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ORIGINAL ARTICLE

Local representation of global diversity in a cosmopolitan lichen-forming fungal species complex (*Rhizoplaca*, Ascomycota)

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ABSTRACT

Aim The relative importance of long-distance dispersal versus vicariance in determining the distribution of lichen-forming fungi remains unresolved. Here, we examined diversity and distributions in a cosmopolitan lichen-forming fungal species complex, *Rhizoplaca melanophthalma sensu lato* (Ascomycota), across a broad, intercontinental geographical distribution. We sought to determine the temporal context of diversification and the impacts of past climatic fluctuations on demographic dynamics within this group.

Location Antarctica, Asia, Europe, North America and South America.

Methods We obtained molecular sequence data from a total of 240 specimens of *R. melanophthalma* s.l. collected across five continents. We assessed the monophyly of candidate species using individual gene trees and a tree from a seven-locus concatenated data set. Divergence times and relationships among candidate species were evaluated using a multilocus coalescent-based species tree approach. Speciation probabilities were estimated using the coalescent-based species delimitation program BPP. We also calculated statistics on molecular diversity and population demographics for independent lineages.

Main conclusions Our analyses of *R. melanophthalma* s.l. collected from five continents supported the presence of six species-level lineages within this complex. Based on current sampling, two of these lineages were found to have broad intercontinental distributions, while the other four were limited to western North America. Of the six lineages, five were found on a single mountain in the western USA and the sixth occurred no more than 200 km away from this mountain. Our estimates of divergence times suggest that Pleistocene glacial cycles played an important role in species diversification within this group. At least three lineages show evidence of recent or ongoing population expansion.

Keywords

BEAST, **biogeography,** BPP, **coalescent, cryptic species, long-distance dispersal,** ***Rhizoplaca melanophthalma*****, speciation.**

INTRODUCTION

Lichen-forming fungi are obligate symbionts with photoautotrophic organisms, mainly green algae and/or cyanobacteria. The lichen symbiosis has been highly successful within fungi, especially Ascomycota, with an estimated diversity greater than 28,000 species (Lücking *et al.*, 2009a). Lichens play a variety of important ecological roles, including the colonization of bare soil and rocks (Nascimbene *et al.*, 2009), stabilization of soil in arid and semi-arid regions (Belnap & Eldridge, 2001), and contributing to nitrogen influx in some ecosystems (Ponzetti & McCune, 2001; Gavazov *et al.*, 2010; Zhao *et al.*, 2010; Raggio *et al.*, 2012). Additionally, lichens are commonly used as bioindicators to assess environmental disturbance (McCune, 2000; Nimis *et al.*, 2002; Bjerke, 2011; Leavitt & St. Clair, 2011).

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Notwithstanding the overall importance of lichen diversity, population structure and distribution patterns are uncertain for most lichen symbionts (Werth, 2010). The assessment of biogeographical patterns in lichens has been complicated by a lack of reliable data (Culberson, 1972). For example, traditional phenotype-based approaches to species recognition have been shown to underestimate diversity in some cases (e.g. Kroken & Taylor, 2001; Divakar *et al.*, 2005; Baloch & Grube, 2009; Molina *et al.*, 2011), and unrecognized lineages may be hidden under widely distributed or disjunct nominal species (Culberson, 1972; Argüello *et al.*, 2007; Thell *et al.*, 2009). In some cases, a careful phenotypic re-examination in light of a molecular phylogeny may reveal previously overlooked characters supporting distinct phylogenetic lineages (McCune & Altermann, 2009; Divakar *et al.*, 2010; Spribille *et al.*, 2011). Owing to the availability of genetic data and related analytical improvements, DNA-based approaches play an increasing role in the recognition of diversity and distributions in lichenized fungi that would otherwise be difficult to discern using traditional phenotypic characters (Divakar *et al.*, 2010; Leavitt *et al.*, 2011a; Rivas Plata & Lumbsch, 2011). Improved species recognition has important implications for a better understanding of biogeographical patterns and factors promoting diversification (Crespo & Lumbsch, 2010; Lumbsch & Leavitt, 2011).

When intercontinental dispersal is common, one should expect to see genetic homogeneity in populations distributed on different continents (Werth, 2011). Broad, intraspecific distributions spanning multiple continents and ecological zones have been confirmed for some lichen symbionts (Crespo *et al.*, 2002; Printzen *et al.*, 2003; Fernández-Mendoza *et al.*, 2011; Leavitt *et al.*, 2012a, 2013a). Disjunct cosmopolitan distributions have been explained alternatively by range fragmentation of ancient species' distributions and widespread long-distance dispersal (Culberson, 1972; Printzen *et al.*, 2003; Geml *et al.*, 2010). However, the role of long-distance dispersal versus vicariance in lichen-forming fungi remains largely unresolved (Printzen *et al.*, 2003; Geml *et al.*, 2010, 2012; Amo de Paz *et al.*, 2011). While evidence for intraspecific long-distance dispersal has been documented (Buschbom, 2007; Geml *et al.*, 2010), assessing diversification and biogeographical patterns within a temporal context remains largely unexplored in nearly all groups of lichenized fungi, with some exceptions (e.g. Otálora *et al.*, 2010; Amo de Paz *et al.*, 2011; Sérusiaux *et al.*, 2011; Leavitt *et al.*, 2012b,c). This is largely due to a poor fossil record for lichenized fungi and uncertainties in the interpretation of the few known fossil records (Taylor & Berbee, 2006; Lücking *et al.*, 2009b; Berbee & Taylor, 2010).

Rhizoplaca melanophthalma (DC.) Leuckert & Poelt is known from largely disjunct populations on all continents except Australia (Fig. 1; Egea, 1996; Ryan, 2001; Castello, 2010; Ruprecht *et al.*, 2012). This species occurs on exposed calcium-poor rock, and ranges in distribution from extremely arid continental habitats to upper montane coniferous forests and the lower portions of the alpine tundra (McCune, 1987; Ryan, 2001). Analyses of molecular sequence data have indicated that traditional phenotype-based species circumscriptions fail to recognize multiple species-level lineages within the nominal mycobiont taxon *R. melanophthalma* (Leavitt *et al.*, 2011b). The *R. melanophthalma* species complex (*sensu* Leavitt *et al.*, 2011b) includes a morphologically and chemically diverse assemblage of growth forms (McCune, 1987; Ryan, 2001). Within *R. melanophthalma sensu lato* (s.l.), Leavitt *et al.* (2011b) circumscribed six 'candidate' species that were supported using multiple lines of evidence

