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Photobiont selectivity and specificity in *Caloplaca* species in a fog-induced community in the Atacama Desert, northern Chile

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ABSTRACT

Little is known about the nature of the association between mycobionts and photobionts in isolated lichen communities. Here we studied the photobiont diversity of different *Caloplaca* species in a fog-induced community in the Atacama Desert. We compared nrDNA ITS sequences of both symbionts, photobionts and mycobionts, along with morphological characters of the different lichen thalli, to investigate the diversity and to assess the degree of selectivity and specificity of photobiont species in a community of *Caloplaca* species. Specimens of six fungal species (*C. orthoclada*, *C. fernandeziana*, and four undescribed species) were sampled along an altitudinal gradient on a coastal bluff with a strong fog presence, 60 km south of Iquique, Chile. The photobiont species in this community belong to three species of the genus *Trebouxia* in the strict sense: *T. arboricola*, *T. decolorans*, and *T. gigantea*. Most of the fungal species were lichenized with photobionts belonging to different haplotypes of *T. arboricola* and *T. decolorans*, although the algae of three specimens, associated with two fungal species (*C. orthoclada* and *C.* sp1), were related to representatives of *T. gigantea*. These results indicate that members of the genus *Caloplaca* in northern Chile have moderate photobiont selectivity and appear to be selective to members of the *T. arboricola* group. Also, at high altitudes, changes in the photobiontal haplotype composition were observed in comparison to lower altitudes, probably generated by a higher water availability given higher fog condensation and precipitation in the upper areas of the bluff. This may suggest that ecological factors, such as altitude and water availability could result in a local shift of the associated photobiont and specialization as a product of local adaptation.

Introduction

As lichens are formed by the symbiotic association of at least one eukaryotic algal or a cyanobacterial species (the photobiont) and one fungal species (the mycobiont), the establishment of a functioning symbiosis requires a mycobiont to meet and associate with a suitable free-living photobiont or to arrest already lichenized photobiont cells (Beck *et al.* 1998; Honegger 2008). The probability of occurrence of this prospective association between a potential mycobiont

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and a compatible free-living algae is considered to be rather low in nature, and is highly increased if the mycobiontal hyphae are able to meet and aggregate with a less compatible photobiont and remain in that state until the association with a compatible biont is generated and a thallus can be formed (Honegger 2008). By this mechanism, after spore germination a mycobiont can survive for a given time on its substrate until it is associated with a more compatible photobiont species (Sanders 2010).

In sexually reproducing fungal species, relichenization is considered to be a necessary event in the life cycle of any lichen, in which germinated spores are expected to associate with free-living algae (Beck *et al.* 2002). In these cases, the photobionts are considered to be horizontally transmitted to the fungus (Honegger & Scherrer 2008). This type of re-lichenization, involving horizontal transmission of photobionts, it is considered to be common (e.g., Beck *et al.* 2002), although it is possible that the photobiontal cells may be provided by lichen thalli already established in the surroundings (e.g., by escaped zoospores) and be arrested by newly germinated spores in the absence of free-living photobionts (Beck *et al.* 1998). This form of horizontal transmission has also been observed in asexual or nearly asexual lichen species (Piercey-Normore 2006; Nelsen & Gargas 2008).

The photobiont may also disperse as part of a vegetative propagule of the thallus, which allows the development of a new lichen thallus with the same pool of photobionts as the initial or parental thallus. This mechanism is known as vertical transmission of photobionts and may explain the photobiont availability in asexual lichen fungi or in those species where the asexual reproduction mode is dominant and the acquisition of free-living algae less probable (Werth *et al.* 2006; Cassie & Piercey-Normore 2008).

Ecological factors can shape the niche of different photobionts, modulating to some degree the composition of different lichen communities (Beck *et al.* 2002; Yahr *et al.* 2006; Hedenås *et al.* 2007; Fernández-Mendoza *et al.* 2011; Marini *et al.* 2011). Thus, in more or less isolated communities the availability of free-living algal cells could be reduced, resulting in a prevailing low photobiont selectivity, understood as the range of possible partners that can be selected by a particular mycobiont, while different lichen species may share the same pool of green algal photobionts (Beck 1999; Helms *et al.* 2001; Beck *et al.* 2002; Romeike *et al.* 2002; Yahr *et al.* 2004). The range of interacting partners of both symbionts (degree of selectivity) determines the specificity of the association (Beck *et al.* 2002). This heterogeneity of photobiont composition may represent multiple re-lichenization events in a community (Beck *et al.* 2002; Hill 2009) where new thalli associate with the available photobiontal strains, either free-living or already associated with other lichen species. This ability to associate with different and locally adapted photobionts has been stated to be of evolutionary advantage as it allows lichen mycobionts to colonize different habitats (Beck 1999; Peršoh *et al.* 2004; Piercey-Normore 2004, 2006, 2009; Honegger 2008; Nelsen & Gargas 2008, 2009).

To understand processes of selectivity and specificity among myco- and photobionts in whole lichen communities it is necessary to examine variation in both bionts (Beck 1999). Here we used nrDNA ITS sequences of both myco- and photobionts to compare the genetic composition of photobionts in different *Caloplaca* species growing together along an altitudinal gradient in a fog-induced community in the Atacama Desert in northern Chile. We use *Caloplaca* as a model as it is distributed along the whole altitudinal and precipitation gradient in this area.

Rich lichen communities develop at the heights of the coastal bluffs along the Cordillera de la Costa (Follmann 1995; Moreira-Muñoz 2011), isolated from each other as they are distributed along the discontinuous top of the coastal range (Rundel 1978). These communities are sustained by a characteristic fog that moves inland from the ocean at altitudes between 500 and 1000 m (Cereceda *et al.* 2002, 2008), and have been the focus of multiple floristic and ecophysiological studies (Follmann & Redón 1972; Redón 1973; Redón *et al.* 1975; Rundel 1978; Redón & Lange 1983; Follmann 1995).

The altitudinal variation in liquid water content of the fog (Cereceda *et al.* 2002, 2008) provides an environmental gradient in the water stress at a small spatial scale (<2 km). Also, stressing conditions can generate variations in the reproductive strategy of lichens, as reduction in soredia production in asexually reproducing species (Mikhailova 2002) and increased apothecia and spore production in sexually reproducing species (Mikhailova 2007). Stressing conditions can also lead to a lower selectivity of the mycobiont towards its photobiont, limited by the availability of photobionts in the environment. Thus, changes in the water availability as a limiting factor in the altitudinal gradient may result in differences in the morphology and reproductive strategies in the species present along the altitudinal gradient and higher photobiont selectivity might be expected.

