RESEARCH ARTICLE

The genetic structure of the cosmopolitan three-partner lichen *Ramalina farinacea* evidences the concerted diversification of symbionts

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Abstract

The epiphytic lichen *Ramalina farinacea* is distributed throughout the northern hemisphere in which the same two algal *Trebouxia* species (provisionally named TR1 and TR9) coexist in every thallus. *Ramalina farinacea* symbionts were characterized based on the two fungal nuclear loci (nrITS and *rpb2*) along with the primary and secondary structures of nrITS from each *Trebouxia* species in the Iberian Peninsula and Canary Islands. The results indicated a noticeable genetic differentiation between mycobionts from these two geographic areas and also suggested concerted changes in the three partners of a lichen symbiosis toward two clearly distinguishable 'holobiont' lineages. Modeling of ITS2 RNA secondary structures suggested their temperature sensitivity in TR1 but not in TR9, which was consistent with the observed superior physiological performance of TR9 phycobionts under relatively high temperatures. Both TR1 and TR9 phycobionts have been also found in a variety of taxonomically distinct lichens with a preferably Mediterranean distribution, being TR1 much more widespread than TR9. Our observations support a model in which ecological diversification and speciation of lichen symbionts in different habitats could include a transient phase consisting of associations with more than one photobiont in individual thalli. Such diversification is likely to be promoted by different physiological backgrounds.

Introduction

Symbiotic life styles constitute an important evolutionary force that is driven by different patterns of associations (Paracer & Ahmadjian, 2000). Variations in symbiotic partnerships depend on multiple factors, including specificity, selectivity, and the mode of symbiont transmission within a selective environment. Specificity refers to the possible taxonomic range of acceptable partners, whereas selectivity denotes the frequency of association between compatible partners (Yahr *et al.*, 2004). Here, we report an interesting variation in the symbiont patterns of lichens that might contribute to their evolutionary and ecological success. Lichens represent major radiations of ascomycetes in a symbiotic stage, characterized by a unique symbiogenetic phenotype of specific biological organization in the thallus (Chapman & Margulis, 1998; Margulis & Barreno, 2003; Barreno, 2004).

The associations of lichens involve at least two very different organisms: a heterotrophic fungus (mycobiont) and a photosynthetic (photobiont) cyanobacterium (cyanobionts) or unicellular green algae (phycobionts). Lichenization allows the mycobiont and photobiont(s) to thrive in habitats that would otherwise be unavailable to either one on its own (Kranner & Lutzoni, 1999). Thus, lichens are found in the most extreme environments, including deserts and high mountains. The important functional interactions between photobionts and mycobionts, and possibly with other symbionts such as bacteria (Grube & Berg, 2009), suggest that these partners evolved simultaneously. This assumption is easily understandable for lichens in which there is a vertical transmission of the photobiont. In such cases, the photobiont is dispersed within vegetative propagules comprising both symbiotic partners. Alternatively, the new photobiont can be incorporated within a fungal-germinating spore or

FEMS MICROBIOLOGY ECOLOGY

vegetative structure, resulting in its horizontal transmission (Zoller & Lutzoni, 2003; Yahr *et al.*, 2004, 2006; Nelsen & Gargas, 2008). Vertical transmission of photobionts has been hypothesized for asexual and nearly asexual lichen-forming fungal species (Cassie & Piercey-Normore, 2008), and horizontal transmission for sexual lichen-forming fungal species (Honegger, 1991; Werth & Sork, 2010).

Lichens with vertically transmitted photobionts would be expected to show genetic structures that tightly match those of their symbiotic partners. However, in some cases, joint dispersal does not imply the maintenance of association patterns, as suggested by Wornik & Grube (2010). For example, changes in a symbiotic organism's reproductive mode may be governed by trade-offs in the fitness of the symbiosis, as proposed by Buschbom & Mueller (2006). Thus, when the relationship between mycobiont and photobiont is optimal in a given environment, the preponderant reproductive mode would be asexual for species with mixed asexual and sexual propagation systems. When changes in the environment make the established symbiosis suboptimal, thus decreasing global fitness, sexual reproduction would be preferred as it provides access to new partners and produces variability through genetic recombination. Successful relichenizations therefore depend upon the mycobiont's ability to form alternative partnerships within a given habitat (Romeike *et al.*, 2002). According to this view, moderate habitats favor the proliferation of a higher diversity of algal genotypes, while more restrictive habitats reduce the available pool of algae owing to the greater selection pressure (Doering & Piercey-Normore, 2009). The ability of lichens to establish new fungus-photobiont associations has enabled them to colonize varied and wide-ranging habitats, assuming that the specificity and ecological selection of the photobionts is supported by a high degree of flexibility. (Grube, 2010).

Very little is known about the mechanisms shaping the associations of lichen symbioses or the relative contributions of phylogenetic history and environmental variation. This is partly owing to poor knowledge of symbiont composition and diversity, as few tools are available for distinguishing and classifying lichen symbionts. However, the refinement of molecular methods has facilitated the detection of potentially novel species of phycobionts and mycobionts, which can then be confirmed on the basis of subtle differences in macromorphological or ultrastructural characters, discovered on their re-evaluation (Casano *et al.*, 2011). This approach to previously hidden species has been successfully applied to lichen mycobionts (Crespo & Pérez-Ortega, 2009) and has brought numerous 'cryptic species' to light. The concept of 'cryptic species' is particularly relevant for algal partners within lichen thalli, given the lack in the lichenized stage of diagnostic characters that could be used to identify distinct photobionts by optical microscopy.

Internal transcribed spacers from the nuclear ribosomal cistron 18S-5.8S-26S rRNA gene (nrITS) have been extensively exploited as a molecular tool to study partner selectivity in lichens (Grube & Muggia, 2010) and to carry out phylogenetic reconstructions of mycobionts (Kelly *et al.*, 2011; Schmull *et al.*, 2011) and photobionts (Piercey-Normore, 2006; Nelsen & Gargas, 2008; Skaloud & Peksa, 2010). Moreover, studies based on nrITS have not been limited to their primary sequences but have been extended to ITS secondary structures, which show strong conservation in both lichen fungi and phycobionts (Beiggi & Piercey-Normore, 2007). In fact, the RNA secondary structure of ITS2 is the most conserved among eukaryotes. It includes canonical domains forming the helices of a 'four-fingered hand' among several conserved features (Mai & Coleman, 1997). Among the four helices, helix II is the most conserved, and almost always shows at least one pyrimidine–pyrimidine mismatch; helix III is usually the longest and often includes a YGGY motif; and helix IV contains a CAGG sequence at its base (Schultz *et al.*, 2005). The maturation of large subunit rRNA includes a series of cleavages that result in the removal of the internal transcribed spacer (ITS2) and thus separates mature 5.8S from 25/28S rRNAs. Several investigations have demonstrated that the formation of a higher-order secondary structure within the assembling pre-ribosomal particle is a prerequisite for accurate and efficient pre-rRNA processing (Cote *et al.*, 2002), and in sum, for functional ribosomes.

Patterns of lichen symbiotic associations within an ecological and a historical framework are highly diverse. They range from a mycobiont species that cultivates the alga species as a monoculture within its thallus (Piercey-Normore & Deduke, 2011) to a fungal species that associates with more than one algal lineage [e.g. *Lecanora* (Blaha *et al.*, 2006) and *Tephromela* (Muggia *et al.*, 2008, 2010)]. In some cases, different algal genotypes coexist within the same thallus [e.g. *Rinodina* (Helms *et al.*, 2001) and *Evernia* (Piercey-Normore, 2006)].

The lichen *Ramalina farinacea* constitutes a particular case in which a fungal species specifically associates with two different *Trebouxia* phycobionts (provisionally named TR1 and TR9) coexisting within each lichen thallus (del Campo *et al.*, 2010; Casano *et al.*, 2011). The two phycobionts differ in their physiological responses to different environmental conditions (Casano *et al.*, 2011), including oxidative stress (del Hoyo *et al.*, 2011). According to Krog & Osthagen (1980), *R. farinacea* originated in the Macaronesian–Mediterranean region and thereafter gradually increased its area to include most of the temperate and boreal regions of the northern hemisphere, with the

Canary Islands as its probable southernmost limit in the Atlantic region.

The primary aim of the present study was to investigate a peculiar symbiotic association found in *R. farinacea* and the possible co-diversification events resulting from a long-term continuous symbiotic association in populations from the Iberian Peninsula and the Canary Islands. We therefore examined the genetic variability of the TR1 and TR9 phycobionts within *R. farinacea* in relation to mycobiont genotypes and geographic location. The symbiont composition and genetic diversity of each sample was evaluated based on the primary and secondary RNA structures of the nrITS from each phycobiont (TR1 and TR9), along with the sequence of fungal nrITS and *rpb2* genes. The obtained algal nrITS sequences corresponding to the TR1 and TR9 algae from *R. farinacea* and other *Ramalina* species were then used to search sequence databases to determine the presence of these phycobionts in other lichen taxa.

