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Phylogenetic distribution and evolution of secondary metabolites in the lichenized fungal genus *Lepraria* (Lecanorales: Stereocaulaceae)

by

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Abstract: Molecular sequence data are used to explore the phylogenetic distribution of secondary metabolites and metabolite classes among species of *Lepraria*. All substance classes examined were phylogenetically widespread, except for β-orcinol *meta*-depsides and anthraquinones, which appeared rare and restricted to a single major clade. Benzyl esters were also found to be rare. Taxa producing each substance class examined were not monophyletic. The ability to regularly produce orcinol *para*-depsides, β-orcinol *meta*-depsides, β-orcinol depsidones, dibenzofurans, benzyl esters, terpenoids and anthraquinones appears to have been gained more than lost, while the ability to produce β-orcinol *para*-depsides and higher aliphatic acids has been lost more than gained. Our results suggest that chemical similarities may not necessarily indicate close phylogenetic relationships. Finally, ancestral state reconstruction at the base of genus *Lepraria* suggests that its ancestor produced β-orcinol *para*-depsides (atranorin) but did not produce orcinol *para*-depsides, β-orcinol *meta*-depsides, benzyl esters or anthraquinones.

Key words: *Lepraria*, secondary metabolites, evolution, ancestral state, lichen.

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Introduction

Despite its apparent lack of sexual reproduction, *Lepraria* represents an evolutionary successful lichenized fungal genus of 45-50 species with a worldwide distribution. *Lepraria* associates with *Asterochloris* algae (Hildreth & Ahmadjian 1981, Nelsen & Gargas 2006 & 2008), and consists solely of individuals forming sterile, sorediate crusts, a growth form thought to be highly adapted and appearing in several other distantly related lineages (Poelt 1987, Ekman & Tønsberg 2002). The lack of ascocarps in *Lepraria* made it difficult to establish its relationship with other taxa based on morphological characters. Ekman & Tønsberg (2002) employed molecular data to determine *Lepraria*'s phylogenetic position, and found that most *Lepraria* species form a monophyletic group in Stereocaulaceae, together with *Stereocaulon* and *Muhria* (note that the monospecific *Muhria* has since been shifted to *Stereocaulon* [Högnabba 2006]). Three species with chemistries different from *Lepraria* sensu stricto, *Botryolepraria lesdainii* (Hue) Canals, Hernández-Mariné, Gómez-Bolea & Llimona, *Lepraria obtusatica* Tønsberg and *Lepraria flavescens* Cl. Roux & Tønsberg (now *Lecanora rouxii* S. Ekman & Tønsberg), were distantly related to *Lepraria* sensu stricto (Ekman & Tønsberg 2002), while the rest of the *Lepraria* species examined formed a monophyletic group in Stereocaulaceae. Ekman & Tønsberg (2002) also illustrated that *Leproloma*, another leprose genus, was not monophyletic, and was embedded within *Lepraria*. Kukwa (2002a) arrived at a similar conclusion, based on morphological and chemical characters, and shifted taxa to *Lepraria*. Increased interest in this group has led to the description of numerous species or new combinations in recent years (Aptroot 2002, Kukwa 2002a, Tønsberg 2002, Sipman 2003 & 2004, Tønsberg 2004, Bayerová et al. 2005, Elix et al. 2005, Elix 2005, Harris in Lendemer 2005, Orange & Wolseley 2005, Baruffo et al. 2006, Crespo et al. 2006, Elix 2006, Kantvilas & Kukwa 2006, Kukwa 2006, Slavíková-Bayerová & Orange 2006, Tønsberg & Zhurbenko 2006, Knudsen & Elix 2007, Knudsen et al. 2007, Lendemer & Harris 2007, Slavíková-Bayerová & Fehrer 2007, Tønsberg 2007).

Reduced morphology in *Lepraria* has made species difficult to define; consequently, secondary metabolites have played a central role in species delimitation. Secondary metabolites fulfill a variety of roles in fungi, including screening harmful UV radiation, and acting as anti-herbivory and anti-microbial agents (Lawrey 1986, Huneck & Yoshimura 1996, Huneck 1999). Within *Lepraria*, nine substance classes are known (Fig. 1). Eight of these substance classes are produced through the polyketide synthetase (acetyl-polymalonyl) pathway: (1) orcinol *para*-depsides, (2) β-orcinol *para*-depsides, (3) β-orcinol *meta*-depsides, (4) β-orcinol depsidones, (5) dibenzofurans, (6) benzyl esters, (7) higher aliphatic acids and (8) anthraquinones. In contrast, the (9) terpenoids are produced through the mevalonic acid pathway. Each secondary metabolite results from the completion of a series of enzymatic steps in a biochemical pathway. Enzyme presence, absence or variation, combined with steps of product modification, result in ultimate secondary metabolite production. Each pathway enzyme is coded for by a gene, and one (or more) genes are regulated by other elements, i.e. genes. As a corollary, a pathway block at any point leads to the accumulation of the preceding product. The genes of the polyketide synthase (PKS) pathway function as modular units (Donadio et al. 1991), interchangeable and useable