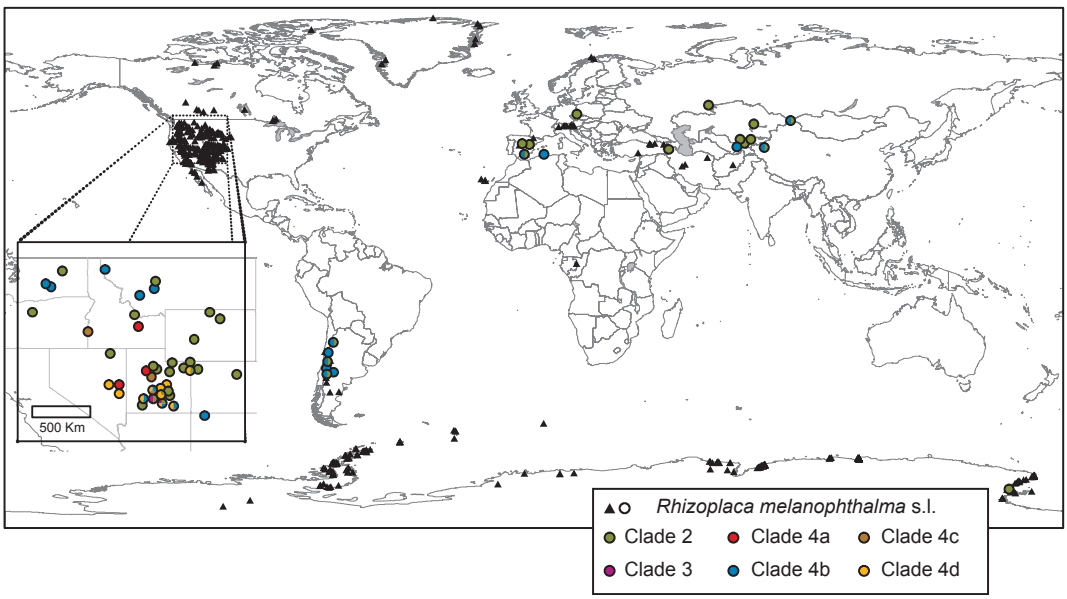

Figure 1 Geographical distribution of *Rhizoplaca melanophthalma sensu lato*. Filled triangles indicate species records from the Global Biodiversity Information Facility database and the Consortium of North American Lichen Herbaria. Coloured circles indicate sampled geographical populations and colours indicate the proportion of sampled lineages within that geographical population.

from molecular sequence data, including: fixed nucleotide characters, genealogical exclusivity, Bayesian population clustering and the coalescent-based species delimitation program BPP (Bayesian Phylogenetics and Phylogeography; Yang & Rannala, 2010). This last method has recently been shown to outperform other species-delimitation methods under a variety of scenarios (Camargo *et al.*, 2012a). Additionally, distinct species-level lineages in the *R. melanophthalma* group are known to occur sympatrically in western North America with strong evidence of reproductive isolation among lineages, and thus *de facto* species status (Leavitt *et al.*, 2011b).

Previous studies have suggested that lineages within the *R. melanophthalma* complex may be broadly distributed (Arup & Grube, 2000; Leavitt *et al.*, 2011b). These may potentially serve as valuable groups for assessing dispersal capacity and landscape-level genetics in response to changing climatic conditions. In addition, *R. melanophthalma* s.l. is frequently used in air quality biomonitoring studies (Dillman, 1996; Ugur *et al.*, 2004) and has been shown to have pharmaceutical potential for treating drug genotoxicity in human blood (Geyikoglu *et al.*, 2007). Therefore, accurate specimen identifications and interpretation of biogeographical patterns may have important implications for biomonitoring and pharmaceutical research.

Currently, molecular species circumscriptions within *R. melanophthalma* s.l. have largely been restricted to collections made in the Intermountain Region of western North America (Leavitt *et al.*, 2011b). Data from a broader geographical sampling are essential for understanding distribution patterns of species-level lineages within this cosmopolitan species complex. The objectives of this paper are: (1) to assess the distribution of candidate species-level lineages within the *R. melanophthalma* complex within a broad geographical context; and (2) to estimate divergence times among species-level lineages using a coalescent-based multilocus species tree approach. In this study, we analysed genetic data generated from *R. melanophthalma* s.l. specimens collected from five continents and report on the distribution patterns of species-level lineages and divergence times within this complex.

MATERIALS AND METHODS

Taxon sampling

Our sampling focused on *Rhizoplaca melanophthalma* s.l. populations from western North America, with supplementary collections from Antarctica, Central Asia, Europe and South America. Poelt (1989) suggested that the arid mountain regions in western North America were one of two centres of distribution for placodioid *Lecanora* diversity, including *Rhizoplaca*. Subsequent studies have supported Poelt's observation (Ryan & Nash, 1993, 1997; Ryan, 2001), and we assume that the *R. melanophthalma* complex follows this pattern. Western North American collections were initially made along an elevational gradient (2200–3400 m) on Thousand Lakes Mountain and the neighbouring Boulder Mountain Plateau, Wayne County, UT, USA (Porter, 1999; Leavitt *et al.*, 2011b). In order to assess distribution patterns for these candidate species within a broader geographical context, we analysed additional specimens from Antarctica (1 specimen), Austria (1), Chile (29), China (12), Czech Republic (3), Iran (16), Kazakhstan (1), Kyrgyzstan (5), Russia (1), Spain (17) and Switzerland (1), in addition to a total of 150 specimens from the USA. We included representatives of vagrant forms identified as *Rhizoplaca idahoensis* Rosentreter & McCune (1 specimen) and *Rhizoplaca haydenii* (Tuck.) W.A. Weber (2 specimens), which have been shown to belong to a monophyletic lineage within the *R. melanophthalma* complex (Leavitt *et al.*, 2011b). *Lecanora novomexicana* H. Magn. was recovered with strong support as the sister group to the remaining lineages within the *R. melanophthalma* group (Leavitt *et al.*, 2011b) and was selected as the outgroup for phylogenetic analyses. A total of 240 specimens were included in the present study (Fig. 1, and see Appendix S1 in Supporting Information).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from a small piece of thallus material using the PrepEase DNA Isolation Kit (USB, Cleveland, OH, USA). Using the primers ITS1F (Gardes & Bruns, 1993), ITS4 (White *et al.*, 1990) and ITS4a (Larena *et al.*, 1999), we amplified the complete internal transcribed spacer region (ITS, *c.* 520 bp) for 104 new specimens collected for this study. For a subset of all specimens (Appendix S2), we amplified fragments from the nuclear ribosomal intergenic spacer region (IGS, *c.* 370 bp) and the protein-coding markers β-tubulin (*c.* 670 bp), elongation factor 1, *EF1* (*c.* 460 bp), *MCM7* (*c.* 540 bp), *RPB1* (*c.* 820 bp) and *RPB2* (*c.* 750 bp). Primers used to amplify the ITS, IGS, β-tubulin and *MCM7* markers are documented in Leavitt *et al.* (2011b). We amplified the *EF1* fragment using EF1-983F with EF1-1567R (Rehner, 2001); the *RPB1* fragment was amplified using gRPB1-A (Stiller & Hall, 1997) and fRPB1-C (Matheny *et al.*, 2002); and the *RBP2* fragment was amplified using a newly designed forward primer, RPB2_Rhizo_F (5′-TDGCRCTSATGTGYTAYATCACWGT-3′), with fRPB2-7cr (Liu *et al.*, 1999). We generated sequence data for all seven loci from three to 13 individuals per lineage. Standard PCR amplifications were conducted in 25-μL reaction volumes. In some cases where standard PCR failed, we used Ready-To-Go PCR Beads (GE Healthcare, Pittsburgh, PA, USA) with improved success. PCR cycling parameters for amplifying the ITS region followed a 66–56 °C touchdown reaction (Lindblom & Ekman, 2006), and cycling parameters for the IGS, β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2* followed a 55–50 °C touchdown reaction (Lindblom & Ekman, 2006). PCR products were visualized on 1% agarose gel and cleaned using ExoSAP-IT (USB, Cleveland, OH, USA). Complementary strands were sequenced using the same primers used for amplifications. Sequencing reactions were performed

