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Source: The Bryologist, 114(2):392-400. 2011.
Published By: The American Bryological and Lichenological Society, Inc.
DOI: 10.1639/0007-2745-114.2.392
URL: http://www.bioone.org/doi/full/10.1639/0007-2745-114.2.392

Hypogymnia phylogeny, including *Cavernularia*, reveals biogeographic structure

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Abstract. We inferred phylogenetic relationships using Bayesian and maximum likelihood approaches for two genera of lichenized fungi, *Hypogymnia* and *Cavernularia* (Parmeliaceae). Based on the combined ITS and *GPD1* dataset from 23 species (49 specimens) of *Hypogymnia* and two species (8 specimens) of *Cavernularia*, we conclude that *Hypogymnia* is paraphyletic, and that it should include *Cavernularia* to retain its monophyly. *Hypogymnia hultenii* (= *Cavernularia hultenii*) and *H. lophyrea* (= *C. lophyrea*) are accepted here. Five species of *Hypogymnia* represented by more than a single individual were found to be monophyletic and significantly supported. The phylogeny reflects a statistically significant biogeographic pattern where continental-scale endemic taxa tend to occur within the same phylogenetic group. Sorediate taxa, which have worldwide or broader geographical ranges than affiliated species lacking soredia, are spread across the phylogenetic tree. *Hypogymnia* contains three species pairs: *H. krogiae* and the sorediate counterpart *H. incurvoides*, *H. minilobata* and the sorediate *H. mollis*, and *H. lophyrea* and the sorediate *H. hultenii*. In the case of *H. minilobata*, both members of the pair are restricted to a small area in southern California. In the other two cases, the fertile counterpart occurs only in North America, while the sorediate species occurs in both North America and Fennoscandia. This suggests but not proves an origin of each species pair in North America, with migration of the sorediate member to Fennoscandia following the prevailing wind direction.

Keywords. Biogeography, *Cavernularia*, DNA sequences, *GPD1*, *Hypogymnia*, ITS, lichenized ascomycetes, Parmeliaceae, species pair.

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DOI: 10.1639/0007-2745-114.2.392

The Bryologist 114(2), pp. 392–400 0007-2745/11/$1.05/0

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The Parmeliaceae is a large, conspicuous family of lichenized fungi, and one of the best studied based on both morphological and molecular data (Blanco et al. 2006; Crespo et al. 2007). This family, as defined by Crespo et al. (2007), appears to be monophyletic. The cupulate exciple is currently the only phenotypic synapomorphy that unites the Parmeliaceae. In this apothecial anatomy, the proper exciple isolates the hypothecium from the medulla. The ''parmelioid'' group within the family appears also to be monophyletic, but *Hypogymnia* (Nyl.) Nyl. and *Cavernularia* Degel. are placed outside that group (Crespo et al. 2007).

Hypogymnia occurs in temperate to subpolar environments with the greatest diversity in oceanic to suboceanic climates. It is found on all continents except Antarctica. In tropical to subtropical latitudes the genus occurs only at high elevations. Approximately 100 species are recognized at present (McCune unpubl.). All *Hypogymnia* species lack rhizines and have thickened lobes (either solid or hollow), bifusiform spermatia, substipitate apothecia, and asci with eight simple, hyaline, ellipsoid to subspherical spores. Most have hollow lobes, a black lower cortex, contain atranorin, physodic acid and related compounds, and have small spores (<9 μm long). A few have solid lobes or a brown to dark brown lower cortex or larger spores or contain usnic acid instead of atranorin.

The related genus *Cavernularia* occurs in oceanic temperate regions of North America and Fennoscandia (Ahti & Henssen 1965; Degelius 1952; Printzen & Ekman 2002). *Cavernularia* is a genus of only two species, appearing like a tiny *Hypogymnia* but with a dense array of small pits in the lower surface. In contrast to the holes in the lower surface, lobe tips, and axils of some species of *Hypogymnia*, the pits in *Cavernularia* do not open into a lobe cavity, but instead open into a small, broadened chamber that is completely lined by the lower cortex. These minute cavities (ca. 0.1 mm diameter) were termed ''cavernulae'' by Degelius (1937), and differentiate *Cavernularia* from all other parmelioid lichens. Otherwise *Cavernularia* resembles *Hypogymnia* in apothecial anatomy, lobe morphology, and chemistry. Both Räsänen (1943) and Krog (1951) merged *Cavernularia* into *Hypogymnia*, but this concept was not adopted by later authors, including later works by Krog.