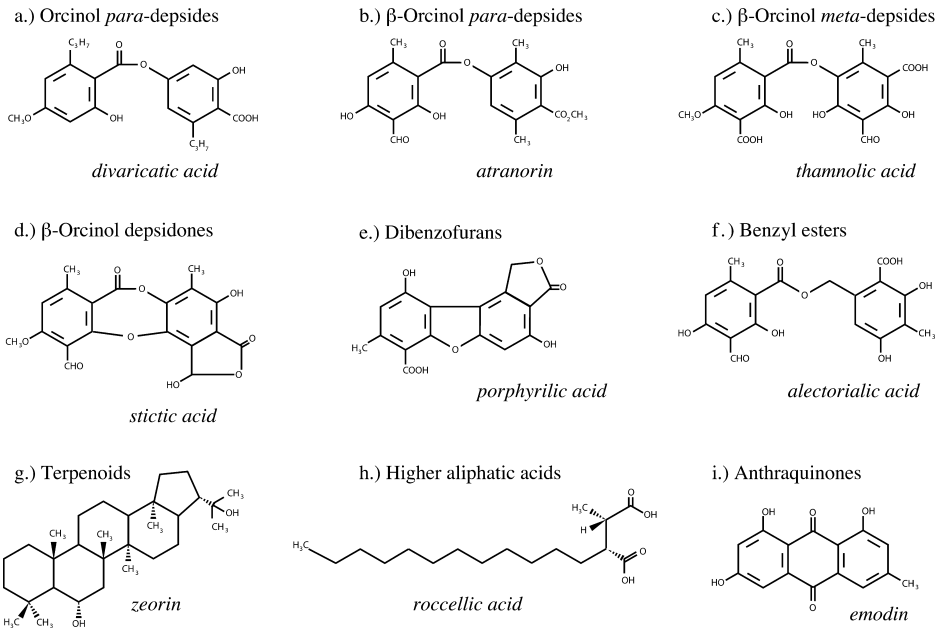

Fig. 1: Structures of secondary metabolite classes produced by *Lepraria*, with chemical examples in italics.

for constructing different pathways (Hopwood 1997). On an evolutionary time scale fungal genomes mix and match PKS units to make various secondary metabolites. These fungal lineages are then subject to putative selective pressures (such as those mentioned above), and secondary metabolites likely facilitate organism survival in a number of ways.

Even though secondary metabolites have long been used in lichen taxonomy to establish taxa at various levels (see Elix 1993, Lumbsch 1998), recent studies employing molecular markers in other genera suggest that some chemotypes, or chemically uniform species, do not necessarily form monophyletic groups or ITS rDNA sequences may be identical between chemotypes (*Usnea*: Articus et al. [2002]; *Porpidia*: Buschbom & Mueller [2006]; *Lepraria*: Myllys et al. [2005]; *Thamnolia*: Nelsen [2005]). Other lichen chemotypes do appear distinct based on molecular data (*Ramalina*: LaGreca [1999]; *Parmeliopsis* Tehler & Källersjö [2001]; *Heterodermia* Lücking et al. [2007]; *Haematomma* Lumbsch et al. [2008]). These varied results suggest that chemical variation should be evaluated on a case-by-case basis.

Here we examine the evolution and phylogenetic distribution of various secondary metabolites and substance classes. We determine whether they are widespread throughout the genus or restricted to certain lineages. If substances/substance classes are narrowly distributed or phylogenetically clustered, they could be used to determine the phylogenetic proximity of species, while if they are widely distributed, their use as a measure of phylogenetic proximity may not be warranted. Furthermore, we

hypothesize which substance classes were present in the ancestor to *Lepraria*, and whether there have been an equal number of gains and losses of these substances.

