Symbiont flexibility in *Thamnolia vermicularis* (Pertusariales: Icmadophilaceae)

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Abstract. Algal and fungal symbionts of the lichenized genus *Thamnolia* typically co-disperse through thallus fragmentation, which may be expected to lead to fungal associations with a restricted range of algal symbionts. Here we examine the range of algae that associate with the fungus *Thamnolia vermicularis*. Phylogenetic analyses of internal transcribed spacer rDNA (ITS) sequences suggest that *Trebouxia* algae associated with *T. vermicularis* are not monophyletic. Algal and fungal phylogenies were compared, and although some congruence was found, a Mantel test found no significant correlation between fungal and algal genetic distances. An AMOVA suggested that ecogeographic factors play a stronger role than fungal genotype in structuring photobiont diversity. Additionally, as a species, *T. vermicularis* associates with a range of algae equal to or greater than that of many other fungal taxa.

Keywords. *Thamnolia*, *Trebouxia*, phylogeny, photobiont, symbiont-switch, re-lichenization, dispersal.

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As with genetic recombination, symbiont-switching generates new associations between lineages creating new combinations of genes. New associations may potentially help symbionts survive changing environmental conditions. Piercey-Normore (2005) suggested that co-dispersing symbioses may share some of the disadvantages of clonal organisms. If symbionts consistently co-disperse, this combination of symbionts may not be able to adapt to changing environmental conditions, colonize new environments or survive various selective pressures. The range of symbionts an organism is able to associate with is also thought to play a role in the survival of a lineage. Low specificity and selectivity have been suggested as life history strategies that help lichen symbionts survive changing or harsh environmental conditions (Piercey-Normore 2006; Romeike et al. 2002; Wirtz et al. 2003). Numerous

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fungal species have been shown to exhibit a preference for certain algal partners (Beck et al. 1998, 2002; Piercey-Normore 2004; Romeike et al. 2002; Yahr et al. 2004), and Yahr et al. (2006) illustrated that this preference can vary both by habitat and by geographic location. The ability to switch symbionts and associate with a wide range of algae may be especially beneficial to organisms with no, or limited, genetic recombination. In clonal organisms, which are presumed to lack genetic recombination, strictly maintaining relationships may be especially perilous, as the mycobiont is less likely to generate new genetic combinations within its own genome.

Thamnolia vermicularis is a widespread, fruticose, lichenized fungus which occurs over soil and stone at high latitudes and altitudes on all continents except Africa and Antarctica. *Thamnolia* is composed of three vegetatively reproducing species, *T. juncea*, *T. papelillo* and *T. vermicularis*, which are distinguished by morphological features (Santesson 2004). Ascomata are unknown from all *Thamnolia* species. Using molecular data, Platt and Spatafora (2000) and Stenroos et al. (2002) demonstrated the placement of *Thamnolia* in the Icmadophilaceae (Pertusariales). Nelsen and Gargas (2009) tested whether genetic evidence for recombination existed in *T. vermicularis* and recovered only weak evidence for it. Miao (in Goward 1999) and Nelsen and Gargas (2009) used molecular data to suggest that chemotypes in *T. vermicularis* do not form strongly supported monophyletic groups, therefore, in this paper, we refer to the thamnolic acid and baeomycesic and squamatic acid chemotypes with the *vermicularis*-type morphology as *T. vermicularis*. Relationships between *T. vermicularis*, *T. juncea* and *T. papelillo* have not been assessed, nor has the monophyly of these three taxa. However, in this study we assume *T. vermicularis* is monophyletic relative to *T. juncea* and *T. papelillo*. In the event that *T. vermicularis* is not monophyletic relative to these taxa, it would still be plausible to suggest that like *Lepraria* (Ekman & Tønsberg 2002), the loss of ascomata originated at the base of *Thamnolia* and was followed by subsequent phenotypic and genetic divergence. This would have eliminated ascospore dispersal and re-lichenization early in the evolution of *Thamnolia*.

Here we investigate whether *Thamnolia vermicularis* has maintained strict associations with its algal symbionts. We test for the monophyly of algal symbionts, compare the phylogentic tree topologies of *T. vermicularis* fungi and their algal symbionts for congruence, and test whether fungal and algal pairwise genetic distances are correlated. In addition, we use an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to assess the magnitude of the role ecogeographic factors and fungal genotype play in determing which algae the fungi associate with. Finally we compare the range of algae *T. vermicularis* associates with to that of other taxa, to see if the predominantly fragmenting *T. vermicularis* associates with a narrower range of algae than other taxa.

Materials and Methods

Taxon Sampling. We collected *Thamnolia vermicularis* samples from China, Costa Rica and Norway, and supplemented these with collections from Alaska, U.S.A. deposited in the University of Wisconsin Herbarium (WIS). We broadened our algal dataset by including rDNA internal transcribed spacer (ITS) sequences from GenBank, representing each of the four major *Trebouxia* clades of Friedl et al. (2000) and Helms (2003). We included GenBank sequences for *Dibaeis baeomyces* as the outgroup for the fungal analyses.

DNA extraction, PCR and sequencing. Specimens used in this study (**Table 1**) have been deposited in WIS. DNA isolations were conducted using the method of Grube et al. (1995) or with the Qiagen DNeasy Mini Extraction Kit, following the modifications of Crespo et al. (2001). The algal ITS was PCR amplified as in Nelsen and Gargas (2006, 2008) using the primers a-nu-ssu-1752-5′ (Nelsen & Gargas 2006) and ITS4T (Kroken & Taylor 2000). Samples were sequenced as in Nelsen and Gargas (2006, 2008) using ITS1 and ITS4 (White et al. 1990). BLAST searches (Altschul et al. 1997) for individual sequence types (**Table 1**) were used to identify the generic placement of algal sequences.

