

**MORPHOLOGICALLY CRYPTIC BIOLOGICAL SPECIES WITHIN THE
 LIVERWORT *FRULLANIA ASAGRAYANA*¹**

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- *Premise of the study:* The *Frullania tamarisci* complex includes eight Holarctic liverwort species. One of these, *F. asagrayana*, is distributed broadly throughout eastern North America from Canada to the Gulf Coast. Preliminary genetic data suggested that the species includes two groups of populations. This study was designed to test whether the two groups are reproductively isolated biological species.
- *Methods:* Eighty-eight samples from across the range of *F. asagrayana*, plus 73 samples from one population, were genotyped for 13 microsatellite loci. Sequences for two plastid loci and nrITS were obtained from 13 accessions. Genetic data were analyzed using coalescent models and Bayesian inference.
- *Key results:* *Frullania asagrayana* is sequence-invariant at the two plastid loci and ITS2, but two clear groups were resolved by microsatellites. The two groups are largely reproductively isolated, but there is a low level of gene flow from the southern to the northern group. No gene flow was detected in the other direction. A local population was heterogeneous but displayed strong genetic structure.
- *Conclusions:* The genetic structure of *F. asagrayana* in eastern North America reflects morphologically cryptic differentiation between reproductively isolated groups of populations, near-panmixis within groups, and clonal propagation at local scales. Reproductive isolation between groups that are invariant at the level of nucleotide sequences shows that caution must be exercised in making taxonomic and evolutionary inferences from reciprocal monophyly (or lack thereof) between putative species.

Key words: biological species; cryptic speciation; *Frullania*; Frullaniaceae; gene flow; population structure; reproductive isolation.

Considerable attention has been given to “the species problem,” and there is widespread application of phylogenetic approaches to circumscription of species. Nevertheless, most species are distinguished because they look different (i.e., are morphologically distinct from other putative species). In small, structurally simple plants such as bryophytes, morphology can be misleading because of convergence to similar morphologies in unrelated plants (e.g., Holyoak and Pedersen, 2007; Feldberg et al., 2010) and confusing levels of morphological plasticity that can make the identification of genetically based differences unclear (e.g., Patterson et al., 1998; Såstad, 1999; Buryova and Shaw, 2005). Some morphologically differentiated species are not resolved as monophyletic on the basis of phylogenetic analyses of nucleotide sequences, possibly because (1) they diverged

recently, (2) they are distinguished by environmentally induced character states, (3) the distinguishing features are polyphyletic in origin, or (4) there is insufficient variation at the loci examined (e.g., Holyoak and Hedenäs, 2006; Hedenäs, 2008). At the other end of the spectrum, morphologically cryptic lineages have been resolved within some widespread bryophytes and these have been interpreted as cryptic species (reviewed in Shaw, 2001; Heinrichs et al., 2009b). An extreme case recently came to light in the liverwort species *Metzgeria furcata* (L.) Dumort. (Metzgeriaceae) (Fuselier et al., 2009). Populations in North America and Europe are morphologically indistinguishable but belong to divergent sister clades that differ by over 60 nucleotide substitutions in sequenced portions of the plastid and nuclear genomes.

Few studies of widespread bryophyte species have rigorously tested for evidence of reproductive isolation between putative cryptic species, although sympatric mixed populations without obvious evidence of recombination suggest that some are distinct biological species. Here, we describe population structure on both local and regional scales in the leafy liverwort *Frullania asagrayana* Mont. (Frullaniaceae) and present analyses of reproductive isolation between two groups of genetically divergent populations. *Frullania* Raddi is one of the largest genera of liverworts, with an estimated 350 species worldwide (Schuster, 1992). *Frullania asagrayana* is part of the so-called *F. tamarisci* complex, which includes plants with elongate leaf lobules (the

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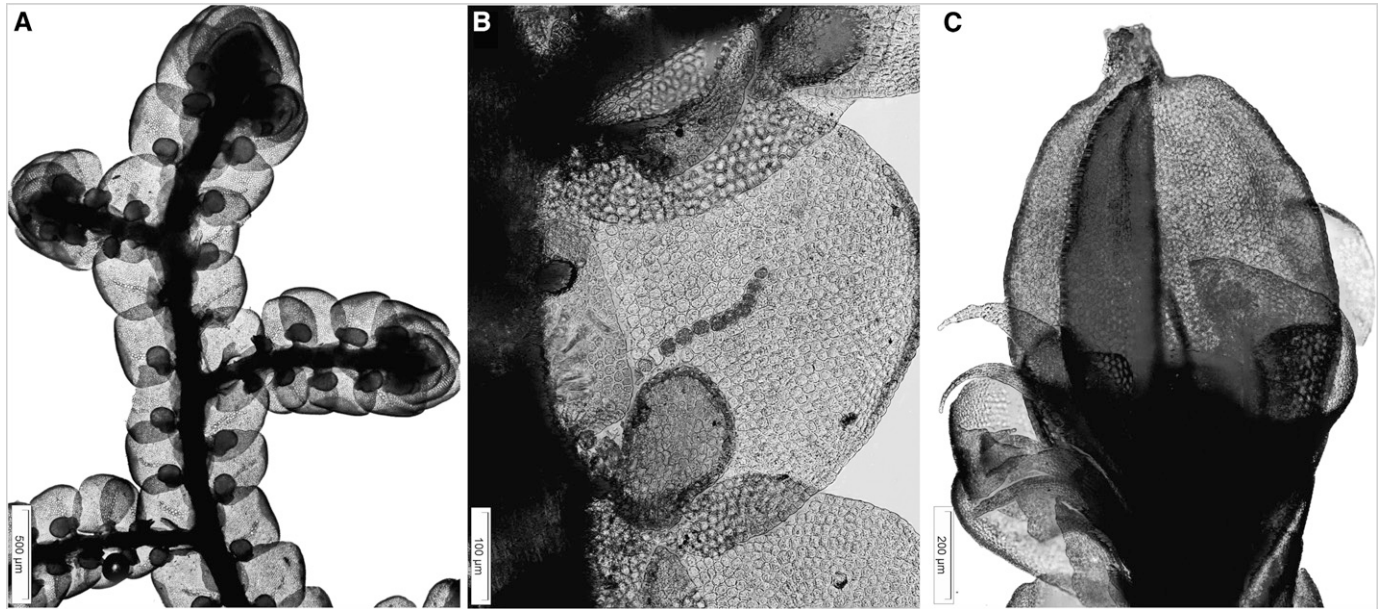


