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Tidying up the genus *Letharia*: introducing *L. lupina* sp. nov. and a new circumscription for *L. columbiana*

Susanne ALTERMANN, Steven D. LEAVITT and Trevor GOWARD

Abstract: Western North America is the global centre of diversity for *Letharia*, a distinctive and cryptically diverse genus of lichenized fungi belonging to the *Parmeliaceae*. The genus is characterized by a shrubby, fruticose habit and presence of vulpinic acid. Previous studies using multiple fungal nuclear loci revealed the existence of two distinct species-level lineages within the traditional concept of *L. vulpina* and four such lineages within *L. columbiana*. Here we use molecular sequence data in an attempt to settle long-standing taxonomic issues in the genus. Our results confirm the widespread existence within *L. vulpina* s. lat. of two distinct species-level groups, each forming a mutually exclusive partnership with a separate algal clade within *Trebouxia jamesii* s. lat. Accordingly, we formally describe the segregate species *L. lupina* sp. nov. Our results also support the evolutionary independence of four candidate species previously circumscribed from *L. columbiana* s. lat. One of these lineages, *L.* 'gracilis', has already received species recognition as *L. gracilis*, while a second, *L.* 'lucida', is epitypified here against *L. columbiana* s. str. Based on results from species delimitation analyses under the multispecies coalescent model, the two remaining lineages, *L.* 'barbata' and *L.* 'rugosa', also warrant formal taxonomic recognition; however, we refrain from describing these species pending additional studies of diagnostic characters, ecological preference, and distributions.

Key words: lichen symbiosis, lichenized Ascomycetes, taxonomy

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Introduction

Letharia (Th. Fr.) Zahlbr. is a distinctive genus of lichenized fungi belonging to the *Parmeliaceae*. They are characterized by a coarse, densely branching, fruticose habit, internal cartilaginous strands, and the production of cortical vulpinic acid (Stephenson & Rundel 1979; Ryan 2002). Vulpinic acid is a brilliant yellow cortical pigment sometimes used as a source of dye (Mead 1972; Turner 1979; Casselman 1996). Consistent with the toxic properties of this substance (Emmerich *et al.* 1993), *L. vulpina* (L.) Hue s. lat. was formerly used in Northern Europe as the active agent in various preparations against foxes and wolves (Schneider 1904; Santesson 1939). Some native peoples of western North America used this species medicinally, for example in the treatment of inflammation and running sores (Chestnut 1902). Its notable resistance to atmospheric pollution (Sigal & Nash 1983) has earned *L. vulpina* s. lat. a prominent role in air quality biomonitoring in regions where it is common (Fenn *et al.* 2007; Geiser & Neitlich 2007; Jovan & Carlberg 2007).

Two species have traditionally been recognized within *Letharia*: *L. vulpina* (L.) Hue with copious isidia/soredia but generally without apothecia, and *L. columbiana* (Nutt.) J. W. Thoms., which lacks abundant asexual reproductive structures but usually bears conspicuous apothecia. Previous work using multiple fungal nuclear loci supported the circumscription of two taxa within *L. vulpina* s. lat and four within *L. columbiana* s. lat. (Kroken & Taylor 2001; McCune & Altermann 2009). Subsequent work employing population assignment tests corroborated

S. Altermann: Whitman College, 345 Boyer Avenue, Walla Walla, WA 99362 USA. Email: altermsm@whitman.edu
S. D. Leavitt: Science & Education, The Field Museum, Chicago, Illinois, USA.
T. Goward: UBC Herbarium, Beaty Museum, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada. Mailing address: Enlichened Consulting Ltd., 5369 Clearwater Valley Road, Upper Clearwater, BC V0E 1N1, Canada.

these six genetic groups, but left their taxonomic rank unclear owing to uncertainty of the most appropriate circumscription of genetic clusters and their evolutionary significance (Altermann *et al*. 2014), possibly due, at least in part, to recent divergence among lineages. However, three lineages within *Letharia*, including the fertile lineage *L.* 'lucida' and both isidio-sorediate groups, *L.* 'lupina' and *L. vulpina*, were consistently circumscribed as distinct genetic groups and clearly merit recognition at the species rank (Altermann *et al*. 2014).

Letharia vulpina s. lat. is widespread in western North America, Europe (Fennoscandia and the Alps), the Caucasus Mountains, and the Atlas Mountains of North Africa (Gams 1955; Deil 1984; Brodo *et al*. 2001), though only in the dry coniferous forests of western North America can it be regarded as abundant. It is listed as rare and threatened in many parts of Europe, especially Fennoscandia (Tønsberg *et al*. 1996; Trass 1997; Vitikainen *et al*. 1997; Gärdenfors 2000).

Goward (1999) was the first to suggest that *L. vulpina* might encompass two species distinguishable on morphological and ecological grounds, for example, branching, colour, density of isidia/soredia, and altitudinal distribution. He referred to the new species as *Letharia* "sp. 1" and gave it a common name: "Mountain Wolf" lichen. Using multi-locus DNA sequencing data and a phylogenetic species recognition approach, Kroken & Taylor (2000, 2001) confirmed that two fungal species-level lineages were indeed present in North America, each of which paired with a distinct algal clade nested within the morphologically-based taxon *Trebouxia jamesii* s. lat. They nicknamed the fungal phylogenetic species *L. vulpina* 'vulpina' and *L. vulpina* 'lupina', but did not go on to formally describe the taxa. They found no overlap in their geographical distribution. Additional work in Europe and the Caucasus detected only *L. vulpina* 'vulpina' (Högberg *et al*. 2002; Arnerup *et al*. 2004), though a single tree trunk in Morocco was found to support both phylogenetic species (Arnerup *et al*. 2004).

The four genetic lineages of *L. columbiana* s. lat. are found primarily on conifers within mountainous regions of western North America, from southern intermontane British Columbia to southern California. At the time of their first molecular circumscription by Kroken & Taylor (2001), they received the herbarium names *L.* 'gracilis', *L.* 'lucida', *L.* 'rugosa', and *L.* 'barbata' based on subspecific epithets proposed by Schade (1955; see also Thomson 1969). *Letharia* 'gracilis' was later formally described at the rank of species as *L. gracilis* McCune & Altermann (McCune & Altermann 2009). More recently, Altermann *et al*. (2014) applied population assignment tests to Kroken & Taylor's dataset and found that each of the four fertile putative species was in some cases supported as a distinct genetic cluster, although only *L.* 'lucida' was consistently recovered as a distinct genetic group across all analyses.

Here we report on a large-scale study of fungal-algal partnerships in *L. vulpina* s. lat. (Altermann 2009), as well as on a more recent field-based and molecular study of *L. columbiana* s. lat., including collections made near its type locality in north-eastern Oregon, USA. DNA sequence data were used to test whether the mutually exclusive fungal-algal partnership pattern in *L.* 'vulpina' and *L.* 'lupina' observed by Kroken & Taylor (2000, 2001) was maintained across the combined range of the species. We set out to determine whether the previously observed morphological and ecological differences between the two lichen phenotypes (Goward 1999) were consistent throughout the ranges of the species. We also attempt to ascertain the evolutionary independence (e.g. species-level status) and stabilize the taxonomy of the four candidate species within *L. columbiana* s. lat.

