Geographic, climatic, and chemical differentiation in the *Hypogymnia imshaugii* species complex (Lecanoromycetes, Parmeliaceae) in North America

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Abstract. *Hypogymnia imshaugii* is one of the most common, conspicuous and morphologically variable epiphytic lichens of the Pacific coastal states and provinces. The species varies greatly in morphology and chemistry, suggesting multiple closely related species or one or more phenotypically plastic species. We sought to determine whether additional ecologically meaningful species might be present within the *H. imshaugii* complex. Improving our species concepts could potentially improve ecological inferences based on community sampling. Three relatively well-defined genetic groups and one residual group in the *H. imshaugii* complex were detected with haplotype networks based on the ITS locus; however, phylogenetic reconstructions on combined ITS, mtSSU, *GPD1* and *TEF1* loci did not reflect this pattern. At present, we have insufficient evidence to support defining any of these groups as new taxa. The four major chemotypes in *H. imshaugii* differed in frequency among the genetic groups. None of the genetic groups was, however, qualitatively uniform in chemotype. Only one chemotype occurred in a single genetic group, but several chemotypes occurred in that group. While broadly sympatric, each chemotype had a distinct geographic distribution, and each chemotype showed its own relationship to climate, as shown by regression of occurrences of chemotypes against climatic variables. The genetic variation detected within *H. imshaugii* did not correspond to geographic variation in morphology, chemistry, or climate. Within the broader *H. imshaugii* complex, we recommend treating *H. amplexa* as a synonym of *H. imshaugii* unless it can be more distinctly separated from the clinal variation in morphology, chemistry, or DNA sequences. In contrast to *H. amplexa*, however, *H. inactiva* and *H. gracilis* are both easily separated morphologically from *H. imshaugii* and do not intergrade with it.

Keywords. Chemotypes, DNA sequences, *Hypogymnia amplexa*, lichenized ascomycetes, lichen substances, nonparametric multiplicative regression, Parmeliaceae.

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Every practitioner of taxonomy has experienced single ''species'' that seem to encompass a bewildering array of variants. What lurks beneath these broad species concepts? They conceal intricate puzzles of morphological and chemical variation that tease the minds of inquisitive scientists. Are we missing an ecological treasure trove by taking a simplistic approach, painting our ecology with broad brush strokes and blurring informative detail? Do simple molecular tools provide greater resolution of species than the traditional morphological and chemical evaluation?

Hypogymnia imshaugii Krog is endemic to North America. This species is one of the most common, conspicuous, and morphologically variable epiphytic lichens of the Pacific coastal states and provinces, inland to Idaho and Montana. Thousands of collections are available from throughout its range. Krog (1968) separated *H. imshaugii* from an extremely broad concept of *H enteromorpha*. She described *H. imshaugii* var. *inactiva* as well. This variety has since been elevated to the species level (Ohlsson 1973). Although superficially similar to the typical form of *H. imshaugii*, *H. inactiva* (Krog) Ohlsson is easily separated by morphology, having lobe cavities that darken and perforations in some lobe tips and axils. *Hypogymnia imshaugii*, on the other hand, has snow white lobe interiors throughout and is always imperforate, except where damaged mechanically, for example by arthropods (McCune 2002).

Hypogymnia imshaugii varies greatly in morphology (**Fig. 1**) and chemistry, suggesting multiple closely related species or one or more phenotypically plastic species. The commonalities among the many variants are snow white lobe cavities and absence of perforations in the lobe tips, axils, and lower surface. The only other North American species of *Hypogymnia* with completely white lobe interiors is the very rare species, *H. subphysodes* (Kremp.) Filson (McCune & Rosentreter 1997), perhaps an recent arrival from South America or Australia, where it is common (Elix 1979).

If the variation in widespread common species such as *Hypogymnia imshaugii* represents ecologically distinctive but unnamed taxa, then recognizing those taxa could enhance our ability to use lichen communities as indicators of air quality and climate change. For example, lichen community sampling on the national forest inventory grid (FIA; Jovan 2008) relies on repeated sampling of lichen communities over large areas and long time spans to indicate changes in forest health and air quality (McCune 2000). If we ignore species that are ecologically meaningful but difficult to define, then we blur our ecological inferences based on community sampling.

At least four morphotypes of *Hypogymnia imshaugii* can be defined:

1. The most typical form (**Figs. 1A & B**), occurring through much its range, has imperforate lobe tips, hollow lobes, open, dichotomous branching, suberect or erect lobes, and medulla PD+ orange. This form is infrequent in southern California but common in British Columbia (BC), western Oregon and Washington, and the northern Rocky Mountains.
2. A second form (**Fig. 1D**) with compact rosettes of contiguous lobes, is PD-. It occurs mainly in upper montane to subalpine areas of western Montana, Idaho, eastern Washington, and eastern Oregon. This form has been referred to by the herbarium name *H.* ''*montana*'' by Pike (unpublished) and McCune and Goward (1995) and was recently published as *H. amplexa* Goward, Björk & Wheeler in Lumbsch et al. (2010). Lawrence Pike and Mason Hale had also segregated a provisional species, using the herbarium name *H.* ''*sierrae*'', from *H. imshaugii* (specimens in US), though we know of no written documentation of the characteristics of this putative taxon.
3. A third form (not illustrated and no recent material available for DNA analysis) from near Los Angeles is extremely compact and appressed, with compressed to folded, hollow lobes, usually PD-, and sometimes a distinctive set of minor unknowns by thin-layer chromatography (TLC).
4. A fourth form (**Fig. 1C**), also restricted to southern California and the Sierra Nevada, has lobes collapsed and ± channeled on the lower surface, semi-solid to solid, with or without extended lobes that are hollow, not black mottled, the central part of the upper cortex strongly rugose, and medulla P+ orange. This form can have sparse subapical and subaxillary perforations, suggesting a relationship to *H. gracilis* McCune (see below).

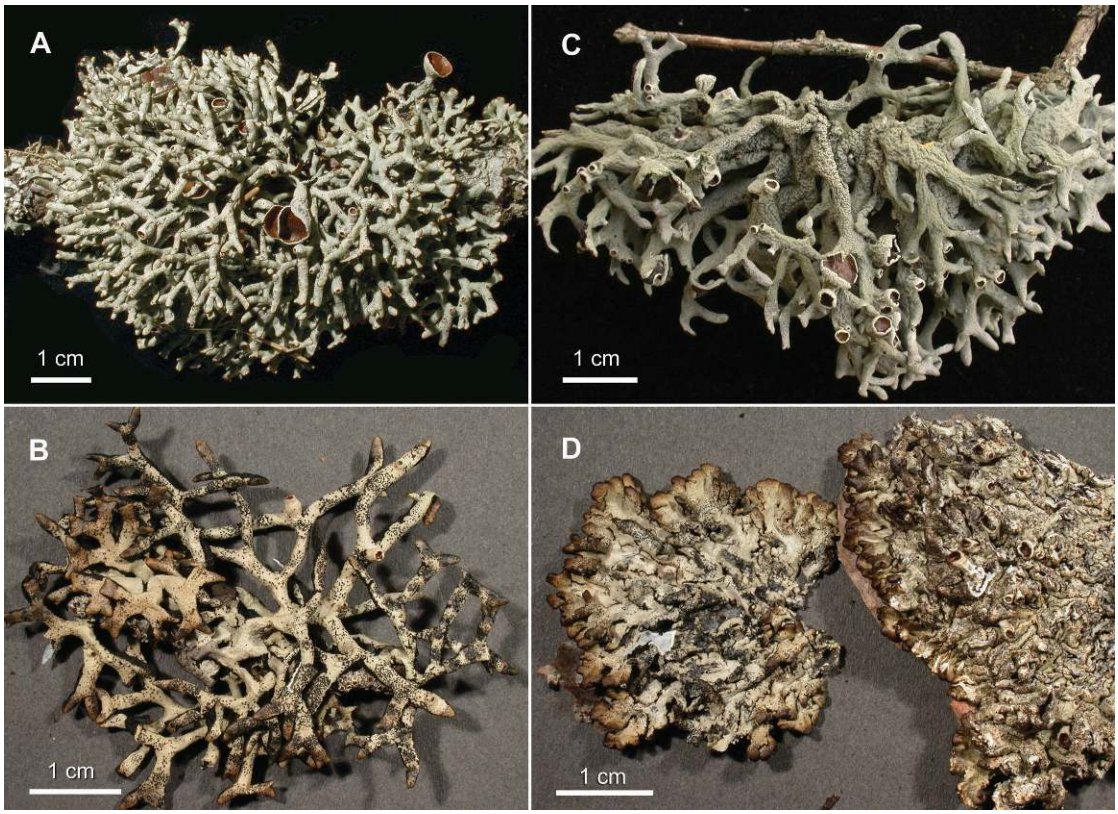

Figure 1. Morphological variation in the *Hypogymnia imshaugii* complex. **A.** *McCune 26671* (Douglas Co., Oregon, exemplifying the typical form and chemistry. **B.** *McCune 26668* (Linn Co., Oregon), another example of the typical form but lacking physodic acid. **C.** *Riddell 07–100* (Riverside Co., California), with collapsed lobe cavities and rugose surface, differing chemically from the typical form in lacking diffractaic acid. **D.** *McCune 26583* (Glacier Co., Montana), with appressed thallus and lacking physodalic and diffractaic acids.