Fig. 1. Gametophyte morphology of *Frullania asagrayana*. (A) Ventral view showing lobules of the lateral leaves. (B) Close-up of one lateral leaf showing the line of specialized cells, called ocelli, each containing one large oil body. (C) Flask-shaped perianth with beaked opening at summit. The perianth surrounds the developing sporophyte until maturity, at which time the seta of the sporophyte elongates rapidly, raising the sporangium or capsule out of the perianth for release of spores.

smaller, ventral segment of a complicate-bilobed leaf, often forming a water sac) and ocelli (specialized leaf cells that contain one large oil body and lack chloroplasts) (Fig. 1). There has been much disagreement about the taxonomic status of morphological variants within the complex, with some authors recognizing one polymorphic species that occurs on multiple continents, and others, multiple species that have more restricted ranges (Hattori, 1972; Crandall-Stotler et al., 1987; Schuster, 1992). Data from nucleotide sequences (Heinrichs et al., 2010) and variation at microsatellite loci (M. Ramaiya et al., unpublished data) strongly support the recognition of separate

species within this complex, and here we adopt such a taxonomy. *Frullania asagrayana* is one of at least eight species of the *F. tamarisci* complex, which is distributed throughout the Holarctic and reaches Java and Sumatra in southeastern Asia. *Frullania asagrayana* is restricted to eastern North America from the maritime provinces of Canada to the Gulf Coast of the United States (Hicks, 1992; Schuster, 1992). The only other species in the complex that occurs in eastern North America is *F. tamarisci* (L.) Dumort. s.str., which grows in the northeastern United States and southeastern Canada but is more common and widespread in Europe (Grolle, 1970; Schuster, 1992).

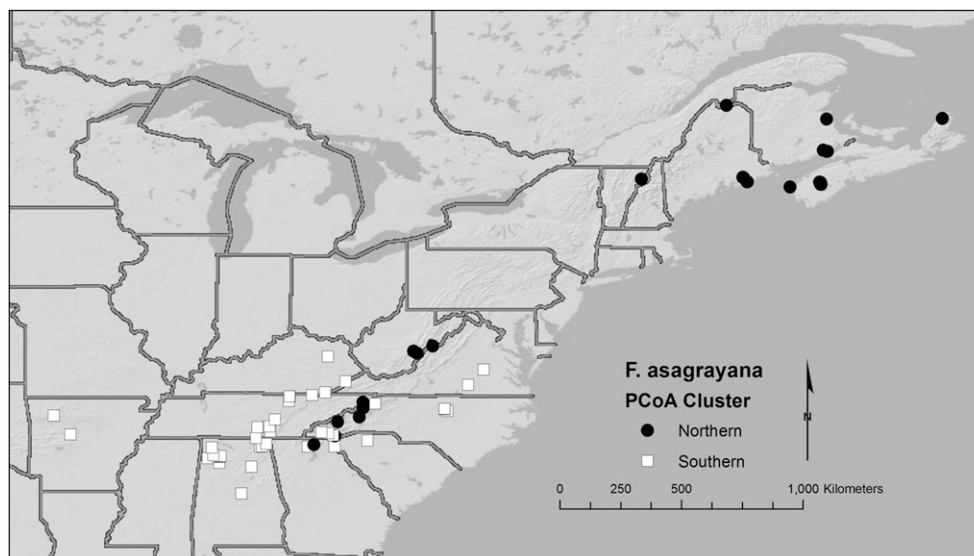


Fig. 2. Map of the eastern United States showing the sampling of plants included in the molecular analyses. Classification of plants as belonging to the Northern or the Southern cluster is based on the principal coordinate analyses (see Fig. 3).

TABLE 1. Primer sequences for microsatellite loci in species of the *Frullania tamarisci* complex.

Primer name	5'	3'
Fru_9	ACACATGACTTGT AGGTGTAGC	ATTCCACAACCAA AGCCCC
Fru_10	AGCTAAGATGCTA ATGCCCC	AGGTCCAAGTCCA CCTTGC
Fru_22	CTCTGGCTCCATC TCCCAC	TGGATGAGTGGCA GTTCAC
Fru_29	ATGGAATTGCAAC CGACATC	TTCTCCGCAGACC CTGAAC
Fru_39	TTGGGTACGTCCG AGGTTG	GGCGATTGATAGC GTGGAG
Fru_53	GGGTTCAAGCCCA AACAAAG	TCAATGAGTGATC CTTCTCTCC
Fru_75	CAGTGAGGGAGAC AGACGC	CCAGCGCCCCATT TTCTAC
Fru_79	GCAGAAGAGGAGG ACGAGG	CTCTCCATCGGGG CCTAAC
Fru_83	CTAACGGCCCAAA CCTTGC	TCCTTCTGAACA CTCTTTTCG
Fru_84	GGAATTGCTCAG TGGGAC	CACCAGGCTCAGA GGAGAYG
Fru_88	CCCGCTCTCCTGC TGATAG	CGATCAGCATC GTCCTC
Fru_93	TTGCGACTCGAAG TTGTGC	GGAGCAGATGCGT TTGGTG
Fru_94	TCAGAATCGAGCG TGGGAG	CATCTGTGCACC TGTTACG

Preliminary evidence from microsatellite analyses of *F. asagrayana* revealed high levels of genetic diversity and suggested that there may be morphologically cryptic groups within the taxonomic species. Here, we report genetic and phylogenetic analyses of populations from throughout most of the range of *F. asagrayana*. We document two strongly differentiated groups based on hypervariable microsatellites that are not resolved by either morphology or sequence data. The goal of this study is to test, using Bayesian and coalescence models, whether these groups represent reproductively isolated biological species.

MATERIALS AND METHODS

Study species—*Frullania asagrayana* has separate male and female gametophytes, as do all other taxa in the *F. tamarisci* complex, and lacks asexual reproduction by caducous leaves or gemmae, which are frequent in the closely related species *F. microphylla* (Gottsche) Pearson and *F. fragilifolia* (Taylor) Gottsche, Lindenb. & Nees (Hentschel et al., 2009). The species forms sporophytes fairly frequently, though mating systems, including gamete dispersal distances and the efficacy of sporeling establishment, are completely unknown. *Frullania asagrayana* typically occurs in deciduous forests, on tree bark, at the bases of trees, and on rocks. The species generally grows in small patches of a few square centimeters. It is likely that mating can occur only between individuals growing on the same tree or rock, as sperm require water for movement from antheridia to archegonia.