using BigDye 3.1 (Applied Biosystems, Foster City, CA, USA). Products were run on an ABI 3730 automated sequencer (Applied Biosystems) at the Pritzker Laboratory at the Field Museum, Chicago, IL, USA.

DNA analyses

Alignment

We assembled and edited sequences using SEQUENCHER 4.10 (Gene Codes Corporation, Ann Arbor, MI). Sequence identity was confirmed using the MEGABLAST search algorithm in GenBank (Wheeler *et al.*, 2006). Sequences were aligned using the program MAFFT 6 (Katoh *et al.*, 2005; Katoh & Toh, 2008). For the β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2* markers, we implemented the G-INS-i alignment algorithm (recommended for sequences with global homology) and '1PAM/K = 2' scoring matrix (recommended when aligning closely related sequences), with an offset value of 0.9 (recommended when long gaps are not expected), with the remaining parameters set to default values. For the IGS and ITS markers, we used the same parameters, with the exception of an offset value of 0.1.

Phylogenetic analyses

In order to assess the monophyly of the candidate species with the increased geographical sampling, we used the program RAxML 7.2.8 (Stamatakis, 2006; Stamatakis *et al.*, 2008) to reconstruct a maximum likelihood (ML) gene tree from the ITS alignment of all 240 specimens. A search combining 200 separate ML searches was conducted, implementing the GTR+G model, and 1000 pseudoreplicates to evaluate bootstrap support for each node. We also performed ML analyses of each individual gene alignment (IGS, β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2*) for a subset of our total sample (Appendix S2). Search parameters and assessment of nodal support were performed as described above.

Relationships among candidate species were estimated from the seven-locus data matrix (Appendix S2) using a total-evidence approach (Kluge, 1989), and a coalescent-based multilocus species-tree approach (Edwards, 2009; Knowles, 2009; Liu *et al.*, 2009; Blair & Murphy, 2011). We conducted an ML analysis of the combined data set using locus-specific model partitions in RAxML. All loci were treated as separate partitions. Search parameters and assessment of nodal support were performed as described above.

Species trees and divergence time estimates

Estimating a species tree using concatenated multilocus sequence data has been shown to be misleading under certain divergence scenarios (Degnan & Rosenberg, 2006, 2009; Leaché, 2009). Therefore, we used the coalescent-based hierarchical Bayesian model *BEAST implemented in BEAST 1.7.4 (Heled & Drummond, 2010) to estimate a species tree for the *R. melanophthalma* complex. *BEAST estimates the species tree directly from the sequence data, and incorporates the coalescent process and the uncertainty associated with gene trees and nucleotide substitution model parameters (Heled & Drummond, 2010). We assigned all individuals with multilocus sequence data to a 'species' group based on the monophyletic groups recovered in the ITS gene tree, which corresponded to previously recognized species-level lineages (Leavitt *et al.*, 2011b). Coalescent-based species tree methods using multiple independent loci have been shown to perform accurately with as few as two to three individuals per species, with increasing performance in speciation histories with deeper total tree depths (Camargo *et al.*, 2012b; Lanier & Knowles, 2012). In the *R. melanophthalma* complex, all species lineages were fully sorted in the ITS topology and in many cases across other independent loci (Leavitt *et al.*, 2011b), suggesting a relatively deep divergence history. We therefore assumed that our sampling was adequate for the molecular analyses described below.

Species tree methods incorporating the process of gene lineage coalescence are likely to provide a more biologically realistic framework for dating divergence events, because they can directly model genetic divergence that pre-dates speciation (McCormack *et al.*, 2011). We therefore estimated divergence dates using a coalescent-based species tree approach implemented in *BEAST. Models of DNA sequence evolution for each marker were selected using the program JMODELTEST 0.1 (Posada, 2008), using the Akaike information criterion. In the absence of relevant fossil evidence for the *R. melanophthalma* complex, we used the molecular evolution rates for the ITS marker [2.43×10^{-9} substitution/site/year (s/s/y)] recently reported for the lichen-forming genus *Melanelixia* (Parmeliaceae, Lecanoromycetes; Leavitt *et al.*, 2012b) to estimate the time to the most recent common ancestor (MRCA) for all clades. This estimated substitution rate is similar to other estimates of ITS substitution rates for both lichen-forming mycobionts (2.38×10^{-9} s/s/y *Oropogon*, Parmeliaceae, Lecanorales; Leavitt *et al.*, 2012c) and a non-lichenized fungus (2.52×10^{-9} s/s/y, Erysiphales; Takamatsu & Matsuda, 2004). Implementing an uncorrelated relaxed lognormal clock (Drummond *et al.*, 2006), we selected a Yule process and gamma-distributed population sizes for the species-tree prior and a piecewise linear population size model with a constant root. Default values were used for remaining priors. Substitution rates for the IGS, β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2* markers were coestimated along the run under a uniform prior (from 0 to 15) relative to the rate for the ITS locus. Two independent Markov chain Monte Carlo (MCMC) analyses were run for a total of 50 million generations, sampling every 2000 steps and excluding the first 12.5 million generations of each run as burn-in. We assessed convergence by examining the likelihood plots through time using TRACER 1.5 (Rambaut & Drummond, 2009) and compared summarized tree topologies from separate runs; the effective sample sizes (ESS) of parameters of interest were all above 200. The posterior

probabilities of nodes were computed from the sampled trees (excluding burn-in samples) using TreeAnnotator 1.7.4 (Rambaut & Drummond, 2010).

