Assessing clonality and chemotype monophyly in *Thamnolia* (Icmadophilaceae)

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Abstract. Here we incorporate molecular sequence data (ITS, IGS, mtLSU, RPB2) to test the hypothesis of strict clonality in the lichen-forming fungus *Thamnolia vermicularis*. Recombination was rejected by most methods, though, a small number did detect recombination. We intepret these analyses as mostly supporting the hypothesis of strict clonality. We were unable to determine if the recombination detected was the result of rare recombination events in recent time, historic recombination, or false positives by certain methods. In addition, we investigated whether chemotypes in *T. vermicularis* formed monophyletic groups. Molecular sequence data suggest that the two chemotypes do not form well-supported, monophyletic lineages. Chemotypes were found with identical haplotypes and some populations were composed of more than one haplotype. Our data suggest that there is not a simple dichotomy between chemotypes in *T. vermicularis*, which may be due to rare or historic recombination, repeated chemotype evolution or incomplete lineage sorting.

Keywords. *Thamnolia*, recombination, secondary metabolites, clonal, incomplete lineage sorting, chemotaxonomy.

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A number of theories support the advantages of genetic recombination, with Muller's Ratchet (Muller 1964), Kondrashov's Hatchet (Kondrashov 1993) and the Red Queen (Bell 1982) appearing among the leaders (Normark et al. 2003). Muller's Ratchet and Kondrashov's Hatchet both argue that deleterious mutations are purged through recombination, while the Red Queen argues that recombination allows organisms to generate new genetic combinations that enable them to escape parasitism (Bremermann 1980; Hamilton 1980; Jaenike 1978; Lloyd 1980). Despite the advantages genetic recombination confers, a number of ancient asexual lineages exist (summarized in Normark et al. 2003). While some of these lineages may truly lack recombination, molecular data have revealed that some putative asexuals exhibit evidence of genetic recombination (Burt et al. 1996; Gandolfi et al. 2003; Geiser et al.

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1998). Among lichen-forming fungi, sex appears to have been lost independently a number of times, based on the absence of ascomatal production. Here we investigate whether evidence exists for cryptic recombination in *Thamnolia*, a presumably sterile genus.

Lichen-forming fungi from *Thamnolia* are known from every continent but Africa and Antarctica (Sheard 1977), growing on soil or stone substrates in high altitudes and/or latitudes. *Thamnolia* fungi associate with *Trebouxia* algae (Brodo et al. 2001; Nelsen & Gargas 2009) and produce thalli with white finger-like stalks, which reproduce vegetatively by fragmentation; apothecia have never been found in this group. Based on molecular data, Platt and Spatafora (2000) and Stenroos et al. (2002) placed *Thamnolia* in the Icmadophilaceae (Pertusariales). The history of species delimitation within *Thamnolia* is somewhat complicated, and has been reviewed by Culberson (1963), Kärnefelt and Thell (1995) and Santesson (2004). At present *Thamnolia* is composed of three vegetatively reproducing species, of which two have only recently been raised to the species level (Santesson 2004). These two species, *Thamnolia papelillo* and *Thamnolia juncea*, were previously considered to be morphological varieties of the third species, *Thamnolia vermicularis*. Each of the three species has a variable chemistry, which is generally not correlated with morphological characteristics, except in the case of *T. papelillo*.