In the present study, we investigate the associations between partners in lichen communities along an ecological gradient. In particular, we postulate that 1) photobiont composition changes with altitude and precipitation along the altitudinal gradient in the coastal hill range in the Atacama Desert; 2) selectivity of photobionts is higher when ecological pressure diminishes (e.g., at high altitudes); 3) changes in the reproductive strategy (i.e., increased vegetative dispersal, reduced spore production) are common at higher altitudes and greater water availability as a response to less stressing conditions in spore-producing species; and 4) horizontal transmission is the main strategy of photobiont acquisition in the coastal hill range of the Atacama Desert.

Materials and methods

Study site

Alto Patache is a fog oasis located at the top of the coastal hill range of Punta Patache, in the Atacama Desert, Northern Chile (24°49′36″S, 70°09′27″W). The highest altitudes range between 750 and 800 m above sea level (Fig 1A). In the lower altitudes (250–400 m) the area is dominated by ephemeral vegetation, mostly *Nolana jaffuelii*, *Leucocoryne appendiculata*, and *Tetragonia ovata* (Pinto & Leubert 2009). Above 600 m there is a shift in the vegetation, with a diminution of ephemeral vegetation and a dominance of perennial shrub-like species (e.g., *Ephedra breana*, *Nolana sedifolia*, *Nolana intonsa*, *Lycium deserti*, *Frankenia*

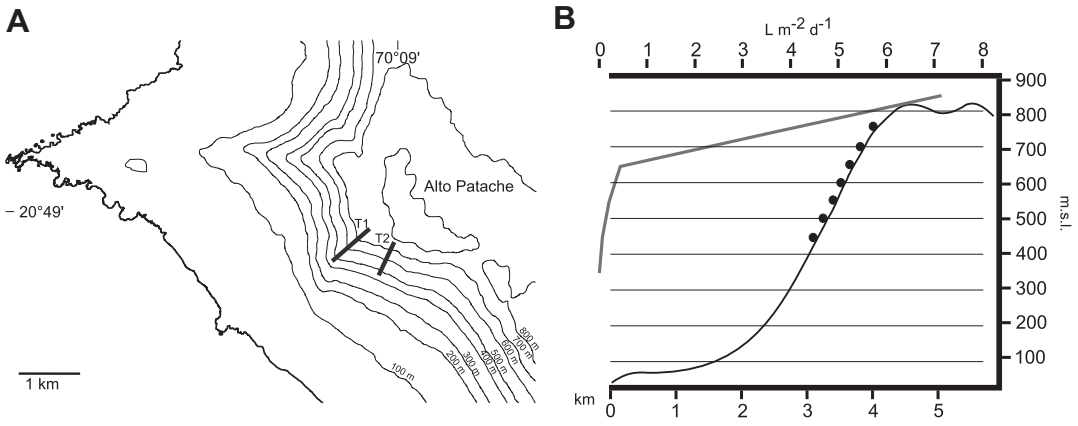

Fig 1 – Location, topology, and fog-water accumulation of the study site. (A) Upper view of Punta Patache and Alto Patache with altitude curves from sea level to 80 m. Transects along the cliff are indicated as grey lines (T1 and T2). (B) Altitudinal profile of Punta Patache with black dots indicating altitudes at which collections were performed. The grey line indicates mean annual fog-water yield in relation to altitude at Alto Patache during 2001–2004 between 350 and 850 m.

chilensis, and *Solanum brachyantherum*) (Muñoz-Schick *et al.* 2001; Pinto & Leubert 2009). This shift it is due to an increase in the water availability, given the condensation of fog coming from the ocean known as 'camanchaca' (Cereceda *et al.* 2008; Moreira-Muñoz 2011). This fog layer carries water droplets that precipitate to different degrees in relation to different altitudes. These values are indicated in Fig 1B.

Lichen samples

Caloplaca specimens were collected on the bluff along two altitudinal gradients on a SW slope between 450 and 750 m above sea level (Fig 1A). Every 50 m in altitude a 15 m horizontal transect was established. Every 1 m of the transect a grid of 20 cm × 20 cm divided into 16 quadrants of 5 cm × 5 cm was used to collect all the *Caloplaca* specimens present on the intersections of the grids on all different substrates. A total of 42 samples were collected. The grids were situated so as to match water collection data acquired by Cereceda *et al.* (2008) and indicated in Fig 1B. All specimens were collected by the first author and Daniel Stanton (Princeton University) and have been deposited in the Botanische Staatssammlung München (M). Vouchers information is presented in Table 1.

DNA extraction, PCR, and sequencing

Total DNA was extracted using Roche High Pure PCR Template Preparation Kit (http://www.roche-applied-science.com/) following the manufacturer's instructions. nrDNA ITS region of both bionts was amplified using the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for the mycobiont, and AL1500bf (Helms *et al.* 2001) and LR1850 (Friedl 1996) for the photobiont. PCR reactions for both myco- and photobionts were as follows: after an initial denaturation step of 95 °C for 10 min, the PCR ran for five cycles (95 °C for 1 min, 53 °C for 30 s, 72 °C for 2 min) and 33 cycles (95 °C for 1 min, 50 °C for 20 s, 72 °C for 2 min) with a final extension step of 72 °C for 10 min. In many cases, a re-amplification step was performed for photobiont samples that did not amplify in the first run. The re-amplification reaction included the primers Al1648 (5′-CAC ACC GCC CGT CGC TC-3′) and ITS4 with a total of 20 cycles (95 °C for 1 min, 50 °C for 20 s, 72 °C for 2 min). Purification of the PCR products was performed using Macherey-Nagel's PCR Clean-Up Gel Extraction Nucleospin II Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The sequencing reaction consisted of 30 cycles (95 °C for 10 s, 50 °C for 15 s, 60 °C for 3 min) with the primers ITS1F and ITS4 for the mycobiont, and Al1648 and ITS4 for the photobiont, using the Big Dye Terminator Reaction Kit 3.1 (Perkin–Elmer Inc., Wellesley, MA, USA). Sequences were obtained using a Perkin Elmer ABI 377 automatic sequencer, using the Big Dye Terminator Reaction Kit (Perkin–Elmer Inc., Wellesley, MA, USA).

Sequence alignment

Sequence fragments obtained from both bionts were subjected to BLAST queries for an initial verification of their identities. Fragments were assembled with the aid of the Staden Package version 1-7-0 (http://staden.sourceforge.net). They were initially aligned using the software Muscle 3.8.31 (Edgar 2004), and manually optimized using the program-packages GeneDoc (Nicholas *et al.* 1997) and Jalview 2.6.1 (Waterhouse *et al.* 2009) without removing ambiguous sites. All newly generated DNA sequences have been deposited in GenBank (Table 1) and alignments of both bionts have been deposited in TreeBASE (accession numbers S12682 and S12683).