> Intermediates between these forms are common, so that many specimens are difficult to assign to particular morphotypes. We therefore focused our analysis on more objective well-defined characters, secondary chemistry and DNA sequences.

Chemically the variation within *Hypogymnia imshaugii* is rich with five major secondary substances showing polymorphisms within the complex, as well as atranorin as a constant. Four of the five major substances showing qualitative variation (physodic, 3-hydroxyphysodic, physodalic, and protocetraric acids) are biosynthetically related, while diffractaic acid is unrelated to the others. The type material of *H. imshaugii* (Krog 1968) is from BC, has dichotomous open branching of hollow lobes (belonging to the first form listed above) and apparently contains atranorin, diffractaic, physodic, physodalic, and protocetraric acids. Krog (1968) reported monoacetylprotocetraric acid (= physodalic acid), physodic acid, and barbatic acid (misidentification of diffractaic acid, which is very close in TLC). Ohlsson (1973) reported five chemotypes (particular combinations of substances) of *H. imshaugii* with various combinations of diffractaic acid (as a "PD+ pale yellow R_f 55" unknown), physodic acid, 3-hydroxyphysodic acid (= conphysodic acid), physodalic acid, protocetraric acid, and a $FeCl_3$+ violet R_f71 unknown substance. The absence of physodalic acid was considered significant in defining the putative species, *H.* "*montana*" (McCune & Goward 1995).

Study of this morphological and chemical variation has so far failed to yield clear separations into segregate species or subspecific taxa, apart from *Hypogymnia gracilis*. This species was recently separated from *H. imshaugii* on morphological

grounds (McCune 2002). *Hypogymnia gracilis* is easily recognized by its whitish to pale brownish or grayish cavity, slender suberect, arcuate lobes, and small holes below the tips and axils. Typical *H. imshaugii* is imperforate and has a snow white medullary cavity. The cavity of *H. imshaugii* darkens only when the thallus is damaged; likewise, the rare perforations seen in the lower surface and lobe tips of *H. imshaugii* usually appear to result from herbivory. *Hypogymnia gracilis* and *H. imshaugii* are allopatric, with *H. gracilis* restricted to low elevations near the coast in southern California and *H. imshaugii* becoming common at mid to higher elevations nearby.

The purpose of this study was to search for correspondences between chemotypes, phylogenetic groups, geography, and climate. We evaluated chemotypes as qualitative presence-absence of lichen secondary substances as detected with traditional TLC techniques and describe their variation with respect to geography and climate. To reconstruct phylogenetic relationships within the *Hypogymnia imshaugii* complex and other representatives from the genus *Hypogymnia* we used four molecular markers: the flanking spacer regions plus the 5.8S nuclear ribosomal RNA (ITS), the small subunit for the mitochondrial ribosomal subunit (mtSSU), partial sequences of the glycerol-3-phosphate-dehydrogenase gene (*GPD1*) and transcription elongation factor alpha (*TEF1*). We attempted to relate phylogenetic and haplotype groupings to the pattern detected in chemical variation, morphology, geography, and climate.

Materials and Methods

Specimen sampling. The distribution of *Hypogymnia imshaugii* was determined by consulting the following herbaria: ASU, BM, COLO, DUKE, H, MIN, MONT, MONTU, NY, OSC, S, SBBG, UBC, UC, UCR, UPS, US, WIS, WTU, and the personal herbaria of B. McCune, & R. Rosentreter. Specimens were selected for chemical analysis to cover the full distributional range and morphology of the species.

Lichen substances. To relate secondary chemistry to genetic and morphological variation we analyzed lichen substances by TLC. Fragments of specimens were extracted in acetone at room temperature, spotted on aluminum-backed silica gel plates (Merck 5554/7 Silica gel 60 F_{254}), run in solvent systems A and C of Culberson et al. (1972), followed by treatment with 10% H_2SO_4 and charred in an oven at 100°C. We performed TLC on all specimens extracted for DNA and many others, for a total of 273 out of 929 georeferenced herbarium specimens examined.

Frequency of individual lichen substances and their combinations were analyzed by comparing expected and observed frequencies. Expected frequencies were based on the marginal totals of a multi-way contingency table, under the null hypothesis of independence of each substance. We expressed departure from expectation as $f \ln\left(f/\hat{f}\right)$, where f is the observed frequency and $\hat{f}$ is the expected frequency. This is a log odds ratio weighted by the frequency of a particular chemotype (Sokal & Rohlf 1995, p. 692). This ratio is zero for the random expectation, negative when the number of items in the cell of a contingency table is less frequent than expected, and positive when more frequent than expected. Twice the sum of these values yields the log likelihood test statistic, which is always non-negative and can be compared to a chi-square distribution for a test of independence between row and column categories (op. cit.).

Independence of genetic groups and individual chemical substances was tested with chi-squares based on two-way contingency tables. Chi-squares rather than log-likelihoods were used in this case to avoid undefined solutions resulting from empty cells. To test whether particular combinations of substances (chemotypes) were equally frequent among genetic groups, we calculated chi-square statistics using expected values of $1/g$ where g is the number of groups.

Sampling and alignments. Specimens were selected for DNA analysis with the following criteria: 1) 33 recent specimens from throughout the range of *H. imshaugii*, spanning a broad range of morphology and chemistry, including six specimens of *H. amplexa* recently segregated from *H. imshaugii*; 2) 26 specimens of *H. imshaugii* from Idaho and Montana sampled on the Forest Inventory and Analysis (FIA) grid in 2006; 3) 26 specimens from other parts of the world in an attempt to sample broadly from

throughout *Hypogymnia*, including *H. inactiva* and *H. gracilis*, close relatives of *H. imshaugii*; and 4) one specimen of *H. subphysodes*, which is unusual in *Hypogymnia* in sharing the white lobe cavities and imperforate lobes of *H. imshaugii*, and could thus be considered as a possible relative from the southern hemisphere.

We evaluated the ITS, mtSSU, *GPD1* and *TEF1* loci for inclusion in phylogenetic analyses. We based our choice of genes after a comparison of sequences available from other members of the Parmeliaceae and Lecanorales deposited in GenBank relying on published primers (Myllys & Thell 2002; Thell et al. 2004).

Fungal genomic DNA was isolated by selecting approximately 5 mm long sections of thalli. DNA was extracted using the FastDNA® kit and The FastPrep® instrument from MPI Biochemicals (Irvine, CA). DNA amplifications were completed using PCR Master Mix from Promega Corporation (Madison, WI) under the following PCR conditions: 94 °C for 2 min; five cycles of 94 °C for 40 s, 55 °C for 45 s lowering by 0.8 °C per cycle and 72 °C for 90 s; 30 cycles of 94 °C for 30 s, 52 °C for 45 s and 65 °C for 120 s and a final cycle for 10 min at 72 °C. The ITS region was amplified with primers ITS4 and ITS5 (White et al. 1990). Primers Gpd1LM and Gpd2LM were used to obtain a partial sequence of the *GPD1* (Thell et al. 2004). The mtSSU was amplified using the primers mrSSU1 and mrSSU3R (Zoller et al. 1999) and *TEF1* with 983 and 2218R (http://AFTOL.org/data.php). DNA alignments were assembled with ClustalX (Thompson et al. 1997) and manually refined using the shareware package BioEdit (Hall 2004). Newly generated DNA sequences were deposited at GenBank (**Supplementary on-line Table S2**).

