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Fungal-algal interactions in *Ramalina menziesii* and its associated epiphytic lichen community

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Abstract: Lichens are a fascinating example of a symbiotic mutualism. It is still uncertain which processes guide fungal-photobiont interactions, and whether they are random or of a more complex nature. Here, the fungal-algal interactions in *Ramalina menziesii* and co-occurring taxa are analyzed by using DNA sequences of the algal Internal Transcribed Spacer region (ITS), to investigate fungal-algal associations in juvenile *R. menziesii* and allied species. Algal species were identified by a combination of BLAST searches, median-joining network analysis, and Bayesian phylogenetics. Fungal-algal networks were analyzed for nestedness, both at the species and haplotype level (fungal species vs. algal haplotypes), and the networks were inspected for evidence of compartmentalization. Bayesian phylogenetic trees indicated that the widespread green alga *Trebouxia decolorans* associated with *R. menziesii*, as well as six other fungal species. Four additional fungal species interacted with four different species of *Trebouxia*. Only in one out of ten samples were algal haplotypes shared with the nearest neighbours of juvenile *R. menziesii*. Fungal-algal species interactions were compartmentalized, while at the level of algal haplotypes, nestedness was found. This pattern is similar to the compartmentalization found in other intimately interacting mutualists.

Key words: compartmentalization, lichen-forming fungi, nestedness, photobiont, species interactions, specificity, symbiosis

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Introduction

Species interactions are a major factor structuring biological communities, and symbiotic interactions can be an important force driving evolution (Margulis & Fester 1991). In lichens, one of the 'textbook examples' of symbiosis, the local composition of symbiotic partnerships between lichen-forming fungi and their photobionts is a fascinating issue (Beck *et al.* 1998, 2002; Yahr *et al.* 2004; Ohmura *et al.* 2006). Progress has been made in recent years due to the development of molecular techniques that allow a straightforward identification of photobiont and fungal genetic strains and species (Scheidegger & Werth 2009; Werth 2010). In this way, photobiont guild structure and one-to-one species, as well as haplotype interactions, have been analyzed in the lichen symbiosis.

The symbiotic mutualistic associations of lichen fungi and their photobionts can be described by their degree of specificity and selectivity. These terms characterize the taxonomic range of the partners with which an organism associates (Smith & Douglas 1987). Following the suggestion by Beck *et al.* (2002), I use the term specificity for the symbiotic association as a whole, that is, from the view of both photobionts and mycobionts. A narrow taxonomic range (e.g., a one-to one relationship) would be indicative of the high specificity of the association. I refer to selectivity as the taxonomic range of the interaction viewed from the perspective of only one biont (Beck *et al.* 2002). Accordingly, a mycobiont would be highly selective in its partner choice if it were found in association with few, closely related photobiont species.

Previous studies have shown that lichen fungi may exhibit a varying degree of selectivity towards their photobionts. Some fungi

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show high selectivity, and they associate with a particular photobiont phylogenetic clade or species (Kroken & Taylor 2000; Helms *et al.* 2001; Beck *et al.* 2002; Piercey-Normore 2004, 2006; Yahr *et al.* 2004; Stenroos *et al.* 2006; Myllys *et al.* 2007). Other fungal species are far less selective towards their photobiont partners (Wirtz *et al.* 2003; Piercey-Normore 2004; Yahr *et al.* 2004; Myllys *et al.* 2007); some may occasionally or always associate with two photobiont species (Piercey-Normore 2006; Casano *et al.* 2011). Some fungal species are able to partner up with photobionts belonging to different kingdoms, and which photobiont partner gets involved in the symbiosis may depend on environmental conditions (Goffinet & Bayer 1997; Tønsberg & Goward 2001; Summerfield *et al.* 2002).

Many open questions remain in the field of fungal-algal associations in lichens: for instance, are fungal-photobiont relationships entirely random or are they structured in a complex way, that is, nested or compartmentalized? In the case of random associations, no apparent pattern of association would be found in species interaction matrices (Bascompte *et al.* 2003; Jordano *et al.* 2003). In nested species interactions, specialists tend to interact with subsets of the species that interact with generalist species (Bascompte *et al.* 2003). In terms of the lichen symbiosis, this would mean that the specialized fungal species are interacting almost exclusively with the photobiont species that the generalistic fungi are interacting with. This nested type of species interaction leads to a pattern in species interaction matrices that resembles an asymptotic curve (Bascompte *et al.* 2003; Jordano *et al.* 2003). Translated to the world of lichen fungi and their photobionts, that would mean that few generalist mycobiont species interact with many photobiont species, and few generalist photobiont species interact with many mycobiont species. Additionally, specialized mycobiont species would interact with a subset of the photobiont species used by the generalists, and vice versa, specialized photobionts would interact with a subset of the mycobiont species. Nestedness can not only be studied at the level of fungal and algal species, but also at the level of haplotypes of each symbiosis partner.

In the case of compartmentalized species interactions (Bascompte *et al.* 2003; Jordano *et al.* 2003), certain fungal species would interact with a defined subset of photobiont species, while other fungal species would interact with a non-overlapping subset of photobiont species; interaction matrices would thus exhibit independent subwebs. Compartmentalization and participation of comparatively few species has recently been documented from networks of mutualistic, symbiotic ant and plant species with a high degree of interaction intimacy, while networks with low interaction intimacy were generally species-rich and nested (Guimarães *et al.* 2007). Results from previous studies indicate that mycobiont-photobiont interactions are not random, and they seem to be independent of the green algal species present in free-living populations at a site (Beck *et al.* 1998; Rambold *et al.* 1998).

Many lichen fungi disperse clonally with symbiotic propagules which contain photobiont cells, which leads to vertical transmission of the photobiont (Nash 1996; Werth *et al.* 2006*a*, *b*) unless the photobiont is exchanged during establishment (Ohmura *et al.* 2006; Wornik & Grube 2010; Werth & Scheidegger 2012). Also, lichen-forming ascomycetes reproduce sexually with ascospores, which are dispersed independently of their photobiont cells (i.e., horizontal transmission), though a few exceptions exist where both partners are co-dispersed (Honegger 2008). Germinating ascospores have to associate with new photobionts (relichenization) in order to form a thallus. This phenomenon is probably common, but has been reported from only a few species (Beck *et al.* 2002; Romeike *et al.* 2002; Sanders & Lücking 2002; Werth & Sork 2008). It has also been suggested that germinating spores could gain photobionts either from vegetative propagules produced by other lichen species, or from adult thalli of other species (Ott 1987*a*; Ott *et al.* 2000; Rikkinen 2003). Alternatively, they may retrieve cells from free-living *Trebouxia* populations (Beck *et al.* 1998).