Speciation probabilities

While morphological and chemical character differences have traditionally served as proxies for identifying reproductively isolated groups, multilocus coalescent-based species delimitation methods can provide a more direct assessment of gene flow and independent lineage status through genetic analysis (Fujita *et al.*, 2012). These coalescent-based methods provide an objective and replicable approach to assess hypotheses of evolutionary independence, regardless of whether putative lineages differ in potentially subjective phenotypic character systems (Fujita *et al.*, 2012). We estimated the marginal posterior probability of speciation using the program bpp 2.1 (Rannala & Yang, 2003; Yang & Rannala, 2010). bpp has recently been shown to outperform other coalescent-based species delimitation methods, with robust performance using a modest number of genetic markers even in cases of recent speciation (Camargo *et al.*, 2012a). We used the gamma prior G(2, 1000) on ancestral population size (θ) and G(2, 1000) was used on root age (τ_0) with algorithm 0. The remaining divergence time parameters were assigned the Dirichlet prior (Yang & Rannala, 2010). Because the prior distribution of θ and τ_0 can result in strong support for models containing more species (Leaché & Fujita, 2010), we also explored two more conservative combinations of priors – the first favouring fewer species by assuming large ancestral population sizes, G(1, 10) and relatively shallow divergences among species, G(2, 2000), and the second assuming intermediate ancestral population sizes, G(1, 100), and relatively shallow divergences among species, G(2, 2000). The maximum clade credibility species tree estimated in the *beast analysis, representing the six candidate species, was used as the fully resolved guide tree. Running a reversible-jump MCMC sampler for 1,000,000 generations with a burn-in of 100,000 produced consistent results across independent analyses initiated with different starting seeds and species trees. Each analysis was run at least twice to confirm consistency between runs. In cases where relationships were not strongly supported in the coalescent-based species tree, exploratory analyses using different guide trees representing alternative topologies resulted in similar speciation probabilities among the topologies (results not shown).

Molecular diversity and population demographics

We used DnaSP 4.50 (Librado & Rozas, 2009) to calculate estimates of genetic diversity for each species (including: number of haplotypes, *h*; haplotypic diversity, *Hd*; number of polymorphic sites, *S*; and nucleotide diversity, π) from the ITS sequence data. To detect possible departures from a constant population size that could be interpreted as a result of a past demographic expansion, we calculated Fu's F_S statistic (Fu, 1997) and Tajima's *D* (Tajima, 1989) for each species (*R. haydenii* and *R. idahoensis* and clades C4a and C4b were excluded due to small sample sizes). Significant and negative values of Tajima's *D* and Fu's F_S are indicative of possible population expansion, and positive values of these sample statistics provide evidence of a recently bottlenecked population or diversifying selection. These statistics were calculated in DnaSP, and significance was determined using the coalescent process implemented in DnaSP (1000 replicates).

RESULTS

The complete ITS data matrix consisted of 240 sequences and 584 aligned nucleotide positions (Appendix S1; TreeBase ID: 13903). The seven-locus data matrix, representing genetic diversity identified from the ITS gene tree, consisted of 40 samples and 4179 aligned base pairs in total (Appendix S2; TreeBase ID: 13903). All new sequences generated for this study have been deposited in GenBank under accession numbers JX948190–JX948294. Table 1 summarizes the patterns of variation in the sampled loci and the best-fitting models of evolution.

Gene trees and distributions

In the ITS gene tree estimated in RAxML, all new sequences generated from this study were recovered within monophyletic clades corresponding to previously identified candidate species-level lineages (Fig. 2; Appendix S3). All candidate species were recovered with strong statistical support [bootstrap support (BS) > 75%], with the exceptions of lineage C4b (BS = 67%) and lineage C4c (BS = 66%). Intercontinental distributions were identified in two lineages, C2 and C4b (Fig. 2; Appendix S1). In many cases, specimens of the broadly distributed lineages (clades C2 and C4b) collected from geographically distinct regions shared identical ITS haplotypes (Table 2). In contrast, only specimens collected in western North America were recovered in clades C3, C4a, C4c and C4d (Fig. 2, Appendix S1). Of the six species-level lineages within the *R. melanophthalma* complex, five were collected from Thousand Lakes Mountain, UT, USA. The single lineage not collected on Thousand Lakes Mountain (C4a) was collected from a site in Juab County, UT, less than 200 km away.

Individual gene trees are shown in Fig. 3. Monophyly and bootstrap support for all clades is summarized for all single-gene topologies in Table 3. Despite the strong support for many lineages in individual gene trees, well-supported relationships among species were largely discordant among gene topologies. In the total-evidence analysis, the partitioned ML analysis of the combined ribosomal and protein-coding genes is presented in Fig. 4. The concatenated ML topology is characterized by well-supported monophyletic lineages corresponding to candidate lineages circumscribed in Leavitt *et al.* (2011b), with the exception of lineage C4d (BS < 50%).

Table 1 Genetic variability of sampled markers in the *Rhizoplaca melanophthalma* species complex, including the number of specimens (*n*) and number of unique haplotypes (in parentheses), alignment length (bp), number of variable sites, number of parsimony-informative (PI) sites for each sampled locus, and the model of evolution identified for each locus using the Akaike information criterion in jModelTest. Collections were made in Antarctica, Asia, Europe and North and South America.