Phylogenetic analyses

Alignments of both the photobionts and mycobionts were subjected to Maximum Parsimony (MP) inference using the program-package PAUP*4.0b4a (Swofford 2000). Maximum Likelihood (ML) search was performed with RAxML v.7.0.3 (Stamatakis 2006), and Bayesian inference analysis with the program-package BAli-Phy (Suchard & Redelings 2006) and MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). For both bionts,

Table 1 – Lichen samples analyzed: identities, haplotypes, voucher specimens, and Genbank accession numbers of both bionts.

Sample	Lichen species	Mycobiont haplotype	Photobiont species	Photobiont haplotype	Altitude (m.s.l)	Voucher	Genbank accession n° mycobiont	Genbank accession n° photobiont
RV001	*Caloplaca orthoclada*	M1a	*Trebouxia arborícola*	P1i	650	RV 2047; M-0102498	JQ993800	JQ993758
RV002	*Caloplaca orthoclada*	M1b	*Trebouxia decolorans.*	P2a	450	RV 1939; M-0102499	JQ993801	JQ993759
RV003	*Caloplaca orthoclada*	M1b	*Trebouxia arboricola*	P1 m	450	RV 1941; M-0102500	JQ993802	JQ993760
RV004	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1u	450	RV 1942; M-0102501	JQ993803	JQ993761
RV005	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1j	450	RV 1943; M-0102502	JQ993804	JQ993762
RV006	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1 m	450	RV 1944; M-0102503	JQ993805	JQ993763
RV007	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1 k	450	RV 1945B; M-0102504	JQ993806	JQ993764
RV008	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1 k	450	RV 1948; M-0102505	JQ993807	JQ993765
RV009	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1v	550	RV 1958; M-1012506	JQ993808	JQ993766
RV010	*Caloplaca orthoclada*	M1b	*Trebouxia arboricola*	P1l	550	RV 1962; M-1012507	JQ993809	JQ993767
RV011	*Caloplaca* sp.1	M2a	*Trebouxia decolorans*	P2b	550	RV 1968; M-1012508	JQ993810	JQ993768
RV012	*Caloplaca* sp.2	M3a	*Trebouxia arboricola*	P1l	550	RV 1974; M-1012509	JQ993811	JQ993769
RV013	*Caloplaca* sp.2	M3a	*Trebouxia arboricola*	P1u	550	RV 1982; M-1012510	JQ993812	JQ993770
RV014	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1o	600	RV 1986; M-1012511	JQ993813	JQ993771
RV015	*Caloplaca orthoclada*	M1b	*Trebouxia gigantea*	P3a	600	RV 1987; M-1012512	JQ993814	JQ993772
RV016	*Caloplaca orthoclada*	M1b	*Trebouxia arboricola*	P1o	600	RV 1987; M-1012513	JQ993815	JQ993773
RV017	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1 n	600	RV 2006; M-1012514	JQ993816	JQ993774
RV018	*Caloplaca* sp.3	M3a	*Trebouxia arboricola*	P1x	600	RV 2010; M-1012515	JQ993817	JQ993775
RV019	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1x	600	RV 2011; M-1012516	JQ993818	JQ993776
RV020	*Caloplaca* sp.1	M2a	*Trebouxia decolorans*	P2a	600	RV 2017; M-1012517	JQ993819	JQ993777
RV021	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1w	600	RV 2020; M-1012518	JQ993820	JQ993778
RV022	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1c	650	RV 2027; M-1012519	JQ993821	JQ993779
RV023	*Caloplaca orthoclada*	M1a	*Trebouxia arboricola*	P1c	650	RV 2032; M-1012520	JQ993822	JQ993780
RV024	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1 p	650	RV 2036; M-1012521	JQ993823	JQ993781
RV025	*Caloplaca orthoclada*	M2b	*Trebouxia gigantea*	P3b	650	RV 2040; M-1012522	JQ993824	JQ993782
RV026	*Caloplaca* sp.1	M2a	*Trebouxia gigantea*	P3b	650	RV 2042; M-1012523	JQ993825	JQ993783
RV027	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1r	650	RV 2048; M-1012524	JQ993826	JQ993784
RV028	*Caloplaca* sp.1	M2b	*Trebouxia arboricola*	P1a	650	RV 2056; M-1012525	JQ993827	JQ993785
RV029	*Caloplaca* sp.4	M4b	*Trebouxia arboricola*	P1b	700	RV 2076; M-1012526	JQ993828	JQ993786
RV030	*Caloplaca* sp.4	M4c	*Trebouxia arboricola*	P1 t	700	RV 2078; M-1012527	JQ993829	JQ993787
RV031	*Caloplaca* sp.1	M2a	*Trebouxia arboricola*	P1q	700	RV 2080; M-1012528	JQ993830	JQ993788
RV032	*Caloplaca* sp.4	M4a	*Trebouxia arboricola*	P1d	750	RV 2101; M-1012529	JQ993831	JQ993789
RV033	*Caloplaca* sp.4	M4a	*Trebouxia arboricola*	P1f	750	RV 2103; M-1012530	JQ993832	JQ993790
RV034	*Caloplaca* sp.3	M5a	*Trebouxia arboricola*	P1f	750	RV 2106; M-1012531	JQ993833	JQ993791
RV035	*Caloplaca* sp.4	M4a	*Trebouxia arboricola*	P1d	750	RV 2127; M-1012532	JQ993834	JQ993792
RV036	*Caloplaca* sp.4	M4a	*Trebouxia arboricola*	P1e	750	RV 2144; M-1012533	JQ993835	JQ993793
RV037	*Caloplaca* sp.4	M4d	*Trebouxia arboricola*	P1y	600	RV 2188; M-1012534	JQ993836	JQ993794
RV038	*Caloplaca* sp.3	M5a	*Trebouxia arboricola*	P1s	650	RV 2217; M-1012535	JQ993837	JQ993795
RV039	*Caloplaca* sp.3	M5b	*Trebouxia arboricola*	P1s	650	RV 2242; M-1012536	JQ993838	JQ993796
RV040	*Caloplaca orthoclada*	M1c	*Trebouxia arboricola*	P1 h	750	RV 2401b; M-1012537	JQ993839	JQ993797
RV041	*Caloplaca orthoclada*	M1c	*Trebouxia arboricola*	P1 h	750	RV 2401a; M-1012538	JQ993840	JQ993798
RV042	*Caloplaca fernandeziana*	M6a	*Trebouxia arboricola*	P1 g	750	RV 2415; M-1012539	JQ993841	JQ993799

MP searches were performed with heuristic searches with tree bisection-reconnection (TBR) swapping. All changes among character states were equally weighted and unordered. Stability of groups was tested using the bootstrap method with 1000 pseudoreplicates (Felsestein 1985).