Materials and methods

Taxon sampling

For this study, 31 thalli from nine populations (Table 1) of *R. farinacea* were analyzed. Four populations were from the Iberian Peninsula: Asturias, Puerto de Maravio (As1–2 samples) and Puerto de Ventana (As3–5 samples); León (Le1–5 samples); Castellón (CS1–5 samples) and Ciudad Real (CR1–5 samples). Two populations were from the Canary Islands (Tenerife): Pinar de la Guancha (Gu1-5 samples) and Los Realejos (Re1-5 samples). Two populations were from California: Mount Hamilton, Santa Clara Co. (CA1 sample) and San Francisco Botanical Garden (CA2 sample). Additional details on the geographic coordinates, altitude, bioclimatic belt and phorophytes are provided in Casano *et al.* (2011). All of the studied materials were deposited in the Herbarium of the Department of Botany, Universitat de Valencia (Valencia, Spain), stored at a temperature of −20 °C.

DNA isolation, amplification, and sequencing

All thalli were surface washed by immersion for 10 min in aqueous sodium hypochlorite (final concentration 3% w/v) and then with sterile distillated water prior to DNA isolation. Total DNA was isolated from each collected lichen thallus following the procedure of Cenis (1992), which was developed to rapidly extract fungal DNA for PCR amplification. The algal nrITS were PCR-amplified using the primers of Kroken & Taylor (2000) and Casano *et al.* (2011). Fungal nrITS were amplified using the primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). A portion of the fungal *rpb2* gene, encoding DNA-directed RNA polymerase II polypeptide 2, was amplified with the primer pair RpolRfF: 5′-TGG TTT GTC CAG CAG AGA CC-3′ and RpolRfR 5′-TCT CAA GAT CTT CCG GAG TC-3′.

Amplification reactions were carried out in a total reaction volume of 50 μL and using Illustra Hot Start Mix RTG (GE Healthcare, NJ). Negative controls, without DNA template, were included in every round of PCR amplifications to eliminate false-positive results caused by contaminants in the reagents. Cycling conditions were as follows: one cycle at 94 °C for 2 min and 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplification products were subjected to electrophoresis through 1.5% agarose gels. The corresponding bands were excised from the gel, purified with a DNA gel extraction kit (Qiaex II; Qiagen GmbH, Hiden, Germany) and directly used for sequence analysis. Purified amplicons were sequenced using the Big DyeTM Terminator Cycle Sequence Ready Reaction kit II (Applied Biosystems Co. Foster City, CA), separated by automated multicapillary electrophoresis, and further analyzed on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems Co.).

Alignments, phylogenetic analyses, RNA secondary structure constructions, database searches, and genetic differentiation estimates

All the sequences determined in this study were aligned using the default parameters implemented in Muscle 3.6 (Edgar, 2004), by using SEAVIEW v4 (Gouy *et al.*, 2010), and then adjusted with BIOEDIT 7.0 (Hall, 1999). Data sets were subjected to maximum-parsimony (MP), maximum-likelihood (ML) using PAUP 4.0b10 (Swofford, 2003), and examined with a Bayesian approach by using BEAST (Heled & Drummond, 2010). Trees were displayed with FIGTREE v1.3.1. (Rambaut, 2008). The model for nucleotide substitution was chosen according to the best score obtained in tests performed with JMODELTEST 0.1 (Posada, 2009). In the MP and ML analyses, a heuristic search was carried out using random stepwise addition, tree bisection-reconnection (TBR), and a branch-swapping algorithm to determine the best tree. For the ML analyses, the transition/transversion ratio was estimated from the minimum evolution tree as implemented in PAUP. Tree length (TL) was used for selecting a true tree that corresponded with the minimum evolution criterion (Pauplin, 2000). For Bayesian analyses, the Markov chain Monte Carlo method from a random starting tree was initiated in the Bayesian inference and run for 10^8 generations. Sampling was every 100th generation. After chain convergence analysis, all samples obtained during the first 50 000 generations were discarded as

Table 1. Locations of the collections of *Ramalina farinacea* samples and algae from data banks used in this study and their GenBank accession numbers

				Algal nrITS			
				Predominant phycobiont		Non-predominant phycobiont	
ID isolation	Country/State-Province/Locality	Fungal nrITS	Fungal *rpb2*	Type	Accession	Type	Accession
As1	Spain/Asturias/Puerto de Maravio	JF414683	JF414695	TR1	GU252192	TR9	JF414630
As2	Spain/Asturias/Puerto de Maravio	JF414684	JF414696	TR1	GU252196	TR9	JF414631
As3	Spain/Asturias/Puerto de Maravio	JF414685	JF414697	TR1	GU252195	TR9	JF414632
As4	Spain/Asturias/Puerto de Ventana	JF414691	JF414698	TR1	GU252203	TR9	JF414633
As5	Spain/Asturias/Puerto de Ventana	JF414692	JF414699	TR1	GU252204	TR9	JF414634
Le1	Spain/León	JF414678	JF414716	TR1	GU252187	TR9	JF414651
Le2	Spain/León	JF414679	JF414717	TR1	GU252188	TR9	JF414652
Le3	Spain/León	JF414680	JF414718	TR1	GU252189	TR9	JF414653
Le4	Spain/León	JF414681	JF414719	TR1	GU252190	TR9	JF414654
Le5	Spain/León	JF414682	JF414720	TR1	GU252191	TR9	JF414655
CS1	Spain/Castellón	JF414664	JF414706	TR1	GU252172	TR9	JF414641
CS2	Spain/Castellón	JF414665	JF414707	TR1	GU252173	TR9	JF414642
CS3	Spain/Castellón	JF414666	JF414708	TR1	GU252174	TR9	JF414643
CS4	Spain/Castellón	JF414667	JF414709	TR1	GU252175	TR9	JF414644
CS5	Spain/Castellón	JF414668	JF414710	TR1	GU252176	TR9	JF414645
CR1	Spain/Ciudad Real	JF414669	JF414702	TR1	GU252177	TR9	JF414637
CR2	Spain/Ciudad Real	JF414670	JF414703	TR1	GU252178	TR9	JF414638
CR4	Spain/Ciudad Real	JF414671	JF414704	TR1	GU252180	TR9	JF414639
CR5	Spain/Ciudad Real	JF414672	JF414705	TR1	GU252181	TR9	JF414640
Gu1	Spain/Canary Islands/La Guancha (Tenerife)	JF414673	JF414711	TR9	GU252182	TR1	JF414646
Gu2	Spain/Canary Islands/La Guancha (Tenerife)	JF414674	JF414712	TR9	GU252183	TR1	JF414647
Gu3	Spain/Canary Islands/La Guancha (Tenerife)	JF414675	JF414713	TR9	GU252184	TR1	JF414648
Gu4	Spain/Canary Islands/La Guancha (Tenerife)	JF414676	JF414714	TR9	GU252185	TR1	JF414649
Gu5	Spain/Canary Islands/La Guancha (Tenerife)	JF414677	JF414715	TR9	GU252186	TR1	JF414650
Re1	Spain/Canary Islands/Los Realejos (Tenerife)	JF414686	JF414721	TR9	GU252197	TR1	JF414656
Re2	Spain/Canary Islands/Los Realejos (Tenerife)	JF414687	JF414722	TR9	GU252198	TR1	JF414657
Re3	Spain/Canary Islands/Los Realejos (Tenerife)	JF414688	JF414723	TR9	GU252199	TR1	JF414658
Re4	Spain/Canary Islands/Los Realejos (Tenerife)	JF414689	JF414724	TR9	GU252200	TR1	JF414659
Re5	Spain/Canary Islands/Los Realejos (Tenerife)	JF414690	JF414725	TR9	GU252201	TR1	JF414660
CA1	USA/California/Joseph Grant Co. Park, Santa Clara	JF414693	JF414700	TR1	GU252205	TR9	JF414635
CA2	USA/California/Botanical Garden, San Fancisco City	JF414694	JF414701	TR1	GU252206	TR9	JF414636