Plant sampling—Regional sampling was based on 88 collections of *F. asagrayana*. The *F. asagrayana* samples represent most of the known range of this species, from New Brunswick south to Georgia, Alabama, Tennessee, and Kentucky (Fig. 2). There is a gap in our sampling along the mid-Atlantic states, and the species is also said to occur westward to Minnesota (Schuster, 1992); future work should address these distributional gaps, but the results are not likely to affect our main goal of assessing reproductive isolation between the two groups of populations resolved by genetic analyses. Voucher information is provided in Appendix 1; specimens are preserved in the following herbaria: DUKE, TENN, and UNAF. Each population included in the regional sampling

was represented by a single herbarium collection, from which one gametophyte was extracted for DNA.

More intensive local sampling at a single North Carolina site was conducted in July 2009. A total of 73 gametophytes of *F. asagrayana* were collected along the north and northwest slopes of Occoneechee Mountain in Orange County, North Carolina, using a hierarchical sampling scheme. Four sampling “sites” separated by ~100 m were identified along a trail through a mixed forest dominated by species of *Quercus* L., *Carya* Nutt., and *Pinus* L. Selection of sites was based on seeing proximate groups of trees and rocks bearing patches of *F. asagrayana* (which is patchily distributed throughout the forest). Within each sampling site, three to five “subsites” were selected; each subsite was a single tree or rock with multiple patches of *F. asagrayana*. Within each subsite, one gametophyte was sampled from each of five “patches.” It was not known whether individual patches represent a single genetic individual or a population of multiple individuals. Analyzing multiple samples from each of the 73 individual patches was not feasible, but we assessed the genetic make-up of three patches by genotyping five separate gametophyte stems from each.

Microsatellite genotyping—Microsatellite loci were newly developed for our study of the *F. tamarisci* complex, and the previously unpublished primer sequences are provided in Table 1. Genetic Identification Services (Chatsworth, California, USA) “enriched” the genomic DNA for microsatellite motifs. Their methods for doing so employ a proprietary process that involves selection from among fragments of genomic DNA, of regions that contain microsatellites of a particular motif (such as –AC). The enriched product is then packaged into a plasmid library for screening and design of primers targeting known microsatellite-containing regions. Loci are isolated from the entire genome, and libraries are constructed for various motifs.

A total of 161 samples of *F. asagrayana* were included in the microsatellite study. DNA extractions were accomplished according to procedures outlined in Shaw et al. (2008). Among the microsatellites developed for ongoing research on *Frullania*, 13 loci were assayed for this study: 9, 10, 22, 29, 39, 53, 75, 79,

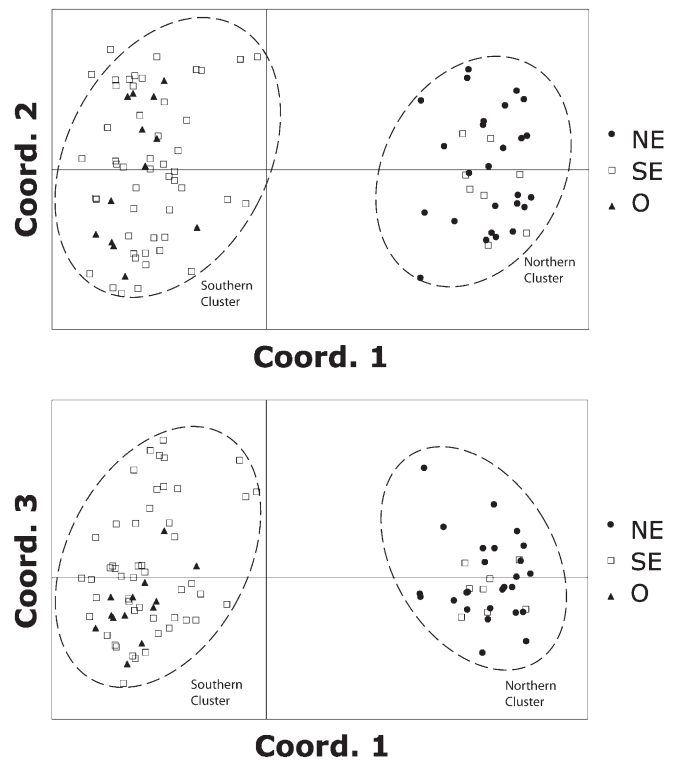


Fig. 3. Principal coordinate (PCo) plot of *Frullania asagrayana* samples from eastern North America. Geographic provenance of samples: NE = northeastern United States, SE = southeastern United States, and O = Occoneechee Mountain. Percentage variance explained by the first three coordinates: PCo1: 49.1%, PCo2: 13.8%, PCo3: 11.6, cumulative total: 74.4%.

TABLE 2. Private alleles and their frequencies in the Southern and Northern groups of *Frullania asagrayana*.

Population	Locus	Allele	Frequency
Southern	83	88	0.419
Southern	83	126	0.081
Southern	83	129	0.048
Southern	83	132	0.081
Southern	83	141	0.048
Southern	84	225	0.021
Southern	84	247	0.021
Southern	84	250	0.042
Southern	84	256	0.021
Southern	84	279	0.021
Southern	10	209	0.952
Southern	10	212	0.048
Southern	9	179	0.066
Southern	9	185	0.721
Southern	9	188	0.131
Southern	9	194	0.016
Southern	75	229	0.085
Southern	75	231	0.017
Southern	75	235	0.017
Southern	75	240	0.051
Southern	22	154	0.016
Southern	22	172	0.016
Southern	53	224	0.054
Southern	53	227	0.161
Southern	53	230	0.536
Southern	53	233	0.089
Southern	53	236	0.143
Southern	53	239	0.018
Southern	88	198	0.067
Southern	88	207	0.033
Southern	88	216	0.150
Southern	88	225	0.483
Southern	88	234	0.133
Southern	88	243	0.050
Southern	88	252	0.050
Northern	83	85	0.029
Northern	83	97	0.029
Northern	83	106	0.029
Northern	84	228	0.065
Northern	84	244	0.032
Northern	10	206	1.000
Northern	29	242	0.567
Northern	29	245	0.333
Northern	29	248	0.033
Northern	94	167	0.033
Northern	22	190	0.031
Northern	22	196	0.031
Northern	79	200	0.032
Northern	53	218	0.970
Northern	53	221	0.030
Northern	39	241	0.030
Northern	39	243	0.061
Northern	39	252	0.061
Northern	88	228	0.030
Northern	88	240	0.121
Northern	88	249	0.061

83, 84, 88, 93, and 94. Locus 22 was included in the study of local genetic structure at Occoneechee Mountain but not in the regional survey. Surveys of primer amplification across a diversity of *Frullania* species indicate that these primers work for taxa within the *F. tamarisci* complex (i.e., *F. asagrayana*, *F. californica* (Austin ex Underw.) A. Evans, *F. nisqualensis* Sull., *F. tamarisci*) but not more broadly across the genus. This specificity contrasts with the situation in the moss genus *Sphagnum*, where microsatellite markers developed for species in one complex amplify effectively without evidence of ascertainment bias across the whole genus (Shaw et al., 2008). DNA extractions and microsatellite genotyping were accomplished according to protocols described by Ricca

et al. (2008). Across all loci and all samples, there was 6% missing data, ranging among loci from 0% (locus 83) to 18% (locus 93).