Materials and Methods

Letharia vulpina s. lat. specimen sampling

Three hundred specimens of *L. vulpina* s. lat. were collected from throughout the range of *Letharia* in western North America, with the addition of one specimen each from Turkey, Switzerland, and Sweden. In an effort to capture maximum genetic diversity in the fungal and algal components, we sampled across a wide variety of altitudes, substrata, and microenvironments. The North American

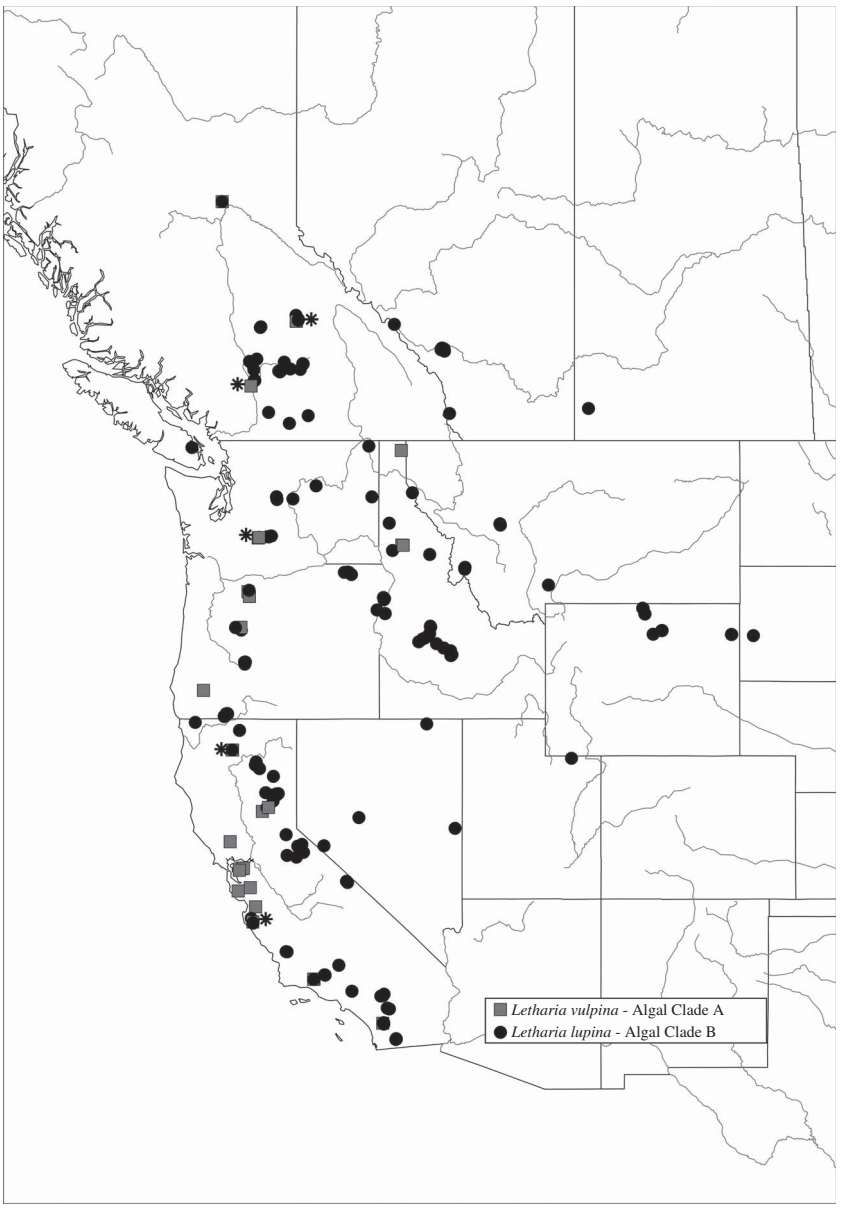

FIG. 1. Geographical distribution of *Letharia vulpina* and *L. lupina* in western North America based on 316 specimens from 105 sites. The map combines location data from this study and that of Kroken & Taylor (2001). Asterisks mark locations where the two fungal species were found within 200 m of each other.

specimens were collected from 105 sites (Fig. 1), with one specimen used from each of 49 sites, and an average of five specimens from each of the remaining 56 sites. Fourteen fertile specimens from 11 sites were included in the study. Of these 300 voucher specimens, 285 have been deposited in the University of California Berkeley (UC) herbarium. The remaining specimens were returned to the lending institutions: Arizona State University (ASU), University of California Riverside (UCR), and the Museum of Evolution Herbarium (UPS). Table 1 provides a summary of collection and other data for 28 representative specimens from throughout the study area.

TABLE 1. *Collection information and GenBank accession numbers for selected specimens of* Letharia vulpina *s. lat. Complete collection information is available on GenBank by accession number and by the PopGen dataset associated with this study.*

	Collection site	Voucher	GenBank Accession Number			
			Fungal ITS	Locus 11	Algal ITS	Actin I Intron
Letharia lupina						
	Canada, Alberta, W. Jumpingpound	*Glass* 07/14/05 (UC)	FJ161370	FJ153288	FJ170467	FJ170822
	Canada, Alberta, Bow Lake	*Glass* 10/10/05 (UC)	FJ161400	FJ153318,	FJ170497	FJ170852
	Canada, British Columbia, Greenstone Mtn.	*Altermann* 77 (UC)	FJ161445	FJ153363	FJ170542	FJ170897
	Canada, British Columbia, 100 Mile House	*Stevenson* 04/28/05 (UC)	FJ161516	FJ153434	FJ170613	FJ170968
	Switzerland, Canton Graubünden	*Goward* 99164 (UC)	FJ161566	FJ153484	FJ170663	FJ171018
	USA, California, Calaveras Co.	*Poulson* 01/01/04 (UC)	FJ161379	FJ153297	FJ170476	FJ170831
	USA, California, Alpine Co.	*Altermann* 15 (UC)	FJ161432	FJ153350	FJ170529	FJ170884
	USA, California, San Diego Co.	*Nash* 44074 (ASU)	FJ161540	FJ153458	FJ170637	FJ170992
	USA, California, Riverside Co.	*Knudsen* 6842 (UCR)	FJ161548	FJ153466	FJ170645	FJ171000
	USA, Idaho, Latah Co.	*Bjork* 04/14/06 (UC)	FJ161458	FJ153376	FJ170555	FJ170910
	USA, Idaho, Shoshone Co.	*Bjork* 12150a (UC)	FJ161463	FJ153381	FJ170560	FJ170915
	USA, Montana, Ravalli Co.	*Pipp* 05/05/05 (UC)	FJ161403	FJ153321	FJ170500	FJ170855
	USA, Montana, Lewis and Clark Co.	*Pipp* 05/03/05 (UC)	FJ161509	FJ153427	FJ170606	FJ170961
	USA, Oregon, Wallowa Co.	*Altermann* 208 (UC)	FJ161614	FJ153532	FJ170711	FJ171066
	USA, Washington, Douglas Co.	*Bjork* 11021 (UC)	FJ161605	FJ153523	FJ170702	FJ171057
	USA, Washington, Douglas Co.	*Bjork* 12185 (UC)	FJ161606	FJ153524	FJ170703	FJ171058
	USA, Wyoming, Sheridan Co.	*Bell* 06/15/05 (UC)	FJ161397	FJ153315	FJ170494	FJ170849
	USA, Wyoming, Crook Co.	*Zimmerman* 07/01/06 (UC)	FJ161465	FJ153383	FJ170562	FJ170917
Letharia vulpina						
	Canada, British Columbia, Teapot Mtn.	*Stevenson* 06/18/05 (UC)	FJ161647	FJ153565	FJ170744	FJ171099
	Turkey, Ala Dag	*Spribille* 06/07/07 (UC)	FJ161663	FJ153581	FJ170760	FJ171115
	Sweden, Dalarna. Idre par. Grundagssätern	*Hermansson* 16600 (UPS)	GQ398408	GQ398410	GQ398411	GQ398412
	USA, California, San Diego Co.	*Knudsen* 04/20/05 (UC)	FJ161654	FJ153572	FJ170751	FJ171106
	USA, California, Yuba Co.	*Dillingham* 12/09/03 (UC)	FJ161649	FJ153567	FJ170746	FJ171101
	USA, Idaho, Boundary Co.	*Bjork* 11201 (UC)	FJ161640	FJ153558	FJ170737	FJ171092
	USA, Idaho, Clearwater Co.	*Bjork* 11191 (UC)	FJ161641	FJ153559	FJ170738	FJ171093
	USA, Oregon, Douglas Co.	*Altermann* 4 (UC)	FJ161653	FJ153571	FJ170750	FJ171105
	USA, Oregon, Linn Co.	*Altermann* 54 (UC)	FJ161665	FJ153583	FJ170762	FJ171117