Algal phylogenetic analyses. Unique algal sequences obtained from *Thamnolia vermicularis*, as well as *Trebouxia* sequences from GenBank (**Table 1**) were manually aligned in Se-Al v. 2.0a11 (Rambaut 1996), and ambiguously aligned regions excluded. A maximum parsimony (MP) analysis were performed in PAUP* 4.0b10 (Swofford 2002), using a heuristic

Table 1. Samples used in this study with GenBank accession numbers. **Bold** accession numbers indicate individuals for which fungal sequences were obtained in Nelsen and Gargas (2009). **Bold** sequence type numbers indicate the representative of that sequence type that was used in phylogenetic analyses. DNA names are given following the name of the fungal sybmiont. Symbols correspond to chemistry: hollow = baeomycesic and squamatic acid, solid = thamnolic acid; and geographic location: circle = U.S.A.; square = Norway; star = China; triangle = Costa Rica. All collections are from or were deposited in WIS.

Alga Partner of: (DNA # & Chemistry/Geog)	Collection Number	GenBank Accession	Seq Type
T. vermicularis (T1) ○	*Talbot KIS 131*, Alaska, U.S.A.	**EU715034**	1
T. vermicularis (T4) ○	*Talbot KIS 165* (1), Alaska, U.S.A.	**EU715035**	1
T. vermicularis (T5) ○	*Talbot 271*, Alaska, U.S.A.	EU715036	1
T. vermicularis (T13) ○	*Talbot NIZ5B-25*, Alaska, U.S.A.	**EU715037**	1
T. vermicularis (T15) ○	*Talbot KIS518*, Alaska, U.S.A.	EU715038	**2**
T. vermicularis (T18) ○	*Talbot OGA1D-17*, Alaska, U.S.A.	**EU715039**	**3**
T. vermicularis (T19) ○	*Talbot RAT1A-12*, Alaska, U.S.A.	**EU715040**	**1**
T. vermicularis (T31) □	*Nelsen 3980*, Troms, Norway	**EU715041**	**4**
T. vermicularis (T32) □	*Nelsen 3981*, Troms, Norway	**EU715042**	**5**
T. vermicularis (TS10) ☆	*Nelsen 2291*, Yunnan, China	**EU715043**	**6**
T. vermicularis (TS11) ☆	*Nelsen 2294*, Yunnan, China	**EU715044**	6
T. vermicularis (TS12) ☆	*Nelsen 2292*, Yunnan, China	**EU715045**	6
T. vermicularis (T3) ●	*Talbot KIS 165 (1)*, Alaska, U.S.A.	**EU715046**	1
T. vermicularis (T7) ●	*Talbot & Schofield 390*, Alaska, U.S.A.	EU715047	**7**
T. vermicularis (T8) ●	*Talbot KIS 161a*, Alaska, U.S.A.	**EU715048**	1
T. vermicularis (T10) ●	*Talbot ADU3B-X-10*, Alaska, U.S.A.	**EU715049**	**8**
T. vermicularis (T11) ●	*Talbot CHA1C-101*, Alaska, U.S.A.	**EU715050**	**9**
T. vermicularis (T20) ●	*Talbot TAG1E-33*, Alaska, U.S.A.	EU715051	9
T. vermicularis (T23) ●	*Talbot KAV2A-08*, Alaska, U.S.A.	EU715052	**10**
T. vermicularis (T25) ●	*Talbot LKI1A-11*, Alaska, U.S.A.	**EU715053**	**11**
T. vermicularis (T29) ●	*Talbot Selawik 7*, Alaska, U.S.A.	EU715054	**12**
T. vermicularis (T30) ●	*Talbot Selawik 291*, Alaska, U.S.A.	EU715055	12
T. vermicularis (TV13) ▲	*Nelsen 3635*, San José, Costa Rica	**EU715056**	**13**
T. vermicularis (TV14) ▲	*Nelsen 3636B*, San José, Costa Rica	**EU715057**	13
T. vermicularis (TV15) ▲	*Nelsen 3630*, San José, Costa Rica	EU715058	13
T. vermicularis (TV16) ★	*Nelsen 2291*, Yunnan, China	EU715059	**14**
T. vermicularis (TV17) ★	*Nelsen 2292*, Yunnan, China	**EU715060**	14
T. vermicularis (TV18) ★	*Nelsen 2413*, Yunnan, China	**EU715061**	1

search with tree-bisection-reconnection (TBR) branch-swapping and random taxon addition with 100 random addition replicates, saving no more than 100 trees with a length greater than 1 step during each replicate. Following this, 1000 bootstrap replicates (Felsenstein 1985) were performed, using identical settings. Additionally, a Bayesian analysis was conducted in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), using the substitution model selected by the Akaike Information Criterion (AIC), implemented in MrModelTest 2.2 (Nylander 2004). Two parallel analyses were run for 2,000,000 generations, using the GTR+G substitution model, at a temperature of 0.048 using four chains and sampling every 100th tree. Acceptance rates between approximately 0.1 and 0.7 were taken as evidence for adequate mixing, similar to the suggestion of Ronquist et al. (2005). The initial 2501 trees were discarded for burnin, and posterior probabilities were estimated by constructing a 50% majority-rule consensus trees of all sampled post burn-in trees in PAUP*4.0b10 (Swofford 2002). Our algal clade nomenclature follows that of Friedl et al. (2000), who recovered four major clades in *Trebouxia*, and Helms (2003), who designated these A ("*arboricola*"), I ("*impressa*"), S ("*simplex*") and G ("*galapagensis*").