In addition to assessing whether evidence for cryptic recombination exists, we also investigated the chemical variation found in *Thamnolia vermicularis*. The phylogenetic distribution and evolution of various substances in other genera or families has been examined (Blanco et al. 2006; Helms et al. 2003; Ihlen & Ekman 2002; Lumbsch et al. 2006; Miadlikowska & Lutzoni 2000; Nelsen & Gargas 2008b; Nordin et al. 2007; Schmitt & Lumbsch 2004), and numerous papers have described sets of principles for how chemical variation can be treated at the species level (Brodo 1986; Culberson 1969; Culberson & Culberson 1994; Elix 1982; Hawksworth 1976; Lumbsch 1998; Rogers 1989). *Thamnolia vermicularis* has been recognized as a single, chemically variable species (Brodo et al. 2001; Filson 1972; Kärnefelt & Thell 1995; Nimis 1993; Purvis et al. 1992; Sheard 1977), or as two chemically distinct species: *T. vermicularis* and *T. subuliformis* (Asahina 1937; Culberson 1963; Galloway 1985; Poelt 1969; Thomson 1984). Morphologically, these two taxa are identical (Culberson 1963; Filson 1972; Kärnefelt & Thell 1995), but differ somewhat in their distribution, with the thamnolic acid chemotype being more common in the southern extent of its range, and the baeomycesic and squamatic acid chemotype being more common in the northern extent of its range (Filson 1972; Sato 1963, 1965, 1968; Sheard 1977). When recognized as two species, the name *T. vermicularis* refers to thamnolic acid-containing individuals, while *T. subuliformis* refers to baeomycesic and squamatic acid-producing individuals. Studies using molecular sequence data have concluded that chemotypes represent distinct species (Platt & Spatafora 2000) as well as that chemotypes represent a single polymorphic species (Miao, in Goward 1999).

Recent studies (Jiang et al. 2002; Wang et al. 2001) have revealed the presence of several other compounds in *Thamnolia vermicularis* and *T. subuliformis*, often in trace amounts. Additionally, carotenoids have also been reported from *Thamnolia* (Czeczuga et al. 1999, 2003). Here we focus on the major compounds: baeomycesic, squamatic and thamnolic acids. These three chemical compounds have never been found together in *Thamnolia* individuals (Kärnefelt & Thell 1995), suggesting that it is unlikely that an individual thallus may possess the ability to switch from the production of one set of secondary metabolites to another. Stocker-Wörgötter (2001) isolated the mycobiont from a baeomycesic and squamatic acid-containing *Thamnolia* individual and found that under laboratory conditions, the culture produced the same chemotype as the parent thallus. All three compounds are β-orcinol depsides, which are structurally similar to one another and are derived from the polyketide synthase (acetyl-polymalonyl) pathway where *para*-depsides (baeomycesic and squamatic acids) are believed to serve as precursors to the production of *meta*-depsides (thamnolic acid) (Culberson & Elix 1989; Elix 1996; Elix & Gaul 1986; Elix et al. 1987). The production of these compounds

is known from other genera, such as *Cladonia*, *Siphula* and *Usnea* (Halonen et al. 1998; Kantvilas 2002; Thomson 1967) and often occur in the same or closely related species, such as *Usnea florida* and *U. subfloridana* (Articus et al. 2002; Halonen et al. 1998).

In the present study, our goals are twofold: (1) to assess whether molecular sequence data provide evidence for cryptic recombination in *Thamnolia vermicularis* and (2) to test whether chemotypes in *T. vermicularis* form separate groups.

Materials and Methods

Taxon sampling. We selected 26 thalli from 19 collections from across a broad geographic range. Samples from the Wisconsin State Herbarium (WIS) were used together with fresh collections made in China, Costa Rica and Norway (deposited in WIS). When possible, we obtained both chemotypes from a particular locality or geographic region. Sequences of *Dibaeis baeomyces* (Icmadophilaceae) were included as the outgroup.

The present study does not include *Thamnolia papelillo* or *T. juncea*, both of which were recently elevated to the species rank by Santesson (2004). Since both chemotypes occur in each of these species, future studies should seek to determine whether these chemical differences are correlated with phylogeny and also whether these two species are phylogenetically distinct from each other and *T. vermicularis*.