Phylogenetic analyses. Outgroups were selected according to Thell et al. (2004) and data sets consisting of ITS, mtSSU, *GPD1* and *TEF1* and a combination of ITS and *GPD1* (trees not shown) were analyzed with missing data present. The combined data set consisted of 86 specimens within *Hypogymnia* (**Supplementary on-line Tables S1 & S2**), but we present an analysis of a subset of these where we had sequences from two or more loci. Phylogenetic trees were obtained by maximum parsimony analysis with the Windows version of T.N.T. (Goloboff et al. 2008) and the strictness level set to 80. Alignment gaps were treated as single gaps by listing all gaps except the first gap character as missing data. Gaps and transversions were given double the weight of transitions. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. This resulted in 18 most parsimonious (MP) trees of 844 steps. Trees were evaluated by 500 jackknife resamplings. Tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996). Maximum-likelihood (ML) analysis was conducted for the same data set using RAxML MPI, v7.0 (Stamatakis 2006) under the GTRGAMMA model setting using a proportion of invariable sites and distribution of rates at variable sites modeled on a discrete gamma distribution with four rate classes. Phylogenetic support was evaluated by 500 bootstrap repetitions in the same program. Conflicts between genes were examined pairwise, excluding specimens with missing genes for each pairing. This was done using the "bootstopping" criterion in RAxML 7.2.3 (Stamatakis et al. 2008) under the CIPRES 2.1 webportal (http://www.phylo.org/portal2) to produce trees of comparative gene sets in all possible combinations where all taxa have the gene present. A conflict among single-locus data sets was considered significant if a well-supported monophyletic group (i.e., bootstrap value $\geq$ 70%; Mason-Gamer & Kellogg 1996) was found to be well-supported as non-monophyletic using a different locus. Because of the low level of genetic variation (many identical sequences and low phylogenetic resolution from each single gene analysis) and no obvious conflict was detected, the different loci were therefore combined. A majority rule consensus tree from the 18 most parsimonious trees was created and MP jackknife and ML bootstrap values plotted on the nodes (**Fig. 4**).

We also performed distance-based analyses within the *Hypogymnia imshaugii* complex, using neighbor joining trees (R 2.6.1 Package APE v. 2.2; Paradis et al. 2004) and hierarchical agglomerative cluster analysis in PC-ORD 5 (McCune & Mefford 2006). We used Ward's method (minimum error

sum of squares) and Kimura's two-parameter distance (Kimura 1980), importing the distance matrix from R (v. 2.6.1 Package APE v. 2.2 Paradis et al. 2004).

Haplotype networks were reconstructed based on aligned ITS sequences using Network 4.600 software (2010 Fluxus Technology, www.fluxus-engineering.com) with the median joining option (Bandelt et al. 1999). Gaps were included as a fifth character state; those within *H. imshaugii* never exceeded one base pair in length and therefore were only counted as one character. Epsilon was set to zero to give a fast approximation to the optimal median joining network. Transitions were weighted equally with transversions. Nine ITS haplotypes were found in two or more specimens, with 35 specimens involved in exact matches as shown in **Supplementary on-line Table S2**.

Habitat analyses. We analyzed presence/absence of lichen substance, combinations of lichens substances, and genetic groups in relation to climate. Each of the dependent variables was binary (0/1). We regressed these against individual climate variables and linear combinations of climate variables using nonparametric multiplicative regression (NPMR; McCune 2006) with a local mean estimator and Gaussian kernel function (Bowman & Azzalini 1997). We chose NPMR over logistic regression to avoid making distributional assumptions about the data and to accommodate a wide range of response surface shapes. Model building sought the best possible combination of predictors of occurrence of chemotypes or lineages. The standard deviation (tolerance) of the kernel function was optimized by maximizing the cross-validated log-likelihood ratio (log*B*), the statistic used to evaluate model quality. Log*B* expresses model strength compared to a naïve model, where the estimated likelihood of occurrence is simply the frequency of the chemotype or lineage in the sample. Model fit was reported with the AUC (area under curve for receiver operating characteristic; 0.5 = random expectation; 1 = perfect fit). In each case, we specified a minimum average neighborhood size of 5% of the sample size. The final models were evaluated with randomization tests using 100 permutations of the response variable in relation to the predictors. Tolerances define how broadly the estimate for a given point is based on the surrounding sample space. A sensitivity of 1.0 means that nudging a predictor results in a response of equal magnitude, when expressed as proportions of the variable ranges; sensitivity of 0.0 means that nudging the predictor results in no response of the dependent variable (McCune 2006). The average neighborhood size, N^*, is the average sum of weights applied to individual data points. This is the average amount of data bearing on the estimate of the response variable at each point.

Climate variables were gridded, modeled values produced by PRISM (Daly et al. 1994) and Daymet (Thornton et al. 1997; http://www.daymet.org/). Climate variables were attributed to geographic locations of the collections using Hawth's Analysis Tools (v 3.27, 2006; www.spatialecology.com) in ArcGIS 9.1. Because the many climate variables were strongly intercorrelated, we reduced them to a small number of synthetic variables (**Table 1**) with Principal Components Analysis (PCA) using PC-ORD 5 (McCune & Mefford 2006). Scatterplots among variables showed more or less linear relationships, justifying the use of PCA. The first three axes explained 87.6 of the variance in the original variables, more variance than expected by chance, based on randomization tests with 999 iterations, using the Rnd-Lambda and the Avg-Rnd criteria (Peres-Neto et al. 2005). We retained the first 6 axes as candidates for predictors in regressions, in case important predictors were not part of the strongest correlation structure among climate variables (**Table 1**).

Geographic vs. genetic variation. We tested the null hypothesis of no relationship between genetic distance, measured as Kimura's two-parameter distance (Kimura 1980), and geographic distance, using a Mantel test (Mantel 1967; McCune & Grace 2002; McCune & Mefford 2006). Geographic distances among specimens were calculated with Hawth's Analysis Tools (v 3.27, 2006; www.spatialecology.com) in ArcGIS 9.1.

Genetic groups vs. climate. We tested for differences in climate among genetic groups with multi-response permutation procedures (MRPP). MRPP is a nonparametric multivariate test of the hypothesis of no difference between groups. Climate

Table 1. PCA axes representing climate variables from Daymet and Prism. The strongest variables are indicated for each axis, separated by columns indicating the direction of the linear relationship. Variables are listed in descending order of strength of relationships with the axis. Arrows under ''Interpretation'' indicates the direction that the climate characteristic increases along the indicated axis. Variables are coded by: Pre = Precipitation, RH = Relative humidity, T = Temperature, Dew = dewpoint temperature, WetDay = number of wet days; Cont = % of precip falling June–August), Ann = Annual, CV = Coefficient of variation, Diff = difference between August maximum and December minimum, Max = Maximum, Mean = Mean, Min = Minimum, Aug = August, Dec = December, Smr = Summer, **Bold** face = Daymet-derived variables; regular font = PRISM-derived variables.

PCA Axis	Variance explained, %	Strongest negative relationships	Strongest positive relationships	Interpretation	Selected by N regression models
1	54.1	TMeanAnn, TMaxAnn, TMaxDec, TMeanDec, **TAnn**, TMinAnn, SmrT, TMeanAug, TMinDec, **TDecMin**	WetDayAug, PreAug, **PreSmr**	← temperature	6
2	25.3	**PreAnn**, PtrDec, PreAnn, WetDayDec, RHAug, RHAnn, WetDayAnn, DewDec, DewAnn	**TDiff**, TMaxAug, **Cont**	← precip → continentality	2
3	8.1	RHDec, RHAnn, DewAug, **ContPre**, RHAug	**PreCV**	→ elevation effect apart from precipitation and temperature, moisture variability.	6
4	3.1	PreAug	RHAug, RHAnn	→ humidity ← cool, wet August	0
5	2.6	**TMaxAug**, WetDayDec	TMinAug	← August temp, Summer temp	6
6	1.7	TMinAug, RHDec	PreAug, RHAug	→ wet summers	1

was represented by the first six principal components of the PRISM and Daymet climate variables. Groups were compared as a whole along with all pairwise comparisons.

Results

Based on 929 herbarium specimens, the *Hypogymnia imshaugii* complex ranges from northern BC to northern Mexico (**Fig. 2**). The northern part of its range extends inland to central Montana, Wyoming, and northern Utah. Southward it is confined to the west side of the crest of the Sierra Nevada.

Although ITS and *GPD1* sequences provided useful information for separating species within the genus *Hypogymnia*, *GPD1* was more difficult to successfully amplify (57 sequences for ITS and 34 for *GPD1* within *H. imshaugii*). The *GPD1* dataset provided 98 potentially parsimony informative characters (PPIC), whereas ITS provided 92. We also found mtSSU to be informative within the genus (27 PPIC), but it had almost no variation within the *H. imshaugii* complex. We included a subset of sequences using data from *TEF1* (15 sequences; 41 PPIC) that did not cover most of the isolates *H. imshaugii* but provided additional variation within the genus.