ML searches on the nrDNA ITS region of both bionts were performed including constant sites. Substitution model and parameter estimation were done utilizing the Akaike Information Criteria (AIC) (Akaike 1973; Posada & Buckley 2004) using the program Modeltest 3.06 (Posada & Crandall 1998). For the mycobionts, Modeltest suggested the TrNef+G (Tamura & Nei 1993) as the best fitting model of evolution for the ML analyses, and the TVM+G model (Posada 2003) for the photobiont data set. Since neither of these models can be implemented in RAxML, both data sets were analyzed using the GTR+G model of evolution (Rodríguez *et al.* 1990) as implemented in RAxML (Stamatakis 2006). We conducted four independent runs from different starting points to assess convergence to the best tree, which was consistently selected within one likelihood unit. ML nodal support was calculated by analyzing 10 000 bootstrap pseudoreplicates.

Bayesian phylogenetic analyses were conducted by sampling trees with a Markov chain Monte Carlo with Metropolis coupling method as implemented in MrBayes v. 3.0b4 (Huelsenbeck & Ronquist 2001). The model of evolution for Bayesian inference was selected utilizing AIC (Akaike 1973; Posada & Buckley 2004) using MrModeltest 2.3 (Nylander 2004). For the mycobionts the software suggested that the SYM+G model (Zharkikh 1994) was the best fitting model for the data, assuming a Gamma distribution for rate heterogeneity among sites. For the photobionts, the software suggested the HKY+G model (Hasegawa *et al.* 1985), assuming a Gamma distribution for rate heterogeneity among sites. Analyses began using random starting trees for one million generations with six chains sampled every ten generations for the fungi and the algae. We examined plots of likelihood for each run using the software Tracer v. 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) to determine the effective sampling sizes and the number of generations required to reach equilibrium values (burn-in) and discarded 25 % of the sampled trees. After discarding the trees collected during burn-in, we assembled all saved trees to calculate posterior probability (PP) as branch support.

For checking the eventual bias that could be operating in the generation of the alignments, we made a simultaneous Bayesian estimation of alignment and phylogeny using the package BAli-Phy (Redelings & Suchard 2005) for the unaligned nrDNA ITS region matrix of both algae and fungi following Gaya *et al.* (2011). Four independent runs of 100 000 generations each were made for both symbionts matrices, sampling the chains every ten generations. We evaluated the convergence and mixing of the runs using the tools provided by the authors (Suchard & Redelings 2006). The first 10 % of the samples of each run was discarded as burn-in after plotting the log-likelihood values against the time generation to determine when the chains reached a stable equilibrium value (Huelsenbeck & Ronquist 2001) using the program Tracer v. 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). The final majority rule consensus tree was obtained by pooling all trees selected from both runs after burn-in.

We compared the results from the Bayesian inference analyses using MrBayes with those resulting from BAli-Phy to detect any misleading estimate of PP and topology as a result of interaction between among-site variations and priors and rate heterogeneity parameters used in the analysis, which can appear despite the use of the appropriate substitution model using Bayesian inference on a given multiple sequence alignment as a result of the inclusion of ambiguously aligned sites (Lemmon *et al.* 2009; Wiens & Morrill 2010). As topological differences were observed in neither the resulting mycobiont (Fig 2) nor the photobiont (Fig 3) trees, we used the complete matrices without removing ambiguously aligned sites for the analyses.

Results

Lichen species

The community studied comprises six *Caloplaca* species: *C. orthoclada*, *C. fernandeziana*, and four undescribed species of *Caloplaca*, named sp1–sp4. The most abundant species is *C. orthoclada* (18 specimens), a lobated species with lecanorine apothecia growing on rocks and small pebbles. This species was originally described by Zahlbruckner (1924) as a crustose saxicolous species with tightly adpressed lobes in coastal environments. *C.* sp1, the second most abundant species with ten specimens, is an epiphyte on shrubs present in medium to high altitudes (550–700 m). It is characterized by its whitish thallus and dark red apothecia. *C.* sp2 (three specimens) is a small saxicolous species. It grows on basic substrata in rock crevices and on mollusk shells at middle to high altitudes (550–600 m). It is characterized by a small crustose whitish thallus (1.8–3.2(4) mm), with very small orange-red to red lecideine apothecia of 0.3–0.5(0.7) mm in diameter. *C.* sp3 (three specimens) is characterized by a small orange-red terricolous thallus ((5)6.8–8(12) mm), with small areoles of (0.1)0.2–0.7(1.0) mm, the presence of medium-sized zeorine apothecia of 3–5(7.5) mm with red discs and a faint whitish pruina. *C.* sp4 (seven specimens) is a terricolous species present between 600 and 750 m growing on horizontal surfaces between rocks in the cliff. *C.* sp4 can be differentiated from *C.* sp3 by its orange-yellow thallus with smaller lecideine apothecia of 2–3(4) mm with orange discs, lacking photobiontal cells at the base of the apothecia and by having smaller spores with a smaller spore length/isthmus ratio. Both *C.* sp3 and *C.* sp4 are terricolous forming a soil crust on fog exposed slopes, but the characters mentioned before allow for both species to be distinguished based on morphology. *C. fernandeziana* (one specimen) is a saxicolous species growing on acidic rocks. It is commonly found at low elevations throughout northern and central Chile and is characterized by a tartareous red thallus which grows up to 9 cm wide with a black prothallus. It develops biatorine apothecia that turn from orange-red to dark ferrugineous red with age. Of the apotheciate species, apothecia were only absent from specimens collected above 750 m, including two specimens of *C. orthoclada*, the only specimen of *C. fernandeziana* and three specimens of *C.* sp4. The presence of pycnidia and conidia was identified in *C. orthoclada*, *C. fernandeziana*, *C.* sp1, *C.* sp3, and *C.* sp4. Two specimens of *C. orthoclada*, growing at 750 m of altitude presented a cushion-like morphology.