One allele was amplified for most loci from most individuals, but in a limited number of cases two alleles amplified such that a gametophyte appeared to be heterozygous. As there is no evidence that gametophytes of *F. asagrayana* are polyploid, these occasional heterozygous patterns were interpreted as localized duplications. No individual in the data set was heterozygous for more than one locus, and 14 individuals (9% of all samples) were heterozygous at one locus. To analyze the data as haploid individuals, we took the conservative approach of eliminating the second allele for individuals as necessary. In most cases the eliminated allele was unique to an individual that carried the duplicated locus. Our approach of deleting these alleles may result in a loss of some information but is conservative and would not affect the outcome of the analyses presented here. Duplicate alleles were eliminated from four loci.

Nucleotide sequencing—In an attempt to test a hypothesis of cryptic speciation suggested by results of the microsatellite analyses, nucleotide sequences were obtained for the nuclear ribosomal ITS2 region and two plastid loci (*trnG* [UCC] intron, *trnL* [UAA] 5' exon-*trnF* [GAA] region; primer information provided in Shaw et al., 2003) from 13 *F. asagrayana*. GenBank numbers for sequences are provided in Appendix 1.

Statistical and phylogenetic analysis—Microsatellite data were analyzed as fragment sizes for analyses of population structure; alleles were coded as numbers of nucleotides rather than repeat numbers. Estimates of variability, Shannon's diversity index, analyses of molecular variance (AMOVA), and principal coordinate analyses (PCoA) of samples were accomplished in GENALEX, version 6.0.3.3 (Peakall and Smouse, 2006). Linkage disequilibrium (LD) among loci was estimated with MULTILOCUS (Agapow and Burt, 2001) with missing data fixed during randomizations. Analyses with other missing-data options did not change the results. Spatial autocorrelations for assessing the relationship between geographic and genetic distances among populations were accomplished with GENALEX. Distance classes were created using the "equal sample sizes" method so that each class had an equal number of comparisons. In all analyses using GENALEX, genetic distance matrices were calculated with the "interpolate missing data" feature off.

After we observed that there were two well-marked clusters of *F. asagrayana* populations on the basis of microsatellite variation, we assessed evidence of reproductive isolation between them using STRUCTURE, version 2.1.2 (Pritchard et al., 2000), to test for genetic admixture within individuals, and MIGRATE-N, version 2.4 (Beerli and Felsenstein, 2001; Beerli, 2005), and IMA2 (Hey, 2010) to test for gene flow between the groups. Shared alleles between the two groups could reflect ancestral polymorphism or gene flow; MIGRATE assumes that all shared polymorphism results from gene flow, whereas IMA2 allows both ancestral polymorphism and gene flow subsequent to divergence.

For MIGRATE and IMA2, microsatellite allele sizes (in nucleotides) were converted to repeat numbers. To arrive at repeat numbers, the lowest allele length at a locus was subtracted from all alleles at that locus. This number was divided by 3, because the microsatellite library was made from trinucleotide repeats. "Repeat number" is, therefore, relative to the shortest allele at a locus. Eight loci (9, 10, 22, 29, 53, 79, 88, 94) showed repeats in regular intervals of three bases without any adjustment. For loci 83 and 84, the allele patterns were regular up to six and eight repeats (above the minimum allele length), respectively. The pattern then skips a single base but continues to be regular (for example, at locus 83 the calculated pattern in repeat number is ...3, 4, 5, 6, 7.333, 8.333, 9.333, 10.333...). At both loci, the repeat numbers were rounded down to the nearest integer and then rescaled so that the lowest integer at each locus was 30; this was done because IMA2 considers repeat numbers below 5 impossible. After adjustment, there were 10 loci treated as having regular allele patterns and that were converted to repeat numbers for IMA2 and MIGRATE.

Admixture analyses using STRUCTURE were run with $K = 2$ through $K = 7$, with 10 replicate runs of 1 million generations (following a burn-in of 250 000 generations) at each value of K . The optimum number of clusters (K) was assessed using the ΔK method (Evanno et al., 2005). MIGRATE was run with 10 replicate runs, each with a 1000-step burn-in, followed by 10 short chains (500 trees sampled) followed by five long chains (5000 trees sampled) per locus. Convergence of the chains was evaluated on the basis of Gelmann's R statistic, which was less than 1.2 for all loci. IMA2 analyses included 3604 sampled genealogies per locus following a burn-in of 40 000 steps. The analyses were based on 150 chains, using a heating scheme suggested in the software manual for sites with high mutation rates. A uniform prior on migration rate was too low in initial runs, resulting in a flat posterior distribution. In the final runs, we

TABLE 3. Regional and local variability within *Frullania asagrayana* at microsatellite loci.

		Locus											
		83	84	10	29	94	9	75	22	79	53	39	88
Southern group	<i>n</i>	62	48	63	60	56	61	59	61	55	56	58	60
	<i>n_a</i>	14	10	2	1	4	5	9	7	4	6	3	9
	<i>n_e</i>	4.757	2.612	1.100	1.000	1.518	1.830	2.389	3.343	1.619	2.904	2.054	3.509
	<i>I</i>	2.079	1.429	0.191	0.000	0.710	0.927	1.348	1.394	0.709	1.351	0.765	1.635
	<i>h</i>	0.790	0.617	0.091	0.000	0.341	0.454	0.581	0.701	0.382	0.656	0.513	0.715
	<i>h_u</i>	0.803	0.630	0.092	0.000	0.347	0.461	0.591	0.713	0.389	0.668	0.522	0.727
Northern group	<i>n</i>	34	31	33	30	30	34	34	32	31	33	33	33
	<i>n_a</i>	12	7	1	4	5	1	5	7	5	2	6	5
	<i>n_e</i>	6.644	2.903	1.000	2.284	1.779	1.000	3.803	1.875	2.169	1.062	2.729	1.683
	<i>I</i>	2.146	1.377	0.000	0.982	0.903	0.000	1.422	1.066	1.063	0.136	1.255	0.848
	<i>h</i>	0.849	0.656	0.000	0.562	0.438	0.000	0.737	0.467	0.539	0.059	0.634	0.406
	<i>h_u</i>	0.875	0.677	0.000	0.582	0.453	0.000	0.759	0.482	0.557	0.061	0.653	0.419
Occoneechee Mountain	<i>n</i>	90	89	89	88	90	89	91	91	91	90	91	91
	<i>n_a</i>	6	5	2	2	3	3	6	4	4	5	4	6
	<i>n_e</i>	2.520	1.292	1.277	1.023	1.046	2.260	1.379	2.485	1.398	2.084	1.634	2.106
	<i>I</i>	1.229	0.513	0.374	0.062	0.122	0.890	0.616	1.010	0.551	0.929	0.655	0.886
	<i>h</i>	0.603	0.226	0.217	0.022	0.044	0.558	0.275	0.598	0.285	0.520	0.388	0.525
	<i>h_u</i>	0.610	0.229	0.219	0.023	0.044	0.564	0.278	0.604	0.288	0.526	0.392	0.531