DNA extraction and sequencing. DNA was extracted from samples listed in **Table 1**, using the methods of Grube et al. (1995) and Crespo et al. (2001). The nuclear ITS rDNA (ITS) and partial nuclear IGS rDNA (IGS), mitochondrial large subunit rDNA (mtLSU) and nuclear second largest subunit of RNA polymerase II (RPB2) loci were amplified using the following primers: ITS1F (Gardes & Bruns 1993) and ITS4A (D. L. Taylor *in* Kroken & Taylor 2001) for ITS, IGS12a and NS1R (Carbone & Kohn 1999) for IGS, ML3.C and ML4 (Printzen 2002) for mtLSU and RPB2-A-MNTH (TGG CAA GAA GCG ACT GGA T) and RPB2-B-MNTH (ATC GGC TGG GAG GTC TTT GTC) for RPB2 (this study). The PCR cocktail concentrations follow Nelsen and Gargas (2008a), and PCR conditions varied by locus. All began with an initial denaturation of 95°C for 5–7 min followed by 10 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, and then 30–35 cycles of 95°C for 1 min, a 1 min denaturation at a locus-specific temperature, an extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The locus-specific annealing temperatures were as follows: ITS and IGS 53°C, mtLSU 58°C and RPB2 55°C. The IGS, mtLSU and RPB2 samples were sequenced with the PCR primers while ITS1 and ITS4 (White et al. 1990) were used to sequence the ITS. Sequencing and cleaning followed the methods in Nelsen and Gargas (2008a).

Recombination detection. Sequences from all *Thamnolia* individuals in **Table 1** were manually aligned using Se-Al v. 2.0a11 (Rambaut 1996) and divided into six datasets (ITS, IGS, mtLSU, RPB2, "full" [all loci concatenated from all individuals] and "restricted" [all loci concatenated from only individuals with all 4 loci sequenced]) and tested for evidence of recombination. Following Posada (2002), we used a number of methods to detect recombination. The RecombiTEST website (http://www.lifesci.sussex.ac.uk/CSE/test/index.php) was used to conduct the Maximum Chi-Square test (Maynard Smith 1992), following the modifications by Piganeau et al. (2004). Additionally, the Phi test for recombination (Bruen et al. 2006) as implemented in SplitsTree 4 (Huson & Bryant 2006) was performed on all datasets. The DATAMONKEY website (http://www.datamonkey.org/), using the HyPhy program (Kosakovsky Pond et al. 2005), was used to test for recombination using Sugiura's (1978) corrected Akaike Information Criterion (AICc) scores from a single breakpoint analysis, as well as a Genetic Algorithm Recombination Detection (GARD) analysis (Kosakovsky Pond et al. 2006). In both cases the Akaike Information Criterion (AIC) (Akaike 1974) was used to determine the best-fit substitution model. Three rate classes were permitted and beta-gamma rate variation (Kosakovsky Pond & Frost 2005) was selected.

Finally, the recombination detection program (RDP) method of Martin and Rybicki (2000), the Maximum Chi-Square test (Maynard Smith 1992; Posada & Crandall 2001) and the Geneconv method (Padidam et al. 1999), as implemented in the

Table 1. Taxa and specimens used in this study with GenBank accession numbers and collection information. Symbols following DNA name reflect the geographic origin and chemistry of samples. Samples from Alaska, U.S.A., are marked with circles, those from Norway are marked with squares, samples from China are marked with stars and those from Costa Rica with triangles. Baeomycesic and squamatic acid-containing *T. vermicularis* individuals are marked with a hollow symbol, while thamnolic acid-containing *T. vermicularis* individuals are marked with a filled symbol. Vouchers are deposited at WIS.