Letharia vulpina s. lat. molecular character sampling

In a previously published companion study that included all the putative *Letharia* lineages, phylogenetic analyses of concatenated multilocus sequence data corroborated a separation between *L. vulpina* and *L. lupina* (Altermann *et al.* 2014). Therefore, we focused here on identifying diagnostic nucleotide position characters in mycobiont markers and investigating patterns of symbiont interactions among *L. vulpina* s. lat. specimens and their associated *Trebouxia* photobionts. In addition to three fungal loci (ITS, anonymous locus 'DO', and anonymous locus '11'; Kroken & Taylor 2001), two algal loci were also amplified and sequenced: algal ITS using primers ITS1T and ITS4T (Kroken 1999) and an actin I intron, using the actin primers 3T and 4T (Kroken & Taylor 2000). Specimen preparation, DNA extraction, and PCR were carried out for all loci in accordance with Kroken & Taylor (2000, 2001). PCR products were cleaned either with a QiaQuick PCR Purification Kit (Qiagen, Venlo, the Netherlands) or using the enzymes Exonuclease I and Shrimp Alkaline Phosphatase (USB Corporation, Cleveland, OH, USA). In both cases, we proceeded according to the manufacturers' instructions. All PCR amplifications yielded single bands with the exception of the actin I intron locus of the Swedish specimen, which yielded two bands. Preliminary sequencing showed that the lighter of these two bands was the target sequence. The band was cut out of a 1% agarose gel and cleaned with a PrepEase Gel Extraction Kit (USB Corporation) according to the manufacturer's instructions.

PCR products from all specimens were bi-directionally sequenced using either an ABI PRISM® 3100 Genetic Analyzer or an ABI 3730 xl DNA Analyzer. Chromatograms were examined and contigs assembled with Sequencher v4.10 (Gene Codes Corporation, Ann Arbor, MI, USA). Multiple sequence alignments were performed for each locus using the program MAFFT v7 (Katoh *et al.* 2005; Katoh & Toh 2008). We implemented the G-INS-i alignment algorithm and '200PAM/K = 2' scoring matrix, with an offset value of 0·0, unalignlevel = 0·7 using the 'Leave gappy regions' setting, and the remaining parameters set at default values.

A maximum likelihood (ML) topology was inferred from representative *Trebouxia* ITS haplotyes listed in Table 1, using the program RAxML v8.2.1 (Stamatakis 2006; Stamatakis *et al.* 2008) in the CIPRES Science Gateway server (http://www.phylo.org/portal2/). The RAxML analysis implemented the 'GTRGAMMA' model and evaluated nodal support using 1000 bootstrap pseudoreplicates. Exploratory phylogenetic analyses under maximum parsimony inference provided highly similar topologies and similar nodal support values (data not shown). Phylogenetic analyses of the actin I intron were not performed due to the excessive ambiguities in the multiple sequence alignment (see Results).

We explored the potential of using restriction digests of the PCR products of the fungal ITS locus for sample identification of *L. vulpina* s. lat. specimens without sequencing. Based on previously published *Letharia* ITS sequences, we identified multiple restriction sites using the restriction enzyme Eco0109I (New England Biolabs). The mycobiont ITS was amplified as described previously (Kroken & Taylor 2001). PCR products were cleaned with QiaQuick (Qiagen Corporation) and digests using Eco0109I following the manufacturer's instructions, in 50 µl reactions at 37°C overnight. Bands were visualized on a 1·5% agarose gel using SYBR Gold stain (Molecular Probes, Inc.).

Letharia columbiana s. lat. sampling and population assignment tests

Unlike *L. vulpina* s. lat, which has been the primary focus of numerous molecular studies (Kroken & Taylor 2000, 2001; Högeberg *et al.* 2002; Arnerup *et al.* 2004; Altermann *et al.* 2014), *L. columbiana* s. lat. has been sequenced on only a few occasions (Kroken & Taylor 2000, 2001; McCune & Altermann 2009). Here we sampled fresh material from a wide geographical range, including near the type locality of *L. columbiana* along the Walla Walla River in north-eastern Oregon (Nuttall 1834; Thomson 1969), along the Spokane River, Washington, and at a number of localities in southern British Columbia. For this portion of the study, molecular data were generated from a total of 49 additional *Letharia* samples, 31 representing *L. columbiana* s. lat. and 18 isidio-sorediate forms. Collection data for these specimens are available in Supplementary Material Table S1 (available online).

Total genomic DNA was extracted from a small piece of thallus material free from visible damage or contamination using the USB PrepEase Genomic DNA Isolation Kit (USB, Cleveland, OH, USA) and following the manufacturer's recommendations. For these specimens, we generated sequence data for three loci, including the internal transcribed spacer region (ITS), and anonymous loci '11' and 'DO' (Kroken & Taylor 2001) which have previously been shown to provide inferences of genetic structure consistent with more comprehensive genetic sampling (Altermann *et al.* 2014). Primers and temperature profiles followed Kroken & Taylor (2001), and PCR amplifications were performed using Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA). Products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB, Cleveland, USA). We sequenced complementary strands using BigDye v3.1 (Applied Biosystems, Foster City, CA, USA) with the same primers used for amplifications. Sequenced PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the Pritzker Laboratory for Molecular Systematics and Evolution at the Field Museum in Chicago.

New sequences were assembled and edited using Sequencher v4.10 (Gene Codes Corporation, Ann Arbor, MI) and combined with Kroken & Taylor's complete 12-marker dataset (2001) representing 51 specimens (retrieved from http://www.treebase.org - ID# S11236). Multiple sequence alignments for each locus were performed using the program MAFFT v7

(Katoh *et al.* 2005; Katoh & Toh 2008). We used the G-INS-i alignment algorithm and '1PAM/K = 2' scoring matrix, with an offset value of 0·9, and the remaining parameters were set to default values.