Materials and methods

TAXON SELECTION: We selected *Lepraria* taxa that had published accounts of their chemical contents and rDNA internal transcribed spacer (ITS) sequences available in GenBank (Table 1). Two *Lepraria incana* (L.) Ach. individuals were included because of their differing chemistries (*L. incana* 1 contains anthraquinones, while *L. incana* 2 does not). Similarly, several *Lepraria caesioalba* (de Lesd.) J.R.Laundon or *L.* cf. *caesioalba* individuals were included, due both to their chemical differences, as well as the potential non-monophyly of *L. caesioalba* as demonstrated by Ekman & Tønsberg (2002). *Stereocaulon tomentosum* Fr. and *Stereocaulon urceolatum* (P.M.Jørg) Högnabba were used as the outgroup.

CHEMICAL COMPOSITION OF LINEAGES: The literature was surveyed for the chemical components of taxa included in this study (Laundon 1989, Kümmerling et al. 1991, Leuckert & Kümmerling 1991, Laundon 1992, Tønsberg 1992, Kümmerling et al. 1993a & b, Lohtander 1994, Kümmerling et al. 1995a & b, Leuckert et al. 1995, Lohtander 1995, Orange 1995, Aptroot et al. 1997, Orange 1997, Saag & Saag 1999, Zedda 2000, Czarnota & Kukwa 2001, Orange 2001, Orange et al. 2001, Ekman & Tønsberg 2002, Kukwa 2002b, Tønsberg 2002, Leuckert et al. 2004, Sipman 2004, Tønsberg 2004, Bayerová et al. 2005, Harris in Lendemer 2005, Crespo et al. 2006, Kukwa 2006, Slavíková-Bayerová & Orange 2006). We focused on examining the distribution of all substance classes, as well as a restricted number of individual substances (atranorin, zeorin and divaricatic, lecanoric, thamnolic, fumarprotocetraric, stictic and alectorialic acids). Species were scored as containing the substance class/substance if it was produced as a constant major or was mostly present in this taxon or in some chemotypes. The substance class/substance was scored as absent if it occurred in trace amounts or was rarely produced as a major. We then looked at how widely distributed these substance classes/substances were from a phylogenetic perspective, by examining the chemical contents over several well-supported clades.

PHYLOGENETIC ANALYSES: Sequences were manually aligned in Se-Al v. 2.0a11 (Rambaut 1996), ambiguous regions were omitted from analysis, and the alignment was deposited in TreeBASE (Accession Number: SN3499). A Bayesian analysis was performed in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001), using the substitution model determined by the AIC in MrModeltest 2.2 (Nylander 2004). The analysis was run for 2,000,000 generations at a temperature of 0.08, and the first 500 trees were discarded for burn-in. A 50% majority-rule consensus tree was constructed of the remaining 19,500 trees. Additionally, a maximum parsimony (MP) analysis was conducted in PAUP* 4.0b10 (Swofford 2002) using a heuristic search with 100 random addition replicates and TBR branch-swapping. Following this, 100 bootstrap replicates were performed using identical settings, except a limit of holding no more than 100 trees per replicate was imposed.

ANCESTRAL STATE RECONSTRUCTION AND HYPOTHESIS TESTING: We randomly selected 10,000 trees from the post-burnin sample of 19,500 trees from the Bayesian analysis using the program RT.PY (Kauff 2002). Characters were then mapped over the 10,000 trees in MacClade 4.05 (Maddison & Maddison 2001), using the maximum parsimony optimality criterion. This approach is similar to that used in Huelsenbeck et al. (2000) and Ihlen & Ekman (2002). The average number of unambiguous state changes for each character, as well as the number of unambiguous gains and losses, was recorded and used to determine if the proportion of gains and losses in each character was equal.

We also sought to determine if the traits showed evidence of phylogenetic conservatism, or alternatively, were randomly distributed (evidence for trait convergence). To determine this, we used a modification of the permutation tail probability (PTP) test (Faith & Cranston 1991), and created 10,000 randomized datasets for each character by using the "shuffle" option in MacClade. The frequency of character states was maintained, and the character states for the outgroup were held constant (Trueman 1996). The 10,000 randomized datasets were then mapped onto the most-likely tree obtained from the Bayesian analysis. If the number of state changes required for the true dataset was significantly less than that required for the randomized datasets, this was interpreted as evidence for a clustering of traits.

Table 1: Taxa used in this study with GenBank accession numbers, characters and character states. Characters are as follows: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzyl esters, 7 = terpenoids, 8 = higher aliphatic acids, 9 = anthraquinones.