Algal monophyly and genetic structure. As a result of symbionts' shared dispersal, it might be

Table 2. List of fungal taxa used in this study with GenBank Accession numbers for each locus. *Thamnolia* fungal sequences were obtained from Nelsen and Gargas (2009), and DNA names are listed for each individual. Symbols behind *Thamnolia* individuals correspond to chemistry: hollow = baeomycesic and squamatic acid, solid = thamnolic acid; and geographic location: circle = U.S.A.; square = Norway; star = China; triangle = Costa Rica.

Fungal Species (DNA #, Chemistry/Geog)	GenBank Accession Numbers			
	ITS	IGS	mtLSU	RPB2
Dibaeis baeomyces	DQ782844			AY641037
T. vermicularis (T1) ○	EU714413	EU714438	EU714456	EU714480
T. vermicularis (T4) ○	EU714416	EU714441	EU714458	EU714482
T. vermicularis (T13) ○	EU714422		EU714464	
T. vermicularis (T18) ○	EU714424	EU714447	EU714466	EU714486
T. vermicularis (T19) ○	EU714425	EU714448	EU714467	EU714487
T. vermicularis (T31) □	EU714430	EU714452	EU714472	EU714490
T. vermicularis (T32) □	EU714431	EU714453	EU714473	EU714491
T. vermicularis (TS10) ☆			EU714474	EU714492
T. vermicularis (TS11) ☆	EU714432		EU714475	EU714493
T. vermicularis (TS12) ☆	EU714434	EU714454		EU714494
T. vermicularis (T3) ●	EU714415	EU714440	EU714457	
T. vermicularis (T8) ●	EU714417	EU714442	EU714459	EU714483
T. vermicularis (T10) ●	EU714419	EU714444	EU714461	EU714485
T. vermicularis (T11) ●	EU714420		EU714462	
T. vermicularis (T25) ●	EU714429	EU714451	EU714471	EU714489
T. vermicularis (TV13) ▲	EU714434		EU714476	EU714495
T. vermicularis (TV14) ▲	EU714435		EU714477	EU714496
T. vermicularis (TV17) ★	EU714436	EU714455	EU714478	EU714497
T. vermicularis (TV18) ★	EU714437		EU714479	EU714498

expected that if associations were strictly maintained over ecological and evolutionary time, fungi would associate with a monophyletic group of algae. In contrast, if relationships were not maintained, fungi might be expected to associate with a polyphyletic assemblage of algae. We tested the hypothesis of symbiont monophyly in a Bayesian framework by constraining the algal topology to be consistent with the monophyly of *Thamnolia*-associated algae. The unconstrained and constrained analyses were compared using Bayes factors, which were calculated by taking twice the difference between the harmonic mean of the unconstrained and constrained analyses. Interpretation of Bayes factor values followed Kass and Raftery (1995). We also employed the Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 2000) and expected likelihood weight (ELW) test (Strimmer & Rambaut 2002), implemented in TreePuzzle 5.2 (Schmidt et al. 2002), to test for monophyly in a likelihood framework. Parameter estimates from the Bayesian analysis were used and the most-likely tree under the maximum likelihood (ML) optimality criterion was obtained in TreePuzzle 5.2 (Schmidt et al. 2002), using the GTR+G model with four variable gamma rates (as employed in the unpartitioned Bayesian analysis). The hypothesis of monophyly was imposed as a topological constraint, and the likelihood of the constrained topology was obtained. Likelihoods of the constrained and unconstrained trees were then compared by means of the SH and ELW tests to determine if these alternate topologies were significantly worse than those obtained in the unconstrained searches.

Fungal-algal and geographic associations. Fungal ITS rDNA, nuclear intergenic spacer rDNA (IGS), mitochondrial large subunit rDNA (mtLSU) and nuclear second largest subunit of RNA polymerase II (RPB2) sequences (**Table 2**) from Nelsen and Gargas (2009) were obtained from most specimens from which algal sequences were derived. Fungal individuals that had corresponding algal sequences were manually aligned in Se-Al v. 2.0a11 (Rambaut 1996), and the incongruence length

difference (ILD) test (Farris et al. 1994) was performed in PAUP* 4.0b10 (Swofford 2002) to test for conflicting datasets, using the settings described for the MP search of the algal dataset.

A MP analysis was conducted on the fungal dataset, using the same settings used for the algal search, and 1000 bootstrap replicates (Felsenstein 1985) were performed. In addition, unpartitioned and partitioned (by locus) Bayesian analyses were performed in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) using the model(s) determined by the AIC in MrModeltest 2.2 (Nylander 2004). The GTR+G model was selected for the unpartitioned dataset, while the SYM+G, GTR, F81 and SYM models were selected for the ITS, IGS, mtLSU and RPB2 datasets, respectively. Two parallel analyses were run for 2,000,000 generations, with four chains each, sampling every 100 generations. Default settings were used, except for the temperature, which was set to 0.12 for the unpartitioned and partitioned analyses. The initial 1001 trees were discarded for each run, and Bayes factors were used to compare the unpartitioned and partitioned analyses (as described above). We then visually compared fungal and algal topologies for congruence and illustrated associations.

Using the models and parameter estimates obtained by the AIC in MrModelTest (Algae: GTR+I; Fungi: GTR), we generated pairwise genetic distance estimates for the algal and fungal datasets in PAUP* 4.0b10 (Swofford 2002). We then used a Mantel test with 10,000 randomizations to determine whether there was a significant correlation between fungal and algal pairwise distances. The Mantel test was implemented using the Isolation by Distance Web Service Version 3.15 (Jensen et al. 2005), which uses the Isolation By Distance (IBD) program of Bohonak (2002).