Locus	*n*	Aligned length	No. of variable sites	No. of PI sites	Model selected
ITS (Total)	240 (110)	584	145	82	TIM2+I+G
ITS	40 (28)	568	68	48	HKY+G
IGS	38 (29)	370	70	51	HKY+I
β-tubulin	40 (20)	668	49	39	K80
EF1	38 (14)	463	34	28	TrNef+G
MCM7	39 (20)	539	49	31	TrN+I
RPB1	39 (18)	819	43	33	K80+G
RPB2	39 (14)	752	53	43	TrN+I

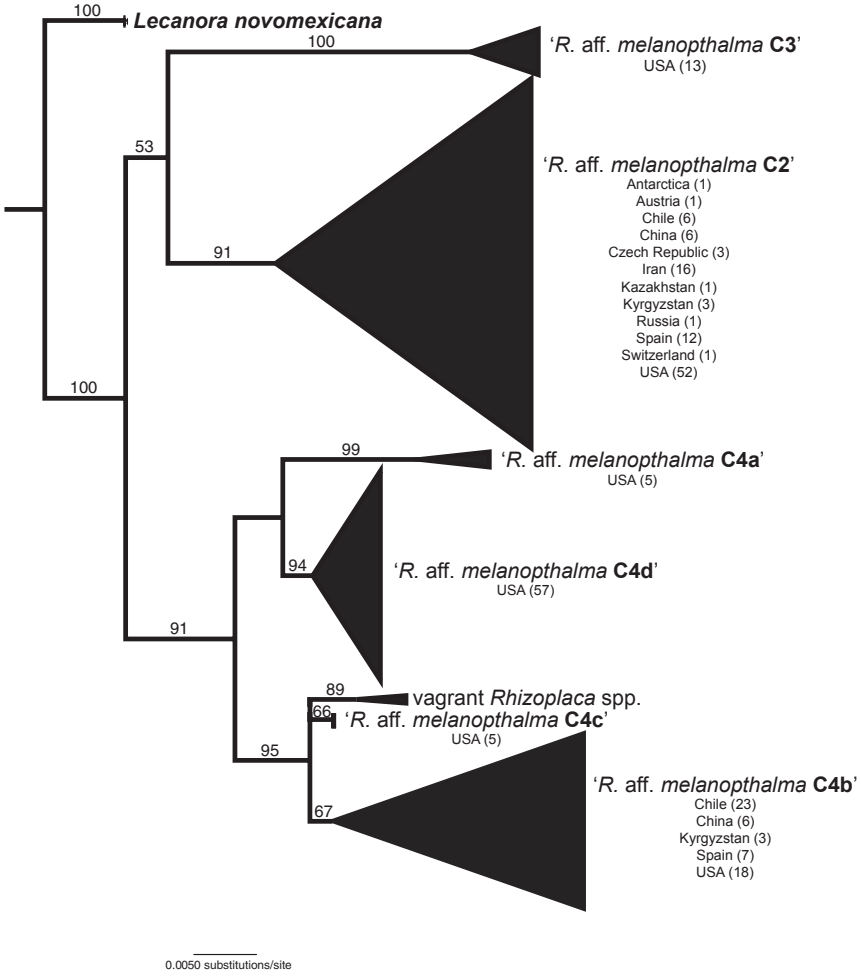

Figure 2 Cartoon representation of the maximum likelihood ITS topology obtained from 240 *Rhizoplaca melanophthalma sensu lato* specimens. Values at each node indicate non-parametric bootstrap support; only support values > 50% are indicated. Tip labels represent the six candidate species-level lineages; the vagrant taxa *Rhizoplaca haydenii* and *R. idahoensis* are combined into a single clade 'vagrant *Rhizoplaca* spp.'. The country of origin for all specimens recovered within each clade is indicated below the tip label.

Coalescent-based species tree and divergence estimates

Large effective sample sizes (ESS > 200) were observed for all parameters in the *beast analyses. The time-calibrated maximum clade credibility chronogram from the multi-locus species tree analysis is shown in Fig. 5. The substitution rates of the seven sampled loci, estimated under a relaxed clock, are reported in Table 4. The initial split between *Lecanora novomexicana* and the *R. melanophthalma* complex was

Table 2 Shared ITS haplotypes across intercontinental populations of *Rhizoplaca melanophthalma sensu lato*. The 'DNA ID no.' refers to an individual representing the shared ITS haplotype.

DNA ID no.	Geographical origin
R. aff. *melanophthalma* 'C2'	
720	USA, China, Chile, Spain, Switzerland
4610	USA & Spain
5186	Chile & Iran
China_1985	Chile, China, Spain
Spain_1983	Spain & USA
R. aff. *melanophthalma* 'C4b'	
551	Chile, Spain, USA
6028	China, Chile, Kyrgyzstan
Chile_6838	Chile & China

estimated to have occurred during the Miocene, *c.* 8.7 Ma (95% highest posterior density, HPD: 5.2–12.7 Ma), and the initial radiation of the *R. melanophthalma* group during the Pliocene, *c.* 4.4 Ma (95% HPD: 2.7–6.3 Ma). Divergence estimates indicate that the majority of the diversification leading to extant species, including the vagrant species, occurred during the Pleistocene (Fig. 5).

Speciation probabilities

Speciation probabilities (SP) estimated using the program BPP are shown in Fig. 5. With the exception of the split between the two vagrant species (*R. haydenii* and *R. idahoensis*), high speciation probabilities (SP $\geq$ 0.95) were estimated at all nodes, using both the default prior gamma distributions for θ [G(2, 1000)] and τ_0 [G(2, 1000)] and a more moderate combination of these priors – G(2, 100) and G(2, 2000) for θ and τ_0, respectively. Under the most conservative combination of priors – θ, G(2, 10) and G(2, 2000) for θ and τ_0, respectively – speciation probabilities match those supported using the default priors, with the exception of lower probabilities for a split between C4d and C4c (SP < 0.50).

Molecular diversity and population demographics

Genetic diversity indices (*Hd*, *S* and π) for species within the *R. melanophthalma* species complex are summarized in Table 5. Tajima's *D* and Fu's F_S statistics were significant ($P < 0.05$) and negative for lineages C2, C4b and C4d (Table 5). No tests were carried out for clades C4a and C4c or the vagrant species *R. haydenii* and *R. idahoensis*, because of their small sample sizes.

DISCUSSION

Our analyses of specimens of *R. melanophthalma* s.l. collected from five continents support the presence of the six species-level lineages within this nominal species identified previously from collections made in western North America (Leavitt *et al.*, 2011b). Despite the increased sampling in this study, including populations from Antarctica, Central Asia, Europe and South America, we did not identify any additional species-level lineages within this complex. Two of the six lineages were found to have broad intercontinental distributions (clades C2 and C4b), and in many cases individuals shared identical ITS haplotypes among geographically disjunct populations (Table 2). Based on the current sampling, the other four lineages were found exclusively in western North America. Surprisingly, of the six known species-level lineages within *R. melanophthalma* s.l., five are found on a single mountain in the western USA and the sixth is known to occur at a distance of no greater than 200 km from that site. Our results highlight a striking case in which the known species diversity in a cosmopolitan species complex is represented in a geographically local region.