Genetic Analyses. Distance based methods, consisting of a haplotype network (**Fig. 3**); neighbor joining, and cluster analysis (the latter two not illustrated) largely agreed in structure for the ITS data. *Hypogymnia inactiva* was isolated in the haplotype network. The remaining specimens fell into three relatively well-defined, but nevertheless indistinct groups. A fourth group was defined from the residual specimens (**Fig. 3**).

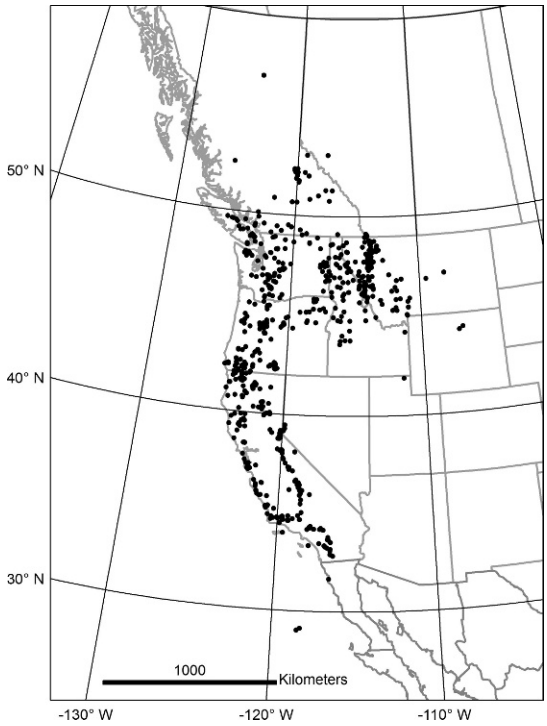

Figure 2. World distribution of the *Hypogymnia imshaugii* complex.

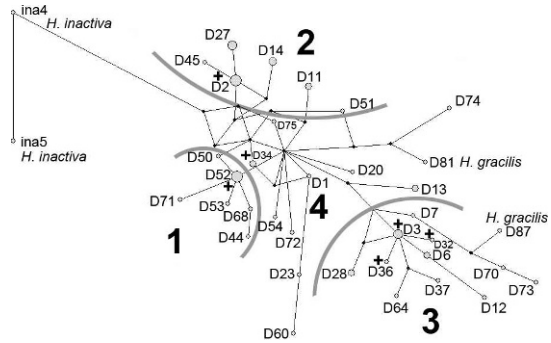

Figure 3. Haplotype network for the *Hypogymnia imshaugii* group based on ITS sequences. Unfilled circles represent one or more specimens, with the diameter of the node proportional to the number of identical haplotypes at that node and the label indicating one of the specimens at that node. Solid dots are hypothetical intermediates in the network. Gray bands enclose four groups of specimens, each consisting of a relatively well defined lineage. Group 4 is the most heterogeneous and is not enclosed by a band. Lengths of the line segments are proportional to the number of changes on one step in the network with the shortest segments indicating one change. Haplotypes containing putative *H. amplexa* from eastern Washington are indicated by +.

Phylogenetic inferences resolved two major sister groups within *Hypogymnia* (**Fig. 4**). One group contains widespread species (e.g. *H. austerodes* (Nyl.) Räsänen, *H. physodes*) and Southern Hemisphere species (e.g. *H. subphysodes, H. antarctica, H.* sp.), while the other group contains all North American endemics (*H. apinnata, H. duplicata, H. enteromorpha, H. inactiva*, etc.) except for *H. incurvoides*, which is known from both North America and Scandinavia. The geographical pattern is elaborated further in Miadlikowska et al. (2011).

Hypogymnia imshaugii forms a well-supported monophyletic group within the North American clade, although it contained two closely related but morphologically well defined species, *H. gracilis* and *H. inactiva*. Within *H. imshaugii*, the phylogenetic structure is ambiguous, with relatively weak support for major groups. High bootstrap support was found for several clades containing only two or three exemplars (**Fig 4**).

Genetic groups vs. geography and climate. Genetic groups (defined by distance-based analysis of the ITS rather than phylogenetic structure in Fig. 4) did not differ by geography, as evidenced by the broad sympatry of the groups (**Fig. 5**) and the absence of a statistically significant relationship between genetic distance and geographic distance (Mantel test; $r = 0.004$, $p = 0.46$). Likewise, we found no relationship between genetic groups and climate variables, as shown by the lack of relationship between genetic distance and scores on PCA axes 1 through 6 combined (Mantel test, $r = 0.039$, $p = 0.28$). We confirmed this result by testing the hypothesis of no difference in climate among genetic groups with MRPP and again found no difference ($A = -0.01$, $p = 0.72$). None of the pairwise contrasts among genetic groups yielded $p < 0.05$ (Group 1 vs. 4 and 1 vs. 3 were strongest at $p = 0.08$ and $p = 0.09$, respectively for axis 3 alone).

Lichen substances. The lichen substances most frequently detected by TLC in the *Hypogymnia imshaugii* complex were atranorin, 3-hydroxyphysodic, diffractaic, physodalic, physodic and protocetraric acids (**Table 2**). We analyzed all of the major substances that were polymorphic within *H. imshaugii*. Additional satellite compounds related to these were occasionally seen but are not reported here, because they were considered to have no