Ramalina menziesii (*Ramalinaceae*) is a common epiphytic lichen in coastal oak savannas of southern California. It is associated with the photobiont genus *Trebouxia* (Brodo *et al.* 2001), which contains some frequent and widely distributed photobiont species. Recently, the local genetic structure was investigated for this lichen fungus across four sites, and high gene flow was found among sites (Werth & Sork 2008). Werth & Sork (2008) reported *Trebouxia decolorans* as photobiont of *R. menziesii*. The reproductive strategy of *Ramalina menziesii* in the study site was determined as almost exclusively sexual, involving relichenizations, whereas clonal reproduction was negligible (Werth & Sork 2008). These results necessitated a more detailed study of fungal-algal relationships using juvenile thalli.

Here, I specifically asked the following questions: 1) What proportion of juveniles of *R. menziesii* had an opportunity to interact with other species (as evidenced by growing in close proximity)? 2) Is the lichen-forming fungus *R. menziesii* selective in its choice of photobiont strains? 3) With which other lichen-forming fungi does *R. menziesii* share a common pool of algal species or haplotypes? If pairs of juvenile *R. menziesii* and its nearest neighbour on a branch have identical photobiont haplotypes, photobiont exchange may have occurred between them. Take-over of algae has been observed in some lichens (Friedl 1987; Ott 1987*a*, *b*; Ott *et al.* 2000). 4) Are fungal species associations and algal species/haplotype associations random in *R. menziesii* and the other members of the lichen community, or are they structured in a more complex way (e.g. nested)?

Materials and Methods

Study site

The study area was located at UCSB Sedgwick Reserve in the Santa Ynez Valley of Santa Barbara County. This field station is administered by UC Santa Barbara as part of the University of California Natural Reserve System (California, United States, 34°42′N, 120°02′W; 2358 ha; elevation 290–790 m). The majority of the reserve is an oak savanna dominated by three tree species: valley oak (*Quercus lobata*; *Fagaceae*), blue oak (*Q. douglasii*), and coastal live oak (*Q. agrifolia*). Sork *et al.* (2002*a*, *b*) provide details of the study site.

Data sampling

To study the potential for interaction with other lichen species, juvenile thalli (≤5 cm long) of *Ramalina menziesii* Tayl. (*Ramalinaceae*) were observed in the field, and it was recorded whether they were growing by themselves or within 2 mm from another lichen species, or from an adult conspecific. A cut-off level of 2 mm was chosen because preliminary observations of distance, between thalli indicated that within this distance, other species were often found. Also, it was assumed that for exchange of algae to happen, physical contact with the neighbouring thallus was required, which was why a rather short distance was used as cut-off level.

Juveniles were observed on multiple branches per tree on a total of 11 trees. The number of juveniles observed per tree varied between 2 and 22, depending on availability of juveniles on branches at an accessible height on the tree (≤2 m). The aim of these observations was to quantify how often *R. menziesii* juveniles had close contact with thalli of other lichen species. Also, these data yielded a qualitative overview of community composition.

The following design was used to study fungal-algal relationships on a total of five trees at the study site: within each tree, 2–3 branches were selected that were more than 1 m from each other; within each branch, 3–4 lichen thalli were collected, depending on availability: 1) a juvenile *Ramalina menziesii* (=0 cm); 2) a lichen species other than *R. menziesii*, situated within 2 mm from the juvenile (the 'nearest neighbour'); 3) a lichen species other than *R. menziesii*, situated at a distance larger than 1 cm from the juvenile, range 1·6–12·3 cm (>1 cm); 4) an additional *R. menziesii*, if present on branch, juvenile or adult. In this way, a total of 45 samples were collected. This design allowed me to test whether pairs of juveniles of *R. menziesii* and other species shared algal DNA sequence haplotypes.

Molecular methods

DNA sequences were obtained from the ITS (Internal Transcribed Spacer) region of the photobiont, situated within the nuclear ribosomal gene cluster. ITS was used because the same locus was used in several other studies of lichen photobionts, including those where *Trebouxia* type cultures or strains from culture reference collections had been sequenced.

Owing to the photobiont specificity of the molecular assay, it was not necessary to obtain axenic cultures prior to sequencing. Instead, the photobiont ITS was amplified by Polymerase Chain Reaction (PCR) from thallus extracts, and sequenced directly.

Each algal-specific PCR contained 1 × Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 200 nM each of forward and reverse primer, 1 µl genomic DNA (0·05–2 ng), and H_2O to a final volume of 30 µl. The Multiplex PCR Master Mix (Qiagen) consisted of an unspecified quantity of dNTPs, HotStarTaq DNA polymerase, and 3 mM $MgCl_2$ (pH 8·7). PCR conditions were as follows: 15 min at 96°C to activate the hot-start polymerase of the multiplex kit, 35 cycles of

30 s at 95°C, 60 s at 56°C, 90 s at 72°C, and a final extension of 10 min at 72°C. The primers used for PCR and sequencing were ITS1T and ITS4T (Kroken & Taylor 2000). PCR purification and sequencing were performed as described in Werth & Sork (2008). DNA sequences were edited with Sequence Scanner Software (Applied Biosystems, Foster City, California, USA) and aligned using ClustalW (Thompson *et al.* 1994) as implemented in MEGA version 4.1 (Kumar *et al.* 2004). Alignments were adjusted manually after the automated alignment had been performed. All unique ITS sequences were deposited in GenBank (accessions EU717911-EU717936; Table 3).

Data analysis

ITS sequences of the photobionts were grouped by tree and branch, and the ITS haplotypes were compared within each hierarchical level (among trees, among branches within tree, within branch). Then, photobiont haplotypes were compared for *R. menziesii* juveniles and their nearest neighbouring lichen fungi. Matches of photobiont haplotypes among thalli indicated photobiont sharing among fungi.

Phylogenetic trees excluding gaps were constructed using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). I ran PAUP version 4.0 to generate model scores (Swofford 1998), and used MrModeltest version 2.2 (Posada & Crandall 1998; Nylander 2004) to infer the appropriate substitution model for the phylogenetic analyses. The best model according to the AIC criterion was selected for all analyses (GTR + G). Four chains were run in parallel in MrBayes with a generation number of 1 000 000, sampling trees every 100th generation. A total of 10 000 trees were sampled, and the first 1000 trees of these were discarded (burn in). Results from a second run of MrBayes were inspected, and tree topology remained consistent across runs (data not shown). In addition, a neighbour-joining analysis was run with 1000 bootstraps in Mega version 4 (Tamura *et al.* 2007) using Jukes-Cantor distance, which resulted in a similar tree topology as the Bayesian analysis (data not shown).

Moreover, median-joining haplotype networks as implemented in the software Network (Bandelt *et al.* 1995, 1999) were constructed. These networks allow a simple inspection of how well a group of sequences is separated from other groups by a plot of the mutated positions on the branches, and hence, to delineate species. Incongruences in the data, for example due to recombination, are shown as loops in the network.

