explored in the future.

599 *Implications for conservation*

600 Our results have clear implications in conservation biology. Interactions among species are
601 certainly influenced by local abiotic conditions, so a certain biont combination would not have
602 the same fitness in two patches with different sizes. Small changes in forest conditions can have
603 drastic consequences and cause existing biont combinations to lose their ability to compete. In
604 addition, if some species can modulate their specialization to a certain degree, then those

605 species would display higher resilience to environmental changes such as those derived from
606 habitat fragmentation than other species. Their ability to adapt to changes in local conditions
607 through changes in specialization towards their photobionts may buffer the effect of more
608 stressful abiotic conditions due to the increase of edge effect in small fragments, at least until
609 phylogenetic constraints prevent interactions in some species. This buffering effect produced by
610 the modulation of specialization may postpone the extinction debt (Berglund and Jonsson,
611 2005), or, depending on the intensity and frequency of perturbation, even avoid extinction, with
612 remarkable implications for biodiversity conservation (Kuussaari et al., 2009). Further studies
613 will show whether the analyses of interaction dynamics (partner fidelity, link conservatism and
614 rewiring) in myco-photobiont symbioses in the context of interaction bipartite networks may be
615 used as suitable tools to understand and predict long-term changes in epiphyte lichen
616 communities.

617 **Author contributions**

618 SPO, MV, MVdP and ABdG; SPO and ABdG designed the study; SPO and ABdG carried out the
619 field sampling; ABdG and MVdP collected laboratory data; MV, SPO, ABdG, MVdP and MV
620 analyzed the data; SPO and ABdG led the writing of the manuscript; All authors contributed
621 critically to the drafts and gave final approval for publication.

622 **Data availability statement**

623 All NGS sequences obtained in this study will be available in the SRA (NCBI). Sequences obtained
624 through Sanger sequencing are available in Gen Bank (Accession numbers provided in
625 Supplementary Material Table 2).

626 **Conflict of interest statement**

627 The authors have no conflict of interests to declare.

628 **Acknowledgments**

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