Specific factors determining distribution patterns for the various distinct lineages within the *R. melanophthalma* species complex remain unclear. However, the broad geographical distributions of clades C2 and C4b and population demographic statistics indicate that these two lineages are likely to have experienced recent population growth (Table 5). In contrast to the broad intercontinental distribution of clades C2 and C4b, clades C3, C4a, C4c and C4d appear to be restricted to western North America. Of these western North American lineages, one clade, C4d, is commonly found on rocks, from lower-elevation pinyon–juniper woodlands to montane coniferous forests and lower alpine tundra. A second lineage, C3, appears to be restricted to subalpine habitats in the south-western USA, where it is locally common. The remaining two lineages known only from western North America occur more rarely throughout lower-elevation habitats in western North America (see Leavitt *et al.*, 2013b). Given the apparent dispersal capacity of other closely related lineages in this species complex (i.e. clades C2 and C4b), it seems unlikely that geographical distributions are restricted to North America due to limited dispersal capacity in these lineages. Although the effective dispersal of lichen-forming fungal species by spores is limited by the availability of appropriate substrata and other ecogeographical factors, other unrecognized dispersal limitations or establishment barriers appear to have limited the distribution of some lineages of *R. melanophthalma* s.l. Alternatively, it has also been proposed that competition for symbiotic partners may be a major driver of diversity in mutualistic relationships (Bruns, 1995; O'Brien *et al.*, 2009), and investigating competition for symbionts may provide insights into mechanisms that may influence distributions.

While previous studies have suggested that Pleistocene glacial cycles played only a minor role in diversification accompanied by speciation in lichen-forming fungi (Otálora *et al.*, 2010; Amo de Paz *et al.*, 2011, 2012; Leavitt *et al.*, 2012a,b,c), the divergence times estimated in this study suggest that the majority of the speciation events in the *R. melanophthalma* complex occurred during the Pleistocene (Fig. 5). The relatively recent diversification history for the *R. melanophthalma* group, its apparent centre of diversity in western North

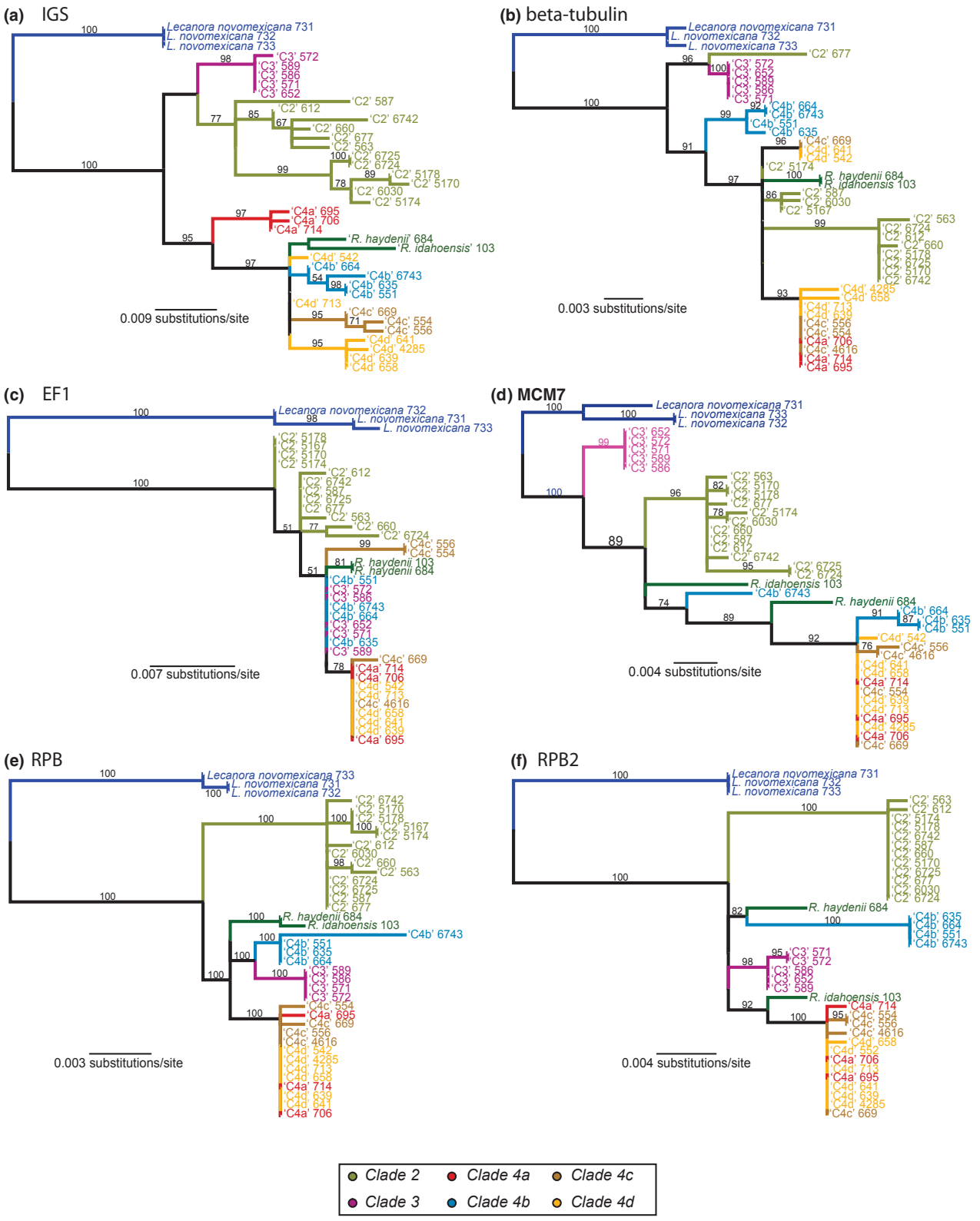

Figure 3 Individual maximum likelihood gene trees (a, IGS; b, β-tubulin; c, *EF1*; d, *MCM7*; e, *RPB1*; f, *RPB2*) inferred from 38–40 specimens representing all candidate species-level lineages in the *Rhizoplaca melanophthalma* species complex using the program RAxML. The ITS topology is shown in Fig. 1 and Appendix S1.

Table 3 Summary of lineage monophyly across the seven sampled loci and a concatenated gene tree for the *Rhizoplaca melanophthalma* species complex. Values indicate nonparametric-bootstrap support estimated in RAxML 7.2.8, and dashes indicate instances where the specific lineage was not recovered as monophyletic. Collections were made in Antarctica, Asia, Europe and North and South America.