To identify photobiont species, BLAST searches (Altschul *et al.* 1997) for the photobiont haplotypes were performed, ITS sequence data of known photobionts were obtained from reference cultures from GenBank, and included in the Bayesian analysis. Photobiont species were considered identical if they were in the same, supported clade as a known species in the median-joining haplotype network and the phylogenetic tree (posterior probability $>0{\cdot}9$), or were considered a 'new' species when clades had a high bootstrap support and did not group within known species. These 'new' algal species may represent either known species for which sequences have not yet been deposited in GenBank, or species new to science. Table 1 gives an overview of the GenBank sequences used in the phylogenetic analysis.

To test for nestedness of the interaction of algal species and their lichen-forming fungi (species-level interaction), a fungi-by-algae matrix was constructed that showed the presence/absence of associations between fungal and photobiont species (rows: fungal species; columns: photobiont species; entries: 1 = algae found as a symbiotic partner of this fungal species, 0 = algae not found as a partner of that fungus). To test for nestedness in the association of algal haplotypes (columns) and fungal species (rows), a second matrix was constructed. The program ANINHADO version 2.03 (Guimarães & Guimarães 2006) was run to determine the matrix temperature T, a measure of how a given presence/absence matrix differs from a perfectly nested matrix, with values ranging from 0 (perfect nestedness) to 100 (no nestedness) (Atmar & Patterson 1993). Nestedness was defined as $N = (100\text{-}T)/100$, and ranged between 0 (no nestedness) and 1 (maximum nestedness) (Bascompte *et al.* 2003; Guimarães & Guimarães 2006). Using ANINHADO, I tested if a respective matrix was significantly nested by comparing the observed matrix temperature with the temperatures of random replicate matrices. These random replicates were constructed using four null models implemented in ANINHADO: 1) In the first null model (hereafter referred to as 'ER'), presences were assigned randomly to any matrix cell, keeping the total number of presences constant. 2) The second null model ('COL') assigned presences randomly within columns, thereby keeping column sums constant. 3) The third null model ('LI') differed by assigning presences randomly within rows, thereby keeping row sums constant. 4) The fourth null model referred to as 'CE' kept both columns and rows constant, and was the most conservative null model because it controlled for the degree of nestedness expected by the heterogeneity of interactions across species (Bascompte *et al.* 2003). For each null model, 1000 random matrices were generated, their matrix temperatures were calculated in ANINHADO, and N was determined, and compared with the nestedness of the observed matrices. Following Bascompte *et al.* (2003), the probability P of a random matrix being equally or more nested than the observed matrix as a test statistic was used.

To compare nestedness among the two matrices (1, fungal species – algal species; 2, fungal species – algal haplotypes), I calculated their relative nestedness as $N^* = (N - N_R)/N_R$, where N was the observed nestedness, and N_R was the averaged nestedness of 1000 random replicates. Positive values of relative nestedness indicate that the network shows asymmetrical specialization and a generalist core (i.e., nestedness), while negative values might be associated with symmetric interaction patterns and the existence of compartments (Guimarães *et al.* 2007).

Nestedness analysis is not an adequate technique to test for non-nested patterns; if associations are not nested, they are either random or, alternatively, they may show a different type of organization (Guimarães *et al.* 2007).

TABLE 1. *Sequences from GenBank included within this study, showing species of* Trebouxia *analyzed, GenBank accession numbers, and references to ITS rDNA sequences*

Trebouxia species	Strain number*	Lichen fungus	Accession	Reference
T. arboricola	SAG 219-1a	Unknown	Z68705	Bhattacharya *et al.* (1996)
T. arboricola	P-83-Ia	*Xanthoria ectaneiodes*	AJ969611	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. asymmetrica	UTEX2507	*Diploschistes albescens*	AF345889	Piercey-Normore & DePriest (2001)
T. asymmetrica	B207	*Toninia sedifolia*	AF344177	Beck *et al.* (2002)
T. asymmetrica	99.024B3	*Fulgensia fulgida*	AF344176	Beck *et al.* (2002)
T. asymmetrica	SAG48.88	*Diploschistes diacapsis*	AJ249565	Friedl *et al.* (2000)
T. corticola	UTEX909	Unknown	AJ249566	Friedl *et al.* (2000)
T. decolorans	P-400-IaSc	*Xanthoria hasseana*	AM159210	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. decolorans	P-69-IaSc	*Xanthoria hasseana*	AJ969534	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. decolorans	*P-319-Ig*	*Xanthoria parietina*	AM159503	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. decolorans	P-164-IXa-2	*Xanthoria parietina*	AJ969563	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. flava	UTEX181	Unknown	AF242467	Kroken & Taylor (2000)
T. galapagensis	UTEX2230	*Ramalina* sp.	AJ249567	Friedl *et al.* (2000)
T. gelatinosa	UBT-86.108B2	*Flavoparmelia caperata*	Z68697	Bhattacharya *et al.* (1996)
T. gelatinosa	UTEX905	Unknown	Z68698	Bhattacharya *et al.* (1996)
T. gelatinosa	P-350a-III	*Xanthomendoza* sp.	AM159213	S. Nyati, S. Scherrer & R. Honegger (unpublished)
T. gigantea	UTEX2231	*Caloplaca cerina*	AF242468	Kroken & Taylor (2000)
T. gigantea	UTEX2231	*Caloplaca cerina*	AJ249577	Friedl *et al.* (2000)
T. higginsae	UTEX2232	*Buellia straminea*	AJ249574	Friedl *et al.* (2000)
T. impressa	UTEX892	*Physcia stellaris*	AF345891	Piercey-Normore & DePriest (2001)
T. impressa	UTEX893	*Physcia stellaris*	AF345890	Piercey-Normore & DePriest (2001)
T. incrustata	UTEX784	*Lecanora dispersa*	AJ293795	Helms *et al.* (2001)
T. jamesii	UBT-86.001E1	Unknown	Z68699	Bhattacharya *et al.* (1996)
T. jamesii	UBT-86.132E2	Unknown	Z68700	Bhattacharya *et al.* (1996)
T. jamesii	UBT-86.156C3	Unknown	Z68701	Bhattacharya *et al.* (1996)
T. jamesii	A(23)	*Umbilicaria umbilicarioides*	AJ431590	Romeike *et al.* (2002)
T. jamesii	A(19)	*Umbilicaria decussata*	AJ431589	Romeike *et al.* (2002)
T. potteri	UTEX900	Unknown	AF242469	Kroken & Taylor (2000)
T. showmanii	UTEX2234	Unknown	AF242470	Kroken & Taylor (2000)
T. usneae	UBT-87.01A1	Unknown	Z68702	Bhattacharya *et al.* (1996)
T. usneae	UTEX2235	*Usnea filipendula*	AJ249573	Friedl *et al.* (2000)

For instance, compartments may exist, which are indicated by isolated subwebs. Therefore, columns and rows were sorted and visually inspected the interaction networks. Moreover, the number of such subwebs within the matrices were counted.