Notes: The Southern and Northern groups are significantly differentiated at $\Phi_{st} = 0.373$ ($P < 0.001$). Locus 22 was included in analyses of data from Occoneechee Mountain but not in the regional analyses. *n* = number of samples, *n_a* = number of alleles, *n_e* = effective number of alleles (estimate of the number of equally frequent alleles if the population were ideal), *I* = Shannon’s information index, *h* = haploid genetic diversity (the probability that two haploid individuals are different), and *h_u* = unbiased haploid diversity.

consequently used an exponential prior on migration rate with a mean of $m/\mu = 4$, based on the mean from analyses using MIGRATE. The exponential prior has its lowest value at zero and no upper bound (with decreasing probability at higher rates, of course). This was considered more informative than a flat, uniform prior because we have no prior knowledge of the “upper bound” of the migration rate, and because the posterior density of migration rate may not reach zero within the bounds (IMa2 manual).

RESULTS

Nucleotide sequence variation—The two plastid loci, *trnG* and *trnL*, were 100% invariant. For ITS2 there was only one substitution within *F. asagrayana*. These results are obviously uninformative with regard to phylogenetic structure within *F. asagrayana*, and no tree is presented.

Regional patterns of microsatellite variation and differentiation—Results from a PCoA of variation at 12 microsatellite

TABLE 4. Multilocus linkage disequilibrium among microsatellite loci within *Frullania asagrayana* and at regional and local scales.

	<i>I_A</i>	<i>R_D</i>	<i>P</i>
All	1.21315	0.112372	0.001
Northern cluster	-0.02355	-0.0024593	ns
Southern cluster	0.14202	0.0133001	0.044
Local: All individuals	1.8116	0.189844	0.01
Local: Genets only	0.711947	0.0605139	0.01

Notes: Analyses were done with the software MULTILOCUS, using the “missing data fixed” option. *I_A* = index of association, a distance-based multilocus estimate of linkage disequilibrium among loci, and *R_D* = another estimate of multilocus disequilibrium that, unlike *I_A*, is not dependent on the number of loci sampled (Agapow and Burt, 2001). For the Occoneechee Mountain (local) data, each index was computed in two ways: on the basis of the raw data, including all samples; and on the basis of a reduced data set that included only the distinguishable multilocus genotypes (genets only).

loci among plants of *F. asagrayana* from eastern North America are shown in Figure 3. Two clusters of plants are significantly differentiated (Φ_{st} [an analogue of *F_{st}*] = 0.373; $P < 0.001$). The group on the left in Figure 3 (negative values in relation to coordinate 1) were all collected in the southern United States, whereas those in the cluster on the right (positive values in relation to coordinate 1) were collected from throughout the range of *F. asagrayana*. All northern populations (from north of Maryland, Delaware, and Virginia) are in the right cluster, though that cluster also includes some southern populations. Representatives of the cluster on the right that occur in the southeastern United States are restricted to high elevations in the Appalachian Mountains. In North Carolina, where the two groups are geographically sympatric, the latter were sampled only from elevations above 1200 m whereas the other group was sampled only from sites below 800 m. We refer to the right and left clusters in Figure 3 as the “Northern” and “Southern” groups, respectively. As noted, these groups do not strictly correspond to northern and southern latitudes; in Figure 3, plants collected north of Delaware, Maryland, and Pennsylvania (i.e., the Mason-Dixon Line) are designated as “northeastern” (NE in Fig. 3) and plants collected south of that boundary are labeled “southeastern” (SE in Fig. 3). Below, we consistently capitalize “Northern” and “Southern” to distinguish these taxon labels from the geographic ranges, which are referred to as “northeastern” and “southeastern.” The geographic distributions of samples representing the two groups are indicated in Figure 2. It is clear that there is higher genetic diversity among southeastern plants of *F. asagrayana* in that they belong to both the Northern and Southern groups, whereas all northeastern plants are in the Northern group.

In addition to the substantial genetic differentiation between the Northern and Southern groups evidenced by a significant Φ_{st} value, the two groups are characterized by 56 private alleles at 10 loci (Table 2). The Northern group has 21 private alleles, and the Southern group has 35 private alleles. Many of these private alleles occur at low frequencies within their groups

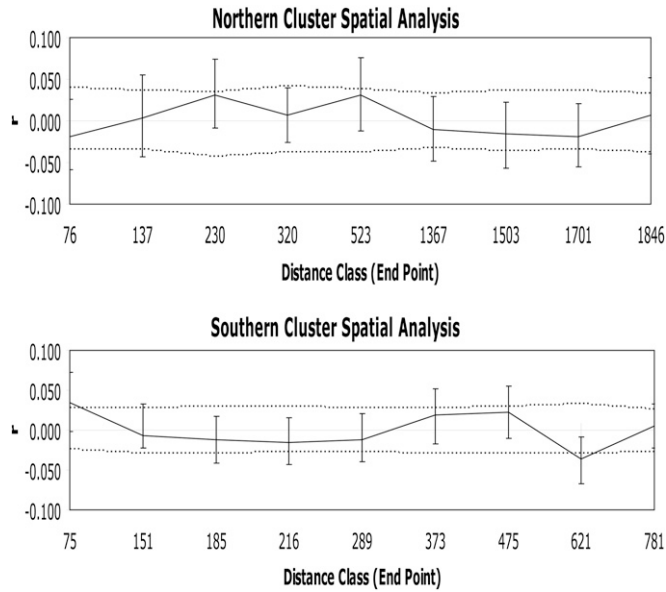


Fig. 4. Spatial autocorrelation between genetic and geographic distances among populations of *Frullania asagrayana* in eastern North America. Distance classes (km) were designed to have equal sample sizes. Dotted lines show confidence intervals for the null distribution (no isolation-by-distance), and the error bars were calculated by permuting the samples 1000 times. A significantly positive R would indicate isolation-by-distance within that distance class.