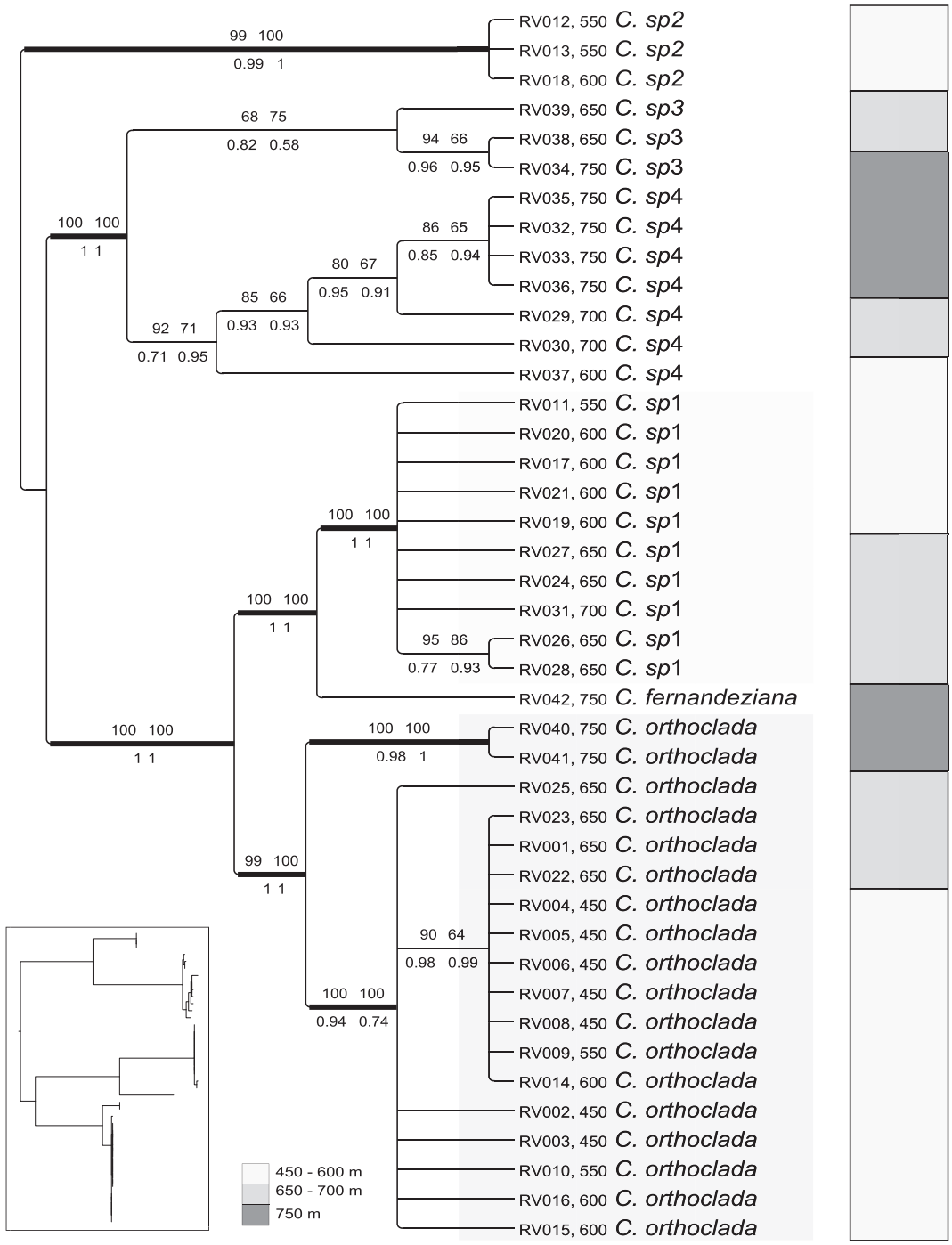

Fig 2 – Unrooted most likely tree of *Caloplaca* ITS nrDNA sequences found using ML in RAxML 7.2.6. Highly supported branches are highlighted by darker lines. Values above branches indicate bootstrap support from ML searches (10 000 replicates) and MP searches (1000 replicates) and posterior probabilities with BAli-Phy and MrBayes support values below the branches. The inset shows the branch lengths from the ML resulting tree. Shaded clades indicate different *Caloplaca* species. Lateral bar indicates height distribution along the altitudinal gradient (light grey = 450–600 m, medium grey = 650–700 m, dark grey = 750 m).

Mycobionts

Forty-two new nrDNA ITS *Caloplaca* sequences were generated. The data matrix included 546 characters, 313 characters were constant, 23 characters were parsimony uninformative and 210 were parsimony informative. The MP analyses yielded 9367 equally most parsimonious trees of 345 steps (CI, excluding uninformative characters = 0.847; RI = 0.979).

ML analysis resulted in a single most likely tree with a log-likelihood value of −2286.60. Rate parameters were estimated