Results

Potential for interaction and diversity

In total, 103 juveniles of *R. menziesii* were surveyed on 11 trees (Table 2). Only four out of the 103 juvenile lichens had no neighbour lichen within a 2 mm distance. At the tree level, the mean number of *R. menziesii* juveniles growing in close proximity to another lichen neighbour ($\bar{x} = 9{\cdot}0$) was 24·8 times larger than that of juveniles growing by themselves ($\bar{x} = 0{\cdot}364$). Thus, many juveniles of *R. menziesii* may have had the potential to interact with other species of lichens during their development.

For the genetic analyses, ten lichen species were sampled from the trees containing *R. menziesii* juveniles (Table 3, Fig. 1). These included two species of *Xanthoria*, *X. hasseana* and *X. tenax*, *Ramalina leptocarpha* and *R. farinacea*, and two *Physcia* species. A total of 26 algal haplotypes were found among the juvenile thalli of *R. menziesii* and the 10 other species (Table 3).

Algal sharing

Four algal haplotypes were shared among the lichen fungi. Juveniles of *R. menziesii* utilized eight algal ITS haplotypes which

TABLE 2. *Species observed in a field survey as nearest neighbours of* Ramalina menziesii, *giving the number of observations and whether fungal species shared photobiont with* R. menziesii *as determined from a DNA sampling survey (specimens included in the DNA sampling are listed in Table 3).*

Species	Observations	Algal sharing
Caloplaca sp.	6	–
Candelaria concolor	–	Yes
Chrysothrix chlorina	4	–
Flavoparmelia caperata	4	No
Lecanora cf. *symmicta*	2	–
Lepraria sp.	3	–
Physcia adscendens	–	Yes
P. tenella	16	No
Physconia isidiigera	5	No
Ramalina farinacea	–	Yes
R. leptocarpha	25	Yes
Rinodina sp.	1	–
Teloschistes chrysophthalmus	12	No
Xanthoria hasseana	11	Yes
X. tenax	10	Yes

– *no data*.

belonged to a single algal species (*Trebouxia decolorans*) (Table 3, Figs 2 & 3). In 90% of the observations, the algal ITS haplotype of the nearest neighbour was different from that of the juvenile *R. menziesii*. On one twig, an algal haplotype was shared between a juvenile and its nearest neighbour *Xanthoria hasseana* (1-3-1b), as well as with a *X. hasseana* thallus growing at a distance of 12·3 cm (1-3-1d). The same haplotype (ITS23) occurred also on two other trees (2-2-1e, 3-1-1a). On another tree, *X. tenax* (2-3-2f) shared an algal haplotype with a *R. menziesii* (2-3-2e) growing at the same spot. Interestingly, the same algal haplotype occurred also on other trees (individuals 1-1-1c, 4-2-1a). Also, another algal haplotype occurred on several trees (ITS1; trees 1, 2, 3, and 4).

Fungal-algal associations

Five species of the genus *Trebouxia* were found to be associated with the lichen fungi studied. *Trebouxia decolorans* was found to be the photobiont of seven lichen-forming fungal species, *Candelaria concolor, Physcia adscendens, Ramalina farinacea, R. leptocarpha, R. menziesii, Xanthoria tenax,* and *X. hasseana* (Table 3, Fig. 2). In contrast, *Physcia tenella* and *Flavoparmelia caperata* (individual 5-1-1c) were each associated with a different, unknown (i.e., either not included in GenBank or new to science) species of the genus *Trebouxia* (Fig. 2). The second thallus of *F. caperata* (5-1-1b) and *Teloschistes chrysophthalmus* were found in association with *T. gelatinosa* (Fig. 2, Table 3). The only photobiont species of *R. menziesii* in the study area was *T. decolorans*. The photobiont of *P. isidiigera* was deemed conspecific with *Trebouxia flava*, and that of *P. tenella* (e.g. 3-1-1g) was distantly related to *T. gelatinosa* (Fig. 2), but represented an unknown species.

Random or structured associations

The species interaction matrix, algal species by fungal species, was not significantly nested ($N = 0{\cdot}564$, a value of 1 indicating perfect nestedness) (Guimarães & Guimarães 2006). The matrix is available in Table 4. This matrix was not significantly nested for any of the null models employed (Table 5). For two null models, the values of relative nestedness, N*, were negative. These negative values may indicate the existence of subwebs (compartmentalization). Visual inspection of the network revealed four reciprocally isolated subwebs, which suggests that

TABLE 3. *Algal – fungal relationships in lichens associated with juvenile* Ramalina menziesii. *The most frequent ITS haplotype, ITS23, is marked in bold*