Taxon	**GenBank Acc. No.**	**Character 123456789**
Lepraria alpina (de Lesd.) Tretiach & Baruffo	AF517888	010000010
Lepraria atlantica Orange	AF517887	010010010
Lepraria atrotomentosa Orange & Wolseley	EU008606	110000110
Lepraria bergensis Tønsberg	AF517900	010000011
Lepraria borealis Lohtander & Tønsberg	AF517908	010000010
Lepraria caesiella R.C.Harris	EU008607	010000100
Lepraria caesioalba (de Lesd.) J.R.Laundon	AF517901	010100010
Lepraria cf. *caesioalba* 1	AF517894	010000010
Lepraria cf. *caesioalba* 2	AF517905	010100010
Lepraria cf. *caesioalba* 3	AF517894	010100000
Lepraria celata Slavíková	DQ401100	000000010
Lepraria crassissima (Hue) Lettau	AF517902	100000100
Lepraria diffusa (J.R.Laundon) Kukwa	AF517903	000010000
Lepraria eburnea J.R.Laundon	AF517918	000101000
Lepraria elobata Tønsberg	AF517909	010100100
Lepraria humida Slavíková & Orange	DQ401101	010000010
Lepraria incana (L.) Ach. 1	AF517899	100000101
Lepraria incana 2	AF517891	100000100
Lepraria isidiata (Llimona) Llimona & Crespo	DQ341281	010100010
Lepraria jackii Tønsberg	AF517911	010000010
Lepraria lobificans Nyl.	AF517913	010100100
Lepraria membranacea (Dicks.) Vain.	AF517915	000010010
Lepraria neglecta (Nyl.) Erichsen	AF517893	000001010
Lepraria nigrocincta Diederich, Sérusiaux & Aptroot	EU008625	100000000
Lepraria nivalis J.R.Laundon	AF517895	0101000?0
Lepraria nylanderiana Kümmerling & Leuckert	EU008626	001000010
Lepraria rigidula (de Lesd.) Tønsberg	AF517914	010000010
Lepraria santosii Argüello & Crespo	DQ341289	010100110
Lepraria sylvicola Orange	DQ401102	010000010
Lepraria toensbergiana Bayerová & Kukwa	AY560835	010000010
Lepraria umbricola Tønsberg	AF517897	001000000
Lepraria vouauxii (Hue) R.C.Harris	AF517906	000010000
Lepraria sp. 1	AF517916	010010000
Stereocaulon tomentosum Fr.	EU008634	010100010
Stereocaulon urceolatum (P.M.Jørg) Högnabba	AF517926	010000000

The likelihood of the ancestral state of each character was also determined at six well-supported nodes, using an approach similar that of Lutzoni et al. (2001) and Miadlikowska & Lutzoni (2004). The maximum likelihood method of Pagel (1999) as implemented in Mesquite 1.12 (Maddison & Maddison 2006), was used to determine the ancestral state at the selected nodes over the 10,000 randomly selected trees used in the parsimony analyses. A likelihood ratio test (LRT) was performed on each character to determine if the Markov k-state 1 parameter (Mk1) model (Lewis 2001) or asymmetrical Markov k-state 2 parameter (AsymmMk) model was the best-fit. Characters were traced over all 10,000 trees, and a likelihood decision threshold of 2.0 (default) was used.

Results

PHYLOGENETIC RELATIONSHIPS: Our ITS rDNA sequence alignment consisted of 460 sites, 188 of which were variable, and 120 parsimony-informative. The GTR+I+G model was chosen as the optimal model, and mixing, as measured by the degree of state exchange acceptance rates, was determined to be adequate (acceptance rates of 10-70%). The most-likely tree from the Bayesian analysis is shown in Figure 2. The MP analysis recovered 127 trees with a length of 506 steps. Clades with strong support from the MP bootstrap analysis (70% or greater) were not in conflict with strongly supported clades in the Bayesian analysis (BPP 0.95 or greater).

Several well-supported clades were recovered (Fig. 2). The L clade (*Lepraria* sensu stricto) was very strongly supported. Several nodes along the backbone of *Lepraria* are weakly supported, however, two major clades were recovered with strong support: the Ln clade and the Li clade. The Ln clade, which consists of an extended version of the *L. neglecta* group, was strongly supported in both Bayesian and MP analyses. This clade contains one strongly supported sub-clade, sub-clade Ln1, while the rest of the taxa in clade Ln formed an unsupported monophyletic group. All other taxa in the genus (besides *L. lobificans*, *L. atrotomentosa*, *L. caesiella* and the Ln clade) form a poorly supported monophyletic group. Clade Li is found in this weakly supported clade, and contains the two strongly supported sub-clades, Li1 and Li2.