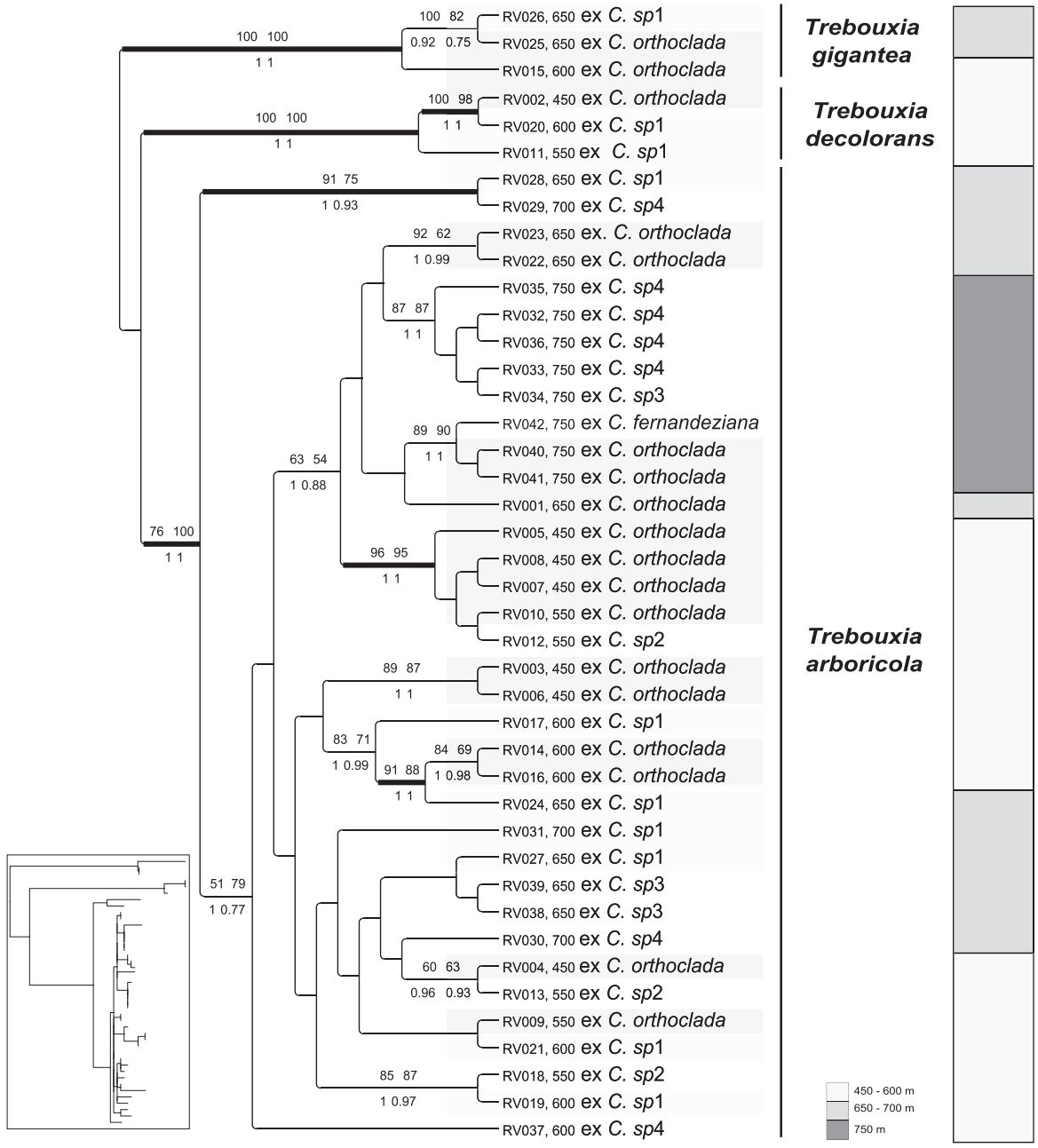

Fig 3 – Unrooted most likely tree found using ML in RAxML 7.2.6 of ITS nrDNA sequences of photobionts associated with *Caloplaca* species in Punta Patache on an altitudinal gradient. Highly supported branches are highlighted by darker lines. Values above branches indicate bootstrap support from ML searches (10 000 replicates) and MP searches (1000 replicates) and posterior probabilities support using BAli-Phy and MrBayes below the branches. The inset shows the branch lengths from the ML resulting tree. Shading indicates relationships with different *Caloplaca* species. Lateral bar indicates height distribution along the altitudinal gradient (light grey = 450–600 m, medium grey = 650–700 m, dark grey = 750 m).

as follows: A–C = 1.000, A–G = 2.174, A–T = 1.000, C–G = 1.000, C–T = 4.987, and G–T = 1.000. Base frequencies were determined as pi(A) = 0.227, pi(C) = 0.279, pi(G) = 0.249, pi(T) = 0.245.

The different analyses yielded similar topologies with only small differences in the support value of the different branches. All analyses resulted in a phylogeny with monophyletic and well separated species (Fig 2).

Several species harbour very similar nrDNA ITS sequences between individuals. The different haplotypes observed are indicated in Table 1. Sequences of *Caloplaca* sp2 were all identical, while most sequences of *Caloplaca orthoclada* showed only one transversion in the ITS1 region generating two distinguishable groups, but larger differences were encountered with samples growing above 750 m. Two samples of *Caloplaca* sp1 showed only one transition in the ITS1 region, and samples of *Caloplaca*

sp3 were separated by one transition in the ITS1 and one transition in the ITS2 regions. *Caloplaca* sp4 was the most variable group, with several transitions and transversions in the ITS1 and ITS2 regions, with a more homogeneous group corresponding to specimens growing above 750 m.

Photobionts

Forty-two new nrDNA ITS *Trebouxia* sequences were generated. The data matrix of these 42 nrDNA ITS sequences included 824 characters, 680 characters were constant, 35 characters were parsimony uninformative and 109 were parsimony informative. The MP analyses yielded 576 equally parsimonious trees of 185 steps (CI, excluding uninformative characters = 0.812; RI = 0.913).

ML analysis resulted in a single most likely tree with a log-likelihood value of −2181.10. Rate parameters were estimated as follows: A−C = 1.990, A−G = 6.752, A−T = 2.446, C−G = 1.413, C−T = 6.752, and G−T = 1.000. Base frequencies were determined as pi(A) = 0.217, pi(C) = 0.233, pi(G) = 0.269, pi(T) = 0.281.

The different analyses yielded similar topologies without major incongruences and with small differences in the support values among analyses. All analyses resulted in a phylogeny with monophyletic, well separated algal species with well supported branches (Fig 3). Each species presented several haplotypes (Table 1) with the largest variations in *Trebouxia arboricola*, the most frequent photobiont species in the data set.

The algal species were identified with highly similar sequences by a BLAST (MegaBLAST) search (Altschul *et al.* 1990) of sequences available in Genbank and a phylogenetic analysis including *Trebouxia* sequences used by Beck *et al.* (2002) (data not shown). Photobionts corresponded to the *T. arboricola* clade, belonging mostly to *T. arboricola* s.l. (including *Trebouxia decolorans*), with the exception of three samples that belonged to *Trebouxia gigantea*. Two of the *T. gigantea* samples were associated with mycobionts of *Caloplaca orthoclada* that had the same mycobiontal haplotype and occurred with a difference in altitude of 50 m (Fig 3). The last one of these three samples was associated with a mycobiont of *C.* sp1 (RV026) that presented one transition in the ITS1 region in relation to the main clade of *C.* sp1. The mycobiont of RV028 shared the same mycobiontal haplotype of RV026, but it was associated to one of the haplotypes of *T. arboricola* (P1a) (Table 1). Both samples were found at the same altitude.