We next examined whether fungal genotype and geographic and ecological factors played roles in structuring the algal symbionts. Algal sequences from *Thamnolia* that had corresponding fungal sequences were partitioned by fungal genotype (six unique genotypes) and then into three broad ecogeographic groups: Boreal/Arctic, East Asian and Tropical Montane. We used the K2P + G ($\alpha = 0.263$) model to estimate genetic distances between algal symbionts, and used an AMOVA to determine what percentage of the variation was explained by each partition. All analyses were performed in Arlequin 3.11 (Excoffier et al. 2005), and 10,000 permutations were used to test significance. In addition, we sought to determine if ecogeographic factors played a role in structuring fungal genotypes, and used an AMOVA and F_{ST} (Weir & Cockerham 1984) values to estimate fungal subdivision between groups, using the K2P model of evolution. Due to the patchiness of the fungal dataset, we included all sites in the genetic distance analysis of the fungal dataset.

Specificity comparison. To compare the range of algae with which *Thamnolia vermicularis* associates to that of other fungal taxa, we compared the maximum pairwise genetic distance of algal symbionts associated with each fungal taxon. Each included fungal taxon had at least five published *Trebouxia* sequences available in GenBank with samples taken from two or more geographic localities. Fifteen fungal taxa (including *T. vermicularis*) from four orders and six families met the criteria for specificity comparison (**Table 3**).

Using default settings in ClustalX 2.0.8 (Larkin et al. 2007), we then aligned published ITS sequences from *Trebouxia* algae associating with these fungal taxa. The following algal sequences were used for each fungal taxon: *Anaptychia ciliaris* (Teloschistales: Physciaceae): AJ293770 (Helms et al. 2001), AF389913–AF389917 (Dahlkild et al. 2001); *Flavocetraria nivalis* (Lecanorales: Parmeliaceae): AY444751–AY444768 (Opanowicz & Grube 2004); *Hypogymnia physodes* (Lecanorales: Parmeliaceae): AJ511355–AJ511363 (Hauck et al. 2007); *Lecanora bicincta* (Lecanorales: Lecanoraceae): DQ166576–DQ166581 (Blaha et al. 2006); *L. rupicola* (Lecanorales: Lecanoraceae): DQ166587–DQ166618 (Blaha et al. 2006); *Parmotrema tinctorum* (Lecanorales: Parmeliaceae): Z68702 (Bhattacharya et al. 1996), AB177817–AB177838 (Ohmura et al. 2006); *Phaeophyscia orbicularis* (Teloschistales: Physciaceae): AJ007386 (Beck et al. 1998), AJ293786 (Helms et al. 2001), AF389928–AF389932 (Dahlkild et al. 2001); *Physcia aipolia/caesia* (Teloschistales: Physciaceae): AJ293775–AJ293776 (Helms et al. 2001), AF389918–AF389922 (Dahlkild et al. 2001); *P. tenella* (Teloschistales: Physciaceae): AJ293788

Table 3. Range of *Trebouxia* algae associating with various fungal taxa. The number of sequences generated in examined studies (#), maximum pairwise genetic distance (Max GD) between algal symbionts associating with a fungal taxon, the maximum pairwise genetic distance divided by the number of sequences (Max GD/#), and sampling locations are shown. Genetic distances are ranked from greatest (1) to least (15).

Fungal Taxon	Sequences	Max GD	Max GD/#	Locations
Lecanora rupicola	32	0.25646 (1)	0.00801 (3)	Australia, Austria, Denmark, France, Greece, Norway, Poland, Portugal, Spain, Sweden
Protoparmeliopsis muralis	40	0.20705 (2)	0.00518 (8)	Austria, Denmark, Estonia, Germany, Italy, Poland, Sweden, Ukraine
Lecanora bicincta	6	0.18554 (3)	0.03092 (1)	Greece, Norway, Spain, Turkey, U.S.A.
Umbilicaria antarctica/kappenii[1]	14	0.15246 (4)	0.01089 (2)	Antarctica
Thamnolia vermicularis	28	0.15078 (5)	0.00539 (6)	China, Costa Rica, Norway, U.S.A.
Tephromela atra[2, 3]	56	0.15041 (6)	0.00269 (10)	Austria, Greece, Italy
Parmotrema tinctorum	69	0.13776 (7)	0.00200 (14)	Japan, U.S.A.
Physconia distorta	6	0.04713 (8)	0.00786 (4)	Finland, Netherlands, Sweden
Phaeophyscia orbicularis	7	0.04387 (9)	0.00627 (5)	Germany, Finland, Sweden
Tephromela grumosa[3]	17	0.03881 (10)	0.00228 (11)	Italy
Anaptychia ciliaris	6	0.03230 (11)	0.00538 (7)	Germany, Sweden
Flavocetraria nivalis	18	0.02487 (12)	0.00138 (15)	Austria, Denmark (Greenland), Finland, Iceland, Norway, Poland, Sweden, Ukraine
Hypogymnia physodes	9	0.02046 (13)	0.00227 (12)	Germany, U.S.A.
Physcia tenella	6	0.01957 (14)	0.00326 (9)	Finland, Germany, Sweden
Physcia aipolia/caesia[4]	7	0.01420 (15)	0.00203 (13)	Germany, Finland, Mexico, Russia, Sweden, U.S.A.

[1] These two taxa have been suggested to form a monophyletic group (Ott et al. 2004).

[2] Includes *T. atra s. str.*, *T. calcarea* and *T. torulosa* – Muggia et al. (2008) demonstrated these three taxa could not be separated at present.

[3] Muggia et al. (2008) demonstrated that *T. grumosa* forms a distinct lineage within the rest of the *T. atra* complex (included in *T. atra* in the present study), and we have treated it as such here. If, however, *T. grumosa* is included with the rest of the *T. atra* complex, maximum genetic distance would increase to 0.15305, and the maximum genetic distance divided by the sample number would decrease to 0.00209.