We used the Bayesian population assignment test implemented in the program STRUCTURE v.2.3.2 (Pritchard *et al.* 2000; Falush *et al.* 2003*a*) to identify genetic groups using all single nucleotide polymorphisms (SNPs) inferred from the aligned sequence data. While STRUCTURE assumes independence across sampled loci such that linkage disequilibrium within regions does not dominate the data (STRUCTURE manual), this approach has also been shown to perform sensibly when using multilocus sequence data and treating all variable sites as independent loci regardless of physical linkage within each locus (e.g. Falush *et al.* 2003*b*; Leavitt *et al.* 2013; O'Neill *et al.* 2013). Furthermore, recombination is likely rampant, even for very short stretches of DNA (Springer & Gatesy 2016), supporting the potential for independence among sites within markers traditionally used for phylogenetic inference. The complete data matrix ($n = 100$) included 51 specimens reported in Kroken & Taylor (2001) and 49 new specimens collected for this study. Our approach to Bayesian clustering followed the methods described in Altermann *et al.* (2014), with the exception of inferring genetic groups strictly for the $K = 6$ model (Kroken & Taylor 2001; Altermann *et al.* 2014). In a previous study of *Letharia*, Bayesian clustering using STRUCTURE consistently recovered Kroken & Taylor's phylogenetic species as distinct genetic groups under a range of scenarios using different combinations of 'SNPs' from multilocus sequence data (Altermann *et al.* 2014). Details, including the number of variable and parsimony-informative sites per locus, potential impact of missing data or different combinations of SNP data, and limitations in inferring the most appropriate number of genetic clusters etc., can be found in Altermann *et al.* (2014).

Empirical species delimitation under the multispecies coalescent

The evolutionary significance and taxonomic status of the six *Letharia* species described by Kroken & Taylor (2001) have remained unclear since their initial circumscription (Altermann *et al.* 2014). Here we used the program BPP v3.2 (Yang & Rannala 2010, 2014; Rannala & Yang 2013) to empirically infer speciation probabilities for the six putative species. BPP incorporates coalescent theory and phylogenetic uncertainty into parameter estimation, and the posterior distribution for species delimitation models is sampled using a reversible-jump Markov chain Monte Carlo (rjMCMC) method. We used the unguided species delimitation algorithm ('A11'; Yang 2015), which explores different species delimitation models and different species phylogenies, with fixed specimen assignments to populations. Under the 'A11' analysis, the program attempts to merge different populations into one species and uses the nearest neighbour interchange (NNI) or subtree pruning and regrafting (SPR) algorithms to change the species tree topology (Yang & Rannala 2014). We used a 12-locus dataset (anonymous locus '14' was represented by a single nucleotide position in Kroken & Taylor's 2001 dataset and therefore excluded) comprised of 100 specimens reported here (TreeBASE study ID S18729). Specimens were assigned to each of the six clusters inferred from the STRUCTURE analyses and three specimens with inferred admixed ancestry were excluded (see Results). A within-model inference (analysis 'A00'; Yang 2015) was used to generate the posterior distribution of the parameters (θs and τs) under the MSC model for a more reasonable combination of priors given the data (Rannala 2015). We used Prior 0, equal probabilities for the labelled histories, to assign probabilities to the models. Rates were allowed to vary among loci (locus rate = 1), and the analyses were set for automatic fine-tune adjustments. In addition to the combination of priors for θs and τs inferred using the 'A00' analysis, we also explored the impact of a wide range of combinations of these priors on speciation probabilities (Table 3). The rjMCMC analysis was run for 100 000 generations, sampling every 2 generations, discarding the first 10% as burn-in. Each analysis was run twice using a different search algorithm (algorithm 0 or 1) to confirm consistency between runs. Speciation probabilities greater than 0·95 were considered supported species delimitations.

Results

Letharia vulpina s. lat. dataset

Three hundred and two specimens of putative *L. vulpina* s. lat. thalli were sequenced at three fungal loci and two algal loci, yielding a total of 1510 sequences. We excluded three sterile specimens that could not be placed despite sequencing at three additional loci (18s, locus 2 and locus 13, see Kroken & Taylor 2001). These three specimens may be members of a new cryptic taxon, or they may be hybrids. Our final dataset therefore consisted of 299 specimens.

New sequences generated for this study were deposited in GenBank under the following accession numbers: FJ161369–FJ161667 and KU745809–KU7458857 (fungal ITS); FJ133287–FJ153585 and KU745739–KU745760 (fungal anonymous locus '11'); FJ041055, FJ041056, and KU745761–KU745808 (fungal anonymous locus 'DO'); FJ170466–FJ170764 (algal ITS); and FJ170821–FJ171119 (algal actin intron I). For the portion of the study investigating *L. vulpina* s. lat., each accession number is linked to the complete locus

alignment through the PopSet database in GenBank, and the alignments and trees were submitted to TreeBASE (S18729). The 12-locus data matrix ($n = 100$) used in the population clustering and Bayesian species delimitation analyses was deposited in TreeBASE (S18729).

The *L. vulpina* s. lat. ITS (551 aligned base pairs) data matrix ($n = 299$) yielded 35 haplotypes, over half of which were unique. All of the ITS sequences could be categorized into two groups based on the residues at sites 69, 388, and 499, based on the GenBank PopSet alignments linked to the accessions associated with this paper. For *L. vulpina* s. lat. specimens, locus 11 (375 base pairs long) yielded 12 haplotypes that could be categorized into two groups based on residues present at sites 29 and 198, and the two most abundant haplotypes differed only by these two base pairs. With the exception of a single unique sequence (sample ID AR1), the DO locus (162 base pairs long) yielded only two haplotypes, and they differed by three base pairs (compare GenBank FJ041055 and FJ041056). These eight fixed polymorphisms across three fungal loci (ITS, '11' and DO) supported the distinction of *L.* 'lupina' from *L. vulpina* s. str. (Table 2). Of the 299 specimens sampled, 261 yielded sequence patterns consistent with *L.* 'lupina', whereas the remainder was consistent with *L. vulpina* s. str. Of the 15 isidio-sorediate specimens examined that were also fertile, 12 proved to be *L. lupina*, while the remaining three grouped with *L. vulpina*.

Sequencing of the algal ITS locus yielded all the haplotypes previously found by Kroken & Taylor (2000), in addition to 61 new haplotypes. We recovered the same two clades ('A' and 'B') previously found by Kroken & Taylor (2000), and the maximum likelihood topology is reported in Fig. 2. Sequences in clade 'A' corresponded to provisional species-level operational taxonomic units (OTUs) 'S03' and 'S05', while those in clade 'B' represented OTU 'S01' (*sensu* Leavitt *et al.* 2015*a*). Based on our data, each of the two fungal species consorts with only one of the two algal clades, respectively. *Letharia vulpina* s. str. was always paired with members of algal ITS clade 'A' and *L. lupina* always with members of clade 'B'.

The actin I intron DNA sequences segregated into two distinct groups that paralleled algal ITS clades 'A' and 'B', and are accordingly referred to here as actin groups 'A' and 'B'. The two groups could not be legitimately aligned as they differ at 50% of intron sites, and therefore phylogenies were not inferred from this data. The strict fungal-algal partnership pattern found with the algal ITS data held true: *L. vulpina* s. str. always paired with algal actin intron group 'A' and *L. lupina* with algal actin intron group 'B'. Our single specimen from Sweden, the type location for *L. vulpina* s. lat., had fungal and algal molecular characters unambiguously associated with *L. vulpina* s. str., as described below.