(e.g., 41 of the 56 alleles have frequencies below 0.10), but some are more common and one allele unique to the Northern group is fixed (at locus 10). An alternative allele at this locus is nearly fixed in the Southern group (one individual has a second, unique allele). The Southern group has three loci (9, 10, and 53)

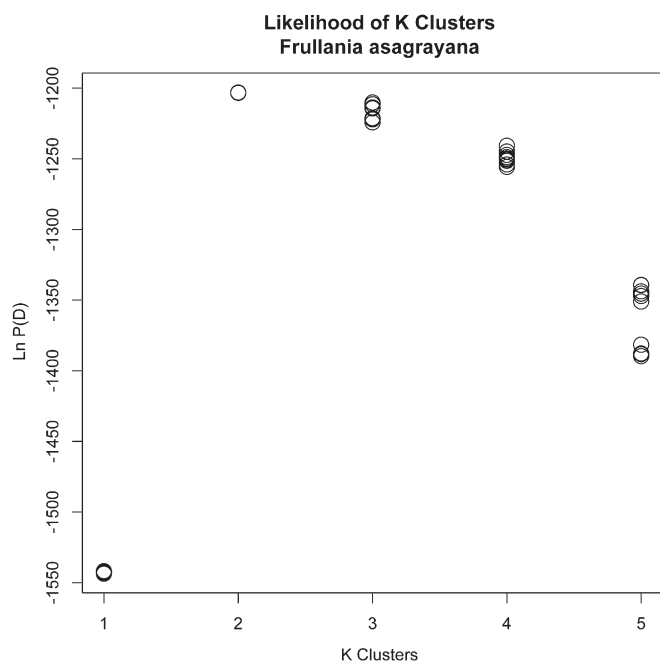


Fig. 5. Plot of $\Delta-K$ (Evanno et al., 2005) in relation to number of clusters resolved in STRUCTURE analyses.

with alleles at frequencies greater than 0.5 that are private to the group, and another (locus 88) that occurs at a frequency of 0.483 (allele 225).

Within *F. asagrayana* overall, 11 of the 12 loci are polymorphic (92%) and the numbers of alleles per polymorphic locus range from 2 (locus 10) to 14 (locus 83). Overall diversity (h) is 0.580 (± 0.068 SE). Diversity statistics are provided separately for the Northern and Southern groups (Table 3). A total of 80 alleles were detected in the Southern group, compared with 60 in the Northern group. Differences in genetic variation between the Northern and Southern groups vary among loci, but overall the two groups are similar in levels of genetic diversity ($I = 1.00$ vs. 0.933, $h = 0.487$ vs. 0.446 for the Southern and Northern groups, respectively, averaged across loci). There is no evidence of linkage disequilibrium among microsatellite loci within the Northern group; LD is very low but significantly different from zero in the Southern group (Table 4). Consistent with the lack or near lack of linkage disequilibrium among loci, there is little evidence of regional population structure within the Northern and Southern groups (Fig. 4). These results are consistent with free recombination within the Northern and Southern groups, along with effective spore dispersal within their regional ranges. LD is significant in the overall data set containing both Northern and Southern plants because of differentiation between the two groups (Table 4).

Gene flow between the Northern and Southern groups—

Analyses using STRUCTURE indicated that two groups of populations best fit the data (Fig. 5), and these correspond to the Northern and Southern groups resolved by PCoA (Fig. 6). Also in agreement with the PCoA results, plants belonging to the Southern group are restricted to lower latitudes in the southeastern United States whereas Northern-group plants in North Carolina are restricted to high elevations. Five plants are characterized by substantial admixture of alleles from the Northern and Southern groups. One is more than 50% Northern in its genetic make-up, another is close to 50% Northern, and three others are predominantly Southern but have substantial Northern genetic components. A few other plants from the southeastern region appear to have limited amounts of genetic influence from Northern plants.

Both MIGRATE and IMA2 converge on a similar result with regard to gene flow between the Northern and Southern groups (Table 5). Both indicate that gene flow is very limited but significantly greater than zero. MIGRATE estimates that gene flow is about 3.5 \times the mutation rate but does not consider the possibility that shared polymorphisms are ancestral. When accounting for ancestral polymorphism in IMA2, the migration rates are still significantly above zero but are much lower. A likelihood ratio test in MIGRATE confirmed that gene flow is significantly greater than zero (AIC = 37716, df = 2) and significantly asymmetric (AIC = -78.14, df = 2), in favor of a higher rate from the Southern group to the Northern group. In IMA2, the posterior distribution of the migration rate from the Southern to the Northern group clearly peaks to the right of zero, whereas the migration from Northern to Southern peaks close to zero (Fig. 7). Further evidence for asymmetric gene flow is that the 95% confidence intervals around the mean of the two migration-rate estimates are nonoverlapping in the MIGRATE results (Table 5). Both methods suggest that gene flow is asymmetric, with flow from the Southern to the Northern group being higher than flow from the Northern to the Southern.

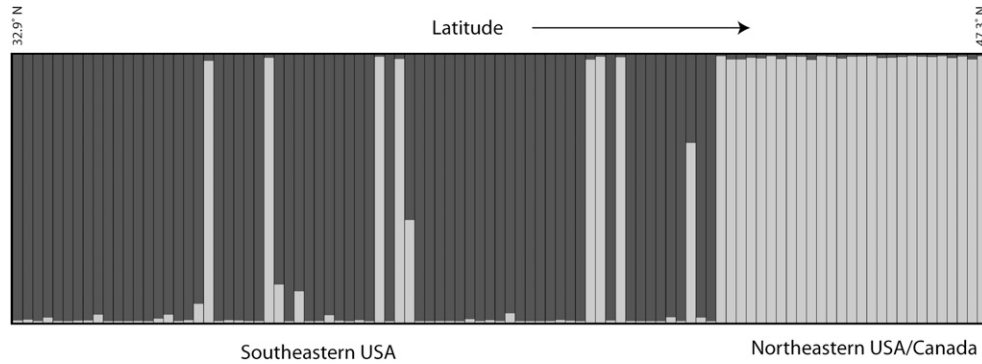


Fig. 6. Log-likelihood ($\text{Pr}(D|K)$) estimates of genetic admixture from 10 independent runs of the program STRUCTURE. Runs at $K = 1-5$ indicate that the data are best explained by two clusters ($K = 2$; see Fig. 5).