CHEMICAL COMPOSITION OF CLADES: Orcinol *para*-depsides, β-orcinol *para*-depsides, depsidones, dibenzofurans, terpenoids and higher aliphatic acids are phylogenetically widespread, occurring across the genus (Fig. 2 and Table 2). The regular production of β-orcinol *meta*-depsides and anthraquinones were quite rare, occurring only in clade Li (however, anthraquinones are produced as a minor substance in *L. humida* [clade Ln] and *L. sylvicola* [clade Li]). Similarly, taxa producing benzyl esters were also rare.

When looking at the chemical composition of the well-supported major and sub-clades (summarized in Fig. 2 and Table 2), the Ln clade contained six substance classes, while the Li clade contained all substance classes known from *Lepraria* except benzyl esters. Sub-clade Ln1 contained a small number of substance classes in comparison to major clade Ln (three of six). Sub-clade Li1 regularly produces six of the eight substance classes found in major clade Li, while sub-clade Li2 regularly produces only four of the eight substance classes of clade Li.

ANCESTRAL STATE RECONSTRUCTION AND HYPOTHESIS TESTING: In the present study, the specific substances listed under each substance class in Table 2 were produced by all taxa producing that substance class (all taxa producing β-orcinol *para*-depsides produced atranorin, etc.). Consequently, ancestral state coding for these particular substances and substance classes were identical, and we therefore drew conclusions on the evolution of specific substances in addition to substance classes.

Based on the present analyses, a small number of character state changes (2) have occurred in β-orcinol *meta*-depsides/thamnolic acid, benzyl esters/alectorialic acid and anthraquinones, while a medium number of character state changes (4-6) have occurred in orcinol *para*-depsides/divaricatic acid, dibenzofurans and terpenoids/

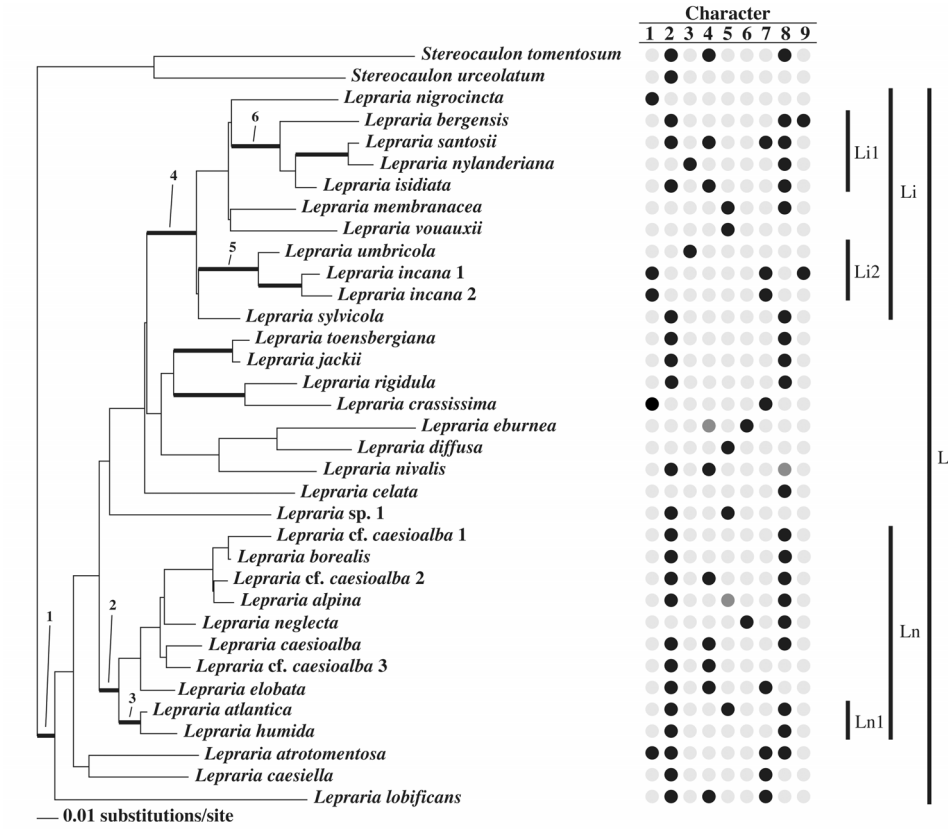

Fig. 2: The most-likely tree from Bayesian analysis, with posterior probabilities. Those branches in bold indicate BPP greater than or equal to 0.95, and MP bootstrap scores of 70% or greater. Strongly supported major and minor clades discussed in the text are marked with a vertical black bar. Presence of the substance class as a constant major is denoted with a black dot; the absence or production of the substance class in trace amounts or rare production of the substance class as a major is marked by a faint grey dot; a dark grey dot indicates that the substance class of interest is mostly present in this taxon or is only present in some chemotypes. Substance class abbreviations: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzyl esters, 7 = terpenoids, 8 = higher aliphatic acids and 9 = anthraquinones.