Discussion

There is a rather high intraspecific homogeneity of nrDNA ITS sequences within the different clades of mycobionts of *Caloplaca* along the altitudinal gradient resulting in medium to high support values for the different species (Fig 2) independent of the inference method used. Only few species showed differences in their genetic structures denoting the presence of different haplotypes, as is the case of *C.* sp4. The phylogenetic tree reveals multiple haplotypes along its altitudinal distribution (600−750 m), all clustering in a monophyletic clade supported in three of four analyses (bootstrap support over 70 % and posterior probabilities over 95 %), separating this species from others in the community. Its habitat is shared with the closely related *C.* sp3, also terricolous. This species is sister to *C.* sp4 and is a monophyletic group, although with variable support. *C.* sp4 can be differentiated from *C.* sp3 by a brighter orange-yellow thallus with smaller apothecia of 2−3(4) mm with orange discs. *C.* sp2, a small saxicolous species, grows in rock crevices at middle to high altitudes (550−600 m). *C.* sp1 is an epiphyte on native shrubs present at medium to high altitudes (550−700 m), where annuals like *Nolana* spp. and *Leucocoryne appendiculata*, along with perennials as *Ephedra breana*, grow in scattered areas and survive by intercepting fog-water droplets with their leaves (Muñoz-Schick *et al.* 2001; Pinto & Leubert 2009). The sole specimen of *Caloplaca fernandeziana* grouped as a sister species to *C.* sp1 with high support. The former species is commonly found at low altitudes and seashore rocks in Mediterranean habitats in central Chile (Zahlbruckner 1917; Follmann & Redón 1972). *Caloplaca orthoclada* covers the whole altitudinal gradient from 450 to 750 m, presenting the largest distribution of the species collected. This species commonly develops apothecia on the lobes and in the centre of the thallus. In our study site, at 750 m, *C. orthoclada* adopts a cushion-like morphology with raised lobes and lacking apothecia. The presence of *C. orthoclada*, *C. fernandeziana*, *C.* sp3, and *C.* sp4 at high altitudes over 600 m seems to be strongly dependent on the precipitation of droplets of the dense fog that collides with the cliff (Fig 1B). This fog supplies water for the smaller crustose forms, enabling the formation of a soil crust that sustains different lichen species that live from the precipitation of water on the rocks (Büdel 2001). The soil crust favours not only lichens, but also other organisms, such as representatives of the genus *Trentepohlia*, both on pebbles and rocks and as an epiphyte, and cyanobacteria (Forest & Weston 1966; Rundel 1978; Büdel 2001). These communities are similar to those that subsist in the Namib Desert and in the coastal Sonoran Desert in Baja California along the fog zone (Rundel 1978; Kappen 1982; Wirth *et al.* 2007; Lalley & Viles 2008).

In contrast to the mycobiontal genetic homogeneity, photobionts exhibit a larger heterogeneity in their genetic inventory (Fig 3). In the algal phylogenetic tree three major clades can be observed. All of them yielded high support values in the four different analyses. The first clade represents specimens of *Trebouxia gigantea*. The second clade includes specimens of *Trebouxia decolorans*, closely related to members of *Trebouxia arboricola*, which is represented in a third clade. Thirty-nine of the specimens collected at the study site were related to members of the *T. arboricola* clade (including *T. arboricola* and *T. decolorans*). The symbiotic associations between members of the lichen genus *Caloplaca* and members of the *T. arboricola* group, as shown in the algae phylogenetic tree, indicate that members of the genus *Caloplaca* in northern Chile have a moderate selectivity and appear to be selective mostly to members of this clade in the sense of Beck (2002). *T. arboricola*, being the photobiont of a great number of mycobionts, has low mycobiont selectivity and, as a consequence, the algae−fungi relationship is moderately specific to unspecific (*sensu* Beck *et al.* 2002).

It is interesting to note that most of the mycobiontal species studied in this community have very little intraspecific variation in their genetic inventory based on nrDNA ITS

sequences, contrasting with the high genetic diversity in the nrDNA ITS found in *Caloplaca* species of the subgenus *Pyrenodesmia* by Muggia *et al.* (2008). Differences with our results may arise from the different sampling; we considered a particular community, isolated from neighbouring communities due to the discontinuous hill range and water availability, while Muggia *et al.* (2008) considered populations along the Italian Peninsula, although in similar substrates and in similar ecological conditions (e.g., sunny exposed surfaces). The low genetic diversity observed in our samples would appear to suggest that asexual reproduction is at the origin of the present population. Nonetheless, preliminary data from mitochondrial and protein-coding markers (e.g., cox1 and mcm7, not shown) discard this possibility as multiple haplotypes are revealed, suggesting a low intraspecific variation of the nrDNA ITS region in these species in comparison with these other markers. Furthermore, the assumption of asexual reproduction is not supported by the genetic structure of the photobionts (Fig 3), since the great number of algal haplotypes is not indicative of vertical photobiont transmission. This pattern can be explained by a low variability in the nrDNA ITS region of the mycobionts, where newly germinated spores associate with free-living photobionts or arrest photobionts from other lichens in the surrounding, raising the number of photobiontal haplotypes in their association. Notwithstanding the high variation in mitochondrial and protein-coding markers found in *Caloplaca* species from our transect (data not shown), we cannot disregard possible algal-switching events that may generate variations in the photobiont composition of the species treated here in the same way as it has been reported for other lichens (Piercey-Normore & DePriest 2001; Yahr *et al.* 2004; Wornik & Grube 2010).

To reveal possible changes in the photobiont composition in the study site, we screened the algal phylogenetic tree along the altitudinal gradient and the gradual accumulation of water precipitation in the bluff. We could find evidence of a shift in the photobiont composition at 750 m in the phylogenetic tree (Fig 3), where all photobiontal haplotypes related to mycobionts of *Caloplaca* species occurring at this altitude are arranged in two monophyletic groups within the same clade in *T. arboricola* (Fig 3). At lower altitudes, the haplotype composition of the photobionts belonging to *T. arboricola* is variable and no evident pattern was observed in the phylogenetic tree, neither in relation to a particular mycobiont nor to altitude and water availability. This change in the photobiont composition at the highest altitude might be due to a greater availability of water as a result of the presence of an almost constant fog layer in the area (Farías *et al.* 2005; Cereceda *et al.* 2008). As the fog moves inland from the ocean and hits the cliff at its higher altitudes (Fig 1B), it generates a very strong altitudinal gradient in fog frequency, with a mean annual fog yield of 0.08 L m^{-2} d^{-1} at 450 m to 3.72 L m^{-2} d^{-1} at 750 m (Cereceda *et al.* 2008). The presence of *T. decolorans* associated with *Caloplaca* species was restricted to altitudes of 600 m or lower, while *T. gigantea* was found associated with *Caloplaca* species from 600 to 650 m only. Nevertheless, it seems impossible to rule out the possible presence of these two algal species at high altitudes (750 m), both free-living or in association with other fungi. Representatives of *T. arboricola* were found at all altitudinal levels from 450 to 750 m in the coastal bluff. The altitudinal change from 700 to 750 m is accompanied by a significant increase in the availability of water (doubling from 2 to 4 L m^{-2} d^{-1}, see Fig 1B) and is correlated with a shift in the morphology of the *Caloplaca* species in the area, as exemplified by *C. orthoclada*. The change in the morphology (from lobated crustose to cushion-like) allows the exposure of a larger surface, which in turn may allow a higher accumulation of water but also changing the control of evaporative water loss (Larson 1981; Cereceda *et al.* 2008; Garreaud *et al.* 2008). No experimental data has been collected to test the capacity of different lichen morphologies to collect fog, although some exploration in this topic has been made (Larson 1979, 1981). In these studies it was found that lichens that present a low ratio between surface area and weight (e.g., crustose lichens) reach water saturation very slowly. This implies slow water evaporation, allowing an optimal use for different metabolic processes, as photosynthesis and respiration, and diminishing damage generated by fast rehydration (Kappen & Valladares 2007; Beckett *et al.* 2008; Kranner *et al.* 2008). This strategy has been widely studied on vascular cushion plants, as they modify the microclimatic conditions in their surrounding, allowing both their own survival while facilitating the establishment of other herbaceous species (see Cavieres *et al.* 2007). In the case of lichens with a larger surface/weight ratio, they can reach water saturation and evaporate their water content very fast (e.g., fruticose and subfruticose forms) (Rundel 1978; Larson 1981). Differences may arise with cushion forming species, as is the case of specimens of *C. orthoclada*. In these cases evaporation rates may become slower (Larson 1981) and desiccation occurs mostly at the tips of the lobes or in the exposed areas, while the bases remain moist (Moser *et al.* 1983; Kranner *et al.* 2008). In this context, changes in the morphology could reflect plasticity as adaptation to more humid conditions, thus favouring a more specific relationship to a clade of photobionts that lives under those conditions, as suggested by Beiggi & Piercey-Normore (2007) for some *Asterochloris* photobionts of *Cladonia* species. Rundel (1978), while discussing morphological adaptations of lichens to desert fog zones, showed that crustose lichens are ecologically successful in these areas by developing morphological adaptations to increase the thallus surface exposed to moist winds. This observed change in the morphology is also concordant with results presented by Wirth *et al.* (2007), who found that species with fruticose and subfruticose morphologies were dominant in more humid areas, being gradually replaced by crustose species in dryer areas in an ocean–inland transect in the Namib Desert.