Local-scale genetic structure at Occoneechee Mountain, North Carolina—Among plants within the Occoneechee Mountain area, 11 of 13 loci are polymorphic, with an overall h of 0.341 ± 0.065 and I of 0.596 ± 0.112 . Variation in the forest is significantly structured, with 52% of the total variation attributable to differentiation among sites and 30% among subsites (individual trees or rocks) within sites when all samples including identical clones were included in the calculations (Table 6). When only distinct genets were included, differentiation among sites, while still significant, accounted for only 16% of the total variation (Table 6). Patches themselves were not sampled systematically, but the five stems sampled from each of three exemplar patches were monomorphic at all 13 loci, although the patches were differentiated (data not shown). Therefore, despite being differentiated (data not shown), the three patches appear to each consist of a single clone.

There was significant multilocus linkage disequilibrium at the local scale. The LD estimates were significant whether clones were included or excluded from the analyses, but LD values were about 3× higher when clones were included (Table 4).

DISCUSSION

The genetic structure of *F. asagrayana* in eastern North America reflects morphologically cryptic differentiation between groups of populations (the Northern vs. Southern groups), near-panmixis within the Northern and Southern groups, and clonal propagation at local scales.

Regional patterns—Differentiation between the Northern and Southern groups was not predicted, because no hint of morphological differentiation was observed among plants sampled for this study and, compared to many liverwort species with intercontinental distributions, the range of *F. asagrayana* in eastern North America is relatively limited. Nevertheless, PhiPt between the groups, 0.373, is substantial and highly significant. Alleles at multiple microsatellite loci that are private to one or the other group imply that Southern and Northern *F. asagrayana* are not exchanging genes to any significant level. Admixture analyses using STRUCTURE indicate that almost all plants belong unambiguously to one group or the other, with less than 10% of the plants exhibiting significant admixture. Moreover, gene-flow analyses from both MIGRATE and IMA2 indicate that hybridization between the groups is very low, and limited hybridization is biased in direction from Southern to Northern plants. All of the analyses suggest that the Northern and Southern groups within *F. asagrayana* represent cryptic, reproductively isolated biological species.

That the Northern and Southern cryptic species do not differ at the nucleotide sequence level despite strong differentiation in microsatellite alleles, combined with the observation that these same loci resolve related species of the *F. tamarisci* complex (Heinrichs et al., 2010), suggest that differentiation between Southern and Northern *F. asagrayana* may be very recent. It is noteworthy that all plants from the northeastern United States are unambiguously Northern in genetic make-up (STRUCTURE results), with no evidence of significant admixture, whereas all plants that display some genetic admixture occur in the southeastern region. In the southeast, the plants that belong genetically to the Northern group have been collected only at

TABLE 5. Estimates of migration (hybridization) between cryptic species of *Frullania asagrayana*.

	Cluster	Theta	5% CI	95% CI	Migration to group	5% CI	95% CI
MIGRATE	Northern	4.331896	3.837317	4.309672	4.646188	4.357323	5.016045
	Southern	2.393806	2.175872	3.468814	3.805425	3.513192	4.168483
IMa2	Northern	1.213	0.75	2.25	0.81	0.06	2.86
	Southern	1.378	0.75	2.25	0.40	0.02	1.22

Notes: $\text{Theta} = 4 * \eta_c * \mu$ and is estimated from each program using a uniform prior with random starting values (IMa2) or from starting values via an F_{st} estimate (MIGRATE). Migration rate is scaled by the mutation rate (m/μ) and can be read as “times higher than the mutation rate.” MIGRATE assumes that all shared polymorphism is the result of ongoing migration, whereas IMa2 explicitly incorporates the presence of ancestral populations. 95% confidence intervals (CI) are provided for theta and migration estimates as output from the posterior distribution (IMa2) or from the final long chain in 10 replicates at each locus (MIGRATE). Although the IMa2 95% CIs for migration rates in opposing directions overlap, the estimates with the highest posterior probabilities are different (Northern to Southern: 0.02, Southern to Northern: 0.38).

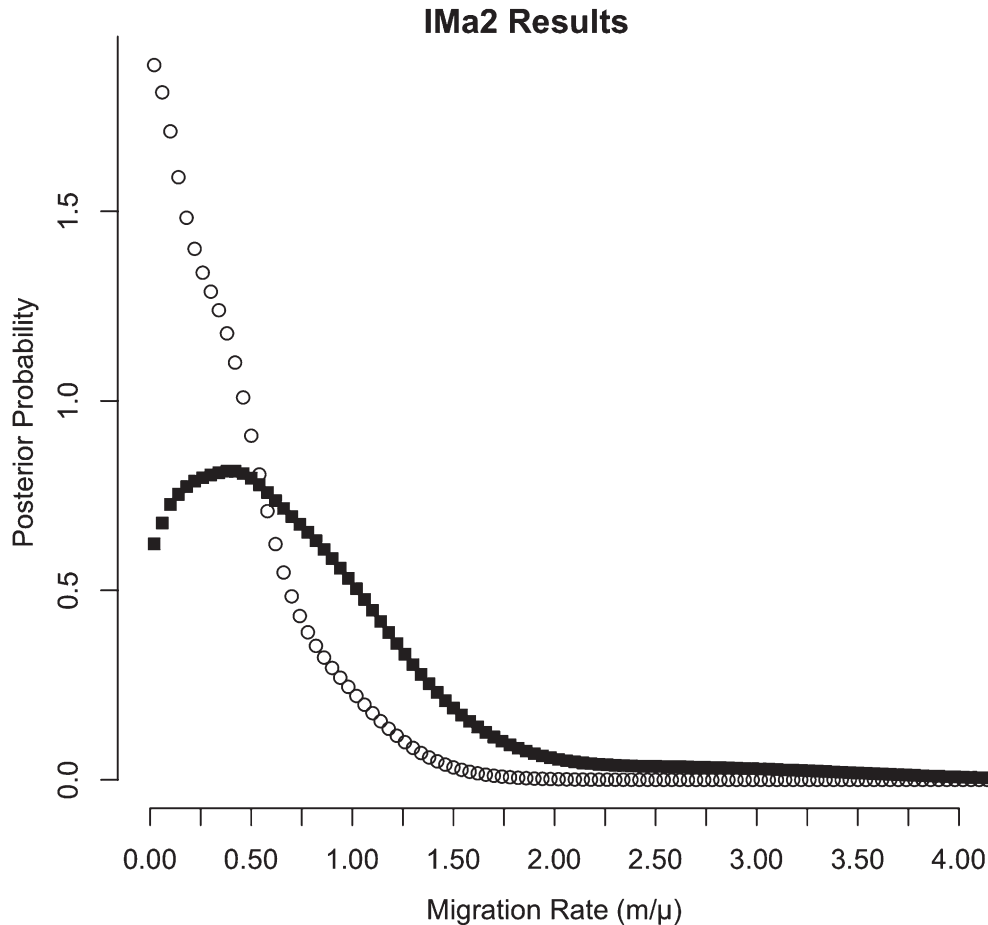


Fig. 7. Distribution of log-likelihood scores for migration-rate estimates from the Northern to the Southern group (circles) and from the Southern to the Northern group (squares), based on IMA2.