burnin. Posterior probabilities for each node were inferred from the resulting consensus tree. Bootstrap probabilities (Felsenstein, 1985) were calculated to estimate the robustness of the clades from 1000 replicates of the data. Statistical parsimony networks were reconstructed using TCS 1.18 (Clement *et al.*, 2000). This program takes into account certain population-level phenomena, such as recombination and the possible persistence of ancestral haplotypes in the population, which are not addressed by traditional methods (MP, NJ, and ML). The inclusion of haplotype networks allows the natural incorporation of the frequently non-bifurcating genealogical information associated with population-level divergences. Database searches were performed by using BLAST (Altschul *et al.*, 1997). RNA secondary structures of algal nrITS sequences were constructed with C software (Matzura & Wennborg, 1996) and modeled at different temperatures (−10, 15, and 37 °C). Temperatures were selected after evaluating their effects of temperature during culture on growth and photosynthesis in isolated TR1 and TR9 phycobionts [partial results are published in Casano *et al.* (2011)]. To estimate genetic differentiation between populations, the DNASP software was used (Librado & Rozas, 2009). This program is able to compute several measures of DNA sequence variation within and between populations. F_{ST} measures the effect of population subdivision, which is the reduction in heterozygosity in a subpopulation owing to genetic drift. F_{ST} values range from zero, indicating panmixis or the absence of genetic divergence, to one, indicating complete isolation or extreme subdivision. Generally, F_{ST} values up to 0.05 imply negligible genetic differentiation, whereas with values > 0.25 very great genetic differentiation within the population analyzed can be expected. Snn is a measure of how often the nearest neighbors of sequences are found in the

same locality. If a population is strongly structured, then the nearest neighbor of a sequence can be expected in the same locality. Thus, the value of Snn will be close to one when the populations at the two localities are highly differentiated. The Snn statistic is more powerful than other statistics such as those of Hudson *et al.* (1992) in cases with small sample sizes (10 or 15), especially in the presence of recombination and low to moderate levels of variation.

Results

Genetic variability of each of the two coexisting phycobionts within *R. farinacea* in relation to mycobiont genotypes

The genetic diversity of the fungal partners in *R. farinacea* lichens was analyzed based on the nrITS and the *rpb2* gene, as described earlier (see Table 1 for information about specimens and GenBank accessions). Phylogenetic reconstructions were performed on 62 new sequences using 31 individuals of *R. farinacea*: 38 sequences from the Iberian Peninsula, 20 from the Canary Islands, and 4 from California. Initial estimations of fungal haplotype diversity (Hd) based on a combination of the two different molecular markers, by implementing the TCS 1.21 program, rendered a single genealogy, thus suggesting that all tested thalli belonged to the same species (data not shown). The obtained haplotype network revealed a clearly differentiated clade including fungal haplotypes of lichens collected in the Canary Islands. The clustering of fungal haplotypes from the Canary Islands was confirmed in phylogenetic reconstructions based on the same molecular markers (Fig. 1). The analysis of the two combined markers included 837 characters: 59 were variable and 11 were parsimony informative. Sequences from *Ramalina complanata* were used as an out-group (accession numbers: FJ356152 for nrITS and DQ883762 for *rpb2*) because of the relative proximity of this species to *R. farinacea* and the availability in GenBank of sequences for the two markers used to construct the phylogeny shown in Fig. 1. MP analyses yielded a tree length (TL) of 62, a consistency index (CI), excluding uninformative characters, of 0.9677, and a retention index (RI) of 0.9512. In the tree depicted in Fig. 1, two main clades are distinguished. Clade I contains several samples from the Iberian Peninsula (Asturias, León, Castellón, and Ciudad Real) and is well supported (bootstrap values of 1000 replicates). Clade II consists of samples from all populations (Iberian Peninsula, Canary Islands, and California) and is supported by a bootstrap value of 74. Samples from León, Castellón, and Ciudad Real had fungal haplotypes distributed throughout the tree of Fig. 1. Samples from Asturias had fungal haplotypes exclusively included in clade I. Samples from the Canary Islands and California

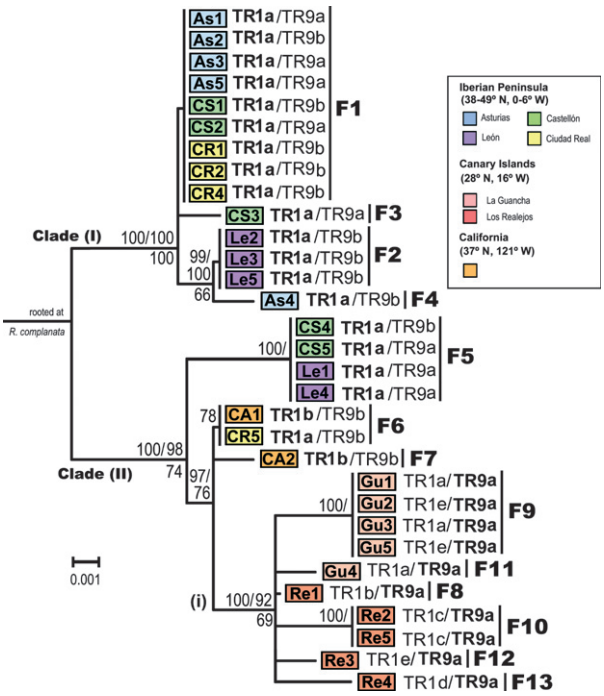

Fig. 1. Phylogram based on the analysis of fungal nrITS and *rpb2* gene sequences in specimens of *Ramalina farinacea*, with *Ramalina complanata* as out-group (accession numbers FJ356152 and DQ883762 for nrITS and *rpb2*, respectively). Vertical bars on the right indicate different fungal haplotypes of F1–F13. Algal haplotypes are indicated at the right of each sample: TR1 haplotypes based on nrITS sequences (TR1a–TR1e) and TR9 haplotypes based on nrITS sequences (TR9a–TR9b) are indicated at the left and the right of the slash-marks, respectively. The predominant phycobiont associated with each fungal haplotype is noted in bold letters. Subclade (i) includes mycobionts from the Canary Islands. The tree was obtained using a Bayesian approach with BEAST. Values at the left and right of the slash-mark on the tree branches correspond to posterior probabilities inferred with Bayesian and MP methods, respectively. Below the branches are the bootstrap values recorded only for those lineages supported by 50% of all (1000) replicates. Subpopulations within the studied populations (the Iberian Peninsula, the Canary Islands and California) and their coordinates are shown in the inset.

had fungal haplotypes exclusively included in clade II. Additional genetic differentiation estimates, obtained with DNASP v5, rendered values of 1.000 and 0.623 for the Snn and F_{ST} statistics (all were $P \leq 0.0001$) carried out for the nrITS, and 0.927 and 0.678 for the Snn and F_{ST} statistics (all were $P \leq 0.0001$) for the *rpb2* gene. These values suggested a high degree of divergence between fungal genotypes from the Iberian Peninsula and the Canary Islands.

Similar genetic differentiation estimates were obtained when DNASP v5 was applied to each separate phycobiont, with values of 0.672 and 0.222 for the Snn and F_{ST} statistics (all them with $0.001 < P < 0.01$) for the TR1 phycobionts and 0.655 and 0.500 for the Snn and F_{ST} statistics (all $0.001 < P < 0.01$) for the TR9 phycobionts. These results

Table 2. Geographic distribution of haplotype combinations of *Ramalina farinacea* phycobionts based on algal nrITS sequences

Haplotype combination	nrITS haplotype of		Geographic locations		
	TR1	TR9	Iberian Peninsula	California	Canary Islands
I	TR1a	TR9b	11*	–	–
II	TR1a	TR9a	8	–	3
III	TR1b	TR9a	–	–	1
IV	TR1c	TR9a	–	–	2
V	TR1d	TR9a	–	–	1
VI	TR1e	TR9a	–	–	3
VII	TR1b	TR9b	–	2	–

*Numbers indicate the observed specimens with a given algal haplotype combination in each studied geographic area. Information on the geographic localities, coordinates, altitude, bioclimatic belt, collection data, and phorophyte are available in Casano *et al.* (2011).

suggested a high degree of divergence between algal genotypes from the Iberian Peninsula and the Canary Islands, as previously reported for mycobionts.

In relation to the genetic diversity of phycobionts, the values of Hd, estimated with the DNASP v5 software, were as follows: absence of polymorphism for TR1 and 0.526 for TR9 phycobionts in the Iberian Peninsula; 0.644 for TR1 and without polymorphism for TR9 phycobionts in the Canary Islands. Regarding the genetic diversity of phycobionts with respect to mycobionts, Fig. 1 shows that the mycobionts from the Canary Islands form a sub-clade (i) within clade II, which is associated with only a single TR9 haplotype (TR9a) but with five algal TR1 haplotypes (TR1a–TR1e). Conversely, all samples from the Iberian Peninsula contained a single TR1 haplotype (TR1a) and two different TR9 haplotypes (TR9a-TR9b). If algal haplotype combinations (TR1 plus TR9) within each lichen thallus are considered, then seven different combinations are obtained (I–VII, Table 2). Among these, only combination II was present in the Iberian Peninsula and the Canary Islands, whereas the remaining combinations were only found either in the Iberian Peninsula (combination I), the Canary Islands (III–VI), or California (VII).