	ITS	IGS	β-tubulin	*EF1*	*MCM7*	*RPB1*	*RPB2*	combined
Lineage C2	91%	77%	—	—	96%	100%	100%	100%
Lineage C3	100%	98%	100%	—	99%	100%	98%	100%
Lineage C4a	99%	97%	—	—	—	—	—	100%
Lineage C4b	67%	< 50%	99%	—	—	100%	100%	100%
Lineage C4c	66%	95%	—	—	—	—	—	84%
Lineage C4d	94%	—	—	—	—	—	—	< 50%

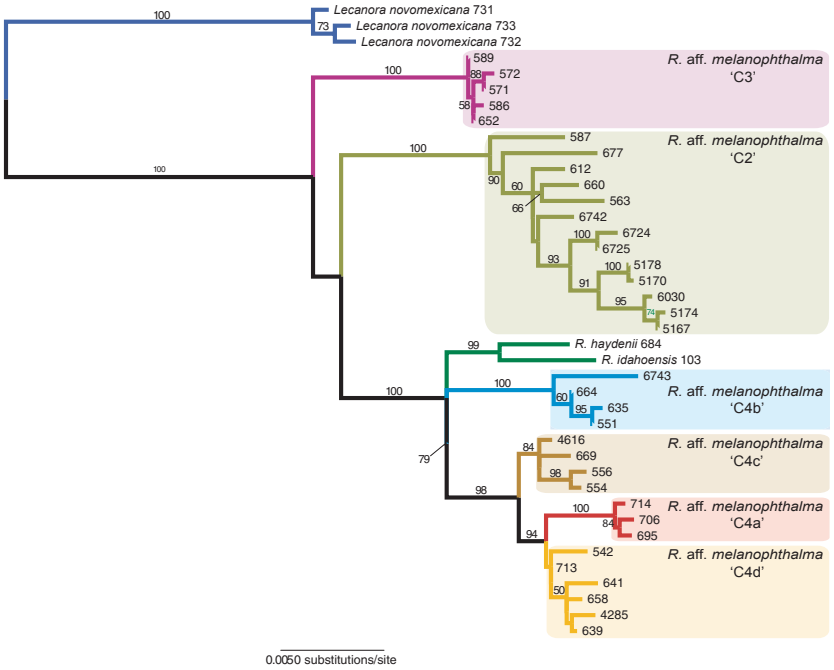

Figure 4 Relationships among 40 specimens representing all candidate species-level lineages in the *Rhizoplaca melanophthalma* species complex inferred from a maximum likelihood analysis of nuclear ribosomal and protein-coding DNA sequence data (ITS, IGS, β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2* markers, 4179 total base pairs). Values at each node indicate non-parametric bootstrap support (percent); only support values > 50% are indicated.

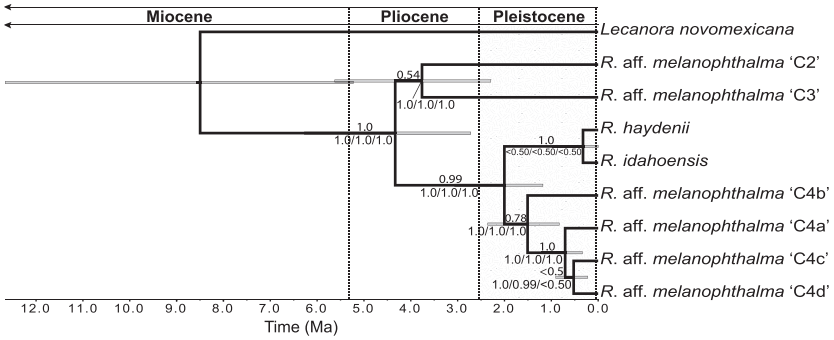

Figure 5 Time-calibrated maximum clade credibility tree for the *Rhizoplaca melanophthalma* species complex. The chronogram was estimated from a multilocus data set (ITS, IGS, β-tubulin, *EF1*, *MCM7*, *RPB1* and *RPB2* markers) within a coalescence-based framework in *BEAST. The divergence times correspond to the mean posterior estimate of their age, in millions of years. The bars indicate the 95% highest posterior density (HPD) interval for the divergence times estimates. Values above branches indicate posterior probability; only values > 0.50 are presented. The three values below branches indicate speciation probabilities estimated using the program BPP 2.1 using default, moderate and conservative priors for the gamma distribution of θ and τ_0 (see text for details).

Table 4 Estimates of substitution rates in the *Rhizoplaca melanophthalma* species complex from the *BEAST analysis of the seven-locus data set estimated under a relaxed molecular clock using fixed substitution rates for the ITS markers (shown in parentheses). Units: substitution/site/10^9 years.

	Multilocus species tree analysis	
Locus	Rate	Rate 95% HPD
ITS	2.41 (2.43)	2.05–2.75
IGS	4.24	2.55–6.13
β-tubulin	1.20	0.69–1.76
EF1	1.19	0.59–1.86
MCM7	1.81	1.03–2.67
RPB1	0.85	0.47–1.30
RPB2	1.26	0.69–1.88

HPD, highest posterior density interval.

Table 5 Estimates of genetic diversity for sampled lineages within the *Rhizoplaca melanophthalma* species complex. Lineages C2 and C4b are represented by collections made in Antarctica (not C4b), Asia, Europe, and North and South America; and lineages C3, C4a, C4c, and C4d were found exclusively in western North America, based on current sampling. Significant values ($P < 0.05$) of Tajima's *D* and Fu's *Fs* are marked in bold type.

Lineage	*n*	*H*	*Hd*	*S*	π	Tajima's *D*	Fu's F_S
C2	98	60	0.974	82	0.00894	**−4.88801**	−70.757
C3	13	5	0.628	5	0.00182	−0.89562	−1.693
C4a	5					n/a	n/a
C4b	57	23	0.757	29	0.00333	**−4.86907**	−22.893
C4c	5					n/a	n/a
C4d	56	10	0.542	11	0.00170	**−3.28120**	−3.272

n, sample size; *H*, number of haplotypes; *Hd*, haplotype diversity; *S*, number of segregating (polymorphic) sites; π, nucleotide diversity.

America, and recent population expansions for at least three of the six lineages (Table 5) support the idea that diversification may have occurred in western North America during the Pleistocene with subsequent long-distance dispersal resulting in the contemporary distribution patterns.