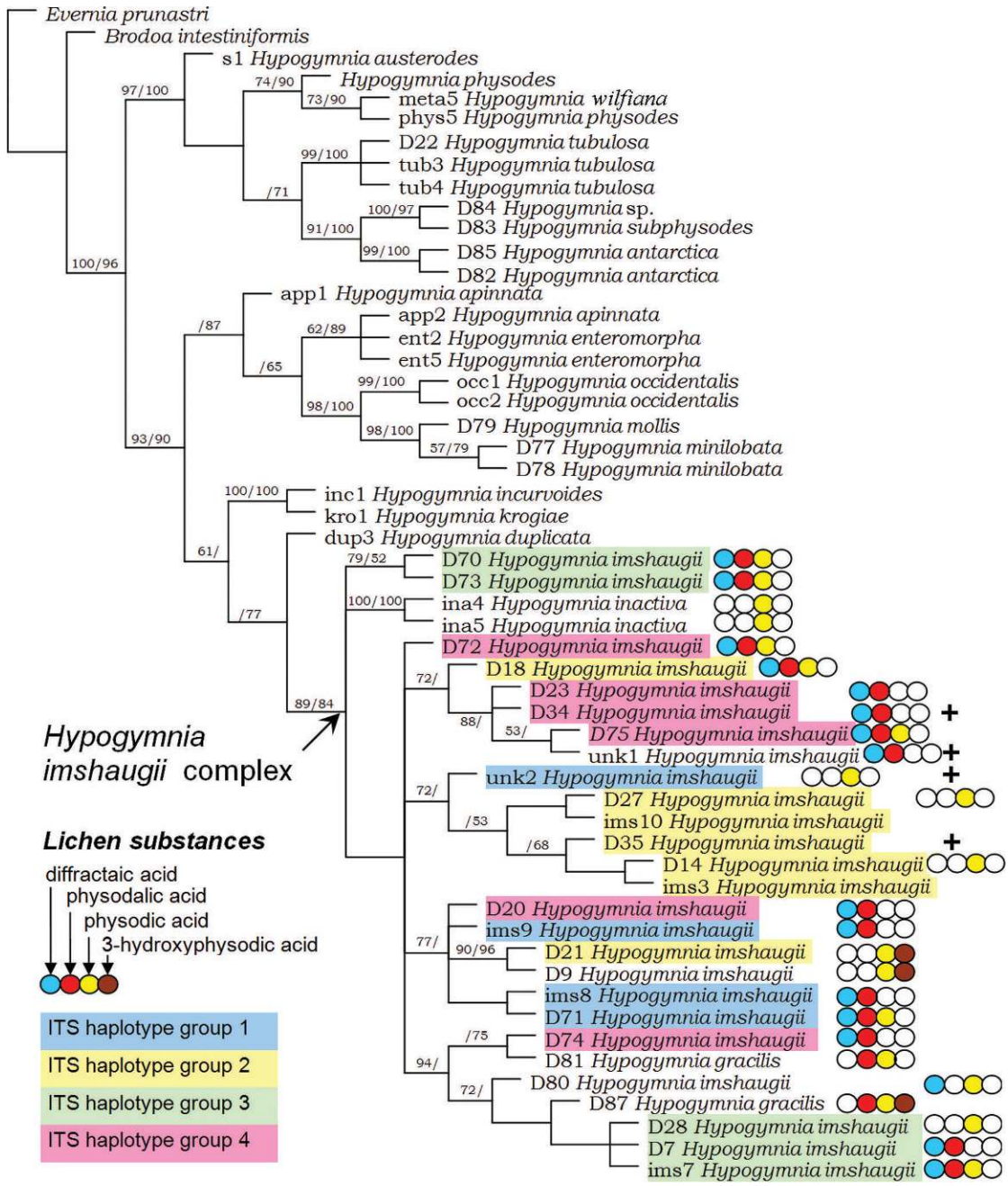

Figure 4. Phylogenetic relationships within the *Hypogymnia imshaugii* complex. A 50% majority rule consensus tree from 18 most parsimonious trees obtained by T.N.T. based on ITS, mtSSU, *GPD1* and *TEF1* (in order of increasing missing data). Specimens were selected to maximize specimens with sequences for multiple loci. Support values are plotted above the nodes. The first number represents 500 jackknife samplings done with parsimony in T.N.T (p=36). The second number is 500 successive bootstrap resamplings done by maximum likelihood using RAxML v7.0. For the parsimony analyses the transition/transversion ratio was set to 2:1. Gaps were regarded as a fifth base with a similar 2:1 transition ratio. Lichen substances in the *H. imshaugii* group are indicated by colored circles, haplotype groups from **Fig. 3** by colored backgrounds. A plus sign indicates putative *H. amplexa* from eastern Washington.

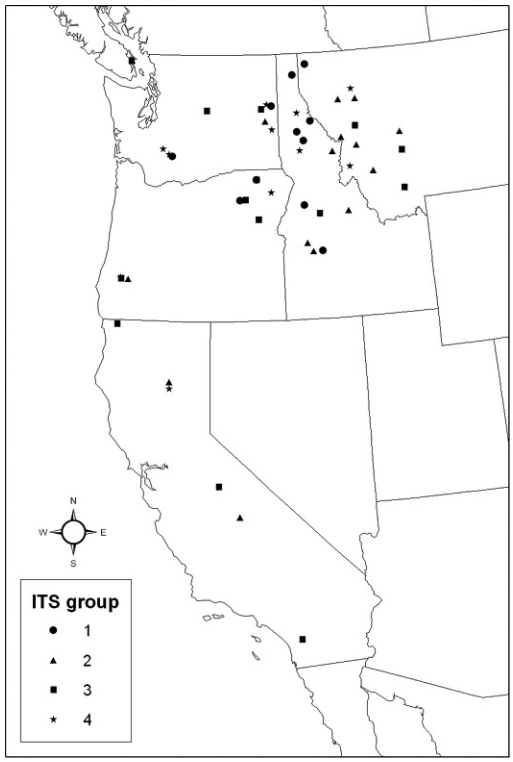

Figure 5. Distribution of genetic groups of *Hypogymnia imshaugii*.

taxonomic or ecological significance. In this species complex, protocetraric acid and physodalic acid always co-occur, and protocetraric is likely the immediate precursor to physodalic acid, so protocetraric acid will not be mentioned further. Nor will atranorin be mentioned, the only constant substance in this group.

Diffractaic acid is often a major substance (**Table 2**), but sometimes only a minor component, barely detectable with TLC. Presence of diffractaic acid is, however, conspicuously patterned with respect to geography (**Fig. 6B**) and climate, being almost universally present in more northern oceanic to suboceanic populations and almost always absent in more southern, continental, and subalpine habitats. In regression models, diffractaic acid was most strongly associated with cool summer temperatures, low elevations, and relatively reliable moisture (**Table 3**; low on climate axes 3 and 5 but uncommon where these axes were both high and where axis 3 was high but axis 5 low). Presence of diffractaic was strongly dependent on ITS groups

Table 2. Expected and observed frequencies of combinations of lichen substances (di = diffractaic, ph = physodic, co = 3-hydroxyphysodic, and pl = physodalic acids) in *Hypogymnia imshaugii*. Expected frequencies are based on marginal totals of a contingency table, assuming independent occurrences of the four primary substances in *H. imshaugii*, other than atranorin. The departure from expectation is the natural logarithm of the odds ratio weighted by the frequency of a particular chemotype. Positive values are more frequent than expected, negative values less frequent, and missing values cannot be calculated because the chemotype was not observed. **Bold** face chemotypes are common and more frequent than expected by chance.

Chemotype	Observed	Expected	Departure
di ph co pl	3	12.23	−4.22
di ph co	0	3.25	—
di ph pl	102	98.13	3.95
di ph	2	26.12	−5.14
di co pl	0	6.32	—
di co	0	1.68	—
di pl	105	50.75	76.33
di	0	13.51	—
ph co pl	15	8.07	9.29
ph co	21	2.15	47.87
ph pl	38	64.80	−20.28
ph	51	17.25	55.29
co pl	0	4.18	—
co	0	1.11	—
pl	15	33.52	−12.06
none	0	8.92	—

($p = 0.0008$) being much less common in group 2 and more common in group 4 than the expected values if diffractaic acid was independent of genetic group (**Table 4**).

3-hydroxyphysodic acid (K+ slow reddish brown) is uncommon in the *H. imshaugii* group (**Table 2**). When it occurs, it is usually without diffractaic acid but with physodic acid. 3-hydroxyphysodic acid is most frequent in southern California and western Montana, is relatively rare in Washington and Oregon and northern California, and nearly absent from BC (**Fig. 6A**). 3-hydroxyphysodic acid was most common in areas that combine relatively cold annual temperatures, high elevations, and cool summer temperatures (high on axes 1, 3, and 5); and in areas with warm annual temperatures but cool summer temperatures at high elevations (low on axis 1 but high on axes 3 and 5; **Table 3**). These two climatic settings correspond to the more northern