The species pair concept (Du Rietz 1924; Mattson & Lumbsch 1989; Poelt 1970) has not been applied within *Hypogymnia*. McCune and Schoch (2009) suggested that the sorediate *H. mollis* was sister to the otherwise similar but esorediate *H. minilobata*, both endemic to southern California. In *Cavernularia*, however, *C. hultenii* and *C. lophyrea* are an obvious species pair. In this study we examine the phylogenetic and geographic relationships of other potential species pair within *Hypogymnia* (e.g., *H. incurvoides* and *H. krogiae*).

The phylogeny of *Hypogymnia* has not been studied. In this paper we focus on selected species of *Hypogymnia* and *Cavernularia*, based on reconstructed phylogeny using the combined entire nuclear ribosomal internal transcribed spacer region (ITS: ITS1, 5.8S, and ITS2) and partial glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*). We address the following questions: 1) are species represented in our data by at least two individuals (12 out of 25) monophyletic? 2) what are the phylogenetic relationships among species, including potential species pairs? 3) does *Cavernularia* represent a separate genus or should it be included in *Hypogymnia* as suggested by Räsänen (1943) and Krog (1951)? 4) can we detect a biogeographic pattern based on their inferred phylogenetic history?

Material and Methods

Sampling and alignments. Fungal genomic DNA was isolated from approximately 5 mm long sections of lichen thalli 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 at 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 at 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 (1 kb) of the *GPD1* gene (Myllys et al. 2002; Thell et al. 2004). One ITS PCR product (for *H. macrospora* 2) had to be cloned (due to the presence of multiple peaks in the chromatograms) using the Topo-TA 5-minute PCR cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. We sampled 23 species of *Hypogymnia* represented by 49 individuals and two species of *Cavernularia* represented by eight individuals. *Brodoa intestiniformis* and *Evernia prunastri* were used as outgroup species. For the 79 ingroup sequences included in our dataset, 40 ITS and 20 *GPD1* were newly generated, whereas 15 ITS and 4 *GPD1* sequences were taken from GenBank. For the 59 specimens included in this study, two ITS sequences and 33 *GPD1* sequences were missing. Sequences were aligned manually with MacClade 4.07 (Maddison and Maddison 2003). Newly generated DNA sequences were deposited in GenBank (**Supplementary Table 1**).

The alignment for the combined 59-OTU dataset consisted of 1654 characters, of which 1080 were *GPD1* and the remaining 574 were ITS. For the *GPD1* data, the alignment of 102 characters was ambiguous and therefore excluded from the analyses, and 806 were constant. For the ITS data, 44 sites were ambiguously aligned and excluded from phylogenetic analyses and 401 were constant. A total of 1508 sites were included in phylogenetic analyses, 978 from the *GPD1* and 530 from the ITS.