ID*	Algal species	Algal ITS	Fungal species	Distance†	Accession
1-1-1a	*T. decolorans*	ITS1	*Ramalina menziesii*	0	EU717911
1-1-1b	*T. decolorans*	ITS24	*Xanthoria tenax*	1	EU717934
1-1-1c	*T. decolorans*	ITS15	*Ramalina farinacea*	480	EU717925
1-1-1d	*T. decolorans*	ITS22	*Xanthoria tenax*	400	EU717932
1-3-1a	***T. decolorans***	**ITS23**	***Ramalina menziesii***	**0**	**EU717933**
1-3-1b	***T. decolorans***	**ITS23**	***Xanthoria hasseana***	**0**	
1-3-1c	‡	‡	*Xanthoria hasseana*	510	
1-3-1d	***T. decolorans***	**ITS23**	***Xanthoria hasseana***	**1230**	
2-2-1a	*T. decolorans*	ITS15	*Ramalina menziesii*	0	
2-2-1b	*T. decolorans*	ITS1	*Ramalina farinacea*	1	
2-2-1c	*T. decolorans*	ITS1	*Xanthoria tenax*	2	
2-2-1d	*T. decolorans*	ITS26	*Ramalina farinacea*	500	EU717936
2-2-1e	***T. decolorans***	**ITS23**	***Xanthoria tenax***	**350**	
2-3-2a	*T. decolorans*	ITS10	*Ramalina menziesii*	0	EU717920
2-3-2b	*T. decolorans*	ITS20	*Xanthoria tenax*	0	EU717930
2-3-2c	*T. decolorans*	ITS21	*Ramalina menziesii*	160	EU717931
2-3-2d	*T. decolorans*	ITS24	*Xanthoria tenax*	160	
2-3-2e	*T. decolorans*	ITS15	*Ramalina menziesii*	410	
2-3-2f	*T. decolorans*	ITS15	*Xanthoria tenax*	410	
2-3-2g	*T. decolorans*	ITS24	*Xanthoria tenax*	760	
3-1-1a	***T. decolorans***	**ITS23**	***Ramalina menziesii***	**0**	
3-1-1b	*T. decolorans*	ITS1	*Candelaria concolor*	0	
3-1-1c	*Trebouxia* sp. 2	ITS2	*Physcia tenella*	1	EU717912
3-1-1d	*T. decolorans*	ITS17	*Candelaria concolor*	730	EU717927
3-1-1e	*T. decolorans*	ITS11	*Ramalina leptocarpha*	700	EU717921
3-1-1f	*T. decolorans*	ITS18	*Candelaria concolor*	800	EU717928
3-1-1g	*Trebouxia* sp. 2	ITS3	*Physcia tenella*	350	EU717913
3-1-3b	***T. decolorans***	**ITS23**	***Ramalina leptocarpha***	**>2000**	
4-1-1a	*T. decolorans*	ITS1	*Ramalina menziesii*	0	
4-1-1b	*T. decolorans*	ITS9	*Physcia adscendens*	0	EU717919
4-1-1c	*Trebouxia* sp. 2	ITS4	*Physcia tenella*	580	EU717914
4-2-1a	*T. decolorans*	ITS15	*Ramalina menziesii*	0	
4-2-1b	*T. decolorans*	ITS16	*Ramalina leptocarpha*	2	EU717926
4-2-1c	*T. decolorans*	ITS25	*Ramalina leptocarpha*	300	EU717935
4-2-1d	‡	‡	*Xanthoria tenax*	0	
4-2-1e	*T. decolorans*	ITS15	*Xanthoria tenax*	490	
4-2-1f	*T. decolorans*	ITS19	*Xanthoria tenax*	210	EU717929
5-1-1a	*T. decolorans*	ITS12	*Ramalina menziesii*	0	EU717922
5-1-1b	*T. gelatinosa*	ITS8	*Flavoparmelia caperata*	0	EU717918
5-1-1c	*Trebouxia* sp. 1	ITS5	*Flavoparmelia caperata*	>2000	EU717915
5-2-1a	*T. decolorans*	ITS13	*Ramalina menziesii*	0	EU717923
5-2-1b	*T. gelatinosa*	ITS8	*Teloschistes chrysophthalmus*	0	
5-6-1a	*T. decolorans*	ITS14	*Ramalina menziesii*	0	EU717924
5-6-1b	*Trebouxia flava*	ITS6	*Physconia isidiigera*	0	EU717916
5-6-1c	*Trebouxia flava*	ITS7	*Physconia isidiigera*	380	EU717917

* sample ID consists of the tree number, branch number, twig number, and sample letter
† the distance from sample a, the juvenile *R. menziesii* [mm]
‡ sequencing failed.

FIG. 1. A typical juvenile, net-formed thallus of the model species *Ramalina menziesii* (lace lichen, *Ramalinaceae*), the dominant epiphytic lichen of a southern Californian oak savannah. The juvenile *R. menziesii* grows next to other members of the epiphytic community, *Xanthoria tenax* and *Ramalina leptocarpha*. Scale = 1 cm. In colour online.

the species-level matrix was indeed organized in compartments. The largest subweb consisted of seven fungal and one algal species, and the remaining three represented one-by-one associations or two-by-two associations.

The nestedness of the matrix of fungal species and algal ITS sequence haplotypes was N = 0·716. Relative nestedness of fungal species and algal haplotypes was higher than that of the fungal-algal species matrix (Table 5). Significant nestedness was found with the ER and COL null model, the LI model was near to significant, and the conservative CE model was not significant.

Overall, the tests above revealed some degree of nestedness in the fungal species-algal ITS haplotype matrix, while the species-by-species matrix was not significantly nested. However, when visually inspecting the haplotype-level network (Table 6), a few isolated subsets nevertheless existed, implying compartmentalization (e.g. for the fungal species *F. caperata*, *T. chrysophthalmus* and *P. tenella*), while the largest part of the network, a group of fungal species including *R. menziesii* and *X. hasseana*, appeared to be nested.

Discussion

First, the potential for *R. menziesii* juveniles to interact with other species was studied using field observations, and I found that juveniles had indeed ample opportunities