		GenBank Acc. No.			
Fungal taxon (DNA Name)	**Collection # (all at WIS)**	**ITS**	**IGS**	**mtLSU**	**RPB2**
Dibaeis baeomyces		DQ782844			AY641037
Thamnolia vermicularis (T1) ○	*Talbot KIS 131*, Alaska, U.S.A.	EU714413	EU714438	EU714456	EU714480
Thamnolia vermicularis (T2) ●	*Talbot KIS 131*, Alaska, U.S.A.	EU714414	EU714439		EU714481
Thamnolia vermicularis (T3) ●	*Talbot KIS 165 (1)*, Alaska, U.S.A.	EU714415	EU714440	EU714457	
Thamnolia vermicularis (T4) ○	*Talbot KIS 165 (1)*, Alaska, U.S.A.	EU714416	EU714441	EU714458	EU714482
Thamnolia vermicularis (T8) ●	*Talbot KIS 161a*, Alaska, U.S.A.	EU714417	EU714442	EU714459	EU714483
Thamnolia vermicularis (T9) ○	*Talbot KIS 161a*, Alaska, U.S.A.	EU714418	EU714443	EU714460	EU714484
Thamnolia vermicularis (T10) ●	*Talbot ADU3B-X-10*, Alaska, U.S.A.	EU714419	EU714444	EU714461	EU714485
Thamnolia vermicularis (T11) ●	*Talbot CHA1C-101*, Alaska, U.S.A.	EU714420		EU714462	
Thamnolia vermicularis (T12) ○	*Talbot CHA1C-101*, Alaska, U.S.A.	EU714421	EU714445	EU714463	
Thamnolia vermicularis (T13) ○	*Talbot NIZ5B-25*, Alaska, U.S.A.	EU714422		EU714464	
Thamnolia vermicularis (T14) ○	*Talbot NIZ5D-24*, Alaska, U.S.A.	EU714423	EU714446	EU714465	
Thamnolia vermicularis (T18) ○	*Talbot OGA1D-17*, Alaska, U.S.A.	EU714424	EU714447	EU714466	EU714486
Thamnolia vermicularis (T19) ○	*Talbot RAT1A-12*, Alaska, U.S.A.	EU714425	EU714448	EU714467	EU714487
Thamnolia vermicularis (T21) ○	*Talbot KAV1A-12*, Alaska, U.S.A.	EU714426	EU714449	EU714468	
Thamnolia vermicularis (T22) ●	*Talbot KAV1A-12*, Alaska, U.S.A.	EU714427		EU714469	
Thamnolia vermicularis (T24) ○	*Talbot LKI1A-11*, Alaska, U.S.A.	EU714428	EU714450	EU714470	EU714488
Thamnolia vermicularis (T25) ●	*Talbot LKI1A-11*, Alaska, U.S.A.	EU714429	EU714451	EU714471	EU714489
Thamnolia vermicularis (T31) ○	*Nelsen 3980*, Troms, Norway	EU714430	EU714452	EU714472	EU714490
Thamnolia vermicularis (T32) ○	*Nelsen 3981*, Troms, Norway	EU714431	EU714453	EU714473	EU714491
Thamnolia vermicularis (TS10) ☆	*Nelsen 2291*, Yunnan, China			EU714474	EU714492
Thamnolia vermicularis (TS11) ☆	*Nelsen 2294*, Yunnan, China	EU714432		EU714475	EU714493
Thamnolia vermicularis (TS12) ☆	*Nelsen 2292*, Yunnan, China	EU714434	EU714454		EU714494
Thamnolia vermicularis (TV13) ▲	*Nelsen 3635*, San José, Costa Rica	EU714434		EU714476	EU714495
Thamnolia vermicularis (TV14) ▲	*Nelsen 3636B*, San José, Costa Rica	EU714435		EU714477	EU714496
Thamnolia vermicularis (TV17) ★	*Nelsen 2292*, Yunnan, China	EU714436	EU714455	EU714478	EU714497
Thamnolia vermicularis (TV18) ★	*Nelsen 2413*, Yunnan, China	EU714437		EU714479	EU714498

Recombination Detection Program 3 (RDP3) (Martin et al. 2005) were used to detect recombination. In all cases, the significance threshold was set to $P = 0.05$ and the Bonferroni correction was implemented. Datasets were permuted 1,000 times and analyses were performed with and without disentangling overlapping events. Default settings were used in all three tests, except that in the RDP analyses, the window size was set to 100 bp and 500 bp and analyses were run with no reference and with internal and external reference. Additionally, gapped sites were stripped from datasets in the Maximum Chi-Square and Geneconv analyses, and a variable window size was used in the Geneconv analysis.

Haplotype networks. Sequences for individual loci from *Thamnolia* individuals in **Table 1** were manually aligned using Se-Al v. 2.0a11 (Rambaut 1996). Alignments for each locus were trimmed so sequences were equal in length, and haplotype networks were constructed for each locus in TCS 1.21 (Clement et al. 2000) using statistical parsimony, with a 95% connection limit and gaps treated as a 5th state. The networks were then visually inspected to determine if chemotypes formed separate clades.