All the *Caloplaca* species studied in this community produce apothecia, or are known to, as is the case of the sole specimen of *C. fernandeziana* (Zahlbruckner 1917; Follmann & Redón 1972). Specimens lacking ascocarps were all located at 750 m in the altitudinal gradient, including specimens of *Caloplaca* sp3, *C.* sp4, and *C. orthoclada* (Fig 1). It is possible that under stressing conditions, like low water availability, smaller thalli are developed and the generation of ascocarps is the result of an adaptive strategy, routing the resources to reproduction by ascospore production, thus confirming our hypothesis 3. A higher availability of water might generate conditions in which drought stress is reduced and the generation and establishment of new thalli might be incremented by thallus fragmentation in

addition of conidia production, as resources are oriented to thallus growth and the possible availability of free-living algae in the environment. Similar patterns were observed in the lichen communities present in the stands of tropical vascular plants (Lücking 1999), where species under stressful conditions dispersed mainly by ascospores. On the other hand, species under less stressful conditions developed conidia as a mean of asexual reproduction. Vegetative dispersal, by production of diaspores (e.g., isidia, soredia) and therefore predominance of vertical transmission of photobionts may explain the presence of only a reduced number of *T. arboricola* haplotypes at higher elevations. In our study site, none of the species developed specialized propagules for vegetative dispersal and all the specimens examined produced pycnidia and conidia. As multiple photobiont haplotypes were observed to associate with few mycobiontal haplotypes, it is possible to assume horizontal transmission as the main strategy in photobiont acquisition. Nonetheless, vertical transmission of photobionts and algal-switching also occur at some scale, modifying the number of photobiont strains related to specific mycobionts along the gradient (Piercey-Normore & DePriest 2001; Beck *et al.* 2002; Yahr *et al.* 2006).

Two species of the altitudinal gradient shared both myco- and photobiontal haplotypes between specimens at 750 m, *C.* sp4 (RV032 and RV036) and *C. orthoclada* (RV040 and RV041). The change in the morphology in *C. orthoclada* at 750 m may allow a fragmentation of the thallus that can lead to the establishment of new thalli, thus partly favouring vertical transmission as a result of a change in the dispersal strategy. These observations are limited by the resolution afforded by the nrDNA ITS region, but preliminary data with different markers indicates that mycobionts of *C. orthoclada* at 750 m present different haplotypes in single-copy genes (data not shown). Because six of the seven specimens of *C.* sp4 were found over 700 m and no representatives were found below the stressing limit of 600 m, where the water accumulation drops abruptly, the potential effect of altitude/water accumulation in the photobiont composition of this species cannot be observed in as much detail as in *C. orthoclada*.

It is possible that few strains of *Trebouxia* are adapted to survive as free living at higher water availability. Therefore, these strains are selected to associate with the different lichen species occurring at high altitudes in the coastal range, as was proposed for *Lepraria* and *Stereocaulon* (Peksa & Škaloud 2011) after evaluating ecological data over genetic distance (e.g., close relationships in a phylogenetic tree). In such a situation, locally adapted photobionts could restrict the availability of partners and seemingly increase the specificity of the association (Beck 1999). Further studies are needed to determine the existence of such free-living photobionts in the investigated transect to shed some light on their availability to and acquisition by the mycobionts and the ecological strategies related to the establishment of new thalli on coastal hills in desert areas.

Environmental factors within lichen thalli may also influence the degree of selectivity of photobiont choice (Beck *et al.* 2002), even between distantly related taxa, and the shared ecological demands may result in a similar photobiont preference along the altitudinal gradient. This hypothesis is supported in the present study by the observed moderate-to-high selectivity in the photobiont choice of the mycobionts because association occurs only with *Trebouxia* species of the *T. arboricola* group. No congruency whatsoever was found between the phylogenies of the symbionts, indicating that possible coevolution did not result in cospeciation.

In conclusion, the available evidence indicates that the multiple associations observed can best be explained by horizontal transmission of photobionts in this lichen community, even if vegetative propagules are produced. Ecological factors, such as altitude, water availability and mode of reproduction, may modulate the availability of photobiontal species and strains at different sites, thus reducing the range of possible associations in an isolated community such as the one studied here. The results obtained indicate an altitudinal and water availability preference in the photobionts of *Caloplaca* species, particularly *C. orthoclada*, in the altitudinal gradient, where a few closely related strains of *T. arboricola* associate with different *Caloplaca* species at higher altitudes, demonstrating a change in the photobiont availability and composition along the altitudinal gradient. Also, these ecological factors can contribute to the limitation of the incorporation and establishment of new photobiontal strains, resulting in a reduction in the potential number of viable partners. At the same time, they can modify the morphology and reproductive strategy of the symbiotic system as a response to stressing conditions. These elements can lead to local adaptation given the reduction of the possible associations, thus increasing the mycobiont selectivity of the photobiont and the specificity between partners that coexist in an isolated community.

Finally, ecological parameters are an important source of information in order to explain availability, selectivity, and specificity and should be considered when performing population structure studies in symbiotic systems.

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