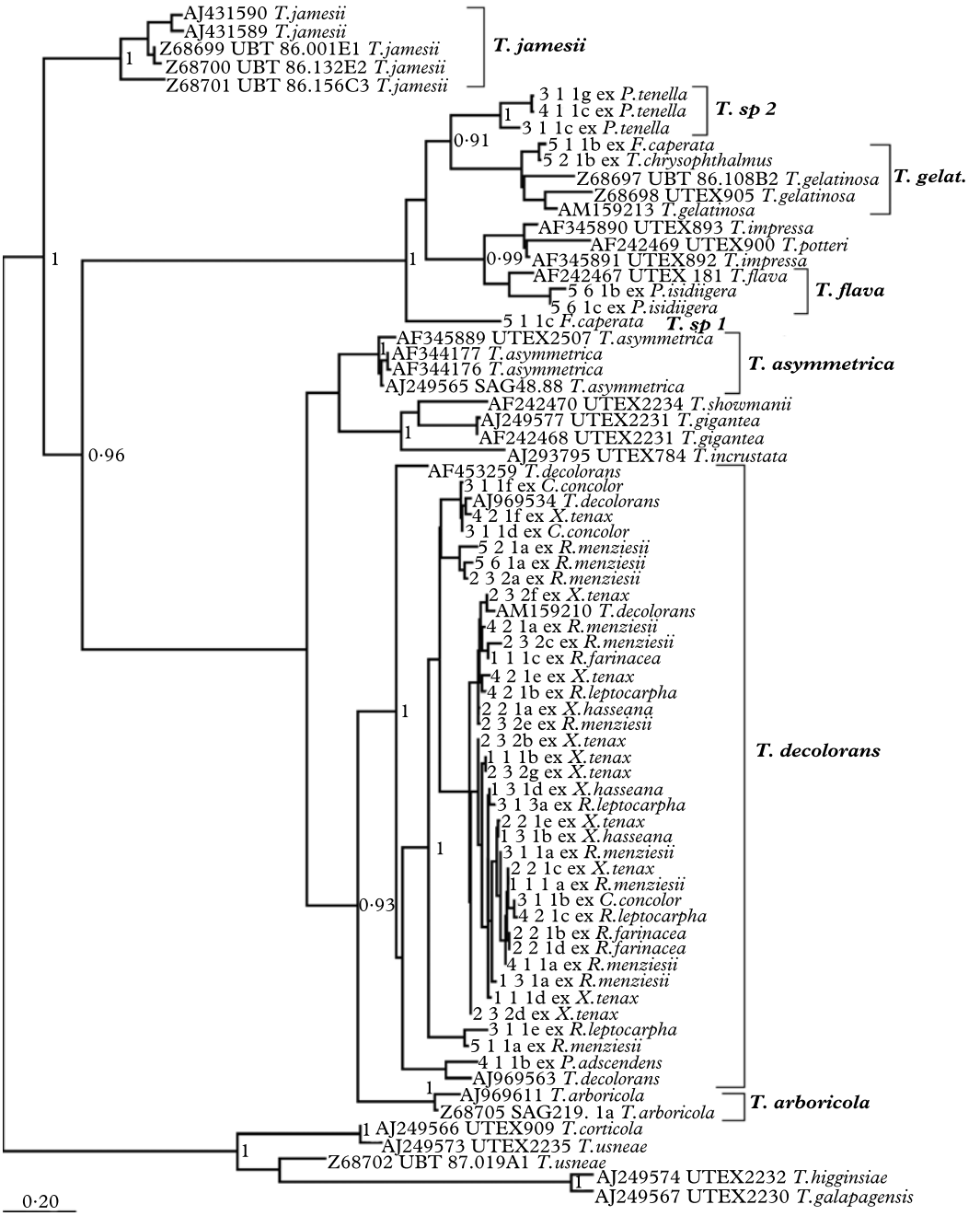

FIG. 2. Phylogenetic tree from Bayesian analysis of the photobionts associated with *Ramalina menziesii* and other lichen fungi from a southern Californian oak savanna. See Table 1 for more information on the sequences from GenBank. The numbers show Bayesian posterior probabilities.

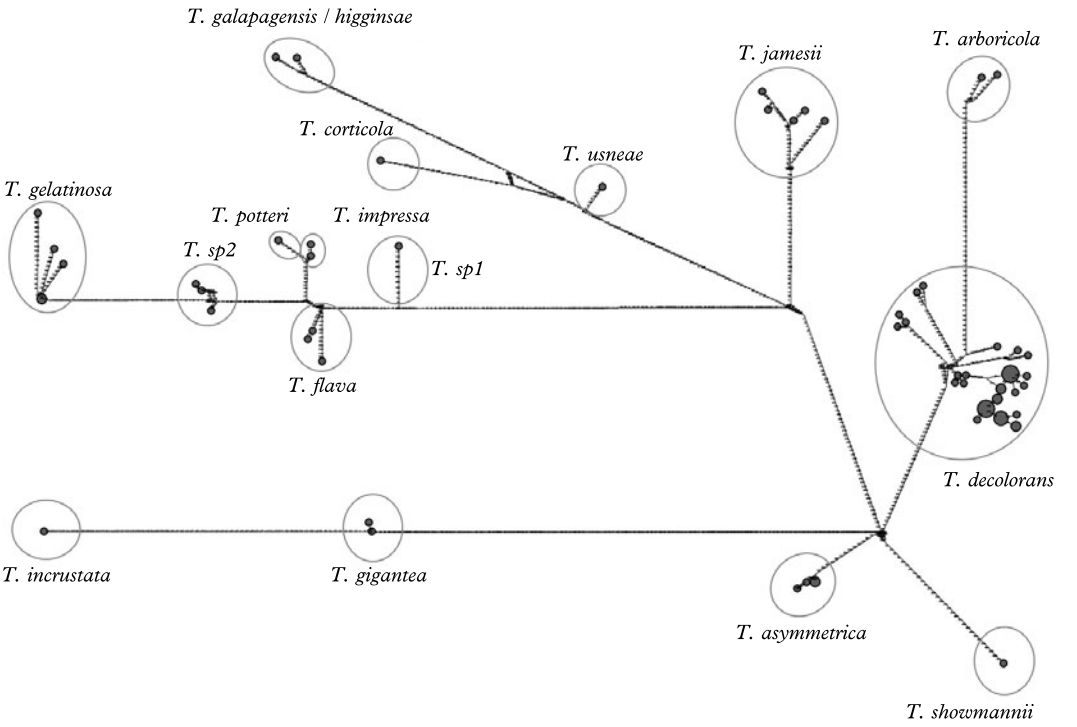

FIG. 3. Median-joining haplotype network of the photobiont community associated with *R. menziesii* and other lichen fungi in a southern Californian oak savanna. Branch length is proportional to the number of mutated positions. Haplotypes are shown as dots, with dot size proportional to a haplotype's frequency. Conspecific individuals are marked with circles.

to interact. Then, I investigated photobiont sharing among fungal species in the epiphytic lichen community with molecular techniques, and found evidence of sharing of one photobiont species (*Trebouxia decolorans*) by several fungal species. There was also some evidence of haplotype sharing among the focal species of the study, *R. menziesii*, and another species growing as its nearest neighbour (e.g., *X. hasseana*), though in most observations (90%), the algal haplotypes of juvenile *R. menziesii* differed from those of their nearest neighbours. The fungal-algal species associations were concordant with compartmentalization, a pattern in the data where groups of fungal species share the same algal partners.

Diversity

The lichen-forming fungi in this study belonged to the orders *Teloschistales* and *Lecanorales*, their photobionts to *Microthamniales* (genus *Trebouxia*). The Bayesian phylogenetic tree based on ITS sequences of *Trebouxia* sp., as well as results from the median-joining haplotype networks, were in agreement with other published phylogenies of lichen photobionts (Beck *et al.* 1998; Dahlkild *et al.* 2001; Helms *et al.* 2001; Hauck *et al.* 2007). The algal species *T. decolorans* associated with multiple fungal species, including the species of *Ramalina* and *Xanthoria*. Only *Flavoparmelia caperata* associated with different algal species (*T. gelatinosa* and *T.* sp1). These observations are consistent with some of these fungi sharing a common pool of algae; they may represent a photobiont-mediated fungal ecological guild. Photobiont-mediated guilds have been reported earlier for cyanobacterial lichens (Rikkinen *et al.* 2002; Rikkinen 2003). The

TABLE 4. *Interaction matrix of fungal and algal species, sorted to reveal potential subwebs. Numbers in parentheses indicate sample sizes.*

	Algal species				
Fungal species	*T. decolorans*	*T. gelatinosa*	*T. sp.1*	*T. sp.2*	*T. flava*
Candelaria concolor (3)	1	0	0	0	0
Physcia adscendens (1)	1	0	0	0	0
Ramalina farinacea (3)	1	0	0	0	0
R. leptocarpha (4)	1	0	0	0	0
R. menziesii (12)	1	0	0	0	0
Xanthoria hasseana (2)	1	0	0	0	0
X. tenax (10)	1	0	0	0	0
Teloschistes chrysophthalmus (1)	0	1	0	0	0
Flavoparmelia caperata (2)	0	1	1	0	0
Physcia tenella (3)	0	0	0	1	0
Physconia isidiigera (2)	0	0	0	0	1

TABLE 5. *Relative nestedness N* of fungal-algal associations for four null models.*

Matrix	ER	CE	LI	COL
Fungal species – algal species	0·0900	−0·1133	0·1440	−0·1950
Fungal species – algal haplotypes	0·2256*	0·1475	0·1324^{+}	0·2554*

* significant at $P = 0·05$.

photobiont community was dominated by the alga *T. decolorans*. This alga is a frequent lichen photobiont and is distributed worldwide. *Trebouxia decolorans* is also known as a photobiont of *Xanthoria parietina*, a common and widespread lichen species (S. Nyati, S. Scherrer & R. Honegger, unpublished data).