Searching for *Trebouxia* TR1 and *T.* TR9 phycobionts beyond *R. farinacea*

After defining the different algal haplotypes for each sampled thallus of *R. farinacea*, we searched for haplotypes similar to those of either TR1 or TR9 in other lichen taxa. Our search initially focused on other members of the *Ramalina* genus analyzed using the same generic primers for algal nrITS as used for *R. farinacea*. Sequence analyses identified at least five species different from *R. farinacea* but with either TR1 or TR9 *Trebouxia* algae as the phycobiont: *R. calicaris*, *R. fastigiata*, *R. fraxinea*, *R. lusitanica*, and *R. siliquosa* (see Fig. 3 for accessions). In all of these species, TR1 was the phycobiont, except *R. fastigiata*, in which TR9 was the phycobiont. Next, we searched for nrITS haplotypes similar to those reported for TR1 and TR9 algae in the NCBI nucleotide database. While approximately 100 sequences very similar to the TR1 haplotypes were found, only two were similar to the TR9 haplotypes in *Tephromela atra* (accessions EU551521 and EU551522), apart from the sequences of *R. farinacea* and *R. fastigiata* as obtained by our group.

To estimate the Hd for TR1 in relation to the taxa of its fungal partner, the genealogy shown in Fig. 2 was constructed with the TCS 1.21 program. As shown in Fig. 2, nrITS haplotypes similar to TR1 were present in a large diversity of lichens from eight different genera accounting for a total of 16 species: *Lecanora* (*L. bicincta*, *L. cenisia*, *L. lojkaeana* and *L. rupicola*), *Ramalina* (*R. calicaris*, *R. farinacea*, *R. fraxinea*, *R. lusitanica* and *R. siliquosa*), *Tephromela* (*T. atra* and *T. grumosa*), *Amandinea* (*A. punctata*), *Anaptychia* (*A. runcinata*), *Evernia* (*E. prunastri*), *Schaereria* (*S. tenebrosa*), and *Lecidea* (*L. roseotincta*). In the genealogy of TR1 haplotypes, a parent node included three different lichen species from different genera (*L. rupicola*, *L. roesotincta*, and *T. grumosa*) from which three main clades could be derived. Lineage L1 included 61 samples from 12 different species from four genera (*Evernia*, *Lecanora*, *Ramalina*, and *Tephromela*). Lineage L2 included 25 samples from five species from four different genera (*Amandinea*, *Anaptychia*, *Lecanora* and *Tephromela*). Lastly, lineage L3 included three samples from the species *T. grumosa*.

To study the relationships among TR1-like phycobionts, 433 characters were aligned with BIOEDIT 7.0, using several sequences of *Trebouxia decolorans* as out-group (accessions: AJ969550, FJ705190, FJ705199, FJ705201, and FJ705206). MP analyses of the algal nrITS sequences resulted in a total of 65 variable characters, of which 42 were parsimony informative. MP analyses resulted in a TL of 95, a CI of 0.7895 and a RI of 0.9736. The phylogram depicted in Fig. 3 included 121 algal nrITS sequences similar to either TR1 (96 sequences) or TR9 (20 sequences) haplotypes and five corresponded with four *T. decolorans* and one *Trebouxia* sp. as an out-group. As expected, TR1 and TR9 haplotypes appeared in two separate clades. Clade I comprised TR1 haplotypes and clade II, TR9 haplotypes, with high bootstrap values of 88 and 100, respectively. The tree topology was consistent with the genealogy depicted in Fig. 2.

Algal nrITS2 RNA secondary structures of *Trebouxia* TR1 and *T.* TR9 phycobionts

To further characterize algal diversity of the two *Trebouxia* phycobionts, which coexist in *R. farinacea* and are also present in other lichen species, we analyzed the RNA

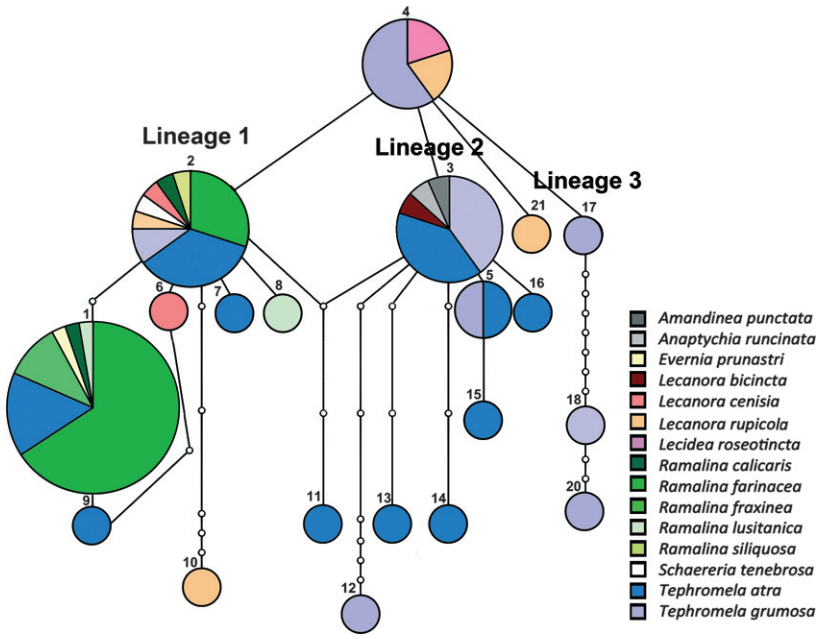

Fig. 2. Parsimony network of algal TR1 haplotypes based on nrITS sequences associated with different fungi. The network was built using the TCS 1.21 program according to the 95% parsimony interval. Each circle corresponds to a particular haplotype. The sizes of the circles are proportional to the number of individuals bearing this particular haplotype. Numerals above circles correspond to algal haplotypes (ITSA) of Fig. 3. Each bar in the network represents a single mutation step and each small empty circles an additional change. Sequences with uncertainness at any nucleotide position have been excluded.

secondary structures of the nrITS2 region. This region comprises the intergenic sequence between 5.8S rDNA and 26S rDNA within the algal nrITS in all specimens noted in Fig. 3. Using the RNADRAW program, we obtained two structural variants of ITS2, ITS2-A, and ITS2-B, when modeled at 15 °C. As shown in Fig. 4, TR1 and TR9 shared these two variants of the ITS2 RNA secondary structure, in which four distinct domains or helices form a 'four-fingered hand', as described in the Introduction. The main difference between ITS2-A and ITS2-B was the presence of an additional helix (IIa) in the ITS2-A structure, resulting in a shortening of helix III.

The secondary structures of both ITS2-A and ITS2-B RNA changed differentially in TR1 and TR9 when the temperature was shifted to higher or lower values (Fig. 4). When the temperature was lowered from 15 to −10 °C, the same two RNA secondary structures, ITS2-A and ITS2-B were obtained in TR1 and TR9 (Fig. 4a and b). However, with an increase in temperature from 15 to 37 °C, a third RNA secondary structure, ITS2-C (Fig. 4b), appeared in TR1, whereas TR9 maintained the ITS2-A structure.

Discussion

Mycobionts and phycobionts from *R. farinacea* are differentially distributed and genetically differentiated between the Iberian Peninsula and the Canary Islands

One of the most remarkable finding of our study is the clear genetic differentiation of mycobionts from *R. farinacea* between the Iberian Peninsula and the Canary Islands. This differentiation was determined by three different and complementary approaches based on the combination of two nuclear-encoded molecular markers (nrITS and the *rpb2* gene). First, a clearly differentiated clade including all samples from the Canary Islands was identified in the haplotype network (data not shown). Second, the clustering of fungal specimens from the Canary Islands within a monophyletic sub-clade (i) was confirmed in the phylogenetic reconstructions shown in Fig. 1. Third, estimates based on F_{ST} and Snn values obtained using the DNASP v5 program corroborated our initial finding of a high degree of genetic divergence between fungal genotypes from the Iberian Peninsula and Canary Islands.