Phylogenetic analyses. Sequences from all taxa in **Table 1** were manually aligned using Se-Al v. 2.0a11 (Rambaut 1996), and the alignment deposited in TreeBASE. The incongruence length difference

(ILD) test (Farris et al. 1994) was performed to test for conflicting datasets. One hundred replicates were performed using a heuristic search with tree bisection-reconnection (TBR) branch swapping and simple taxon addition, with 100 random stepwise addition replicates, saving no more than 100 trees greater than 1 step per replicate, and gaps treated as missing data.

A maximum parsimony (MP) analysis of the full dataset was performed in PAUP* 4.0b10 (Swofford 2002), using the same search settings described for the ILD test. Confidence was assessed by means of 1000 bootstrap replicates. Unpartitioned and partitioned (by locus) Bayesian analyses were performed in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) using the best-fit model(s) as 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 3,000,000 generations, with four chains each, sampling every 100 generations. Default settings were used, except for the temperature, which was set to 0.1 for the unpartitioned analysis and 0.08 for the partitioned analysis. Acceptance rates between 0.1 and 0.7 were taken as evidence for adequate mixing, and initial burn-in trees were discarded for each run. Unpartitioned and partitioned analyses were compared using Bayes factors, which were calculated by taking twice the difference between the harmonic mean of the unpartitioned and partitioned analyses. Interpretation of Bayes factor values followed Kass and Raftery (1995).

Topological hypotheses were then tested in a Bayesian framework using the partitioning scheme proposed by the Bayes factor. Topologies in which one chemotype was monophyletic and the other was not, were enforced as constraints, and the hypotheses of chemotype monophyly were tested by using Bayes factors to compare the constrained and unconstrained analyses (as described above). 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 chemotype monophyly in a likelihood framework. Parameter estimates from the combined Bayesian analysis were used and the most-likely tree under the maximum likelihood (ML) optimality criterion was obtained in TreePuzzle 5.2, using the GTR+G model with four variable gamma rates (as employed in the unpartitioned Bayesian analysis). The hypotheses of individual chemotype monophyly or reciprocal monophyly were then imposed as topological constraints, and the likelihood of each 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.

Results

Recombination detection. Analyses typically revealed a lack of recombination in all datasets as shown in **Table 2**. The full dataset could not be analyzed with a number of methods due to the lack of sequence data for many loci, and the Modified Maximum Chi-Square test could not be performed on a number of datasets due to a shortage of variable positions. Recombination was detected in the ITS dataset with the Modified Maximum Chi-Square method, but no other method detected recombination in this dataset. Additionally, recombination was detected in the full dataset with the HyPhy single breakpoint and GARD analyses, with the most likely breakpoint occurring in the IGS, however, the other two methods used to detect recombination on this dataset did not recover evidence for it, and no evidence of recombination was found in the IGS dataset or the restricted dataset by any method.

Haplotype networks. Single networks were recovered for all loci, except ITS, in which two networks were recovered (**Fig. 1**), one consisting solely of baeomycesic and squamatic acid-containing individuals from Alaska, U.S.A, and the other network consisting of both chemotypes from Costa Rica, China, Norway and Alaska, U.S.A. Chemotypes did not form separate clades in the haplotype networks, and at least one haplotype per locus was recovered from both chemotypes (ITS haplotypes 2 and 4, IGS haplotype 3, mtLSU haplotype 1 and

Table 2. Datasets tested for recombination using a number of methods, and whether recombination was detected. If recombination was detected, the p-value or AICc score is listed. Abbreviations are as follows: RDP = Recombination Detection Program Method; GeneConv = GeneConv method for recombination detection; MC^2 = Maximum Chi-Square Test; MMC^2 = Modified Maximum Chi-Square Test; HPS = HyPhy Single Breakpoint Analysis; HPG = HyPhy GARD Analysis; Phi = Phi Test for Recombination.