In the *L. vulpina* s. lat. group, specimen identification of both the myco- and photobiont partners was successful by running agarose gels with the *Trebouxia* actin intron PCR product and restriction digests of the fungal ITS PCR product. Algal clade membership could be quickly and accurately diagnosed by the size of the PCR amplicon of the actin I intron, inasmuch as the two actin intron clades differ in PCR product size by about 70 bp, clade 'A' having the longer

TABLE 2. *Eight fungal DNA sequence sites that consistently differ between* Letharia vulpina *and* L. lupina.

	ITS			11		DO		
Locus Position	69	388	499	29	198	113	122	156
L. vulpina	C	C	C	C	C	A	T	T
L. lupina	T	T	T	T	T	G	C	C

ITS and locus 11 position numbers are based on the GenBank PopSet alignments linked to the accessions associated with this paper. A cytosine at ITS position 388 yields an Eco0109I restriction site.

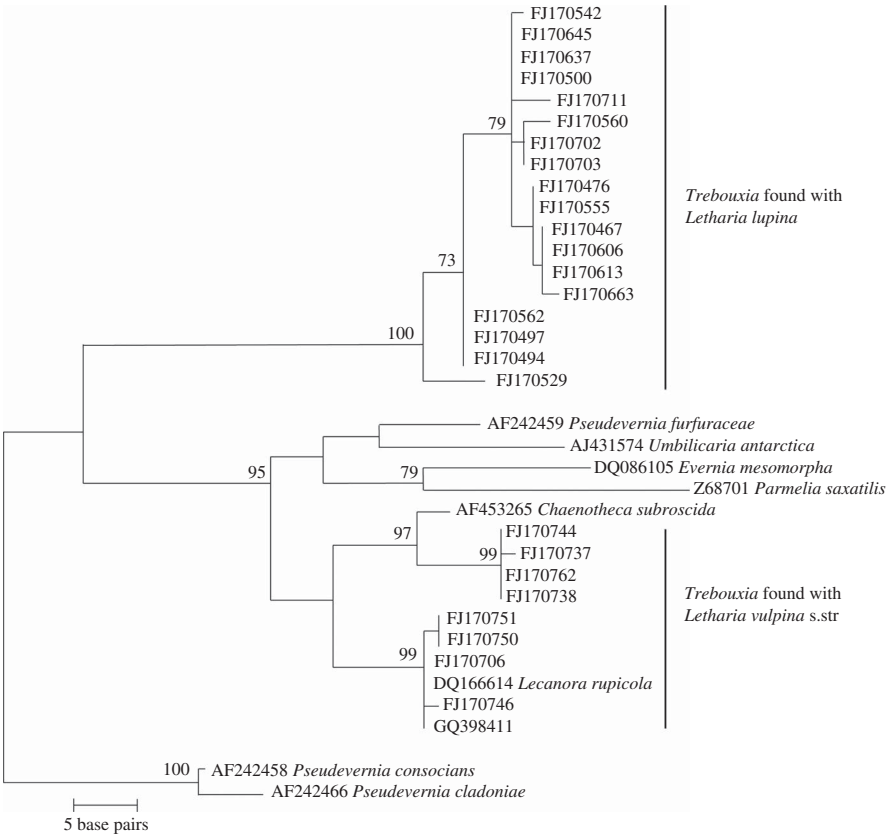

FIG. 2. An ITS maximum likelihood topology for *Trebouxia* associated with *Letharia vulpina* and *L. lupina* (specimens listed in Table 1). GenBank accession numbers identify all specimens, and the ITS alignment comprised 716 aligned nucleotide position characters. *Trebouxia* sequences associated with *L. vulpina* s. str. were exclusively recovered in clade 'A', representing two species-level OTUs – 'S03' and 'S05'; *Trebouxia* associated with *L. lupina* were exclusively recovered in clade 'B' and represented a single OTU – 'S01'. Latin names on other terminal taxa refer to the fungal partner of sequenced algae found in GenBank. The tree is rooted with algae that partner with *Pseudevernia consocians* and *P. cladonia*. Numbers at nodes represent bootstrap support for the clade. Only bootstrap values over 70% are shown.

fragment. Restriction digests of *L. lupina* ITS sequences yielded two fragments, 156 bp and 535 bp in length, while those of *L. vulpina* yielded three fragments, 156 bp, 246 bp, and 289 bp in length.

Letharia columbiana s. lat. sampling and population assignment tests

The complete, 12-marker dataset representing 100 individuals comprised a total of 3849 aligned nucleotide position characters, including 248 variable positions: 38/552 in the ITS, 13/193 in locus DO, 19/375 in locus 11, and 178/2729 in the other nine loci originally reported in Kroken & Taylor (2001). The Bayesian clustering analysis recovered genetic clusters consistent with previously published analyses, with each of Kroken & Taylor's phylogenetic species recovered as a distinct genetic group (Fig. 3). *Letharia columbiana* s. lat. specimens collected from the type locality of *L. columbiana* were recovered in three different genetic groups, *L.* 'barbata' ($n = 5$), *L.* 'lucida' ($n = 4$), and *L.* 'rugosa' ($n = 1$),

while all *L. columbiana* s. lat. specimens from the Spokane River were inferred to belong to the *L.* 'barbata' group (Fig. 3). At both sites, isidio-sorediate forms were recovered in the *L. lupina* group. Most *L. columbiana* s. lat. specimens from UBC were also recovered in three different genetic groups, *L.* 'barbata' ($n = 8$), *L. gracilis* ($n = 1$), and *L.* 'lucida' ($n = 1$), although three specimens were inferred to have admixed ancestries (Fig. 3). Isidio-sorediate forms from UBC were recovered in two distinct clusters, *L. lupina* and *L. vulpina*, consistent with initial morphologically based identification.

Speciation probabilities

BPP analyses provided unambiguous support for a six species model (posterior probability = 1·0), with each of Kroken & Taylor's six provisional species supported by speciation probabilities = 1·0 (Table 3). The branching pattern of all species-level lineages in *Letharia* was unresolved in the BPP analyses, with no single topology inferred with posterior probability >0·25 in any of the analyses (Fig. 4).

Discussion

Our results confirm the finding of Kroken & Taylor (2001) that *L. vulpina* in its traditional circumscription includes two distinct species-level lineages. The three fungal loci examined yielded eight fixed polymorphisms (Table 2), making it possible to distinguish unequivocally between the two isidio-sorediate fungal species. However, this molecular approach alone cannot reliably be employed to distinguish between the two isidio-sorediate fungal species and the four primarily apotheciate taxa because *L. vulpina* shares some of these polymorphisms with *L. columbiana* 'rugosa,' and *L. lupina* shares some polymorphisms with *L. gracilis*. We also confirmed that *L. vulpina* and *L. lupina* consort consistently and uniquely with genetically distinct clades of *Trebouxia jamesii* s. lat. *Letharia lupina* and *L. vulpina* can also be distinguished using genetic information