Factors influencing juvenile development in *R. menziesii*

Why do most *R. menziesii* juveniles grow close to other lichen thalli, even though they mostly do not seem to utilize the algae of other lichens? This is particularly intriguing as the branches I observed were not completely covered with lichens, seemingly leaving ample space for new colonizations.

Interestingly, this observed pattern is the opposite of what one would expect if interspecific competition played a major role. In this case, juveniles of *R. menziesii* should avoid growing close to adult lichen thalli that may outcompete them in the pursuit of light. Competition by other epiphytic species has been identified as an important mortality factor during juvenile development (Sillett *et al.* 2000*a*; Zoller *et al.* 2000). Allelopathic effects (Fahselt 1994), phenolic compounds leached from other lichen species hindering the growth of juveniles, do not seem to play a major role in the development of *R. menziesii* in the study area. If allelopathic effects were important, I would expect to see juveniles of *R. menziesii* growing at a larger distance from other lichens on the branches, rather than within a few millimetres from their neighbours, as observed in most juvenile thalli included in this study. Juveniles require a particular microclimate for their development (Schuster 1985). Possibly, the area within 2 mm from an occupied spot on a

TABLE 6. *Interaction matrix of fungal species and algal ITS sequence haplotypes, sorted by their frequency in columns and rows to reveal potential subwebs; for names of fungal genera and species, see Table 4*

	Algal ITS sequence haplotypes																									
Fungal species	H1	H23	H15	H21	H10	H12	H13	H14	H19	H20	H22	H24	H11	H16	H25	H17	H18	H26	H2	H3	H4	H6	H7	H8	H5	H9	**Freq**
R. menziesii	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**8**
X. tenax	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**7**
R. leptocarpha	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	**4**
C. concolor	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	**3**
R. farinacea	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	**3**
X. hasseana	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**
P. tenella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	**3**
P. isidiigera	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	**2**
F. caperata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	**2**
T. chrysopht.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	**1**
P. adscendens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	**1**
Frequency	**4**	**4**	**3**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**1**	

branch may contain microclimatic conditions suitable for spore germination and juvenile development of *R. menziesii*, so that specimens nearby have a facilitation effect on young thalli. For instance, the presence of a nearest neighbour might favour shading of a germinating spore, and increase the water potential of the site relative to an 'empty' site. While the morphology of juvenile development and survival rates of diaspores have been studied in detail (Ott 1987*a*; Scheidegger 1995; Ott *et al.* 2000; Sillett *et al.* 2000*a*, *b*; Zoller *et al.* 2000; Hilmo & Såstad 2001; Hilmo & Ott 2002), the environmental conditions required for the development of lichens from symbiotic propagules and from ascospores are still poorly understood, though the latter are key to the population dynamics of sexual lichen fungi.

Algal sharing

The many juvenile thalli of *R. menziesii* that were found growing close to neighbours highlight the potential of *R. menziesii* juveniles to have interacted with other species in the course of their development, and it cannot be ruled out that presence of neighbouring thalli leads to a facilitation effect, that is, increased establishment success. While the observational data show that *R. menziesii* juveniles grow most often in proximity with other species, this is obviously no indication that an interaction has indeed occurred with the neighbouring lichen species. I therefore used genetic data to investigate haplotype sharing between juvenile *R. menziesii* and neighbouring thalli.

Juveniles of *R. menziesii* used 31% of the algal haplotypes found in this study. In only one case was there evidence for algal sharing with a nearest neighbour (i.e. *X. hasseana*, 1-3-1a and 1-3-1b). In branch 1-3, the same photobiont was also found within the same branch at a larger distance, suggesting that the juvenile *R. menziesii* may have associated with a locally abundant, free-living algal haplotype, rather than a true algal exchange having taken place. In all other cases, there was no evidence of algal exchange among the juvenile and its nearest neighbour (Table 3), despite an apparently ample opportunity for interactions.

Several factors could explain these results. Firstly, the alga of the nearest neighbour may or may not be compatible (Yahr *et al.* 2004) with a developing *R. menziesii* sporeling. For instance, all nearest neighbouring algae found on tree number five were apparently not photobionts of *R. menziesii*. Secondly, *R. menziesii* may have retrieved its algae either from free-living algal populations or from vegetative, symbiotic lichen propagules. There is evidence that some species of *Trebouxia* can occur in free-living populations (Bubrick *et al.* 1984; Mukhtar *et al.* 1994). Thirdly, the algal layer of another lichen thallus may not be easily accessed by a *R. menziesii* sporeling. Many lichens, including the foliose and fruticose species investigated, have a dense upper cortex of fungal hyphae (Honegger 2008). Algal cells are usually situated in the algal layer below the cortex, and are often surrounded by fungal hyphae, and haustoria connect to the algal cells (Honegger 2008). Therefore, few algal cells may be available from an adult lichen thallus, unless for some reason the upper cortex is lacking. This could be the case in sorediate lichens, where the upper cortex breaks up to release soredia. Also, as a thallus gets damaged, for example by herbivores, the algal layer might become exposed leading to algal release. Indeed, it has been shown that in some cases *Trebouxia* cells can be released from damaged lichen thalli, given that sufficient moisture is available (Richardson 1999). However, for a sporeling or a juvenile lichen thallus, algae might also be available from propagules, free-living algae, or thallus fragments (Bubrick *et al.* 1984; Ahmadjian 1988; Mukhtar *et al.* 1994; Rikkinen 2003). Fourthly, algal sharing due to clonal propagation is not a likely explanation of the pattern found, as juveniles were investigated in the species-level study, and *R. menziesii* does not have any particular structures facilitating vegetative dispersal (e.g. soredia, isidia). Juveniles therefore have to result from sexual propagation, which is in accordance with the findings of a previous study (Werth & Sork 2008). Fifthly, the

juvenile thalli studied were between 0·5 cm and 5 cm long; these size classes may represent different ages. A species which was the nearest neighbour during the time of sampling, may not actually have been present on the branch when an ascospore was germinating and incorporating its alga. Nevertheless, if algal 'takeover' by *R. menziesii* was a common phenomenon, one would expect more observations of algal sharing; at least algal sharing among neighbouring thalli containing the compatible algal species, *T. decolorans*.