Phylogenetic analyses. Maximum likelihood (ML) bootstrap analyses were performed on the ITS 57-OTU and *GPD1* 27-OTU datasets separately using RAxML version 7.0.4 (Stamatakis 2006a) for 1000 replicates, implementing a GTR model (GTRCAT; Stamatakis 2006b) for each of the six data partitions (ITS1, 5.8S, ITS2, *GPD1* 1st, 2nd, and 3rd positions). To detect topological incongruence among single gene phylogenies, a reciprocal 70% ML bootstrap support criterion was implemented (Mason-Gamer & Kellogg 1996; Reeb et al. 2004). A conflict was assumed to be significant if a group of taxa was supported at $\geq$ 70% as monophyletic in one majority rule bootstrap tree, but supported as non-monophyletic in another. No conflict was detected, therefore, the two single locus datasets were concatenated in an ITS+*GPD1* 59-OTU combined dataset (TreeBASE; http://purl.org/phylo/treebase/phylows/study/TB2:S11110). A maximum likelihood search using RAxML, 1000 replicates, GTR model with gamma distribution and four discrete rate categories (GTRGAMMA) was performed on the 59-OTU combined dataset. Phylogenetic confidence was estimated for the combined dataset with ML bootstrapping (RAxML), using the same settings as for the single-locus analyses. In addition to ML bootstrap support, posterior probabilities (PP) were obtained from a Bayesian analysis conducted with MrBayes v3.1.1 (Huelsenbeck & Ronquist 2001), which was run with 4 independent chains for 50,000,000 generations, sampling every 500th tree, estimating a six-parameter model for nucleotide substitution (GTR; Rodríguez et al. 1990) with a gamma distribution approximated with four categories, and a proportion of invariable sites for six out of the seven data partitions. For the 5.8S, the Kimura-2-parameter model was used (Kimura 1980). All model parameters were unlinked. Models of evolution for Bayesian analysis were selected using the hierarchical Likelihood Ratio Test as implemented in Modeltest v3.7 (Posada & Crandall 1998). Two independent Bayesian runs were conducted to ensure that stationarity was reached and the runs converged at the same log-likelihood level (verified by eye and with the AWTY option; Nylander et al. 2008; Wilgenbusch et al. 2004). After discarding the burn-in, 25,000 trees of each run were pooled to calculate a 50% majority-rule consensus tree. Bootstrap proportions $\geq$ 70%, and posterior probabilities $\geq$ 0.95, were considered significant.

Testing the phylogeographic structure. We tested the null hypothesis of no relationship between continental-scale endemism and supported monophyletic multiple-species groups with a Pearson chi-square (χ^2) test of independence of continental groups vs. phylogenetic groups. Each of the 25 species of *Hypogymnia* included here (approximately 25% of total number of species in the genus), was assigned to one of four groups of endemism: Asian,

Austral (including South America, Australia, and New Zealand), North American, and widespread species occurring in two or more of the preceding three groups (= biogeographic coding; **Fig. 1**). Three species primarily on one continent but with a small number of disjuncts on one other continent were assigned to the primary continent (*H. hultenii*, *H. incurvoides*, and *H. subphysodes*). Alternative biogeography coding where these three species were classified as widespread was also tested.

Species were also assigned to one of four groups (groups 1 to 4; **Fig. 1**) corresponding to mutually exclusive multi-species significantly supported clades in our phylogenetic tree. Three additional species (*H. physodes*, *H. pulverata*, and *H. wilfiana*) not falling within one of those monophyletic multi-species groups were assigned to a fifth group (group 0; **Fig. 1**). We also evaluated an alternative coding, where the group of primarily North American species was divided into its four supported mutually exclusive multi-species subgroups, for a total of eight groups.

Results and Discussion

Well-supported phylogenetic relationships revealed by the ML and Bayesian analyses are in agreement. Overall, slightly more internodes were significantly supported by ML bootstrap compared to Bayesian analysis (**Fig. 1**). Five of ten *Hypogymnia* species represented by more than one specimen (*H. minilobata*, *H. occidentalis*, *H. physodes*, *H. pruinosa* and *H. tubulosa*) were revealed as monophyletic, with high support. *Hypogymnia imshaugii* becomes monophyletic if *H. inactiva* (represented by a single specimen) is included. Recently the species complex of *H. imshaugii* was the focus of a separate study (McCune et al. 2011). *Hypogymnia apinnata* and *H. enteromorpha* were intermixed within a single well-supported clade. More sampling with more variable loci is needed to evaluate species delimitation within these groups.