[4] These two taxa have been suggested to form a monophyletic group (Myllys et al. 2001).

(Helms et al. 2001), AF389933–AF389937 (Dahlkild et al. 2001); *Physconia distorta* (Teloschistales: Physciaceae): AF242467 (Kroken & Taylor 2000), AF389923–AF389927 (Dahlkild et al. 2001); *Protoparmeliopsis muralis* (Lecanorales: Lecanoraceae): AY703898–AY703907, DQ133473–DQ133502 (Guzow-Krzeminska 2006); *Tephromela atra* (Lecanorales: Mycoblastaceae): EU551473–EU5514796, EU551508–EU551510, EU551512–EU551515, EU551517–EU551522, EU551524, EU551526–EU551528, EU551530–EU551534, EU551541–EU551550 (Muggia et al. 2008); *T. grumosa* (Lecanorales: Mycoblastaceae): EU551497–EU551507, EU551511, EU551535–EU551537, EU551539–EU551540 (Muggia et al. 2008); *Thamnolia vermicularis* (Pertusariales: Icmadophilaceae): EU715034–EU715061 (this study); *Umbilicaria antarctica/kappenii* (Umbilicariales: Umbilicariaceae): AJ315854–AJ315855, AJ318779–AJ3187780, AJ431574–AJ431582, AJ431591 (Romeike et al. 2002). Sequences were manually adjusted and we used the AIC as implemented in MrModeltest 2.2 (Nylander 2004) to determine the substitution model. The GTR+I+G model was selected, and we used the parameter estimates generated by MrModeltest 2.2 (Nylander 2004) to estimate maximum likelihood pairwise genetic distances in PAUP 4.0b10 (Swofford 2002). For each fungal taxon, we recorded the maximum pairwise genetic distance value between its algal symbionts and used this an estimate of the maximum range of algae each fungal taxon associates with.

Because sampling efforts were not equal across fungal taxa, we also sought to determine whether a relationship existed between the number of

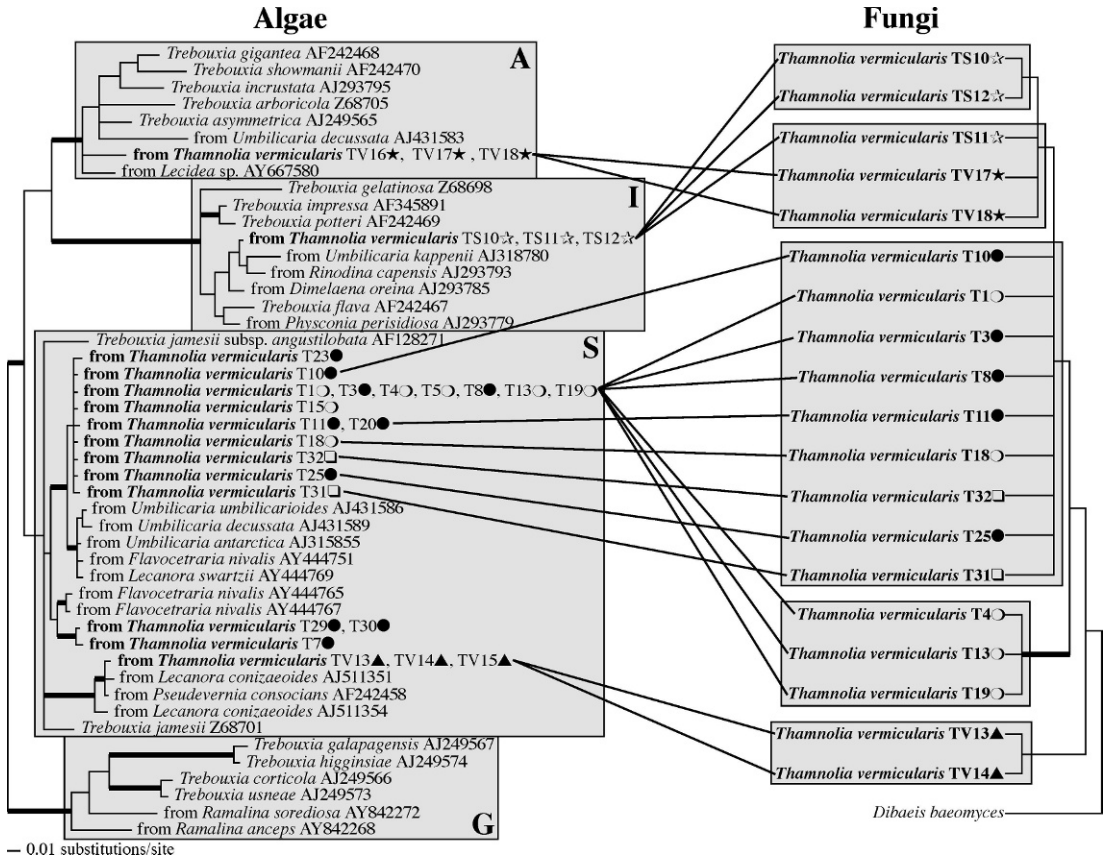

Figure 1. Majority-rule consensus trees from Bayesian analyses of algal and fungal datasets. Thickened branches indicate Bayesian posterior probabilities of 0.95 or higher and MP bootstrap support of 70% or higher. Branch lengths are provided for the algal topology, and sequences derived from *Thamnolia vermicularis* fungi and their associated *Trebouxia* algae are **bold-faced**. The DNA name, geographic origin (circle = U.S.A.; square = Norway; star = China; triangle = Costa Rica) and mycobiont chemistry (filled symbols = thamnolic acid; hollow symbols = baeomycesic and squamatic acid) are shown for *T. vermicularis* fungi and their associated algae. Algal clades A, I, S and G *sensu* Friedl et al. (2000) and Helms (2003) are shown in shaded boxes on the algal topology, while shaded boxes on the fungal topology indicate unique fungal genotypes. Lines connect algal and fungal symbionts from individual thalli.

sequences generated, and the range of algae each fungal taxon associates with (Poulin 1992). We compared the number of algal sequences/fungal taxon with maximum genetic distance using a linear regression.