Studies on symbiosis have generally concentrated on a single primary symbiont and its host. Consequently, the focus of studies of heritable changes in development has been on the host genome and, occasionally on the genome of a specific primary symbiont (often in the context of co-evolution studies). Nowadays, the hologenome theory of evolution considers the holobiont with its hologenome, acting in consortium, as a unit of selection in evolution (Rosenberg & Zilber-Rosenberg, 2011). The holobiont has been defined as the host organism together with all of its symbiotic microbiota (Gilbert *et al.*, 2010). Consideration of the hologenome brings forth certain modes of variation in the holobiont, which are unique to this entity. One such variation is 'microbial amplification', in which there is an increase in one group of symbionts relative to others, which can occur when conditions change. An increase in the abundance of a particular microbe is actually equivalent to gene amplification (Rosenberg & Zilber-Rosenberg, 2011). In our study, when 'universal primers' for *Trebouxia* algae

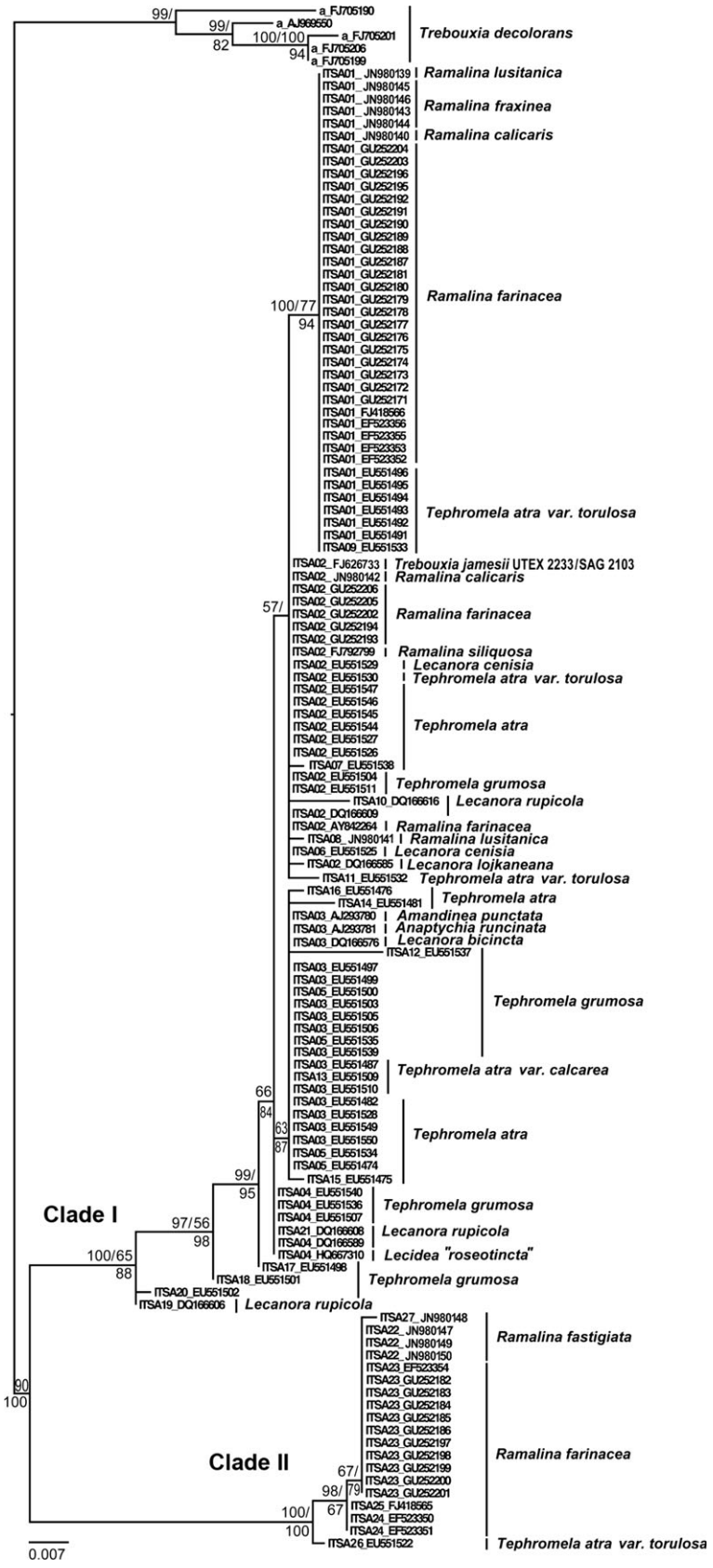

Fig. 3. Phylogram based on the analysis of algal nrITS from TR1 and TR9-like phycobionts associated with different lichen taxa, using several sequences of *Trebouxia decolorans* as out-group (accession numbers: AJ969550, FJ705190, FJ705199, FJ705201 and FJ705206). Vertical bars on the right indicate different lichen species containing a phycobiont whose nrITS sequence is very similar to that of either TR1 or TR9 phycobionts from *Ramalina farinacea*. The tree was obtained using a Bayesian approach with BEAST. Values at the left and right of the slash-mark correspond to posterior probabilities inferred with Bayesian and MP methods, respectively. Below the branches are the bootstrap values recorded only for those lineages supported by 50% of all (100) replicates. The scale bar indicates substitutions/site. Each sample is named according to the number of algal haplotype (ITSA) followed by their GenBank accession number.

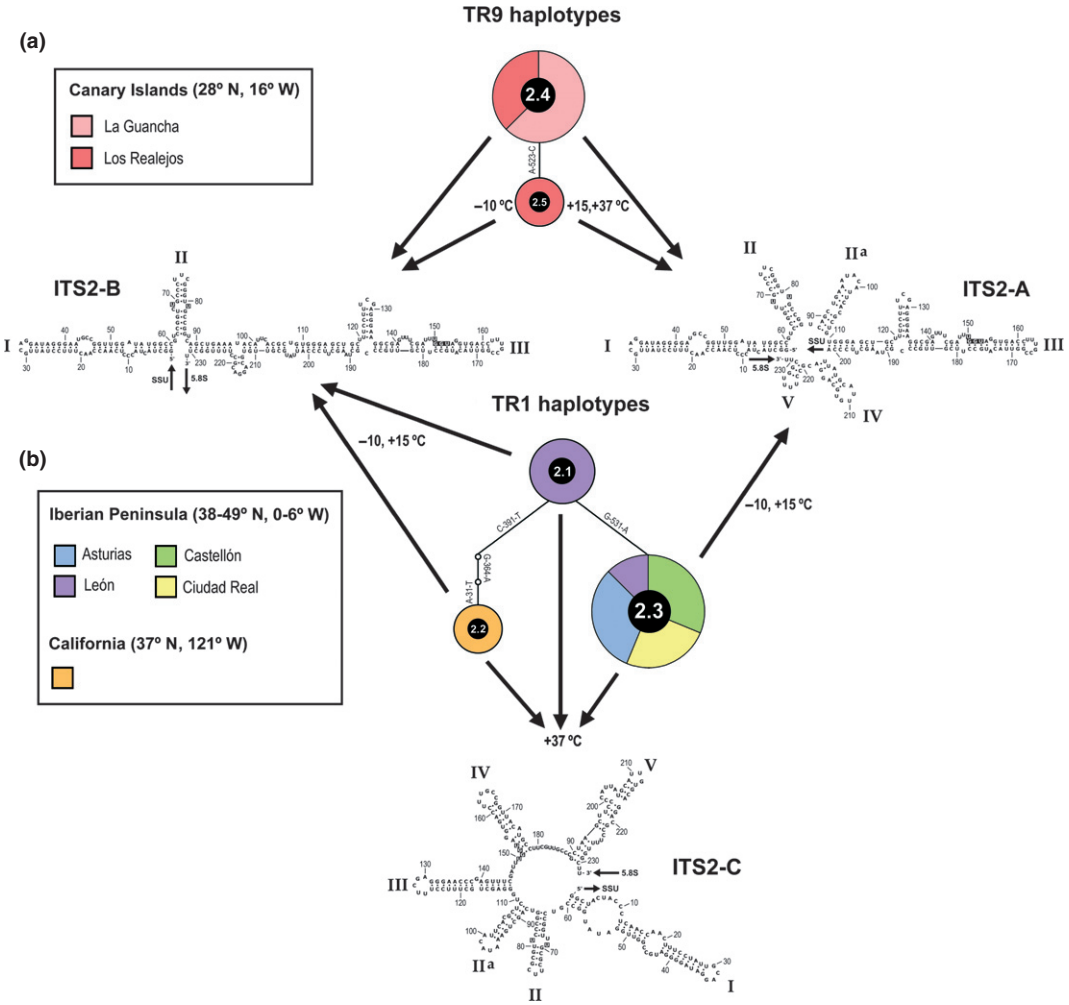

Fig. 4. Haplotype genealogies and RNA secondary structures of *Ramalina farinacea* phycobionts constructed on the basis of nrITS2 RNA sequences. (a) Haplotype genealogy and RNA secondary structures of ITS2 RNA from samples collected in the Canary Islands. (b) Haplotype genealogy and RNA secondary structures of ITS2 RNA from samples collected in the Iberian Peninsula and California. Haplotype genealogies were built using the TCS 1.21 program. The depicted RNA secondary structures were constructed on the basis of the sequences of TR1 phycobionts with the RNADRAW program. The ITS2-A and ITS2-B RNA secondary structures based on the sequences of TR9 phycobionts (not shown) were identical to those of TR1 phycobionts at each given temperature. Subpopulations within the studied populations (the Iberian Peninsula, the Canary Islands, and California) and their coordinates are shown in the insets.

were used, a single algal nrITS sequence per thallus, which clearly corresponded to either TR1 or TR9 phycobionts, was consistently obtained, and never a mixed nrITS sequence. We interpret these results as reflecting the occurrence of 'microbial amplification', with an increase in either TR1or TR9 as suggested in previous studies (Casano *et al.*, 2011). Accordingly, in this study we define the 'predominant phycobiont' to be the alga whose nrITS sequence is obtained after amplification with 'universal or generic primers' (Kroken & Taylor, 2000) for *Trebouxia* algae and the 'non-predominant phycobiont' to be the alga whose nrITS sequence is obtained only after amplifying with primers specific for either TR1 or TR9 (Casano *et al.*, 2011).