Dataset	RDP	GeneConv	MC^2	MMC^2	HPS	HPG	Phi
ITS	No	No	No	Yes, $P = 0.035$	No	No	No
IGS	No	No	No	N/A	No	No	No
mtLSU	No	No	No	N/A	No	No	No
RPB2	No	No	No	N/A	No	No	No
Full	N/A	N/A	N/A	No	Yes, AICc=1.907	Yes, AICc=27.955	No
Restricted	No	No	No	N/A	No	No	No

RPB2 haplotype 2), while others appeared as private alleles, restricted to a single chemotype (**Fig. 1**). Some haplotypes (ITS haplotype 4, IGS haplotype 3, mtLSU haplotype 1 and RPB2 haplotype 2) were found to be geographically widespread. In some instances, both chemotypes were found in the same collection or population; when this was found, some (T1/T2, TS10/TV17) had identical haplotypes, while others (T3/T4, T8/T9, T21/T22, T24/T25, TS10/TV17) had different haplotypes (**Fig. 1**). These samples provided evidence for genetic variation within populations.

Phylogenetic analyses. Our alignment consisted of 2368 sites from ITS, partial IGS, mtLSU and RPB2 loci from 26 *Thamnolia* isolates. We found low variation, as measured by variable and parsimony-informative sites, within the ingroup (29 and 21 sites, respectively). **Table 3** illustrates the number of individuals included in each data set, the length of each alignment and the number of variable and parsimony-informative sites. Data sets were not significantly incongruent as determined by the ILD test ($P = 1.0$), and the datasets were combined for analysis.

The unconstrained MP analysis yielded 5000 trees with a length of 261 steps. In the Bayesian analysis, the partitioned analysis was found to be the best-fit for the Bayesian analysis of the full data set, with a Bayes factor value of 118.1 (Bayes factor values greater than 10 suggest very strong evidence against

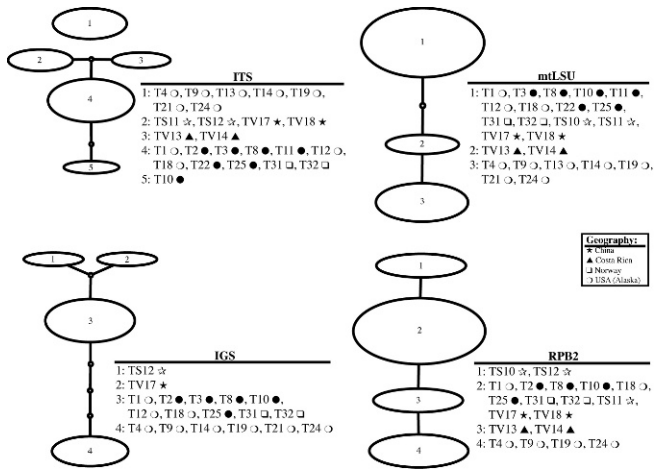

Figure 1. The 95% probability haplotype network reconstructions for each locus with haplotype number, corresponding DNA name and geographic origin (see insert) of each sample. Filled symbols refer to samples with thamnolic acid while hollow symbols refer to those with baeomycesic and squamatic acid. The size of the ovals with haplotype numbers is proportional to the number of individuals with each haplotype. Small circles represent missing haplotypes, and the distance between each circle/oval represents one mutational step.

Table 3. Alignment length and number of variable and parsimony-informative sites for each data partition. Parentheses indicate the number of variable and parsimony-informative sites for the ingroup only.

Name	Length	# variable sites	# PI sites
Full	2368	256 (29)	24 (21)
ITS	522	112 (14)	13 (10)
IGS	316	8 (8)	5 (5)
mtLSU	804	4 (4)	3 (3)
RPB2	726	132 (3)	3 (3)

the null hypothesis). There was no topological incongruence between the 50% majority-rule consensus tree from the Bayesian analysis and the strict consensus from the MP analysis. The 50% majority-rule consensus of all sampled trees (after burn-in) is shown in **Fig. 2**, where *Dibaeis baeomyces* was used to root the tree and the ingroup was made monophyletic. Our tree suggests four groupings of *Thamnolia* representatives, although none of these is strongly supported. Group I includes isolates from China, of both chemical types; the paraphyletic group II includes isolates from the U.S.A. and Norway of both chemical types; group III includes isolates from the U.S.A. of baeomycesic and squamatic acid chemotype only and group IV includes isolates from Costa Rica of the thamnolic acid chemotype only.