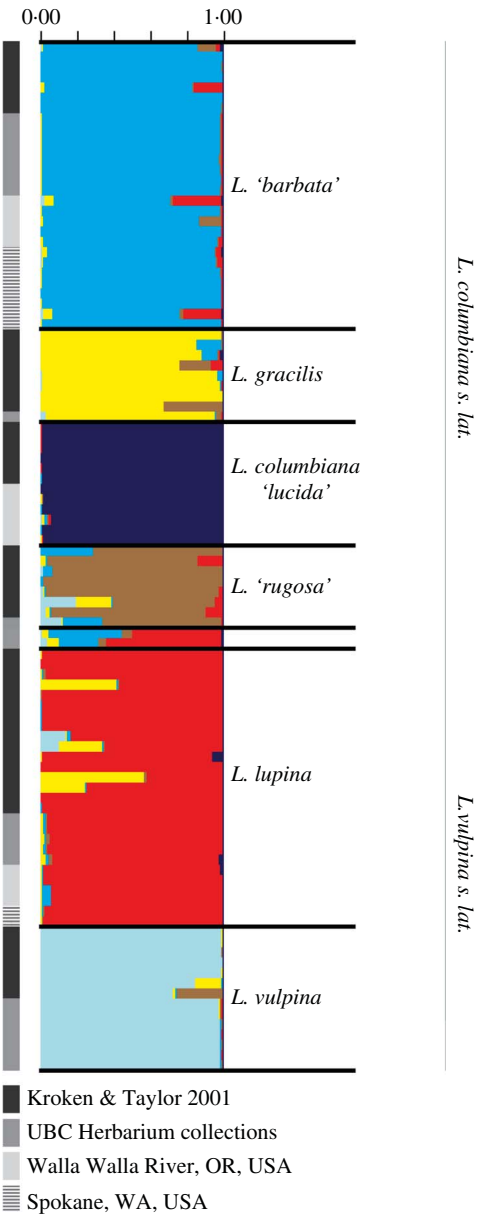

FIG. 3. Results from a Bayesian genetic clustering analysis of *Letharia* specimens. Individual population assignments were inferred using a STRUCTURE analysis of single nucleotide polymorphisms from multi-locus sequence data from 100 *Letharia* specimens under a model assuming six genetic groups (see Kroken & Taylor 2001). Horizontal bars represent individual assignment probability into different genetic clusters depicted with distinct colours.

TABLE 3. *Results from the unguided species delimitation ('A11') analyses implemented in the program BPP and posterior probabilities for the supported species delimitation model. The combinations of species divergence times (τ_0) and population size parameters (θ_0) priors for four separate analyses are provided, along with the posterior support for the six-species model (P_6). In all cases, each of Kroken & Taylor's (2001) putative species received unambiguous support. The bold combination of priors represents the most reasonable combination of priors given the data.*

τ_0	θ_0	P_6
~ G(2,2000)	~ G(2,1000)	1·0
~ G(2,2000)	~ G(1,100)	1·0
~ G(1,100)	~ G(2,2000)	1·0
~ G(1,1000)	**~ G(4,260)**	**1·0**

P_6: *L.* 'barbata'; *L. gracilis*; *L.* 'lucida'; *L.* 'lupina'; *L.* 'rugosa'; *L. vulpina*

without sequencing by running PCR products directly on an agarose gel (algal actin 1 intron locus) and gels of restriction digests of fungal ITS PCR product, bringing genetic identification of *L. lupina* within reach of taxonomic end-users with access to basic molecular tools.

We found no evidence of fungi switching between algal clades, even in localities that supported the two fungal species in close proximity (Fig. 2). From these observations, we infer that phylogenetic constraint, not ecological opportunity, determines each fungal species' selection of algal partner (Yahr *et al.* 2004; Otálora *et al.* 2010; Leavitt *et al.* 2015*a*). Although it is assumed that reproduction in both *L. lupina* and *L. vulpina* is predominantly clonal, previous studies have shown that joint dispersal of both symbionts as asexual diaspores does not imply maintenance of partnerships (Wornik & Grube 2010). While maintenance of symbiotic associations is an option for lichens with predominantly clonal reproductive strategies, algal switching during vegetative development of the thalli appears to be common (Nelson & Gargas 2008; Wornik & Grube 2010; Leavitt *et al.* 2015*a*).

DNA sequencing is increasingly being used to clarify phylogenetic relationships among lichenized fungi, especially above the rank of species (Lutzoni *et al.* 2001, 2004;

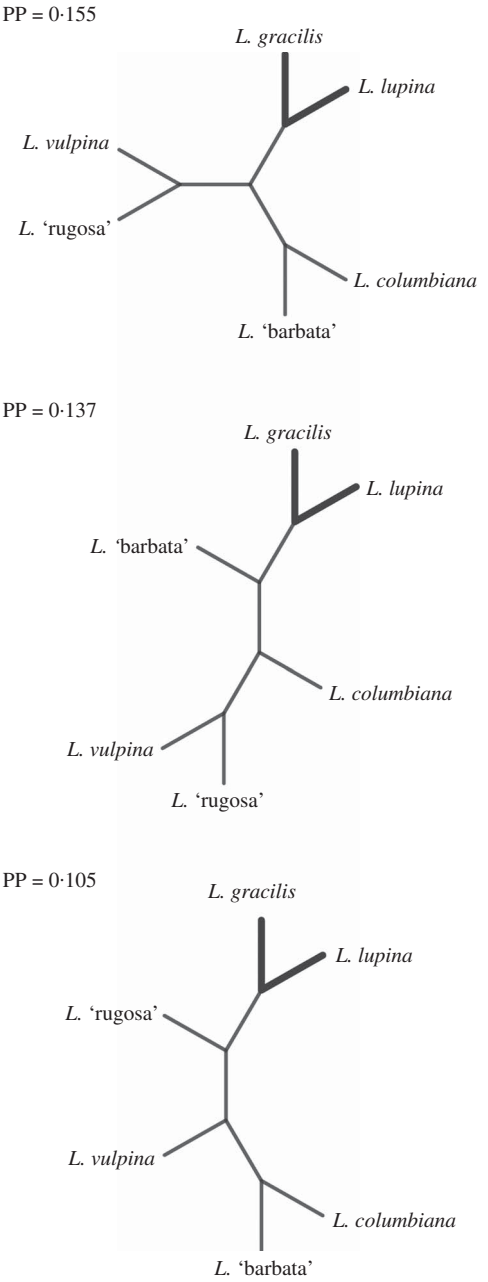

FIG. 4. *Letharia* species trees receiving the highest posterior probabilities (PP) inferred under the multispecies coalescent model. Under the most appropriate combination of priors, no single topology was supported by (PP) >0·15, although *L. vulpina* and *L.* 'rugosa' were consistently recovered as sister lineages in the majority of trees.

Crespo *et al.* 2007). DNA sequencing is also being used to support morphological species recognition (Tibell & Beck 2001; Miadlikowska *et al.* 2002; Molina *et al.* 2004; Divakar *et al.* 2005*a*, *b*; Seymour *et al.* 2007), as well as species distinguished primarily on secondary chemistry (LaGreca 1999; Bayerová *et al.* 2005; Slavíková-Bayerová & Orange 2006). In their synopsis of the 9th International Mycological Congress symposium regarding cryptic speciation in lichens, Crespo & Lumbsch (2010) report a consensus "to recognize species formally when the phylogeny was unequivocal and other evidence supported their separation". Examples of "other evidence" typically include thallus morphology but can also extend to ecology, geographical distribution, fixed differences in nucleotide sequence data and ultramicroscopic features (Lumbsch & Leavitt 2011; Leavitt *et al.* 2015*b*).