After analyzing all the obtained algal nrITS sequences of the predominant and non-predominant phycobionts, we found that the differentiation of *R. farinacea* lichens between the Iberian Peninsula and the Canary Islands was also reflected in their TR1 and TR9 phycobionts. TR1 was the predominant phycobiont in all samples from the Iberian Peninsula and California, whereas TR9 was the predominant phycobiont in all samples from the Canary Islands, as previously reported (Casano *et al.*, 2011).

Additionally, the predominant TR9 phycobiont in the Canary Islands was found to be linked with a series of fungal haplotypes that represent a clearly distinguishable sub-clade (i), shown in Fig. 1. If this finding implies an incipient specialization of *R. farinacea* fungal partners

(enclosed in sub-clade i) in different geographic areas, then this is an important contribution to our understanding of lichen evolution. The differential specialization of symbionts is accompanied by a higher genetic diversity of both the mycobiont and the non-predominant phycobionts in the Canary Islands (Hd = 0.822 and Hd = 0.644, respectively) than in the Iberian Peninsula (Hd = 0.432 and Hd = 0.526). The higher diversity and the apparent specialization of Canary Islands populations may be explained by the island immaturity-speciation pulse model of island evolution, which posits that the opportunities for speciation have a broadly predictable relationship to the life cycle of oceanic islands (Whittaker *et al.*, 2007). Opportunity drives the speciation rate and is greatest at a relatively early stage in the life cycle of an island, when the intrinsic carrying capacity exceeds species richness by the greatest margin. Tenerife is one of the youngest islands within the Canarian Archipelago, which is generally thought to have emerged initially as two or possibly three separate islands, the oldest dating back to *c.* 11 Ma. These proto-islands fused intro the single present-day island only within the last 3.3 Myr (Fernández-Palacios *et al.*, 2011). Moreover, during their life span, volcanic oceanic islands are often subject to a series of catastrophic events. Thus, in terms of their geological dynamics, the Canary Islands are clearly different from other island groups (Whittaker *et al.*, 2007).

ITS2 RNA temperature-dependent secondary structure variations provide new physiological clues regarding the persistence of TR1 and TR9 phycobionts within *R. farinacea*

The thermal variations of the ITS2 RNA secondary structure provided new physiological clues regarding the persistence of TR1 and TR9 phycobionts within *R. farinacea*. The RNA secondary structure of ITS2 is highly conserved among eukaryotes showing canonical domains forming a 'four-fingered hand' and several conserved features (Mai & Coleman, 1997). Maturation of the large subunit rRNA includes a series of cleavages that result in the removal of ITS2, separating mature 5.8S and 25/28S rRNAs. Several investigations have demonstrated that the formation of a higher-order secondary structure within the assembling pre-ribosomal particle is a prerequisite for accurate and efficient pre-rRNA processing (Cote *et al.*, 2002). It has long been known that temperature-related alterations in rRNA processing greatly inhibit the production of large ribosomal subunits in the cytoplasm (Toniolo *et al.*, 1973). To find a possible link between the different nrITS sequences of TR1 and TR9 phycobionts and distinct physiological traits, models of the RNA secondary structures of the ITS2s from all the TR1 and TR9 haplotype variants at different temperatures were created. This approach yielded important physiological insight into the persistence of TR1 and TR9 phycobionts within *R. farinacea* thalli. As reported in the Results, the RNA secondary structure of this key intergenic region seems to be highly thermally stable in TR9 phycobionts but thermally labile in TR1 phycobionts. In this study, two variants of the ITS2 RNA secondary structures were noted to occur at −10 and 15 °C in TR1 and TR9 phycobionts (Fig. 4). These two structures, ITS2-A and ITS2-B, may be comparable with the two alternative secondary structures previously proposed for ITS2 (Yeh & Lee, 1990; Joseph *et al.*, 1999). Elements of both structural models are important in efficient rRNA processing (Cote *et al.*, 2002). However, when the same structures were modeled at 37 °C, we obtained a third variant in the TR1 phycobionts (ITS2-C) but not in the TR9 phycobionts. This ITS2-C RNA structure lacked the elemental features necessary for the effective removal of ITS2 after transcription. These structural models are hypothetical, and biochemical experimentation is necessary to support their configurations. Nevertheless, it may be that the predicted thermal dependence of the ITS2 RNA secondary structure and its probable consequences on nuclear ribosomal cistron processing contribute to explain the superior physiological performance of TR9 phycobionts under relatively high temperatures, in contrast to TR1, which thrives at relatively low–moderate temperatures (Casano *et al.*, 2011). It is therefore possible that the *R. farinacea* fungus utilizes the two algae to broaden its physiological behavior in response to changing environmental conditions.

Together, these results can be interpreted as an incipient specialization of mycobionts along with a bias in phycobiont predominance (TR1 in the Iberian Peninsula and TR9 in the Canary Islands). This specialization is probably imposed by gross ecological traits related to latitude (see inset of Fig. 1) and/or the continentality/insularity of geographic locations. The latitude of the two Californian localities was similar to that of the localities from the Iberian Peninsula (see inset of Fig. 1) and likewise subject to a Mediterranean climate. Interestingly, fungal haplotypes from California (F6 and F7) were included within clade II but outside sub-clade (ii) in Fig. 1. These two samples had TR1 as predominant phycobiont (as in all samples from the Iberian Peninsula) but the TR1b haplotype (as several samples from the Canary Islands). The adjacent positions of the Californian and Canarian samples (F6) in Fig. 1 and their phycobiont composition may suggest an intermediate diversification status of holobionts. However, a higher number of samples from California must be analyzed to confirm this assumption.

Changes in fungal genomes seem to be encompassed with changes in each algal genome, resulting in concerted

changes in the three genomes. Such changes may illustrate a transient phase toward specialization by the fungal and algal partners in the different habitats and seems to be facilitated by the association with more than one phycobiont in each thallus.

The coexistence of two or perhaps more physiologically different *Trebouxia* taxa within the same lichen thallus migth be more common than previously thought

It has been shown that *Ramalina* species from different climates prefer distinct lineages of *Trebouxia* algae (Cordeiro *et al.*, 2005). In *Tephromela atra* and *T. grumosa*, a number of lichen thalli were shown to have TR1 as the phycobiont (named *Trebouxia* spec. 2 in Muggia *et al.*, 2008, 2010) while in only two samples of *T. atra* was TR9 the phycobiont. Interestingly, these two samples were collected from Ischia, a volcanic island lying at the northern end of the Gulf of Naples, in Italy. Preliminary studies in our laboratory with specimens of *R. fastigiata* (Catalá S, unpublished results) and *T. atra* (del Campo *et al.*, unpublished results) from different localities of the Iberian Peninsula, using the same molecular markers as in *R. farinacea*, revealed the coexistence of TR1 and TR9 in all the analyzed samples. Therefore, the TR1/TR9 binomial may be more frequent than previously thought suggesting its successful colonization of at least Mediterranean and Macaronesian regions.

Lecanora rupicola also has TR1 its phycobiont referred to as *Trebouxia* spec. 2 in Blaha *et al.* (2006). Recently, an association between *Lecidea roseotincta* and two different *Trebouxia* species was reported (Schmull *et al.*, 2011). One of the *Trebouxia* species has been named *T. roseotinctae* (accession HQ667310) but seems to be TR1, based on its nrITS sequences. In addition, at least six different *Trebouxia* phycobionts have been detected, including one that seems to be TR1, in *R. fraxinea* (Catalá *et al.*, unpublished results). Interestingly, more than two of these algae occupied (by TEM) the same thallus. These considerations along with the results of this study suggest that the coexistence of at least two physiologically different *Trebouxia* strains or species within the same lichen thallus is a not uncommon occurrence.