One of the three clones of an ITS amplicon for *Hypogymnia macrospora* 2 (C) did not group with the three other ITS sequences of *H. macrospora* that form a well supported monophyletic group, but no significant support was obtained for the placement of *H. macrospora* 2C outside of the monophyletic *H. macrospora* species. It is very likely that different sequences of *H. macrospora* 2 represent different ITS copies within this individual and/or contamination, such as spores, from other nearby *Hypogymnia* species. ITS sequencing from single-spore isolated strains are needed to determine which of these two interpretations is most likely, if both factors are not at play.

Our phylogeny confirms monophyly (significant ML and PP support) of three putative species pairs, *Hypogymnia krogiae*/*H. incurvoides*, *H. minilobata*/*H. mollis*, and *C. lophyrea*/*C. hultenii*. Both known species for *Cavernularia*, *C. hultenii* and *C. lophyrea*, were found to be monophyletic, however, not well-supported (**Fig. 1**).

Three main points emerged from our analyses: (1) The monophyletic genus *Cavernularia* is nested within *Hypogymnia* and, therefore, should be treated as part of the genus *Hypogymnia*; (2) major groups within *Hypogymnia* are structured biogeographically; and (3) it is very likely that two instances of species pairs appear to have originated in North America, with the sorediate member migrating to Fennoscandia. Each of these points is discussed below.

Cavernularia was erected by Degelius (1937) to accommodate two peculiar little hypogymnioid lichens, the esorediate *Parmelia lophyrea* Ach. and an undescribed sorediate but otherwise similar counterpart. The two species, *C. lophyrea* (Ach.) Degel. and *C. hultenii* Degel., are a species pair that share an unusual morphology of the lower surface. Instead of the smooth or irregularly wrinkled surface typical of *Hypogymnia*, *Cavernularia* was separated solely on the basis of having an array of pronounced but small depressions in the lower surface. Räsänen (1943) formally named *Cavernularia* as a section within *Hypogymnia* (''Sekt. 1. *Cavernularia* (Degelius) Räs.''), but did not introduce new combinations of species. Krog (1951) later shifted both species of *Cavernularia* to *Hypogymnia*, forming the new combinations *Hypogymnia hultenii* (Degel.) Krog and *Hypogymnia lophyrea* (Ach.) Krog. Krog (1951) did not cite the basionym or the original place or date of publication, instead just making an indirect reference. The combinations are, however, legitimate based on Art. 33.2 of the Vienna Code