Large areas of North America were subject to global cooling, aridification and major glaciation events during the Pleistocene (Van Devender & Spaulding, 1979; Graham, 1999; Osborn & Bevis, 2001; Pierce, 2003). These major climatic shifts had a substantial impact on vascular plant communities in western North America (Thompson & Anderson, 2000; Pierce, 2003; Coats *et al.*, 2008; Loera *et al.*, 2012) and are also likely to have had an impact on lichen communities (Leavitt *et al.*, 2012b). Our results suggest that diversification in the *R. melanophthalma* group was driven by major climatic changes during the Pleistocene in heterogeneous habitats in western North America, probably serving as a 'centre of origin' for this complex (Darwin, 1859; Briggs, 2000).

The centrifugal theory of geographical speciation argues that, as a species undergoes successive geographical expansions and contractions, speciation may occur in refugial populations during contraction phases, and in the next expansion phase, the central species may overwhelm peripheral populations, causing their extinction (Brown, 1957; Briggs, 2000). In the *R. melanophthalma* group, multiple lineages show evidence of recent or ongoing population expansions (clades C2, C4b and C4d; Table 5), while others appear to be rare or lack evidence supporting population expansion (clades C3, C4a and C4c). The centrifugal theory of geographical speciation provides a plausible explanation for the biogeographical species distribution patterns observed in this study, including the closely related lineages that occur in sympatry in western North America. In the *R. melanophthalma* group, we hypothesize that speciation may have occurred in refugial populations in western North America created by climatic changes during the Pleistocene, and the dominant, advanced species subsequently spread over large geographical areas. The long-term evolutionary success of the lineages restricted to western North America may be limited by small population sizes and reduced genetic variation (Table 5).

Most of the collections of *R. melanophthalma* s.l. from western North America used in this study resulted from rigorous, systematic sampling along an elevational gradient on the Aquarius Plateau in southern Utah (Leavitt *et al.*, 2011b). The absence of comparable sampling outside western North America calls into question whether the apparent absence of some lineages in Central Asia, Europe and South America may simply be a product of our biased sampling. We found that two *R. melanophthalma* s.l. lineages (clades C2 and C4b) occurred sympatrically in restricted geographical regions (i.e. Chile, China, Iran, Kyrgyzstan and Spain), and intense local sampling in other regions may ultimately reveal diversity similar to that found in western North America. However, a total of 54 samples, representing five of the six species-level lineages, were still recovered in North America after excluding the collections made from the systematic sampling on the Aquarius Plateau. To further assess the potential impact of our biased sampling, we also artificially decreased our North American sampling to levels similar to other comparable geographical regions. In this study, a total of 35 individuals were sampled from Central Asia, 24 from Europe, and 29 from South America. Of the 150 specimens from western North America, we randomly chose 30 individuals and assessed the average number of lineages recovered in the subsamples over 100 randomizations. Based on this simple comparison, we found that with a similar sampling effort (number of individuals) across comparable geographical regions, at least five species-level lineages were consistently recovered in North America (data not shown). In contrast, only two broadly distributed lineages were found in similarly sized samples from Central Asia, Europe and South America (clades C2 and C4b). Ultimately, rigorous sampling in other regions, such as Central Asia, Europe and North and South America, will be needed to more accurately assess the distribution patterns of all lineages within this group.

Elucidating the geographical origin of the lineages within the *R. melanophthalma* complex may be challenging, given the apparent dispersal capacity for at least two lineages within this group. Also, divergence estimates must be taken with some caution. Although estimated substitution rates for the nuclear ribosomal ITS region in fungi are largely similar across the limited number of studies currently available (Takamatsu & Matsuda, 2004; Leavitt *et al.*, 2012a,b,c), rate heterogeneity can be expected among distinct lineages (Bromham, 2011; Gaut *et al.*, 2011; Nygren *et al.*, 2011). We are aware of the potential bias of using a substitution rate from an unrelated lineage to estimate divergence times in the *R. melanophthalma* complex, but our study provides a valuable hypothesis of the timing of diversification that merits additional study.

Our results clearly demonstrate that defining 'populations' in lichen-forming fungi is not a straightforward task. For example, with our sampling of *R. melanophthalma* s.l., we show that an individual sampled at random from a single plot on a mountain in the south-western USA may be more closely related to an individual collected in Asia, Europe, South America, or even Antarctica, than to another individual sampled from the same plot (see Leavitt *et al.*, 2011b; Appendix S3). Lineages within the *R. melanophthalma* complex may potentially serve as valuable groups for assessing dispersal capacity and landscape-level genetics in response to changing climatic conditions. Recognition of distinct species-level lineages will aid in these population-level genetic studies, including assessing landscape-level gene flow by minimizing the bias resulting from comparisons based on geographically defined populations that may contain multiple independent lineages.

Our study of the *R. melanophthalma* complex provides a striking example of the complex biogeographical patterns found in some lichen-forming ascomycetes, where the global diversity within a morphologically cryptic lichen-forming fungal species complex with a cosmopolitan distribution can be found in local geographical areas. We provide additional evidence that the six lineages within the *R. melanophthalma* complex merit formal recognition as species. Formal species descriptions and epitypification of *R. melanophthalma* are presented in a companion paper (Leavitt *et al.*, 2013b). Ultimately, the recognition of cryptic species diversity will aid in a more appropriate interpretation of biogeographical and ecological patterns in lichen-forming ascomycetes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Collection information for all specimens of *Rhizoplaca melanophthalma sensu lato* included in the present study.

Appendix S2 Selected specimens representing sampled genetic diversity, including GenBank accession numbers for the seven sampled loci: nuclear ribosomal internal transcribed spacer (ITS) region and intergenic spacer (IGS) region, and the protein-coding markers *EF1*, β-tubulin, *MCM7*, *RPB1* and *RPB2*.

Appendix S3 Maximum likelihood ITS gene tree of the 240 sampled specimens of *Rhizoplaca melanophthalma sensu lato*. Bootstrap support is indicated at the nodes.

BIOSKETCH

Steven D. Leavitt is a research associate at Brigham Young University (Provo, Utah, USA) and The Field Museum (Chicago, Illinois, USA). His research interests include diversity, speciation, biogeography and ecology in lichen symbionts in arid regions and bio-monitoring. The research outlined here was the result of an international collaboration effort among lichenologists from three continents.

Author contributions: S.D.L. and L.L.S. conceived the research ideas; F.F. and H.T.L. contributed with the conceptual development of the work; P.K.D., F.F., S.P.O., J.V., S.D.L., L.L.S., M.S. collected the data; F.F. and S.D.L. analysed the data; and S.D.L. led the writing.

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