TR1 phycobionts are widespread among lichens, with a preferably Mediterranean distribution

Our search results produced approximately 100 nrITS sequences very similar to those from *Trebouxia* phycobionts in a large variety of lichen taxa within *Lecanorales* but in distinct families, such as *Lecanoraceae* (Schmull *et al.*, 2011) *Parmeliaceae* (*Evernia prunastri*, this work), *Physciaceae* (Helms *et al.*, 2001), *Ramalinaceae* (several *Ramalina* species in this work), and *Tephromelataceae* (Muggia *et al.*, 2008, 2010). As shown in Fig. 2, the majority of Hd in TR1 phycobionts descended from a single haplotype (ITSA-4). This ancestral haplotype, although not highly represented, seems to be widely distributed both phylogenetically and geographically. Phylogenetically, it is shared by three lichen species belonging to three distinct genera (*Lecidea*, *Lecanora*, and *Tephromela*) but it is absent in *Ramalina*. Geographically, it has been found within median–high latitudes, ranging from 38 to 43° in Spain and Italy to higher than 54° in Denmark and Norway. The observation of multiple pathways, including three distinct lineages and interconnections resulting in a network linking haplotypes, is consistent with recombination. In this network, haplotypes of intermediate ancestry, such as ITSA-1, ITSA-2, and ITSA-3, are associated with a number of fungi. Further down in the genealogy, a radiation of new haplotypes is observed, especially in *Tephromela atra*. It is noteworthy that the genealogy includes a similar number of samples from *R. farinacea* and *T. atra*, which belong to the same order (*Lecanorales*) and suborder (*Lecanorineae*) but to different families, that is, *Ramalinaceae* and *Lecanoraceae*, respectively. The distribution of these two lichen species is rather cosmopolitan, as they have been studied in samples collected in similar geographic areas in the Mediterranean regions of Italy and Spain. As shown in Fig. 2, in *R. farinacea* there are only two different haplotypes (ITSA-1 and 2), both rather ancestral and constrained to lineage 1. This contrast with the higher number of different haplotypes in *T. atra*, most of which belong to lineage 2 (e.g. haplotypes ITSA-7, 9, 11–16). This difference might reflect the different modes of reproduction of *T. atra* and *R. farinacea*, sexual and asexual, respectively. However, the presence of diverse and in some cases shared (e.g. ITSA-3 and 5) haplotypes in *T. grumosa*, which is closely related to *T. atra*, suggests an alternative explanation since *T. grumosa* reproduces asexually.

Most of the sequences shown in Figs 2 and 3 were from lichens collected in Mediterranean regions, with TR1 being more widespread than TR9. In the case of TR1, the same algal genotypes are found in association with taxonomically different fungi whose habitats have a preferred Mediterranean distribution (e.g. ITSA-1, ITSA-2, and ITSA-3 in Fig. 3). These observations, along with the physiological differences determined in the two phycobionts (Casano *et al.*, 2011; del Hoyo *et al.*, 2011), are consistent with an eco-physiological adaptation to Mediterranean conditions. Associations of phycobionts enclosed in particular algal clades with taxonomically different but ecologically similar lichens also have been

observed in *Asterochloris* algae (Peksa & Skaloud, 2011). These associations include two distinct genera (*Lepraria* and *Setereocaulon*) belonging to a single family (*Stereocaulaceae*). In the present work, the same phycobionts were found to be shared in several distinct genera from five distinct families (*Lecanoraceae*, *Parmeliaceae*, *Physciaceae*, *Ramalinaceae*, and *Tephromelataceae*).

Experimental evidence indicates that the lichen association between photobionts and mycobionts increases tolerance to stress conditions (Kranner *et al.*, 2005; Kosugi *et al.*, 2009; Catalá *et al.*, 2010). By changing the composition (qualitatively and quantitatively) of their algal partners, lichenized fungi may expand their ability to adapt to a broad range of ecological conditions. Sun & Friedmann (2005) noted that the ratio of consumer (fungi) vs. producer (algae) can be adjusted depending on the climatic conditions. According to their 'community adaptation hypothesis', the temperature of the habitat correlates with a higher proportion of producers to account for the respiration of both partners. Apart from this mode of adaptation, there is some evidence that widely distributed lichens can adjust their algal partner according to their presence in different habitats, following the concept of 'habitat-adapted symbiosis' (Rodriguez *et al.*, 2008). For example, *L. rupicola* has different photobiont species in alpine vs. Mediterranean habitats (Blaha *et al.*, 2006), and a similar situation was shown to occur in *Lecidea fuscoatra* (unpublished results). Our results suggest a third possibility of adaptation, in which physiologically distinct strains of the same or sister species are present in the same thallus. Whether two or perhaps more *Trebouxia* species coexist in the same lichen thallus in other lichen taxa remains the subject of further studies using approaches similar to those applied in *R. farinacea*. Additional physiological studies are also necessary to confirm our hypothesis that the coexistence of physiologically complementary photobionts within the same lichen thallus increases tolerance to changing and often stressful environments. Another question that remains to be answered is whether the proportion of these strains varies according to the ecological conditions.

Finally, it will be of interest to determine whether the ecological diversification and speciation of lichen symbionts in the different habitats includes a transient phase, consisting of associations between more than one phycobiont in individual thalli. Alternatively, the coexistence of multiple phycobionts may be crucial for increasing ecophysiological fitness under changing environments and therefore might be the rule in lichens living on a wide variety of substrata and in diverse habitats. These considerations along with the results of this study suggest that the coexistence of at least two physiologically different *Trebouxia* strains or species within the same lichen thallus is a not uncommon occurrence, even though the relative abundance of strains may vary substantially in different species or ecological settings.

Acknowledgements

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References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Barreno E (2004) Hongos simbiontes: Líquenes, Micoficobiosis y Micorrizas. *Botánica* (Izco J, ed.), pp. 309–340. MacGraw-Hill Interamericana, Madrid.

Beiggi S & Piercey-Normore MD (2007) Evolution of ITS ribosomal RNA secondary structures in fungal and algal symbionts of selected species of *Cladonia* sect. *Cladonia* (Cladoniaceae, Ascomycotina). *J Mol Evol* **64**: 528–542.

Blaha J, Baloch E & Grube M (2006) High photobiont diversity associated with the euryoecious lichen-forming ascomycete *Lecanora rupicola* (Lecanoraceae, Ascomycota). *Biol J Linn Soc* **88**: 283–293.

Buschbom J & Mueller GM (2006) Testing "species pair" hypotheses: evolutionary processes in the lichen-forming species complex *Porpidia flavocoerulescens* and *Porpidia melinodes*. *Mol Biol Evol* **23**: 574–586.

Casano LM, del Campo EM, Garcia-Breijo FJ, Reig-Armiñana J, Gasulla F, Del Hoyo A, Guera A & Barreno E (2011) Two *Trebouxia* algae with different physiological performances are ever-present in lichen thalli of *Ramalina farinacea*. Coexistence versus competition? *Environ Microbiol* **13**: 806–818.

Cassie DM & Piercey-Normore MD (2008) Dispersal in a sterile lichen-forming fungus, *Thamnolia subuliformis* (Ascomycotina: Icmadophilaceae). *Botany* **86**: 751–762.

Catalá M, Gasulla F, Pradas del Real AE, Garcia-Breijo F, Reig-Armiñana J & Barreno E (2010) Fungal-associated NO is involved in the regulation of oxidative stress during rehydration in lichen symbiosis. *BMC Microbiol* **10**: 297.

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res* **20**: 2380.

Chapman MJ & Margulis L (1998) Morphogenesis by symbiogenesis. *Int Microbiol* **1**: 319–326.

Clement M, Posada D & Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Mol Ecol* **9**: 1657–1659.

Cordeiro LMC, Reis RA, Cruz LM, Stocker-Wögöter E, Grube M & Iacomini M (2005) Molecular studies of photobionts of selected lichens from the coastal vegetation of Brazil. *FEMS Microbiol Ecol* **54**: 381–390.

Cote CA, Greer CL & Peculis BA (2002) Dynamic conformational model for the role of ITS2 in pre-rRNA processing in yeast. *RNA* **8**: 786–797.

Crespo A & Pérez-Ortega S (2009) Cryptic species and species pairs in lichens: a discussion on the relationship between molecular phylogenies and morphological characters. *Anales del Jardín Botánico de Madrid* **66S1**: 71–81.

del Campo EM, Gimeno J, De Nova JPG, Casano LM, Gasulla F, García-Breijo F, Reig-Armiñana J & Barreno E (2010) South European populations of *Ramalina farinacea* (L.) Ach. share different *Trebouxia* algae. *Together and Separate: The Lives of the Lichen Symbionts* (Nash THI, ed.), pp. 1–2. J. Cramer in der Gebrüder Borntraeger Verlagsbuchhandlung, Stuttgart.

del Hoyo A, Álvarez R, del Campo EM, Gasulla F, Barreno E & Casano LM (2011) Oxidative stress induces distinct physiological responses in the two *Trebouxia* phycobionts of the lichen *Ramalina farinacea*. *Ann Bot* **107**: 109–118.