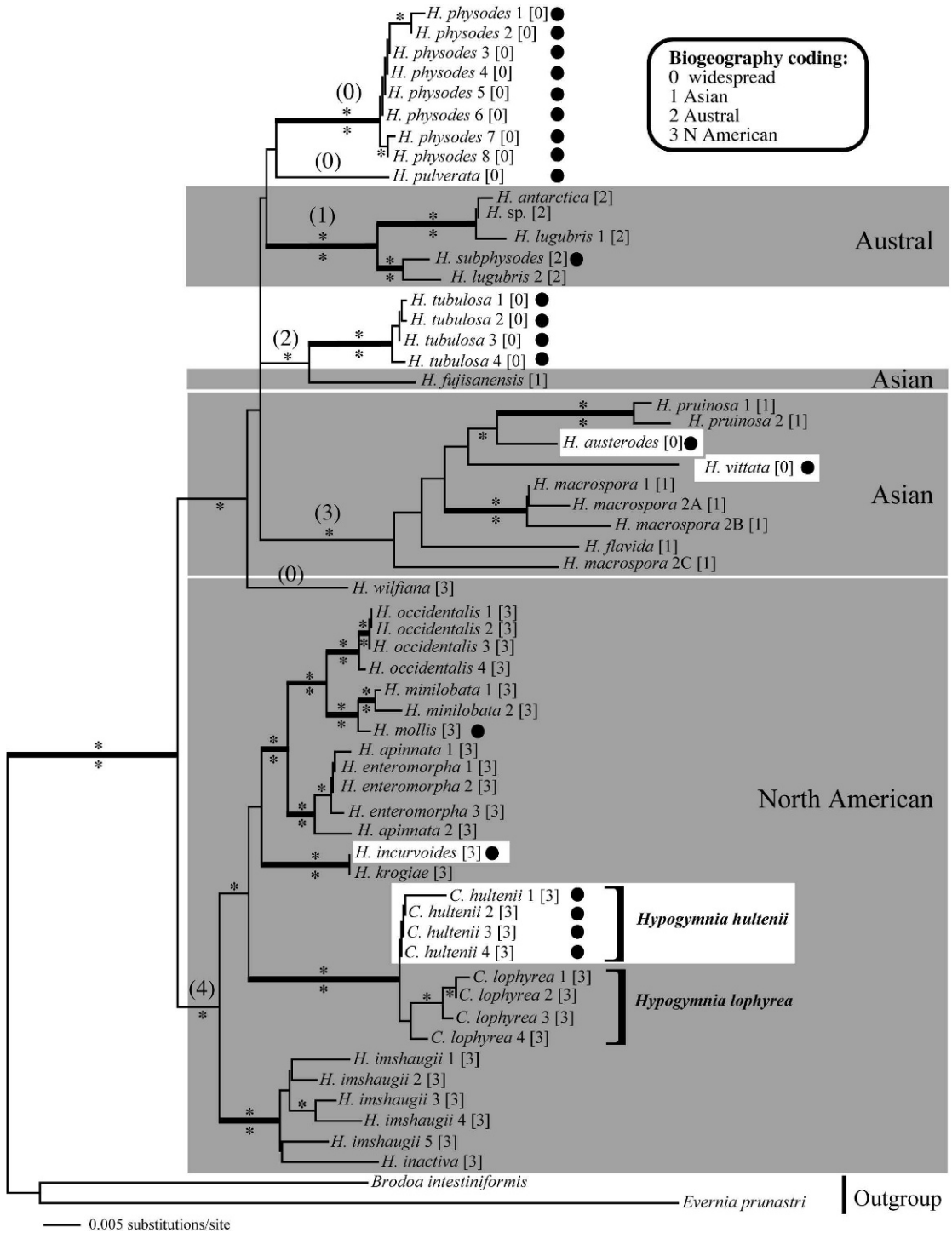

Figure 1. Phylogenetic relationships among 57 members of *Hypogymnia*, including *Cavernularia*, resulting from a maximum likelihood search (RAxML) using combined ITS and *GPD1* dataset (-lnL 5013.4). *Evernia prunastri* and *Brodoa intestiniformis* were used as outgroup. Stars indicate significant support: above internodes indicate ML bootstrap values ≥ 70%, and below internodes represent PP values ≥ 0.95. Black dots indicate taxa with soredia. Numbers in square brackets following taxon names indicate coding for the geographic distribution whereas numbers in parenthesis above internodes indicate coding for the phylogenetic groups used in chi-square test for the phylogeographic structure (see Material and Methods; *Testing for the phylogeographic*

(McNeill et al. 2006) because they were published before January 1953. Although Krog (1968) and Dahl and Krog (1973) later accepted *Cavernularia*, Räsänen's and Krog's earlier inclusion within *Hypogymnia* was supported here. Based on the phylogeny shown in **Fig. 1**, *Cavernularia* should not be retained as a separate genus and we accept its synonymy with *Hypogymnia*.

The unique most recent common ancestry of the two *Cavernularia* species is well supported but their respective monophyly is not (**Fig. 1**). Very short branches within the widely distributed *C. hultenii* reflect little genetic variation resulting from clonal reproduction (through soredia) of this mostly asexual species. The strictly North American, *C. lophyrea* shows higher intragenic variation perhaps due to common recombination, as it is always dispersed by sexually generated propagules (ascospores). The limited distribution of *C. lophyrea* may reflect shorter viability of ascospores coupled with higher sensitivity to desiccation, compare to soredia, and difficulties finding the appropriate algal partners when dispersed by fungal ascospores (horizontal transmission of the photobiont from generation to generation), compared to long distance simultaneous dispersal of both partners (algal and fungal) by soredia in *C. hultenii* (vertical transmission of the photobiont).