Results

Algal diversity and tests for monophyly. All *Thamnolia vermicularis* individuals associated with *Trebouxia* species as their photobiont, as identified by BLAST sequence comparisons (93–99% identity). Fourteen ITS sequence types (89.6% similiarity or greater) were recovered from algae associating with *T. vermicularis* (**Table 1, Fig. 1**). The alignment of the algal dataset consisted of 551 sites, 192 of which were variable and 137 parsimony-informative. Twelve most-parsimonious trees of 446 steps were obtained. No incongruence was found between the strict consensus of the most-parsimonious trees and the 50% majority-rule consensus tree from the Bayesian analysis. The 50% majority-rule consensus tree from the Bayesian analysis, rooted with clade G (following Romeike et al. 2002), is shown in **Fig. 1**.

Thamnolia vermicularis associated with a polyphyletic assemblage of algae from clades S, A and I (**Fig. 1**). Arctic individuals associated with two sub-clades in clade S, Costa Rican individuals associated with a separate sub-clade in clade S, and thamnolic

acid-containing individuals from China associated with clade A, while baeomycesic and squamatic acid-containing individuals from China associated with clade I. The majority of the *Thamnolia* samples examined associated with algae from three sub-clades in clade S. In clade S, no strong symbiont preference was seen between chemotypes, and some algae with identical ITS sequences were shared by both chemotypes. One sub-clade in clade S, with which *T. vermicularis* associates, has also been recovered from other high latitude or high altitude taxa, from both the Northern and Southern Hemispheres. The majority of the algal sequences obtained from *T. vermicularis* in clade S were from coastal locations in Alaska and Norway, however, a small number of individuals (T7, T29 and T30) were from inland or more northern locations in Alaska, and these individuals form a separate clade. Not all populations were found to be homogeneous with respect to algal individuals, as demonstrated by one of the Chinese populations (TS10, TS12, TV16 and TV17 were within 3 m of one another), where individuals from clades A and I were recovered. Monophyly of *Thamnolia*-associated algae was rejected with a Bayes factor value of 531.36 (a value greater than 10 is considered ''very strong'' evidence against the constrained hypothesis). Similarly, the SH and ELW tests also rejected this hypothesis ($P < 0.0001$ for both).

Fungal-algal and geographic associations. Six *Thamnolia vermicularis* fungal genotypes were recovered. Individual loci showed low sequence divergence with 97.8%, 97.7%, 99.3% and 99.6% similarity or greater over the ITS, IGS, mtLSU and RPB2, respectively. The aligned fungal dataset (including the outgroup) consisted of 2348 sites, 260 of which were variable and 24 parsimony-informative. No incongruence between loci was found in the fungal dataset (ILD: $P = 1.0$), and loci were combined. In the MP analysis, 5000 most-parsimonious trees of 265 steps were recovered. In the Bayesian analyses, the partitioned analysis was used as a Bayes factor value of 115.86 was recovered, which was taken as ''very strong'' evidence against the unpartitioned analysis. Some incongruence was detected between the strict consensus of the most-parsimonious trees and the 50% majority-rule consensus tree from the Bayesian analysis. The T4/T13/T19 genotype was sister to the rest of *T. vermicularis* in the MP analysis, while the TV13/TV14 genotype was sister to the rest of *T. vermicularis* in the Bayesian analysis (shown in **Fig. 1**). Fungal and algal trees from Bayesian analyses are shown in **Fig. 1**, illustrating the symbiotic associations. Costa Rican fungal individuals formed a weakly supported monophyletic group, which associated with a group of algae in clade S. The *T. vermicularis* fungi from China formed a weakly supported monophyletic group, which associated with algae in clades A and I. Arctic fungal individuals formed a paraphyletic grouping, that associated with algae in clade S. Some congruence between topologies is suggested, but support in the fungal topology is low, and algae associated with *T. vermicularis* clearly are not monophyletic, as demonstrated earlier. In addition, the Mantel test showed a non-significant relationship between algal and fungal genetic distance matrices ($r = -0.0485$, $P = 0.5865$).

The AMOVA analysis provided evidence for the role of ecogeographic factors in structuring which algae the fungi associate with, as 69.31% ($P = 0.01832$) of the variation in algal symbionts was found between ecogeographic regions ($F_{CT} = 0.69310$), 14.32% ($P = 0.32376$) among fungal genotypes within ecogeographic regions ($F_{SC} = 0.46657$), and 16.37% ($P = 0.00010$) within fungal genotypes ($F_{ST} = 0.83629$). While most of the algal variation was found among ecogeographic regions, fungal genotypes were weakly, but significantly structured by ecogeographic factors as 28.14% ($P = 0.03703$) of the variation was found among ecogeographic regions, while 71.86% was found among genotypes ($F_{ST} = 0.28138$).