Doering M & Piercey-Normore MD (2009) Genetically divergent algae shape an epiphytic lichen community on Jack Pine in Manitoba. *Lichenologist* **41**: 69–80.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**: 1792–1797.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fernández-Palacios JM, de Nascimento L, Rüdiger O, García-del-Rey E, Arévalo JR & Whittaker RJ (2011) A reconstruction of Palaeo-Macaronesia, with particular reference to the long-term biogeography of the Atlantic island laurel forests. *J Biogeogr* **38**: 226–246.

Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol Ecol* **2**: 113–118.

Gilbert SF, McDonald E, Boyle N, Buttino N, Gyi L, Mai M, Prakash N & Robinson J (2010) Symbiosis as a source of selectable epigenetic variation: taking the heat for the big guy. *Philos Trans R Soc Lond B Biol Sci* **365**: 671–678.

Gouy M, Guindon S & Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**: 221–224.

Grube M (2010) Die Hard: *Lichens. Symbioses and Stress* (Seckbach J & Grube M, eds), pp. 509–523. SpringerLink, Dordrecht-Heidelberg.

Grube M & Berg G (2009) Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol Rev* **23**: 72–85.

Grube M & Muggia L (2010) Identifying algal symbionts in lichen symbioses. *Tools for Identifying Biodiversity: Progress and Problems* (Nimis PL & Vignes Lebbe R, eds), pp. 295–299. Edizioni Università di Trieste, Trieste.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**: 95–98.

Heled J & Drummond AJ (2010) Bayesian inference of species trees from multilocus data. *Mol Biol Evol* **27**: 570–580.

Helms G, Friedl T, Rambold G & Mayrhofer H (2001) Identification of photobionts from the lichen family *Physciaceae* using algal-specific ITS or rDNA sequencing. *Lichenologist* **33**: 73–86.

Honegger R (1991) Fungal evolution: symbiosis and morphogenesis. *Symbiosis as a Source of Evolutionary Innovation* (Margulis L & Fester R, eds), pp. 319–340. The MIT Press, Cambridge, Massachusetts.

Hudson RR, Boos DD & Kaplan NL (1992) A statistical test for detecting geographic subdivision. *Mol Biol Evol* **9**: 138–151.

Joseph N, Krauskopf E, Vera MI & Michot B (1999) Ribosomal internal transcribed spacer 2 (ITS2) exhibits a common core of secondary structure in vertebrates and yeast. *Nucleic Acids Res* **27**: 4533–4540.

Kelly LJ, Hollingsworth PM, Coppins BJ, Ellis CJ, Harrold P, Tosh J & Yahr R (2011) DNA barcoding of lichenized fungi demonstrates high identification success in a floristic context. *New Phytol* **191**: 288–300.

Kosugi M, Arita M, Shizuma R, Moriyama Y, Kashino Y, Koike H & Satoh K (2009) Responses to desiccation stress in lichens are different from those in their photobionts. *Plant Cell Physiol* **50**: 879–888.

Kranner I & Lutzoni F (1999) Evolutionary consequences of transition to a lichen symbiotic state and physiological adaptation to oxidative damage associated with poikilohydry. *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization* (Lerner HR, ed.), pp. 591–628. M. Dekker, New York.

Kranner I, Cram WJ, Zorn M, Wornik S, Yoshimura I, Stabentheiner E & Pfeifhofer HW (2005) Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. *P Natl Acad Sci USA* **102**: 3141–3146.

Krog H & Osthagen H (1980) The genus *Ramalina* in the Canary Islands. *Norw J Bot* **27**: 255–296.

Kroken S & Taylor JW (2000) Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist* **103**: 645–660.

Librado P & Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452.

Mai JC & Coleman AW (1997) The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. *J Mol Evol* **44**: 258–271.

Margulis L & Barreno E (2003) Looking at lichens. *Bioscience* **53**: 776–778.

Matzura O & Wennborg A (1996) RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. *Bioinformatics* **12**: 247–249.

Muggia L, Grube M & Tretiach M (2008) Genetic diversity and photobiont associations in selected taxa of the *Tephromela atra* group (Lecanorales, lichenised Ascomycota). *Mycol Prog* **7**: 147–160.

Muggia L, Zellnig G, Rabensteiner J & Grube M (2010) Morphological and phylogenetic study of algal partners

associated with the lichen-forming fungus *Tephromela atra* from the Mediterranean region. *Symbiosis* **51**: 149–160.

Nelsen MP & Gargas A (2008) Dissociation and horizontal transmission of codispersing lichen symbionts in the genus *Lepraria* (Lecanorales: Stereocaulaceae). *New Phytol* **177**: 264–275.

Paracer S & Ahmadjian V (2000) *Symbiosis: An Introduction to Biological Associations*. Oxford University Press, Oxford, New York.

Pauplin Y (2000) Direct calculation of a tree length using a distance matrix. *J Mol Evol* **51**: 41–47.

Peksa O & Skaloud P (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). *Mol Ecol* **20**: 3936–3948.

Piercey-Normore MD (2006) The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. *New Phytol* **169**: 331–344.

Piercey-Normore MD & Deduke C (2011) Fungal farmers or algal escorts: lichen adaptation from the algal perspective. *Mol Ecol* **20**: 3708–3710.

Posada D (2009) Selection of models of DNA evolution with jModelTest. *Methods Mol Biol* **537**: 93–112.

Rambaut A (2008) *FigTree: Tree Figure Drawing Tool Version 1.3.1 2006–2009*, Institute of Evolutionary Biology, University of Edinburgh. http://tree.bio.ed.ac.uk/.

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim YO & Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. *ISME J* **2**: 404–416.

Romeike J, Friedl T, Helms G & Ott S (2002) Genetic diversity of algal and fungal partners in four species of *Umbilicaria* (Lichenized Ascomycetes) along a transect of the Antarctic peninsula. *Mol Biol Evol* **19**: 1209–1217.

Rosenberg E & Zilber-Rosenberg I (2011) Symbiosis and development: the hologenome concept. *Birth Defects Res C* **93**: 56–66.

Schmull M, Miadlikowska J, Pelzer M, *et al.* (2011) Phylogenetic affiliations of members of the heterogeneous lichen-forming fungi of the genus *Lecidea* sensu Zahlbruckner (Lecanoromycetes, Ascomycota). *Mycologia* **103**: 983–1003.

Schultz J, Maisel S, Gerlach D, Muller T & Wolf M (2005) A common core of secondary structure of the internal transcribed spacer 2 (ITS2) throughout the Eukaryota. *RNA* **11**: 361–364.

Skaloud P & Peksa O (2010) Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta). *Mol Phylogenet Evol* **54**: 36–46.

Sun HJ & Friedmann EI (2005) Communities adjust their temperature optima by shifting producer-to-consumer ratio, shown in lichens as models: II. Experimental verification. *Microb Ecol* **49**: 528–535.

Swofford DL (2003) *PAUP. Phylogenetic Analysis Using Parsimony (and other Methods), Version 4*. Sinauer Associates, Sunderland, MA.

Toniolo D, Meiss HK & Basilico C (1973) A temperature-sensitive mutation affecting 28S ribosomal RNA production in mammalian cells. *P Natl Acad Sci USA* **70**: 1273–1277.

Werth S & Sork VL (2010) Identity and genetic structure of the photobiont of the epiphytic lichen *Ramalina menziesii* on three oak species in southern California. *Am J Bot* **97**: 821–830.

White TJ, Bruns T, Lee S & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sninsky JJ & White TJ, eds), pp. 315–322. Academic Press, Inc, New York.

Whittaker RJ, Ladle RJ, Araújo MB, Fernández-Palacios JM, Delgado JD & Arévalo JR (2007) The island immaturity – alternative to the "diversity begets diversity" model. *Ecography* **30**: 321–327.

Wornik S & Grube M (2010) Joint dispersal does not imply maintenance of partnerships in lichen symbioses. *Microb Ecol* **59**: 150–157.

Yahr R, Vilgalys R & DePriest PT (2004) Strong fungal specificity and selectivity for algal symbionts in Florida scrub *Cladonia* lichens. *Mol Ecol* **13**: 3367–3378.

Yahr R, Vilgalys R & DePriest PT (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytol* **171**: 847–860.

Yeh LC & Lee JC (1990) Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J Mol Biol* **211**: 699–712.

Zoller S & Lutzoni F (2003) Slow algae, fast fungi: exceptionally high nucleotide substitution rate differences between lichenized fungi *Omphalina* and their symbiotic green algae *Coccomyxa*. *Mol Phylogenet Evol* **29**: 629–640.