zeorin (Table 3). A large number of state changes (7+) were required for β-orcinol *para*-depsides/atranorin, β-orcinol depsidones and higher aliphatic acids (Table 3). Gains were more likely to occur for orcinol *para*-depsides/divaricatic acid, β-orcinol *meta*-depsides/thamnolic acid, β-orcinol depsidones, dibenzofurans, benzyl esters/alectorialic acid, terpenoids/zeorin and anthraquinones, while losses were more likely for β-orcinol *para*-depsides/atranorin and higher aliphatic acids (Table 4). Taxa producing the examined substance classes were not monophyletic, a result which was confirmed with Templeton tests (Templeton 1983) and the BPP of this topology (Nelsen & Gargas, unpublished data). Furthermore, the number

TABLE 2: Secondary metabolite substance classes and select substances found in *Lepraria* clades. Presence (+) or absence (-) of various substance classes in well-supported clades (Fig. 2) are shown.

Class	Substance	*Lepraria* s. str. clade Major clade Sub-clade	L	L n	L n 1	L i	L i 1	L i 2
Polyketide pathway								
Orcinol *para*-			+	-	-	+	-	+
depsides	Divaricatic acid		+	-	-	+	-	+
β-Orcinol *para*-			+	+	+	+	+	-
depsides	Atranorin		+	+	+	+	+	-
β-Orcinol *meta*-			+	-	-	+	+	+
depsides	Thamnolic acid		+	-	-	+	+	+
β-Orcinol depsidones			+	+	-	+	+	-
Dibenzofurans			+	+	+	+	-	-
Benzyl esters			+	+	-	-	-	-
	Alectorialic acid		+	+	-	-	-	-
Higher aliphatic acids			+	+	+	+	+	-
Anthraquinones			+	-	-	+	+	+
Mevalonic acid pathway								
Terpenoids			+	+	-	+	+	+
	Zeorin		+	+	-	+	+	+

of character state changes required for the dataset used in this study was not significantly fewer than the number of state changes when the dataset was shuffled 10,000 times (Table 5).

At the base of *Lepraria* sensu stricto (node 1 in Fig. 2), the ancestral state reconstructions (Table 6) suggest the presence of β-orcinol *para*-depsides/atranorin, and the absence of orcinol *para*-depsides/divaricatic acid, β-orcinol *meta*-depsides/thamnolic acid, benzyl esters/alectorialic acid and anthraquinones. The presence or absence of β-orcinol depsidones, dibenzofurans, terpenoids/zeorin and higher aliphatic acids at node 1 could not be determined. The ancestral state reconstructions suggest a presence of β-orcinol *para*-depsides/atranorin and the absence of orcinol *para*-depsides/divaricatic acid, β-orcinol *meta*-depsides/thamnolic acid, benzyl esters/alectorialic acid and anthraquinones at the base of clade Ln (node 2 in Fig. 2), while the ancestral states of β-orcinol depsidones, dibenzofurans, terpenoids/zeorin and higher aliphatic acids could not be determined. Similar ancestral states were suggested for the base of clade Ln1 (node 3 in Fig. 2), except the absence of terpenoids/zeorin was confirmed. The base of clade Li (node 4 in Fig. 2) was found to not produce orcinol *para*-depsides/divaricatic acid, β-orcinol *meta*-depsides/thamnolic acid, benzyl esters/alectorialic acid, terpenoids/zeorin and anthraquinones, while we were not able to determine whether it regularly produced β-orcinol *para*-depsides/atranorin, β-orcinol depsidones, dibenzofurans or higher aliphatic acids. The base of clade Li1 (node 6 in Fig. 2) had an identical ancestral state reconstruction to the base of Li. The base of clade Li2 (node 5 in Fig. 2) yielded similar results, except the absence of β-orcinol *para*-depsides/atranorin was confirmed.