Specificity comparison. When comparing the maximum genetic distance between algal symbionts (**Table 3**), *Thamnolia vermicularis* associated with a wide range of algae, in comparison to other fungal taxa. *Lecanora rupicola*, *Protoparmeliopsis muralis*, *L. bicincta* and *Umbilicaria antarctica/kappenii* associated with a wider range than *T. vermicularis*, while all Mycoblastaceae, Physciaceae and Parmeliaceae taxa included associated with a narrower range than *T. vermicularis* (**Table 3**). There was, however, a significant, positive relationship (n

= 15, $r^2 = 0.302$, $P = 0.034$) between sequence number and the maximum pairwise genetic distance. We attempted to account for this, by dividing the maximum genetic distance by the number of algal sequences for each fungal taxon. When this was done, *T. vermicularis* was still found to associate with an intermediate range of algae when compared to other fungal taxa (**Table 3**). This method of correcting for sample size is a coarse approach, and may be more appropriately adjusted for using other methods. Additionally, accumulation or saturation curves could be generated for each fungal taxon to identify which were undersampled.

Discussion

Algal diversity. *Thamnolia vermicularis* was found to associate with *Trebouxia* algae, confirming previous reports (Brodo et al. 2001; Galloway 1985). Its association with *Trebouxia* is interesting as most Icmadophilaceae genera are known to associate with other algal genera. For instance, *Dibaeis* and some *Icmadophila* species associate with *Coccomyxa* (Brodo et al. 2001; Galloway 1985; Jaag 1933), while *Siphula* and other *Icmadophila* species associate with *Elliptochloris* (Dahlkild et al. in prep). The wide range of algae this family associates with is quite interesting and deserves further study.

Algae from most *Thamnolia vermicularis* thalli were from clade S, a geographically widespread group, occurring in the Arctic, Antarctic and several regions in between. Many of the lichens harboring these algae live in colder environments, consistent with our samples of *Thamnolia*. Some individuals associated with algae from clades A and I, but none associated with clade G. Algal individuals from clade G do not appear frequently in the high altitude or high latitude environments where *T. vermicularis* is found (Cordeiro et al. 2005; Helms 2003; Helms et al. 2001; Ohmura et al. 2006), potentially explaining absences of association with this lineage.

Loss of ascomata and polyphyly of algal symbionts. If a fungal lineage lacking ascomata arises through the evolution of a single fungal individual that is female- and male-sterile (unable to be fertilized or fertilize), then polyphyly of algal symbionts would indicate symbiont-switching. Polyphyly of algal symbionts, however, may not necessarily be indicative of recent symbiont-switching. If there has been repeated evolution of individuals that are both female- and male-sterile, algal symbionts would not necessarily be monophyletic, as the ascomata-lacking fungal taxon itself is not monophyletic, and each origin of asexuality could be associated with a separate alga. If, however, the loss of ascomata arises through the evolution of female-sterile individuals (individuals which can fertilize, but cannot be fertilized) and the subsequent spread and fixation of the associated mutation(s), polyphyly may not necessarily be indicative of recent symbiont-switching (though it may be indicative of historic symbiont-switching). Leslie and Klein (1996) have discussed situations in which fungal female-sterility could evolve in populations of the *Gibberella fujikuroi* complex. If the loss of ascomata in *T. vermicularis* originated through female-sterility, then algal polyphyly could be due to multiple historic re-lichenization events. If female-sterile individuals fertilized female-fertile individuals, the progeny (female-fertile and female-sterile) would attempt to re-lichenize, and these historic, separate re-lichenization events could be preserved through time, making the range of algae polyphyletic. However, if ascomata were lost in the ancestor to *Thamnolia* through female-sterility, and *T. vermicularis* subsequently arose from only one of these individuals (instead of several), the polyphyly of algae associated with *T. vermicularis* would be indicative of recent symbiont-switching (unless the parent thallus harbored multiple algal genotypes). At present, it is unclear which of these situations, if any, best describes the mechanism by which ascomata were lost in *Thamnolia*.

Although ascomata are unknown from *Thamnolia*, *T. vermicularis* occasionally produces pycnidia with conidia (A. Knight, pers comm.; Ozenda & Clauzade 1970). Conidia are thought to function as spermatia, fertilizing other individuals (Pöggeler et al. 2006), therefore, female-sterility could be a possible explanation for the polyphyly of the algal symbionts with which *T. vermicularis* associates, but much more work is needed to elucidate the loss of ascomata and origin of asexuality in *Thamnolia*. At present, we are not able to determine the proportion of symbiont-switching that

is historic vs. recent, however, the association of a fungal genotype (TS11/TV17/TV18) with two very different algal individuals (from clades A and I) strongly suggests the presence of some recent switching.

Fungal-algal and geographic associations. Although there was some congruence between fungal and algal topologies, we do not consider this strong evidence for co-diversification. Support for the fungal topology is weak (most likely a result of the low level of genetic variation detected in the examined loci), but the algae associated with *Thamnolia vermicularis* are clearly polyphyletic. Additionally, if clade G is not basal, topologies between symbionts will be more discordant. The Mantel test also rejected a correlation between pairwise genetic distance of algal and fungal partners. Studies in a number of other lichen symbioses have also demonstrated that strong evidence for strict parallel diversification between lichenized fungi and their green algal partners is lacking (Dahlkild et al. 2001; Kroken & Taylor 2000; Nelsen & Gargas 2008; Piercey-Normore & DePriest 2001; Zoller & Lutzoni 2003).

Yahr et al. (2004) demonstrated that several *Cladonia* species (including the clonal and co-dispersing *C. perforata*) associate with significantly different groups of algae over their geographic range, and also that the frequencies of associations between fungal and algal lineages varies with habitat and geography in *Cladonia subtenuis* (Yahr et al. 2006). The ability to switch symbionts permits the symbiosis to be fine-tuned over geographic and environmental gradients, while the strict preservation of relationships may lead to its termination. Our results suggest that the identity of the associated algal symbiont differs by geography and habitat, and possibly also by fungal genotype. Some of the fungi appear to form monophyletic groups based on their geography, and weak, but significant subdivision was suggested in the fungi by the AMOVA analysis. At the species level, *Thamnolia vermicularis* associates with a wide range of *Trebouxia* symbionts; at the smaller scale of *T. vermicularis* clades, we found a narrower range of associated symbionts. The AMOVA test and F_{ST} values confirmed this trend, suggesting greater variation between ecogeographic regions than within. This trend, however, must be further investigated with larger sample sizes.