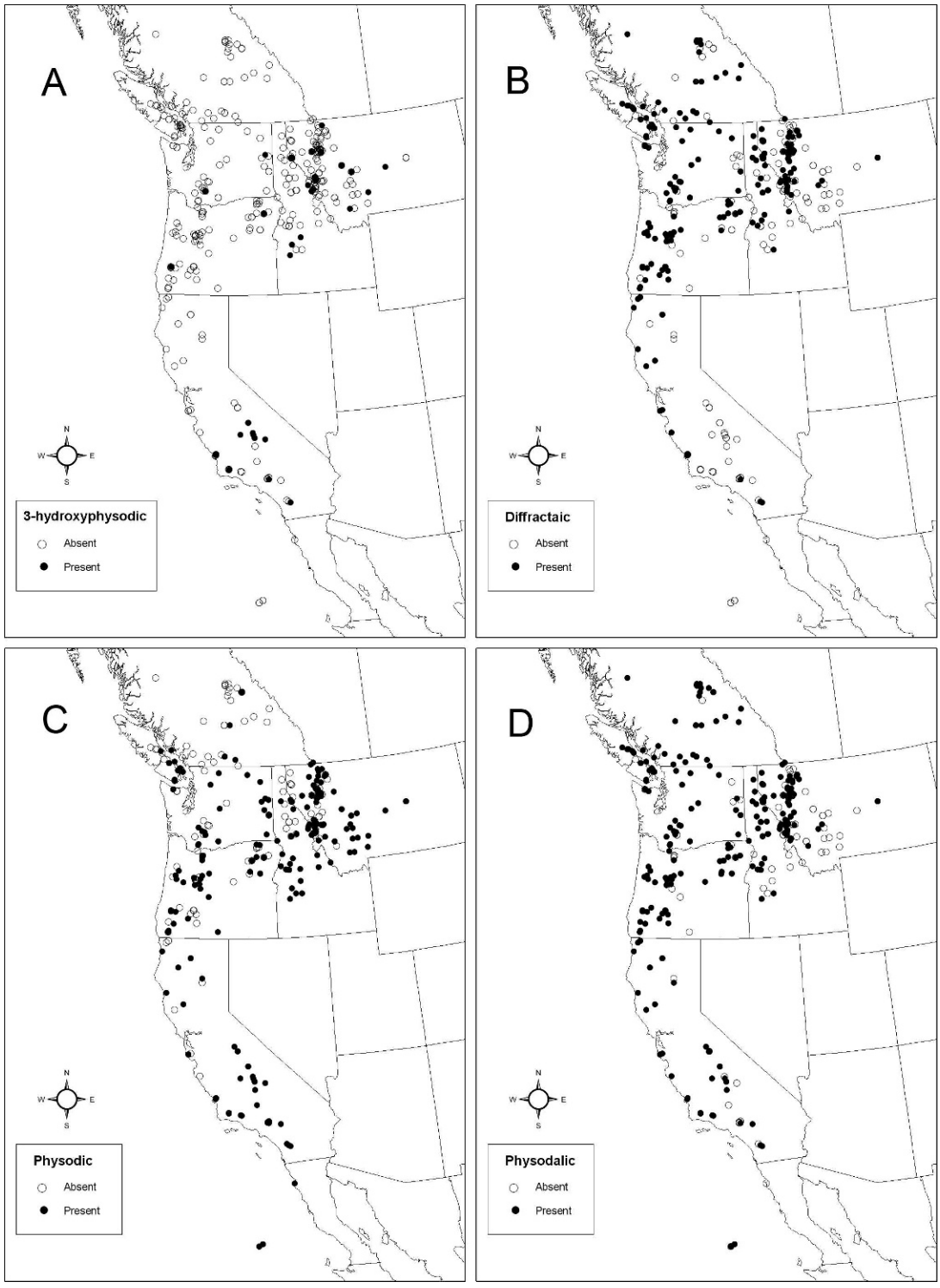

Figure 6. Distribution of occurrences of single lichen substances in *Hypogymnia imshaugii*. **A.** 3-hydroxyphysodic acid. **B.** Diffractaic acid. **C.** Physodic acid. **D.** Physodalic acid.

Table 3. Nonparametric multiplicative regression of occurrence of lichen substances in *H. imshaugii* against climate variables. Climate variables were first reduced by PCA into 6 independent synthetic variables (Table 1). Model fit was evaluated with the AUC (area under curve for receiver operating characteristic; 0.5 = random expectation; 1 = perfect fit); N^* is the average amount of data bearing on an estimate; p value is derived from a randomization test; tolerance decreases with importance of the predictor; sensitivity of 1.0 means that nudging a predictor by 5% of its range results in a 5% change in the frequency of occurrence. All except the final model were fitted with 273 specimens. The last model has a subset of 155 observations, using only the specimens with diffractaic + physodalic ± physodic acid. Presence of physodic acid was then used as the response variables. This model therefore addresses the question of whether or not presence of physodic acid is associated with climate, given the presence of diffractaic and physodalic acids.

Dependent variable	Fit AUC	N^*	p	Climatic predictors	Tolerance	Sensitivity
INDIVIDUAL SUBSTANCES						
Diffractaic acid	0.671	23.1	0.01	Axis 5	0.272	0.839
				Axis 3	0.973	0.666
3-hydroxy-physodic acid	0.699	22.0	0.01	Axis 1	2.550	0.364
				Axis 3	0.619	0.659
				Axis 5	1.087	0.151
Physodic acid	0.713	13.7	0.01	Axis 1	0.850	2.159
				Axis 2	4.159	0.217
				Axis 5	0.634	0.677
Physodalic acid	0.774	14.6	0.01	Axis 1	1.869	0.519
				Axis 3	1.946	0.196
				Axis 5	0.317	0.879
CHEMOTYPES						
diffractaic + physodalic acids	0.675	16.5	0.01	Axis 1	1.360	0.944
				Axis 6	0.235	1.400
3-hydroxyphysodic + physodic acids	0.807	15.9	0.01	Axis 2	0.717	0.850
				Axis 3	0.708	0.533
Physodic acid only	0.730	19.6	0.01	Axis 1	1.529	0.406
				Axis 3	1.857	0.151
				Axis 5	0.544	0.446
physodic given diffractaic + physodalic acids)	0.715	8.5	0.01	Axis 1	6.116	1.487
				Axis 3	1.703	0.269
				Axis 5	0.668	0.467

(e.g., western Montana) and southern (southern California) populations, respectively. 3-hydroxyphysodic acid was more common in ITS Group 2 than expected and less common in groups 1 and 3 ($p = 0.005$, **Table 4**).

Physodalic acid and the co-occurring protocetraric acid, are the substances responsible for the PD+ orange or red reaction of many specimens in the *Hypogymnia imshaugii* group. Physodalic acid occurs throughout the range of *H. imshaugii*, but is relatively rare in southwestern Montana and central Idaho (**Fig. 6D**). In BC, Washington, Idaho, and Montana, most specimens either have physodalic as a major substance or it is lacking. In California, however, particularly in the Sierra Nevada and coast ranges of central and southern California, the variation in physodalic acid appears to be quantitative, ranging from abundant to absent and everything in between. In single populations or packets, one commonly finds a polymorphism with some specimens PD+ orange, some PD- and some PD+ yellow. These spot test results are borne out with TLC data, with PD+ yellow specimens showing a weak spot for physodalic acid. Regression models showed that physodalic acid was common in a broad range of climates, but notably uncommon at high elevations with low annual temperatures and low summer temperatures (axis 3 high but axes 1 and 5

Table 4. Presence-absence of major lichen substances in each genetic group and occurrences of major chemotypes (combinations of lichen substances) in each genetic group; "3-hydroxph" = 3-hydroxyphysodic. Rare combinations of secondary substances are not shown. The hypotheses of independence between genetic groups and individual substances are tested with chi-squares (χ^2). For chemotypes, the null hypothesis is that chemotypes are equally likely in all groups, with expected values as 0.25*total number of each chemotype.

		Genetic Group					
		1 (n=11)	2 (n=17)	3 (n=15)	4 (n=12)	χ^2	*p*
INDIVIDUAL SUBSTANCES							
Diffractaic	+	9	3	8	10	16.7	<0.001
	−	2	14	7	2		
Physodic	+	5	16	10	5	11.14	0.011
	−	6	1	5	7		
3-hydroxyph.	+	0	7	0	2	12.74	0.005
	−	11	10	15	10		
Physodalic	+	10	8	11	12	12.43	0.006
	−	1	9	4	0		
CHEMOTYPES							
Diffractaic + physodic + physodalic		4	2	3	3	0.67	0.880
Diffractaic + physodalic		5	1	5	6	3.47	0.320
Physodic + 3-hydroxyph. + physodalic		0	4	0	1	8.60	0.035
Physodic + 3-hydroxyph.		0	3	0	0	9.00	0.029
Physodic + physodalic		0	1	3	0	6.00	0.110
Physodic only		1	6	4	0	8.27	0.041

low; **Table 3**). Physodalic acid was also uncommon at medium annual temperatures but cool summer temperatures, regardless of elevation (axis 1 intermediate and axis 5 low, regardless of axis 3). Physodalic acid was less common in ITS group 2 and more common in group 4 than expected ($p = 0.006$, **Table 4**).

Physodic acid (KC+ orange or pink), the most common substance in *Hypogymnia* worldwide (apart from atranorin), is almost always present in *H. imshaugii* if physodalic acid is absent. It can, however, be present even when physodalic acid is present. Physodic acid occurs in *H. imshaugii* throughout its range (**Fig. 6C**). Regression models suggested a checkered pattern of occurrence, with physodic acid associated with both low and high elevations, but not middle elevations (common on either end of axis 3 but rare in the middle regardless of axis 2 or 5; **Table 3**). This pattern suggests that the presence of physodic acid does not represent a single unified relationship to climate, but that it occurs as parts of multiple chemotypes differing in their relationships to climate. Physodic acid was less common in areas that were most oceanic and with low summer temperatures (low on axes 2 and 5). Physodic acid was more common than expected in ITS Group 2 and less common than expected in Groups 1 and 4 ($p = 0.01$, **Table 4**).

Combined chemotypes. The acids were not independent in their assortment ($p < 0.001$, **Table 2**). For example, 3-hydroxyphysodic acid was much more likely to be present when diffractaic acid was absent. The four unexpectedly common combinations (bold in **Table 2**) of lichen acids (henceforth chemotypes) were modeled separately under the assumption that the combinations of acids may be more informative than their individual occurrences. We also found that these combinations were associated with ITS groups ($p = 0.007$, **Table 4**).

Regressing chemotypes against climatic variables yielded models that were as strong or stronger than regressing individual acids against climate. The chemotype 3-hydroxyphysodic + physodic acids showed the biggest improvement. For this chemotype the fit (AUC) against climate was better than the

models for either of the individual substances, 3-hydroxyphysodic or physodic acid, even though the chemotype model included only two predictors while the individual substance models both had three predictors (**Table 3**).