From this perspective, *L. vulpina* and *L. lupina* clearly qualify as separate fungal species. The existence of fixed multi-locus polymorphisms on both sides of the symbiosis points to two separate symbioses, and hence to two divergent fungal ecologies shown here to be consistent throughout their respective geographical ranges. At larger spatial scales, it seems reasonable to infer that this same ecological distinction is causally related, at least in part, to the different patterns of distribution observed in these two species (Fig. 2), especially in terms of elevation. Other lines of support can also be advanced in favour of recognizing *L. lupina* and *L. vulpina* as distinct species. First, molecular data (Kroken & Taylor 2001; Altermann *et al.* 2014) suggest that *L. lupina* and *L. vulpina* s. str. are not each other's closest relatives, the latter species being genetically more similar to the *L. columbiana* 'rugosa' lineage than to *L. lupina*; hence, adopting a subspecific rank for *L. lupina* would be inappropriate. While *L. vulpina* and *L. lupina* have until now been treated as a single taxon, and would thus qualify as cryptic species (Crespo & Pérez-Ortega 2009), they are nonetheless usually allopatric in distribution and exhibit distinct ecological preferences. The general pattern of differing ecological requirements for each taxon further supports their recognition as separate species.

The type material of *L. columbiana* s. lat. has been examined by us and unfortunately presents several obstacles to a precise circumscription of *L. columbiana* s. str. First, the type material contains multiple thalli, only a few of which are in fact attributable to *L. columbiana* s. lat. as currently accepted; the rest belong to *L. vulpina* s. lat. Second, molecular testing would be necessary to link the fertile specimens with any of the cryptic lineages currently united within *L. columbiana* s. lat. And third, successful molecular testing appears to be precluded in this case by 1) the age of the material (collected in 1833), and 2) widespread infection by zygomorphic fungi. It can be noted here that an attempt by S. Kroken to extract usable DNA from a promising apothecium failed (as annotated on the holotype).

Under these circumstances, we believe that the interests of nomenclatural stability are best served by epitypifying *L. columbiana* against a sequenced specimen of known identity that has been collected from the type locality. We take this step in full agreement with Ariyawansa *et al.* (2014) that epitypification should not be undertaken except in cases where it can be shown that the holotype in question is demonstrably ambiguous (Jørgensen 2014).

Although multiple *Letharia* lineages are present at the type locality, *L. columbiana* 'lucida' makes the strongest candidate on which to base the species name. Firstly, *L. columbiana* 'lucida' always lacks isidiosoredia, secondly it is chemically synapomorphic by the absence of norstictic acid in the apothecial hymenium (the holotype of *L. columbiana* does not contain norstictic acid), and lastly, it receives the most consistent molecular support of any of the *L. columbiana* s. lat. lineages (Altermann *et al.* 2014). While the other three fertile phylogenetic lineages circumscribed by Kroken & Taylor (2001) are also corroborated (Fig. 3; Table 3), we choose to refrain from further taxonomic innovation pending additional studies of potentially diagnostic characters,

ecological preferences, and distributions. Until then, we believe the interests of taxonomic stability are best served by retaining the current provisional treatment of *L.* 'barbata' and *L.* 'rugosa'. A schematic figure summarizing the phylogenetic relationships among the six *Letharia* species is provided in Fig. 4.

The Species

Letharia lupina Altermann, Leavitt & Goward sp. nov.

MycoBank No.: MB811441

Resembles *Letharia vulpina*, but is distinct at a molecular level on both the fungal and algal side of the symbiosis. *Letharia lupina* is also more common than *L. vulpina* in North America.

Type: USA, Oregon, Umatilla County, Umatilla National Forest, Lincton Mountain Road, 45°47'40"N, 118°9'41"W, 1354 m, on wood, 29 June 2006, *Altermann* 226 (UC 2049992—holotype; UCSC, US, UPS—isotypes). GenBank fungal accession numbers: FJ161594 (ITS), KJ565799 (nuclear ribosomal large subunit (28S)), KJ565519 (elongation factor 1-alpha), KJ564492 (anonymous locus 11), and algal accessions: FJ170688 (ITS) and FJ171043 (actin I intron).

Thallus shrubby-fruticose, highly variable, brilliant lemon yellow (typical material) to chartreuse green, except usually brown to black at the branch tips and whitish (or at least not black) towards the base (corticate portions only; non-corticate portions can be black), varying from loosely subpendent (typical) to tightly tufted, at maturity 5–20 cm long and 4–8 cm wide, copiously branched, the branches terete to more often angular-ridged, coarse, 1–3 mm wide in basal portions, mostly irregularly branching except more or less isotomic-dichotomous towards the tips, lined on ridges with pseudocyphellae, these bearing sparse (typical) or dense globular to weakly cylindrical isidia 0·1–0·3 mm long, these generally concolorous with the adjacent stem cortex, often at length replaced by weakly corticate gymnidia (*sensu* Jørgensen & Kashiwadani 2001).

Apothecia rare, appearing late in development, usually only on large thalli, 0·75–1·50 (–5·00) mm across, solitary, sessile; *disc* pale brown to dark brown, dull to shiny, deeply concave when young, becoming less so with age; thalline margin strongly raised and inflexed when young, with no to few 1–3(–5) mm long fibrils, longer fibrils sometimes branching, underside of margin strongly foveolate and always sorediate; *epihymenium c.* 13 µm, brown; *hymenium* 45–58 µm, hyaline; *ascospores* ellipsoid, 5–7 × 3–4 µm, simple, hyaline.

Pycnidia rare, laminal, immersed, usually with a dark brown to black rim; *ostioles c.* 75 µm diam. *Conidiophores* ± type V (Vobis 1980). *Conidia* 7–9 × 1 µm, straight.

Chemistry. Vulpinic acid and atranorin in the cortex, with norstictic acid in the hymenium of the apothecia (Culberson 1969; Kroken & Taylor 2001).

Etymology. Lupinus alludes to the traditional common name 'Wolf Lichen' (Latin 'lupus' = wolf), which in turn alludes to the former North European custom of mixing *Letharia* (in which the main cortical pigment, vulpinic acid, is toxic) with fat and shards of glass, and applying it to carcasses as bait for wolves (Santesson 1939).

Distribution and ecology. Letharia lupina is widely distributed in western North America (Fig. 1), where it is much more frequently encountered than *L. vulpina*. Of 297 North American specimens, 260 (88%) proved to be *L. lupina* whereas only 37 (12%) belonged to *L. vulpina* s. str. *Letharia lupina* is also the more widespread of the two, with it alone occurring east of the continental divide into Alberta, Saskatchewan, Montana, Wyoming, south-western South Dakota, Utah and Nevada. In mountainous regions, it likewise has a much broader elevational range, extending from valley bottom upwards to treeline (190 m to 3370 m). By contrast, *L. vulpina* appears to be restricted mostly to valley elevations below *c.* 1600 m in the southern portions of its range and only up to *c.* 800 m further north. In the Old World, *L. lupina* is currently known only from mountainous areas of Morocco (Arnerup *et al.* 2004) and Switzerland (Table 1).