Mechanisms of symbiont-switching. In lichens, symbiont-switching could occur at several stages in the life cycle. Thorough discussions on the mechanisms of symbiont-switching can be found in Nelsen and Gargas (2008), Ohmura et al. (2006), and Yahr et al. (2006), and are briefly summarized here.

Lichen propagules, such as soredia, are known to fuse upon germination, forming a single thallus derived from multiple propagules (Honegger 1992; Jahns 1972; Schuster et al. 1985), suggesting that a single thallus may be composed of more than one fungal or algal individual. Recent molecular work on other species have recovered multiple fungal (Murtaugh et al. 2000; Robertson & Piercey-Normore 2007) and algal (Bhattacharya et al. 1996; Guzow-Krzeminska 2006; Helms et al. 2001; Piercey-Normore 2006; Romeike et al. 2002) genotypes or sequences from a single thallus; although genetic heterogeneity within a thallus does not necessarily indicate multiple individuals (Robertson & Piercey-Normore 2007; Simon et al. 2005). We found no intrathallus heterogeneity in *Thamnolia vermicularis*, which is in agreement with Platt and Spatafora (2000) and Cassie (2006). However, we only sequenced fungal and algal DNA from one portion of the thallus. Future studies should include multiple samples from a single thallus to confirm homogeneity within a thallus.

Independent dispersal of each symbiont provides another opportunity for switching. Fungal pycnidia have been observed in *Thamnolia vermicularis* (A. Knight, pers comm.; Ozenda & Clauzade 1970). Diahyphae, which are groups of hyphae or chains of cells produced by Gomphillaceae fungi, were able to germinate and begin re-lichenization (Sanders & Lücking 2002), and conidial germination varied by taxa under laboratory conditions (Vobis 1977). Germination and re-lichenization has not yet been demonstrated for *Thamnolia* conidia, but this would provide a plausible means for symbiont switching. If the fungus could survive in a free-living state, as do ascospore cultures from representatives of *Anaptychia*, *Physcia*, *Physconia* and *Xanthoria* (Etges & Ott 2001), it could re-lichenize with free-living *Trebouxia* cells (Hedenås

et al. 2007; Mukhtar et al. 1994; Sanders 2005; Sanders & Lücking 2002; Tschermak-Woess 1978), associate with or parasitize an incompatible alga until meeting a preferable partner (Honegger 1992), or obtain *Trebouxia* algae from other propagules or lichens (Friedl 1987; Gaßmann & Ott 2000; Lücking & Grube 2002; Ott 1987a, b; Ott et al. 1995; Rambold & Triebel 1992; Stenroos 1990).

Specificity comparison. Our results suggest that despite their predominantly shared mode of dispersal, *Thamnolia vermicularis* fungi associate with a wide range of algal symbionts. This is not entirely surprising as Yahr et al. (2004) investigated the range of algae with which several *Cladonia* species associate, and found that asexually reproducing species did not strictly associate with a narrower range than sexually reproducing species. Similarly, Nelsen and Gargas (2008) illustrated that *Lepraria* fungi have not maintained strict associations with their *Asterochloris* algal symbionts. The majority of lichenized fungal taxa investigated (n = 9–10) associated with a narrower range of *Trebouxia* algae, and a small number (n = 4–5) associated with a wider range than *T. vermicularis* (**Table 3**). While the metric used in this study to measure specificity is coarse and can certainly be improved upon, it nevertheless suggests that *T. vermicularis* does not associate with an extremely narrow range of algae.

Symbiont range and the evolutionary advantages of flexibility. Associating with a wide range of symbionts may help species survive in harsh environmental conditions (Piercey-Normore 2006; Romeike et al. 2002; Wirtz et al. 2003). The wide range of symbionts associating with *Thamnolia vermicularis* supports this hypothesis, although associating with a wide range of algae was not restricted to taxa living in extreme environments (as shown by *Lecanora bicincta* and *L. rupicola*). Associating with a wide range of symbionts provides a fitness advantage as it may ease the re-lichenization process and allow the symbiosis to be fine-tuned over geographic and environmental gradients, as well as evolutionary timescales.

Co-dispersal avoids the perilous symbiont reässociation step in the life cycle, but it has disadvantages common to clonal lineages lacking recombination (Piercey-Normore 2005). The absence of sexual reproduction is thought to be detrimental to the longevity of a species (Muller 1932). The inability to create new genetic combinations can prevent a species from surviving ecological changes, colonizing new environments and surviving parasites. Many corals undergo a process known as adaptive bleaching, in which they purge their suboptimal algal symbionts and replace them with new algal symbionts. This process aids corals in surviving ecological changes (Baker 2001), thereby illustrating the advantages of symbiont-switching.

As stated above, the lack of recombination is thought to have negative effects on the longevity of a species, and its ability to adapt to changing selective pressures. Nelsen and Gargas (2008) investigated the evolutionary persistence of relationships between clonally reproducing *Lepraria* fungi and their algal symbionts, and concluded that symbiont switching had occurred, and associations were not strictly maintained. Thus far, recombination has not been detected with strong support in *Thamnolia vermicularis* (Nelsen & Gargas 2009). Symbiont-switching may provide an alternative mechanism to maximize fitness in fungi lacking or rarely undergoing genetic recombination. Shuffling relationships between fungal and algal symbionts may lead to fungi associating with algal symbionts more capable of surviving various selective pressures, thereby aiding the survival and persistence of these fungi, and the association as a whole.

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