The diffractaic + physodalic acid chemotype is common in the northern part of the range of *Hypogymnia imshaugii*, and predominates in BC (**Fig. 7A**). This chemotype had a bimodal relationship to climate (**Fig. 8**). Each hump in this diagram corresponds to a region of the climate space where this chemotype is relatively frequent. The bimodal response surface indicates that the chemotype does not have a homogeneous relationship to climate, suggesting the expression of different genotypes within the chemotype, disequilibrium in the distribution relation to climate, or the action of historical or other factors that have disrupted the distribution of this chemotype. The chemotype was most common in two climates: cool overall with high August precipitation and humidity (high on axes 1 and 6; **Table 3**; **Fig. 8**) and moderate temperatures with low August precipitation (middle of axis 1, low on axis 6). The first of these occurs primarily in BC and the northern Rockies while the second peak has a more Mediterranean climate and occurs in the drier parts of western Oregon and Washington. This chemotype was much less common than expected in ITS group 2 and more common than expected in ITS groups 1 and 4 (**Table 4**).

The combination of 3-hydroxyphysodic and physodic acids was fairly rare, but much more common than expected given the overall rarity of 3-hydroxyphysodic acid (**Table 2**). It occurs primarily in western Montana, with scattered occurrences south and west of there (**Fig. 7D**). The chemotype was most abundant at higher elevation suboceanic climates (high on axis 3, moderately low on axis 2; **Table 3**; **Fig. 8**). This chemotype was more common than expected in ITS Group 2 and less common than expected in the other ITS groups (**Table 4**).

The chemotype with only physodic acid was strongly associated with relatively warm high-elevation areas (low on axis 1 and high on axis 3; **Table 3**; **Fig. 8**). The chemotype is geographically widespread, occurring in the northern Rockies, the Cascade Range, and the Sierra Nevada (**Fig. 7C**). This chemotype was more common than expected in ITS groups 2 and 3 (**Table 4**).

We considered lumping all chemotypes with diffractaic and physodalic acids into a single chemotype regardless of the presence of physodic acid. However, within this group, physodic acid was strongly associated with environmental variables (**Table 3**). We detected this by selecting the subset of the specimens containing both diffractaic and physodalic acid (155 specimens), then regressing presence-absence of physodic acid was against climate (**Table 3**). Physodic acid was more likely to be found with diffractaic and physodalic acids in a bimodal pattern (**Fig. 8**), with peaks in moderately warm and moderately cool climates but in both cases with moderate to warm summers. This chemotype occurred throughout the range of the species, except that it did not occur on the eastern fringe of the range (absent from Sierra Nevada, rare in central Idaho and southwestern Montana).

Apart from the variation in these major substances, a few rare chemotypes were found. The most unusual was the presence of placodiolic acid as a major substance, along with physodalic and protocetraric acids. This chemotype was found in a single specimen (Oregon: Josephine Co., Tyee Rapids on Rogue River, 200 m, *McCune 24572*), a specimen that appeared otherwise typical. Placodiolic acid is not known from any other species of North American *Hypogymnia*. Elsewhere in the world it is a rare substance in the genus, occurring as a major substance only in *H. sikkimensis* G. P. Sinha & Elix from India (2003).

Segregates in the Hypogymnia imshaugii complex. The morphotype recently described as *H. amplexa* (Goward et al. in Lumbsch et al. 2010) was represented in our data by six specimens collected by Björk (**Supplementary on-line Tables S1 & S2**) in eastern Washington, all of which had ± appressed, ± contiguous lobes. We obtained ITS sequences from five of these. These specimens fell in all four ITS haplotype groups (see "+" in **Fig. 3**). Furthermore, the four specimens from which we sequenced multiple loci did not segregate into a supported phylogenetic group (see "+" in **Fig. 4**). Other specimens that we studied belonged to this

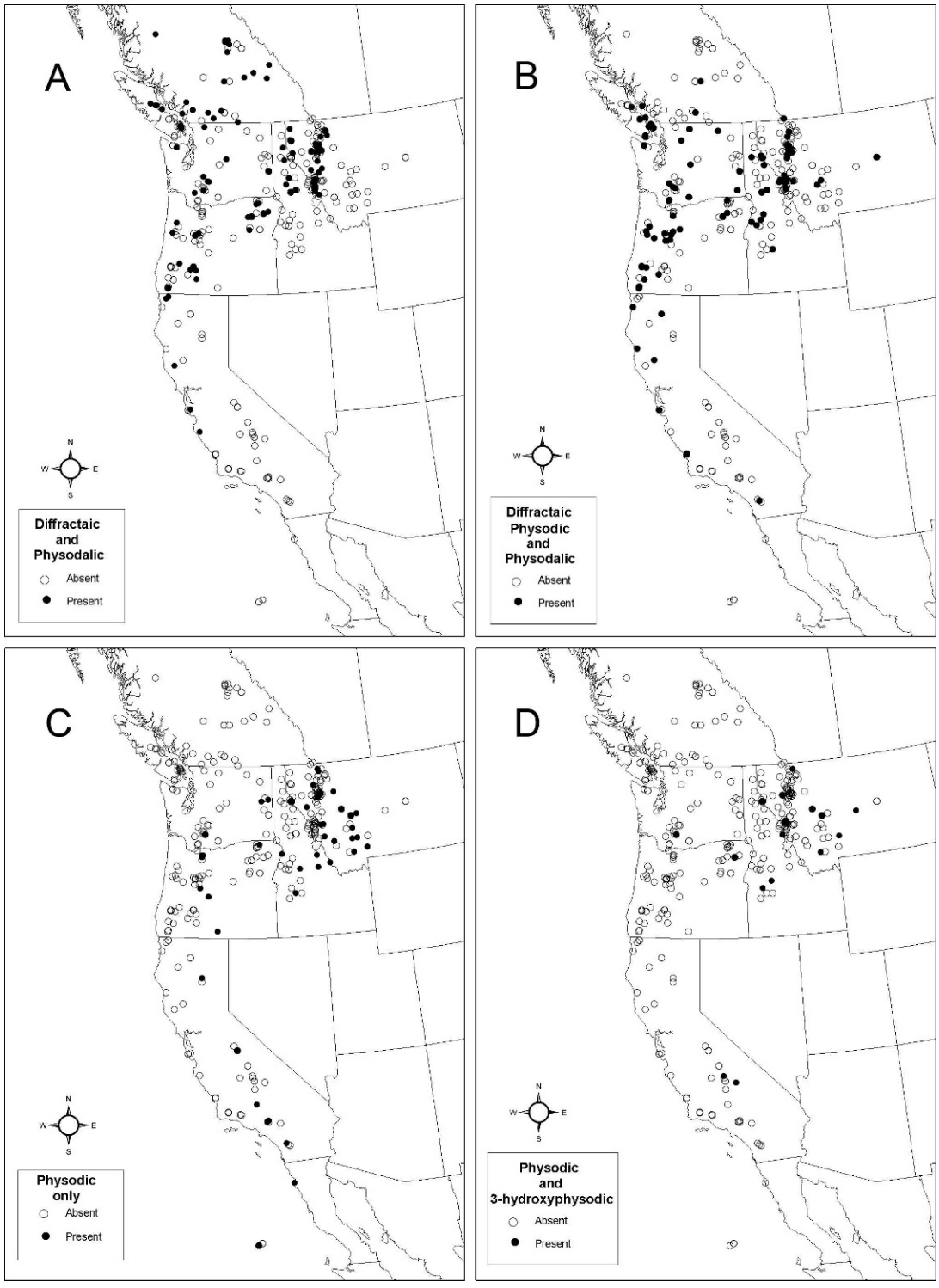

Figure 7. Distribution of combinations of lichen substances in *Hypogymnia imshaugii*. **A.** Diffractaic and physodalic acids. **B** Diffractaic, physodic and physodalic acids. C. Physodic acid only. **D.** Physodic and 3-hydroxyphysodic acids.