Significantly supported multi-species monophyletic groups were strongly associated with continental-scale endemism ($\chi^2 = 44.3$, *d.f.* $= 12$, $p < 0.001$ for coding shown in **Fig. 1**; $p < 0.001$ for all alternative coding) with endemic taxa tending to occur within the same monophyletic group. Two conditions seem necessary to explain the grouping of continental-scale endemics within the phylogenetic framework. First, speciation within *Hypogymnia* has mainly occurred after separation of the continents. Second, gene flow via long-distance dispersal has been largely ineffective at homogenizing the genus across continents.

North American endemics restricted to a single clade (with the exception of *H. wilfiana*) are sister to the remaining clades encompassing Australasian and widespread taxa (significant PP support). Endemic Asian, Austral (Australia, New Zealand, and South America), and North American species represent independent phylogenetic lineages (**Fig. 1**). Several exceptions to the revealed geographical trend are apparent. Widespread species, such as *H. physodes* and *H. tubulosa* (two sorediate species), are placed outside those endemic groups. A Swedish specimen of *H. vittata* (another sorediate species) is nested within a group of species that is mostly endemic to Asia (significant ML bootstrap support), suggesting an origin in Asia. *Hypogymnia vittata* is known from North America, Europe, and Africa; more sampling is needed to test if specimens from distant geographical localities are similar genetically to the specimen used here. Likewise, *H. austerodes*, a widespread sorediate species (**Fig. 1**) in the northern Hemisphere, as well as Africa, is placed within the group dominated by Asian endemics. Only one Asian endemic, *H. fujisanensis* (Asahina) Kurok. (Kurokawa 1971), is placed outside this group. It showed an affinity with *H. tubulosa*, despite a markedly different morphology and chemistry. *Hypogymnia fujisanensis* has narrow, appressed, richly branched esorediate lobes with the cavity collapsed and lower surface greatly folded and intricate, forming a thick layer; medulla K+ slow reddish brown, KC+ orange red, P+ orange red (physodalic acid). This contrasts with the sorediate, suberect, P- lobes of *H. tubulosa* (physodalic acid lacking).

Hypogymnia wilfiana is a North American endemic recently segregated from the Asian *H. metaphysodes* (Goward et al. 2010). It shares morphological similarities with *H. antarctica* (South American) and *H. physodes* (widespread). Its phylogenetic placement remains unclear (**Fig. 1**).

Two species pairs had similar continental-scale distributional patterns. North America hosts both the fertile and sorediate counterparts for *Hypogymnia lophyrea* /*H. hultenii* and *H. krogiae*/*H. incurvoides*, while only the sorediate species (*H. hultenii* and *H. incurvoides*) occur in Fennoscandia. A possible interpretation of this pattern is an origin of all four species in North America followed by long-distance

←

structure). Gray boxes delimit members in different biogeographic groups. Taxa with a white background have broad distributions. This schematic representation of distributional patterns applies only to the ingroup.

dispersal of the sorediate members to Fennoscandia, in keeping with prevailing winds. A population-level analysis of ITS and IGS for *H. hultenii* suggests, however, that the pattern may not be as simple as that (Printzen & Ekman 2002). Based on haplotype networks, they hypothesized that the current distribution of *H. hultenii* resulted from fragmentation of a formerly continuous range. Nested clade analysis of a dataset with more individuals was, however, equivocal in supporting various scenarios of dispersal and range expansion (Printzen et al. 2003).

Our results suggest that sexually reproducing species of *Hypogymnia*, without soredia, speciate locally, and perhaps often sympatrically, because reproduction with ascospores seems to constrain their geographical expansion; whereas mostly asexually reproducing (sorediate) species exhibit geographical range expansions and long-distance dispersals. Long distance dispersal by soredia could allow the initiation of local (endemic) speciation at remote locations, if sexual reproduction is not completely lost and a reversal to a mostly sexual reproductive mode occurs, as it seems to be the case for *Umbilicaria* species endemic to the Andes (Hestmark et al. 2011). The older these long dispersal events are, the longer the time periods are for speciation, and resulting species diversification, to take place. This dual dispersal strategy (short- and long-distance) directly associated with two reproductive modes (sexual and asexual, respectively) alternating back and forth (cyclic) through time leading to endemic speciation of mainly sexually reproducing populations, could be at work in many lichen genera that include sorediate species. Sexually reproducing individuals are often found, although at extremely low frequencies, in populations of most lichen species reproducing mostly with soredia (e.g., Tønsberg 1992). The older the age of the ancestor for clades including sorediate species, the more cycles of long dispersal followed by local speciation could take place, with the potential of resulting in more complex macroevolutionary histories, as it seems to be the case for *Umbilicaria*, compare to relatively young genera, such as *Hypogymnia*.