Fungal-algal associations

The fungal species included in this study utilized only one algal species, with the notable exception of *Flavoparmelia caperata,* which used two algal species. A pool of seven fungal species belonging to the orders *Teloschistales* (e.g., *Xanthoria hasseana*) and *Lecanorales* (e.g., *Ramalina menziesii*) each utilized the same algal species, *T. decolorans*. A second group of fungal species of the same orders used several *Trebouxia* species of an algal clade including *T. gelatinosa*. Such high fungal selectivity for particular algal species has also been reported in other studies (Beck 1999; Beck *et al.* 2002; Piercey-Normore 2004, 2006; Yahr *et al.* 2004). Doering & Piercey-Normore (2009) documented the photobionts of a lichen community on Jack pine, and found that fungal species were associated with at least five algal species. Similar to findings in the present study, taxonomically different fungal species (e.g. those belonging to *Physciaceae* and *Parmeliaceae*) associated with the same algal species.

Random or structured associations

The results demonstrate that *Ramalina menziesii* interacts with a specific subset of the photobiont species present in the lichen community. The interaction between mycobionts and photobionts is specific at the level of individual species, thus corroborating the results of other studies (Beck *et al.* 2002; Piercey-Normore 2004, 2006; Yahr *et al.* 2004; Ohmura *et al.* 2006; Hauck *et al.* 2007).

Most interestingly, the structure of fungal-algal associations changed with decreasing scale. The organization of fungal and algal species was consistent with compartmentalization, a pattern where several independent subsets of species interact with one another. This pattern was different from what has been observed in less intimate mutualistic interactions, such as plant-pollinator or plant-herbivore interactions, in which the observed species associations were significantly nested, that is with few generalist species interacting with many species, and many specialists interacting with subsets of the species interacting with the generalists (Bascompte *et al.* 2003; Jordano *et al.* 2003; Bascompte & Jordano 2007). However, symbiotic mutualisms of ant and plant species exhibited compartmentalized interactions as well (Guimarães *et al.* 2007). In the present study, fungal and algal species showed evidence of compartmentalization. However, it has to be considered that the sample sizes were relatively small and the communities were certainly not completely sampled. Further studies that employ a more intensive sampling scheme are necessary to investigate whether the patterns reported here hold with increased sample sizes, and in different lichen communities.

The test for nestedness was significant at the smaller spatial scale, that is for the network of fungal species and algal haplotypes, probably because a large part of the network was comprised of the fungal species associating with haplotypes of *T. decolorans*, and, at this scale, some evidence for nestedness appeared to be present in the data (i.e. *T. decolorans* haplotypes interacting with many fungal species). The nestedness within the *T. decolorans*-mediated subweb may have masked the compartmentalized structure of the small remaining part of the network, making the statistical test of nestedness significant in 50% of the permutation tests performed. To conclude, also at the scale of haplotypes, the network showed significant organization, which differed slightly from the organization at the species level.

For the analyses of fungal-algal interactions, I investigated only part of a community (i.e. the lichens that were found on the same

branches as juvenile *R. menziesii*). An increased sampling effort would have revealed more associations among species, which might have made it more likely to observe significant nesting at the haplotype level. The statistical significance of nestedness, despite the limited sample size, implies that some nested structure is indeed present in the haplotype-level data. Increased sampling could also have revealed more subwebs in the species-level data, as soon as additional species were included.

One important question is what are the mechanisms leading to the compartmentalized organization of fungal and algal species? A possible explanation is that the fungi may not be compatible with all algal species. Indeed, most fungal species in the present study were associated with only one algal species, suggesting high fungal selectivity. A wide range of algal partners indicating low selectivity has been found for some lichen fungi (Piercey-Normore 2004; Yahr *et al.* 2004), whereas others appeared to be more specific (Beck 1999; Kroken & Taylor 2000; Yahr *et al.* 2004; Piercey-Normore 2006; Hauck *et al.* 2007; Doering & Piercey-Normore 2009). Even within the same fungal genus, selectivity for the photobiont partner can vary dramatically (Piercey-Normore 2004). Moreover, the mode of photobiont transmission might be a major factor influencing the nature of fungal-algal interactions at the community scale (Beck *et al.* 1998; Yahr *et al.* 2004). If photobionts were predominantly vertically transmitted, the same photobiont haplotype should be found repeatedly within the same fungal species. This might lead to compartmentalization of haplotype associations. However, here I found that the recurrent algal haplotypes were often shared among fungal species, with a few recurrences within species. For *R. menziesii*, a previous study found evidence of sexual reproduction involving relichenizations, that is, horizontal transmission of photobionts (Werth & Sork 2008). Also, *R. menziesii* was associated with multiple algal haplotypes, indicating horizontal transmission of algae. Relichenization and horizontal transmission mode has also been reported for other species (Piercey-Normore & DePriest 2001; Beck *et al.* 2002; Romeike *et al.* 2002; Sanders & Lücking 2002; Nelsen & Gargas 2008). The relative frequency of vertical and horizontal transmission of photobionts may have a pronounced effect on the structure of fungal-algal associations (Werth & Scheidegger 2012). However, this would not necessarily be reflected in the present nestedness analyses which do not consider the frequency of associations. Last but not least, differential selection could create the observed compartmentalized structure of fungal-algal associations, if selection favoured some associations over others; recent studies have provided evidence for this hypothesis (Blaha *et al.* 2006; Nelsen & Gargas 2009; Werth & Sork 2010; Fernández-Mendoza *et al.* 2011; Peksa & Skaloud 2011; Werth 2011; Werth & Scheidegger 2012).

It would be interesting to test if the compartmentalized pattern of fungal-algal associations observed in the present study can be confirmed in other lichen communities, for example in communities where cyanobacterial photobionts predominate, or in communities where *Dictyochloropsis* sp. or *Trentepohlia* sp. (green algal) photobionts are frequent; these communities might show altogether different patterns in fungal-algal associations, relative to the communities associating with *Trebouxia* spp. included in the present study.

Conclusions

While juveniles of *R. menziesii* had indeed ample opportunities to interact with other lichen species, for most observations, the algal haplotypes of juvenile *R. menziesii* differed from those of their nearest neighbours, indicating that algae are not taken up from the thalli of other species growing in the vicinity. However, the observation that most thalli of *R. menziesii* grew in the vicinity of other lichen thalli indicates that facilitation might take place, that is increased juvenile survival due to the beneficial effect of neighbours. *Ramalina menziesii* was specific in its choice of a photobiont species, but shared this with several other lichen fungi.

Fungal-algal associations at the species level were not random but concordant with compartmentalization, similar to the pattern found in other intimately interacting mutualists. However, at a finer taxonomic scale (fungal species & algal haplotypes), a different pattern was observed, with interaction matrices showing some evidence of nestedness. Hence, network organization in communities of mycobionts and their photobiont partners depends on the scale of the study.

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