In the partitioned Bayesian analyses, topologies consistent with individual chemotype monophyly were rejected, with a Bayes factor value of 61.3 for monophyly of the thamnolic acid chemotype, and a Bayes factor value of 52.9 for monophyly of the baeomycesic and squamatic acid-containing chemotype. Similarly, in ML analyses, the SH and ELW tests rejected individual chemotype monophyly and reciprocal chemotype monophyly at $P < 0.0001$.

Discussion

Recombination. Our data do not present strong evidence for recombination in *Thamnolia vermicularis*. One of seven methods detected recombination in the ITS dataset, and two of four detected recombination in the IGS of the full dataset, however, none of the six methods detected recombination in the IGS dataset or the restricted dataset. Taken together, we feel the evidence for recombination in our data is weak and do not feel that the data at hand present a strong case for the presence of cryptic recombination. We were unable, however, to determine if the recombination detected was due to rare recombination events in recent time, historic recombination, or false positives by various methods. More individuals and loci must be screened to validate the results presented here, but at present, these data suggest that *Thamnolia* may have persisted in the absence of recombination (or with little recombination).

Chemotype monophyly. Our study found no simple dichotomy between the two chemotypes (**Figs. 1, 2**), although there is weak support throughout most of the phylogenetic tree. This finding is in agreement with some studies, which have also found a lack of chemotype monophyly (*Usnea*: Articus et al. 2002; *Porpidia*: Buschbom & Mueller 2006), but in conflict with other studies which have suggested that chemotypes may be reciprocally monophyletic (*Ramalina*: LaGreca 1999; *Heterodermia*: Lücking et al. 2008; *Haematomma*: Lumbsch et al. 2008; *Parmeliopsis*: Tehler & Källersjö 2001). Taken together, these studies suggest that no universal rule exists for delimiting these taxa and that each group should be dealt with on a case-by-case basis.

Our findings of low sequence diversity and chemotypes with identical haplotypes are in contrast with those of Platt and Spatafora (2000), who described the genetic distance (of partial nuclear large subunit rDNA sequences of ca. 600 bp) between a *Thamnolia* individual of each chemotype (both from western U.S.A.) as being greater than that between other species or even between genera. In addition, Cassie (2006) detected high levels of genetic variation within and between populations of the baeomycesic and squamatic acid-containing *T. vermicularis* chemotype using ISSR microsatellites. In contrast, our study found relatively low levels of sequence variation among 26 *Thamnolia* thalli for four loci.

Why are chemotypes not monophyletic? A number of possible explanations exist for why chemotypes are not monophyletic. First, it could be possible that cryptic recombination is occurring, but we were unable to detect it with high confidence in the present dataset. Alternatively, few genetic changes