Table 3: Frequency of the number of changes associated with MCMC MP reconstruction of 10,000 randomly selected trees (from the original MCMC sampling of 19,500 trees). Frequencies in bold correspond to the number of changes when the dataset is mapped onto the most-likely tree from the Bayesian analysis. Characters are as follows: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzyl esters, 7 = terpenoids, 8 = higher aliphatic acids, 9 = anthraquinones.

Character	**# Changes**														**Avg # of Changes**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
1				**99.99**	0.01										4.00
2						0.15	55.04	**44.78**	0.03						7.45
3		**100**													2.00
4								2.06	**96.43**	1.51					9.00
5				**31.65**	68.36										4.68
6		**100**													2.00
7					32.56	**66.74**	0.70								5.68
8								0.33	11.56	56.41	**31.70**				10.20
9		**100**													2.00

TABLE 4: Average number of gains and losses of each character and the gain:loss ratio. MCMC MP reconstruction of 10,000 randomly selected trees (from the 19,500 MCMC trees). Character abbreviations: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzyl esters, 7 = terpenoids, 8 = higher aliphatic acids and 9 = anthraquinones.

Character Ratio	Avg # of Gains	Avg #of Losses	Gain:Loss
1	4.00	0.00	-
2	0.91	3.45	0.26
3	2.00	0.00	-
4	2.33	0.19	12.15
5	4.09	0.00	-
6	2.00	0.00	-
7	4.50	0.16	27.75
8	1.42	2.06	0.69
9	2.00	0.00	-

Discussion

The presence or absence of secondary metabolites is frequently used as a taxonomic character in *Lepraria*. However, the production of these compounds can also be homoplasious. Ekman and Tønsberg (2002) demonstrated that dibenzofuran-containing species formerly placed in the genus *Leproloma* were not monophyletic, and that two species with identical chemistry (*L. lobificans* and *L. elobata*) were not closely related. Ihlen & Ekman (2002), Blanco et al. (2006) and Lumbsch et al. (2006) have all investigated the evolution of secondary metabolites in various taxa and have concluded that there have been gains and losses of the ability to produce various substances. These results are all consistent with Culberson (1986), who suggested that convergent gains in the ability to produce a particular substance are not unlikely, as few biosynthetic steps are often needed to produce a particular substance from a primary product. In the present study, character states do not appear monophyletic or significantly phylogenetically clustered. Additionally, a number of changes (gains and losses) were also suggested for various substance classes. Taken together, these results suggest several cases of convergence, and that chemical similarities may not necessarily indicate a close phylogenetic relationship.

The analyses based on the current taxon/data sampling suggest that the ancestor to extant *Lepraria* taxa contained β-orcinol *para*-depsides/atranorin, but did not contain orcinol *para*-depsides/divaricatic acid, β-orcinol *meta*-depsides/thamnolic acid, benzyl esters/alectorialic acid or anthraquinones. Atranorin is the most common β-orcinol *para*-depside in lichens (Culberson 1969) and the ability to produce this substance has also been gained and lost numerous times in Parmeliaceae (Blanco et al. 2006). Although a number of substances were absent at the base of *Lepraria*, a number of taxa later gained the ability to regularly produce these compounds (Table 5). Similarly, some taxa lost the ability to produce substances present at the base of *Lepraria* or substances that were later gained (Table 5).

Table 5: Frequency of the number of changes in each character when 10,000 randomly shuffled datasets are mapped onto the most-likely tree from Bayesian analysis. Frequencies in bold correspond to the number of changes when the non-randomized dataset is used. Characters are as follows: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzyl esters, 7 = terpenoids, 8 = higher aliphatic acids, 9 = anthraquinones.

Character	**# Changes**														**Avg # of Changes**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	
1		0.11	1.20	**20.36**	78.33										4.77
2				0.01	0.04	0.50	2.02	**7.11**	19.41	31.98	28.17	10.76			10.08
3	2.02	**97.98**													1.98
4				0.01	0.10	0.79	4.35	17.96	**41.41**	35.38					9.06
5		0.07	1.08	**19.43**	79.42										4.78
6	1.95	**98.05**													1.98
7				0.28	1.73	**11.68**	39.39	46.92							7.31
8					0.02	0.06	0.29	2.02	6.80	16.71	**26.23**	28.10	15.66	4.11	11.34
9	2.05	**97.95**													1.98

Table 6: Probability of each character state at 6 nodes of interest. MCMC ML sampling of 10,000 trees. Models correspond to Mk1 (M) or AsymmMk (A). Nodes correspond with those shown in Figure 2. Probabilities greater than or equal to 95% are in bold. States of 1 and 0 refer to the presence and absence of substance classes. Characters are as follows: 1 = orcinol *para*-depsides, 2 = β-orcinol *para*-depsides, 3 = β-orcinol *meta*-depsides, 4 = β-orcinol depsidones, 5 = dibenzofurans, 6 = benzylesters, 7 = terpenoids, 8 = higher aliphatic acids, 9 = anthraquinones.