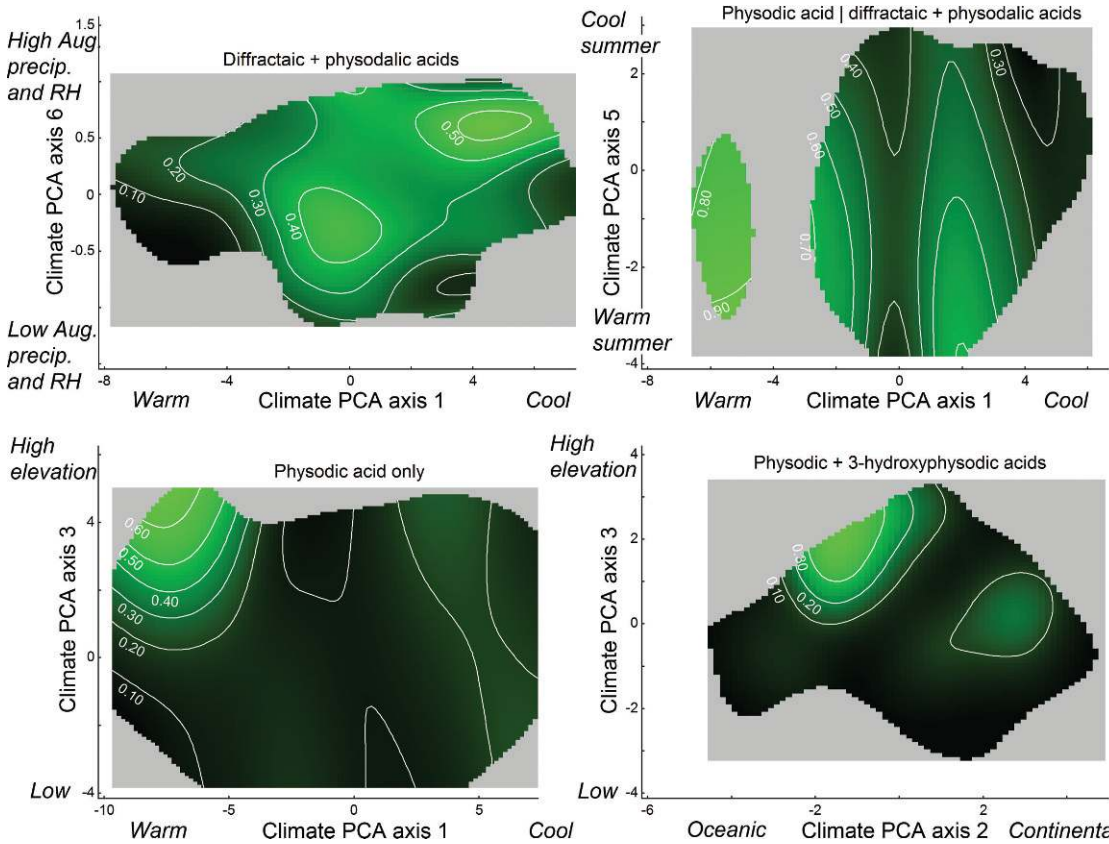

Figure 8. Relationships between occurrences of major chemotypes and climatic variables, showing the fitted surfaces from NPMR models (**Table 3**). Shading and contours indicate the relative frequency of occurrence (0.0 = never, 1.0 = always) under the specified climatic conditions. The climatic axes derived by PCA are labeled with their interpretations (**Table 1**). Background gray areas had insufficient data to make estimates.

morphotype, but we did not try to place them in *H. amplexa* vs. *H. imshaugii* because the morphological and chemical differentiating characters are clinal and difficult to apply. Separation of *H. amplexa* from *H. imshaugii* was not supported by our analyses.

Both *Hypogymnia gracilis* and *H. inactiva* also were placed within the *H. imshaugii* complex. Unlike *H. amplexa*, however, *H. gracilis* and *H. inactiva* are clearly separable morphologically from *H. imshaugii* and do not intergrade with it. Furthermore, *H. gracilis* and *H. inactiva* have some degree of segregation on the tree (**Fig. 4**). *Hypogymnia inactiva* was monophyletic but ambiguously placed relative to *H. imshaugii*. The two *H. gracilis* specimens fell within a well-supported group but were accompanied by several *H. imshaugii* specimens.

Discussion

All *Hypogymnia imshaugii* and *H. amplexa* specimens are grouped in a single well-supported monophyletic group encompassing additional species identified as *H. inactiva* and *H. gracilis* (**Fig. 4**). Within *H. imshaugii*, three relatively tight genetic groups and one poorly-resolved residual group were detected with haplotype networks. Of the four lineages resolved in the ITS network (**Fig. 3**) and other distance-based analyses, none was reconstructed and supported by bootstrap or jackknife analyses in the phylogeny inferred from multiple loci (**Fig. 4**). Whether or not these groups are reproductively isolated is an open question that could potentially be answered with sequences from more loci and analyses of coalescence. At present, we have insufficient evidence to support defining any of these groups as new taxa.

The four major chemotypes in *Hypogymnia imshaugii* differed in frequency among the genetic groups. None of the genetic groups was, however, uniform in chemotype. Only one of the chemotypes occurred in a single genetic group. Our results are, therefore, intermediate on the spectrum of degree of chemotypic variation correspondence with phylogeny (reviewed by Printzen 2010). In *H. imshaugii*, chemotypic variation appears to be ecologically important, as shown by the strong relationships between chemotype occurrences and climate.

Ecological differences among chemotypes have been commonly observed in lichens (e.g. Culberson & Culberson 1967). We cannot usually determine whether the pattern derives from ecological roles of the chemotypes, or whether it simply indicates differences in ancestry – the imprint of past divergences, founder effects, or other barriers to gene flow. In the case of *H. imshaugii*, however, genetic differences in a non-coding region of DNA are partly discordant with chemotypic variation, suggesting that ecological differences among chemotypes, not just ancestry, is responsible for the geographic and climatic patterning of chemotypes.

Chemotypes differed among genetic groups and in their relationships to climate, yet genetic groups had no detectable relationship to geography or climate. The sample size was large enough to provide a reasonable degree of power in the statistical tests. Yet both Mantel tests and MRPP showed no difference in climate among genetic groups. This was true overall and for pairwise contrasts between genetic groups, whether climate variables were considered one at a time or together. The genetic variation within *Hypogymnia imshaugii*, as expressed in our data primarily in ITS and *GPD1*, does not reflect the geographic variation in morphology, chemistry, and climate. The ITS region appears to evolve slowly in *Hypogymnia*, based on the relative uniformity of the ITS across the genus (McCune & Schoch 2008). While it is possible that the morphological variation and quantitative variation in lichen substances in *H. imshaugii* are due to environmental plasticity, the qualitative chemotypic variation is more likely to be genetically controlled. Polymorphisms in lichen substances that differ in biogenesis, and are associated with a biogeographic difference, are often assumed to be genetically-based grounds for separating species (Elix & Stocker-Wörgötter 2008). While these criteria might be met in the case of *H. imshaugii*, the geographic and ecological separation among chemotypes is clinal, rather than sharp, discouraging the taxonomic recognition of chemotypes. Reciprocal transplant experiments are needed to explore the limits of genetic vs. environmental control of morphology and secondary chemistry.

Because the four genetic groups do not separate by geography and climate, recognizing these lineages as distinct taxa is unlikely to improve the utility of lichen communities as indicators of air quality and climate change. The lineages are broadly sympatric, and do not appear to separate by elevation or other habitat factors where they overlap.

Segregates from Hypogymnia imshaugii. Although the great morphological and chemical diversity within *H. imshaugii* suggests the presence of additional unnamed species within the complex, none of the genetic markers we examined revealed them. Nor were the genetic groups within *H. imshaugii* well-differentiated ecologically. Even morphologically and ecologically distinct species within the *H. imshaugii* complex, such as *H. inactiva* and *H. gracilis* cannot be reliably separated from the remainder of the complex based on the chosen loci.

Segregation of the morphotype described as *Hypogymnia amplexa* is not supported by our chemotype or ITS groupings. These specimens fell into two chemotypes, and occurred in all four haplotype groups. We recommend treating *H. amplexa* as a synonym of *H. imshaugii* unless it can be more distinctly separated from the clinal variation in morphology, chemistry, or DNA sequences. In contrast to *H. amplexa*, however, *H. inactiva* and *H. gracilis* are both easily separated morphologically from *H. imshaugii* and do not intergrade with it.

Further resolution of the *Hypogymnia imshaugii* complex requires the use of genetic markers other than those that we included in haplotype and phylogenetic reconstructions. The available data suggest that a broader genomic approach is needed. Testing genetic differentiation within the *H. imshaugii* complex by identifying and amplifying

microsatellite loci could be a future approach (Magain et al. 2010; Widmer et al. 2010).

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Supplementary on-line tables:

Table S1. Specimens used for DNA sequencing. Voucher information and chemical profile.

Table S2: GenBank accession numbers for specimens used for DNA sequencing.