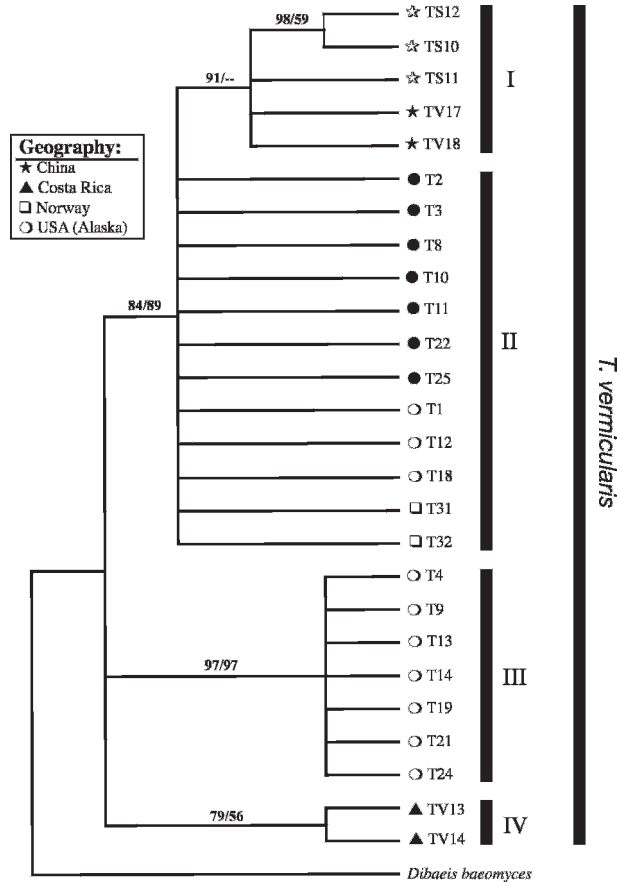

Figure 2. The 50% majority-rule consensus tree from 57,000 sampled trees in the Bayesian analysis. Bayesian posterior probabilities followed by maximum parsimony bootstrap scores above 50 are listed on branches. The DNA name, geographic origin (see insert) and chemistry (filled symbols = thamnolic acid; hollow symbols = baeomycesic and squamatic acid) of each *Thamnolia* sample are shown. Groups I–IV refer to those discussed in text.

may be required to generate chemical differences, and the repeated evolution of new chemotypes might easily occur. Elix and Gaul (1986) and Elix et al. (1987) have demonstrated that *para*-depsides may serve as precursors for the production of *meta*-depsides. This suggests that either a mutation could have arisen which blocked the conversion of *para*-depsides (baeomycesic and squamatic acids) to *meta*-depsides (thamnolic acid), resulting only in the production of the *para*-depside precursors, or alternatively, a new pathway evolved which converted *para*-depsides to *meta*-depsides (Culberson & Culberson 2001). A loss of function seems more likely than the evolution of a new pathway, but this hypothesis needs further testing.

Another plausible explanation for the lack of monophyly may be due to incomplete lineage sorting. This explanation requires a deeper investigation into the clonal origins of *Thamnolia*. Honegger and Zippler (2007) suggested that the absence of ascomata in *T. vermicularis* may be due to mutations in genes involved in sexual reproduction, and we support this hypothesis. If a single ancestral individual lost the ability to fertilize and be fertilized, the hypothesis of incomplete lineage sorting alone would not be satisfactory, as this individual would have produced either thamnolic acid or baeomycesic and squamatic acids. In the absence of recombination, this explanation would require multiple losses of the ability to fertilize and be fertilized and/or the evolution of a second chemotype, not just once (because chemotypes would either be reciprocally monophyletic or one monophyletic and the other paraphyletic), but

several times. However, incomplete lineage sorting should not be ruled out as an alternate situation exists.

Thamnolia occasionally produces pycnidia with conidia (A. Knight, pers comm.; Ozenda & Clauzade 1970), and conidia are thought to function as spermatia, fertilizing other individuals (Pöggeler et al. 2006). One way in which the loss of ascomata may proceed is through the evolution of female-sterile individuals (individuals which can still fertilize, but cannot be fertilized), which could increase in abundance and outnumber the female-fertile individuals (Leslie & Klein 1996). Leslie and Klein (1996) have discussed how this could occur in populations of the *Gibberella fujikuroi* complex; a situation such as this would have also allowed for genetic individuals of both *Thamnolia* chemotypes to have lost the ability to produce ascomata, and for incomplete lineage sorting to be the source of chemotype non-monophyly.

With the present data, we were unable to determine the source of chemotype non-monophyly. However, we demonstrate that with the data presented here, there is little evidence for cryptic recombination, and there does not appear to be a simple dichotomy between chemotypes. Instead, a more complicated situation is suggested. A number of possible explanations exist, including rare recombination events, repeated chemotype evolution and incomplete lineage sorting. The recent surge in molecular studies focusing on the criteria used to the identify species of lichen-forming fungi (see DePriest 2004; Grube & Kroken 2000), and the genes responsible for chemical production in lichens (Grube & Blaha 2003; Miao et al. 2001; Opanowicz et al. 2006; Schmitt et al. 2005) will help elucidate both the source and value of chemical variation within and between taxa. Since chemical variation is used as a taxonomic character for many fungal lineages, these studies have the potential to have a profound impact on the taxonomy of lichen-forming fungi.

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