Within *Hypogymnia*, sorediate taxa do not cluster together (**Fig. 1**). This pattern, along with the taxon sampling for this study, suggests multiple independent transitions from the absence to the presence of a vegetative mode of reproduction. Within *Porpidia s.l.*, a lichen genus rich in species pairs, losses of soredia were reconstructed as more frequent than gains (Buschbom & Barker 2006). The authors reported that the unequal rates of change in the reproductive mode were independent of taxon sampling, however, the reconstructions of the ancestral states were not. Because our dataset is highly incomplete (contains only 25 of ca. 100 recognized species) and the resulting phylogeny partly inconclusive (many phylogenetic relationships are poorly supported), the reconstructions of the evolutionary history of the vegetative reproductive trait within the genus *Hypogymnia* using maximum likelihood option in Mesquite (Maddison & Maddison 2010) were highly inconclusive. More loci and a more complete taxon sampling are needed to clarify relationships within the genus and to track evolutionary transitions between sorediate and non-sorediate taxa, including an exhaustive sampling of outgroup taxa to unravel the reproductive trait of the ancestor to *Hypogymnia*. For example, we need to sample more species with rimmed holes (SW China; McCune et al. 2002; represented here by *H. macrospora*), the sorediate *H. pseudophysodes* complex in Far East Asia along the north Pacific (*H. bullata*, *H. pseudophysodes*, and *H. submundata*), south Asian species (*H. pseudobitteriana*, *H. zeylanica*), the *H. austerodes* group (with *H. bitteri* and *H. subobscura*), the solid-lobed Austral species (*H. pulverata*, *H. mundata*, *H. billardieri*, and *H. tubularis*), island endemics (*H. tavaresii*, *H. madeirensis*, *H. guadalupensis*), and unusual species that are difficult to place (*H. rugosa* and *H. bryophila*; endemic to North America and Portugal, respectively).

Acknowledgments

We thank the curators of ASU, BM, COLO, H, MIN, NY, OSC, S, SBBG, UBC, UC, UCR, UPS, US, WIS, and WTU who kindly assisted with loans and visits, Desirée Johnson for laboratory assistance, and Joey Spatafora for advice and the use of his lab. Thanks to the many people who provided specimens, including Frances Anderson, Curtis Björk, Trevor Goward, Jill Grenon,

Sarah Jovan, Kerry Knudsen, Dave Kofranek, Peter Nelson, Eric Peterson, Andrea Pipp, Jennifer Riddell, Jim Riley, Heather Root, Toby Spribille, Li-song Wang, Tim Wheeler, and the Forest Inventory and Analysis program of the USDA Forest Service. We are thankful to Tom Milledge and John Pormann for their generous assistance with the Duke Scalable Computing Support Center and to Molly McMullen for proofreading this manuscript. Thanks to David Hawksworth for pointing out Räsänen's early placement of *Cavernularia* within *Hypogymnia*. Many sequences used here were generated through support of the USDA Forest Service PNW Station to McCune. CLS was supported in part by the Intramural Research Program of the NIH, National Library of Medicine. JM and KM were supported in part by the Assembling the Fungal Tree of Life (AFToL) project, funded by NSF Assembling the Tree of Life (AToL) award DEB-0228668 to FL.

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ms received August 18, 2010; accepted March 11, 2011.

Supplementary Table S1: Voucher information and GenBank accession numbers.