The identity of the algal partner in the single Swiss specimen has been checked by us, and belongs to 'clade B', as in North America. *Letharia lupina* is commonly found on old fence posts as well as on the decorticated trunks and branches of a variety of conifers, especially *Pinus*. Over much of its range it also occurs on conifer bark, including that of *Abies*, *Calocedrus*, *Picea*, *Pinus*, and *Pseudotsuga*. On hardwood trees it most commonly colonizes wood, as in the case of *Arbutus*, *Arctostaphylos*, *Populus*, and *Salix*, though we have also seen it on the corticated branches of *Betula* and *Quercus*. Saxicolous forms of this species are also occasionally found on granitic outcrops.

In an early attempt to distinguish *L. lupina* from *L. vulpina*, Goward (1999) called attention to the former's more vivid yellow thallus, looser branching, and sparser production of isidia. Although these characters do tend to correlate with *L. lupina* in northern portions of its range, subsequent examination of sequenced material of both *L. lupina* and *L. vulpina* from across their respective distribution areas has shown that thallus colour, branching and isidia production are highly plastic in these species, and span a similar range of variability (Kroken & Taylor 2001; Ryan 2002; Arnerup *et al.* 2004). Hence we conclude that thallus morphology does not in this case provide a reliable character for species identification, except perhaps locally.

Letharia vulpina (L.) Hue

MycoBank No.: MB393671

Hue, *Nouv. Arch. Mus. Hist. Nat.* **1:** 27 (1899); *Evernia vulpina* Ach. *Lichenogr. Universalis* (1810); *Lichen vulpinus* L., *Sp. Pl.* **2:** 1155 (1753); type: Sweden, designated by Jørgensen *et al.* (1994) (LINN 1273298—lectotype, upper left specimen).

Thallus morphology in *L. vulpina* exhibits the same range of variation as *L. lupina* and cannot be used to distinguish the species (see above). Ryan (2002) provides a detailed description.

Chemistry. Vulpinic acid and atranorin in the cortex, with norstictic acid in the hymenium of the apothecia (Culberson 1969; Kroken & Taylor 2001).

Distribution and ecology. *Letharia vulpina* s. str. is widespread in western North America west of the continental divide, where it ranges south from central British Columbia to southern California (Fig. 2). Throughout its range it is most common in dry coniferous forests (also chaparral), mostly at elevations below *c.* 800 m in the north and 1600 m in the south. Whereas *L. vulpina* appears to be much less common than *L. lupina* in North America (see above), the opposite seems to be true in the Old World, where molecular studies have confirmed its presence in Italy, Sweden, Switzerland, Turkey, Morocco, and the Caucasus (Högberg *et al.* 2002; Arnerup *et al.* 2004). *Letharia lupina*, by contrast, has so far been documented in the Old World only from Morocco and Switzerland (see above). In North America, *L. vulpina* is most commonly found on fence posts and on the wood and bark of *Pseudotsuga menziesii* and *Pinus ponderosa*. We have also found it on *Pinus coulteri*, *P. jeffreyi*, *Pseudotsuga macrocarpa*, *Juniperus californicum* and *Arctostaphylos*. In the Old World, we have confirmed *Letharia vulpina* on *Larix*, *Cedrus*, and on *Pinus sylvestris*. In common with *L. lupina*, *L. vulpina* is most frequent in sunny, summer-dry regions, where both species likely benefit from frequent exposure to night-time dew or fog.

Remarks. Molecular data suggest a close relationship between *L. vulpina* and *L. columbiana* 'rugosa' (Altermann *et al.* 2014). According to Kroken & Taylor (2001), the branches of *L. columbiana* 'rugosa' are more rugose than those of *L. vulpina*, though whether this reflects mere ecotypic variation remains uncertain. On the other hand, the apothecia of *L. vulpina*, when present, are consistently smaller relative to branch width than those of any of the four *L. columbiana* s. lat. species-level lineages. Speciation probabilities inferred under the multispecies coalescent provided unambiguous support for recognizing *L. vulpina* as a lineage distinct from *L.* 'rugosa' (and all other candidate *Letharia* species; Table 3).

Letharia columbiana (Nutt.) Thoms.

MycoBank No.: MB345259

Thomson, *Taxon* **18:** 535 (1969); *Borrera columbiana* Nutt., *J. Acad. Nat. Sci Philadelphia* **7:** 5 (1834).

Type: USA, Oregon, Umatilla County, Umatilla National Forest, Lincton Mountain Road, 45°49' 45·8"N, 118°12'43·7"W, 1221 m, 4 June 2014, *Altermann & Hutchison* 280B (UC—epitype designated here). GenBank accession numbers: KT453735 (ITS), KT453811 (nuclear ribosomal large subunit (28S)), KT453878 (DNA replication licensing factor (*Mcm7*)); KT453919 (RNA polymerase II largest subunit (*RPB1*)); KT453939 (RNA polymerase II second largest subunit (RPB2)); and KT453855 (mitochondrial small subunit ribosomal RNA (mtSSU)). USA, Oregon, Walla Walla, 1833, *Wyeth* (PH—holotype).

Letharia columbiana s. str. appears to vary across the same range of variation as *L. columbiana* s. lat., for example, including *L.* 'barbata' and *L.* 'rugosa', though further work is required to confirm this. A full morphological description of *L. columbiana* s. lat. is given in Ryan (2002). *Letharia gracilis* can be recognized by its sparse, slender, smooth, and drooping branches (McCune & Altermann 2009).

Chemistry. Vulpinic acid and atranorin in the cortex. In contrast with all other *Letharia* lineages (i.e. including 'barbata,' 'rugosa,' and *L. gracilis*), norstictic acid is absent in the apothecial hymenium in this species (Kroken & Taylor 2001, and see Culberson 1969).

Etymology. '*Columbiana*' refers to the Columbia River along which Nathaniel Wyeth travelled and collected in 1833. However, the holotype specimen was collected not along the Columbia River itself but along its tributary, the Walla Walla River, probably in northern Oregon where pine trees are common.

Distribution and ecology. Letharia columbiana s. lat. is widely distributed in western North America west of the cordillera, but is less frequently encountered than *L. vulpina* and *L. lupina*. As far as is currently known, its range extends north to southern intermontane British Columbia, south to southern California, and east to Wyoming (see map in Brodo *et al.* 2001). Sequenced collections include Crater Lake, Oregon; Donner Pass, California; Toiyabe National Forest, California; Stanislaus National Forest, California; Umatilla National Forest, Oregon; Okanagan, Washington; Spokane River, Washington; Bighorn National Forest, Wyoming; and Coldwater River drainage, British Columbia (Kroken & Taylor 2001, and see Supplementary Material Table S1). *Letharia columbiana* s. lat. has been found on old fence posts as well as on the decorticated trunks and branches of conifers such as *Picea engelmannii*, *Pinus contorta*, *P. lambertiana*, *Abies concolor*, and *A. magnifica*. S. Kroken found that *L. columbiana* s. lat. fungi pair only with *Trebouxia* 'clade B' algae, as *L. lupina* does (Kroken & Taylor 2000), but we have since found *L. columbiana* pairs with both clades of algae (unpublished data).

Remarks. The holotype consists of two heterogeneous tufts. Most of the material belongs to *L. vulpina* s. lat., which obscures the apotheciate thalli upon which J. Thomson based the name *L. columbiana*. According to annotations on the holotype sheet, S. Kroken attempted and failed to extract DNA from one of the apothecia. The holotype is also compromised by zygomycete conidiophores at the base of each clump.

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Supplementary Material

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0024282916000396

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