	Character	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	Model	**M**	**M**	**A**	**A**	**A**	**A**	**M**	**M**	**A**
Node	**State**									
1	% Node Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% No Uniquely Best State	2.39	0.10	0.00	**100.0**	69.6	0.00	77.14	**99.88**	0.00
	% 1 is Present	0.00	**99.90**	0.00	0.00	0.00	0.00	16.18	0.00	0.00
	% 0 is Present	**97.61**	0.00	**100.0**	0.00	30.40	**100.0**	6.68	0.12	**100.0**
2	% Node Absent	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
	% No Uniquely Best State	0.00	0.01	0.00	**99.94**	**99.89**	0.00	17.95	**99.94**	0.00
	% 1 is Present	0.00	**99.96**	0.00	0.00	0.00	0.00	0.00	0.03	0.00
	% 0 is Present	**99.97**	0.00	**99.97**	0.03	0.08	**99.97**	82.02	0.00	**99.97**
3	% Node Absent	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31
	% No Uniquely Best State	0.00	0.00	0.00	74.61	**99.69**	0.00	0.00	84.27	0.00
	% 1 is Present	0.00	**99.69**	0.00	0.00	0.00	0.00	0.00	15.42	0.00
	% 0 is Present	**99.69**	0.00	**99.69**	25.08	0.00	**99.69**	**99.69**	0.00	**99.69**
4	% Node Absent	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	% No Uniquely Best State	0.01	**98.12**	0.00	**93.76**	79.54	0.00	0.01	**99.99**	0.00
	% 1 is Present	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% 0 is Present	**99.98**	1.78	**99.99**	6.23	20.45	**99.99**	**99.98**	0.00	**99.99**
5	% Node Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% No Uniquely Best State	1.76	0.13	0.00	52.05	51.57	0.00	2.50	**97.50**	0.00
	% 1 is Present	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% 0 is Present	**98.24**	**99.87**	**100.0**	47.95	48.43	**100.0**	**97.5**	2.50	**100.0**
6	% Node Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% No Uniquely Best State	0.00	85.4	0.00	100.0	60.15	0.00	0.00	87.50	0.00
	% 1 is Present	0.00	14.6	0.00	0.00	0.00	0.00	0.00	12.50	0.00
	% 0 is Present	**100.0**	0.00	**100.0**	0.00	39.85	**100.0**	**100.0**	0.00	**100.0**

Loss or gain of a particular chemical/secondary metabolite on any branch of this phylogenetic tree may be the result of several possibilities including: 1) this ITS rDNA sequence analysis does not correctly reconstruct *Lepraria* phylogeny. Future phylogenetic hypotheses may be better refined by analyzing more genes (Myllys et al. 2005, Nelsen & Gargas 2008); 2) *Lepraria* species may undergo cryptic mating or pseudosexuality resulting in genome hybridization; 3) sorting of ancestral polymorphisms of gene presence; or 4) although unlikely, there may be true horizontal gain of pathway enzyme genes from distant lineages.

This study has demonstrated that chemical similarities do not necessarily indicate close phylogenetic relationships. Future studies should include more taxa and examine the phylogenetic distribution of other secondary metabolites, such as thamnolic

and lecanoric acids by determining how closely related *Lepraria lecanorica* Tønsberg is to *L. atrotomentosa* (both produce lecanoric acid) and how closely related *Lepraria aurescens* Orange & Wolseley and *Lepraria pulchra* Orange & Wolseley (both with thamnolic acid) are to one another and *L. nylanderiana* and *L. umbricola* (both with thamnolic acid). Additionally, the evolution of secondary metabolites in this group should also be further examined by incorporating more sequence data and individual substances, and using the likelihood optimality criterion to investigate gain:loss ratios. Future work should also investigate the exact mechanisms responsible (such as mutation versus gain or loss of a gene) for gains and losses by investigating the polyketide synthase genes (Grube & Blaha 2003; Kroken et al. 2003; Miao et al. 2001; Opanowicz et al. 2006; Schmitt et al. 2005). We hope that the results presented here will serve as a preliminary estimate of the evolutionary history of these substances in *Lepraria*.

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