high elevations in the southern Appalachian Mountains (e.g., greater than ~1200 m elevation). If divergence of the Northern and Southern groups was associated with Pleistocene glaciation, that divergence would presumably have been in the southeastern United States. On the basis of the STRUCTURE results, admixed plants could have alleles characteristic of both the Northern and Southern groups because they retain polymorphism that antedates their primary divergence or because of subsequent interbreeding.

The algorithm underlying MIGRATE assumes that all shared alleles reflect migration (secondary hybridization, in this context) rather than retained ancestral polymorphism, but some component of the signal identified by MIGRATE could result from recent shared ancestry. The observation that gene flow appears to be biased from the Southern to the Northern group is consistent with an origin of Northern plants from Southern plants. On the other hand, IMA2 attempts to sort out shared ancestry from hybridization subsequent to isolation, and IMA2 also detects a signal of asymmetric interspecific hybridization. It appears that some mixing of genetic types in the southeastern United States reflects retained polymorphism, along with limited hybridization between the groups. Because sexual mating involves swimming sperm, hybridization would have to require that Northern and Southern plants grow in intimate sympatry. We did not detect any such mixed colonies, but our sampling was not sufficient to make any conclusions

about their occurrence. Current collections indicate that in mountainous areas, Southern *F. asagrayana* plants occur at lower elevations than Northern ones, but additional field work is required to determine whether Southern and Northern plants occur sympatrically at scales relevant to the possibility of interbreeding. Analyses of mixed populations are critical to determine whether reproductive isolation between Northern and Southern plants is due to prezygotic barriers other than spatial isolation.

The Southern and Northern groups within *F. asagrayana* clearly represent evolutionarily significant units, and our results show that evolutionarily significant units of biodiversity may not be resolved as reciprocally monophyletic groups based on sequences from commonly used loci and emphasize that tests for phylogenetic species may be inadequate, especially in cases of very recent species. A striking case of this comes from two species of crows (*Aves: Corvus*). Mitochondrial DNA sequences, AFLP markers, isozymes, and microsatellites all failed to reveal any differentiation between the black carrion crow and the hooded crow, yet the two morphologically distinguishable taxa differed in expression profiles when the transcriptomes were compared using pyrosequencing of mRNA (Wolf et al., 2010). The two morphologically defined taxa appear to differ primarily in regulatory genes that control the expression of other protein-coding genes, which themselves may not differ between species at the nucleotide level.

TABLE 6. Hierarchical partitioning of genetic variation within *Frullania asagrayana* among sites, subsites, and patches at Occoneechee Mountain, North Carolina (* $P < 0.01$, ** $P < 0.001$).

All individuals	df	SS	MS	Estimated variance	Percent variance	PhiPT
Among sites	3	354.488	118.163	5.617	52%	
Among subsites	10	227.242	22.724	4.181	38%	
Among patches	59	63.283	1.073	1.073	10%	
Total	72	645.014		10.870	100%	0.901
Genets only						
Among sites	3	53.783	17.928	2.019	16%	
Within sites	11	115.283	10.480	10.480	84%	
Total	14	169.067		12.499	100%	0.162

Genetically and phylogenetically divergent population-groups with species have been documented in diverse groups of organisms, including plants, animals, and microorganisms. Patterns of infraspecific genetic structure in plants of eastern North America are highly diverse and likely reflect multiple causes (Soltis et al., 2006). Latitudinal divergence and differences in genetic diversity have been documented, although the patterns are not as congruent across species as in Europe, where the main mountain systems run east to west (Soltis et al., 2006). In the mosses, regional genetic structure within eastern North America has rarely been detected (though studies have been limited); no evidence of differentiation was detected in the peat moss *Sphagnum torreyanum* Sull., sampled from Newfoundland to the Gulf Coast using microsatellites (Shaw et al., 2009). On the other hand, the eastern North American species, *S. macrophyllum* Brid., includes reciprocally monophyletic, highly differentiated northern and southern clades (M. G. Johnson et al. unpublished data).

Within-region patterns—The importance of sexual reproduction and spores in the life cycles of mosses and liverworts has been extensively debated (Anderson, 1963; Longton and Miles, 1982; Sundberg and Rydin, 2002). Although many or most liverworts (like mosses) have the capacity for asexual propagation of gametophytes, the absence of linkage disequilibrium within the Southern and Northern groups of *F. asagrayana* demonstrates that sexual reproduction is an important process in these taxa. Indeed, evidence of extensive recombination among microsatellite loci, combined with the absence of any sign of isolation-by-distance among populations, suggests that the Southern and Northern groups each represent panmictic or nearly panmictic population systems over quite broad geographic ranges. These results present a picture of outcrossing among gametophytes and effective dispersal and establishment by spores. Observations that suggest sexual recombination and effective regional mixing within the Southern and Northern groups reinforce the significance of apparent reproductive isolation between the two cryptic species.

Local patterns—While regional patterns within the Northern and Southern groups suggest genetic recombination and effective migration associated with spore production and dispersal, local genetic structure at Occoneechee Mountain documents clonality and differentiation among proximate genetic neighborhoods. There might seem to be a contradiction between panmixis at the regional scale and marked population structure at the local scale, but plotting the Occoneechee Mountain plants within the PCoA analysis of all Southern and

Northern samples (Fig. 3) shows why this seeming contradiction occurs. The plants at Occoneechee Mountain represent a broad sample of genetic types within the Southern group; apparently, genetic diversity at the Occoneechee site represents a random sample of genetic variation in the Southern group overall, derived by independent colonization of Occoneechee Mountain from disparate source populations. Subsequent to these colonizations, clonal propagation with limited dispersal of clones, along with restricted gene-flow distances via gamete dispersal, create genetically differentiated genetic neighborhoods. The observation that linkage disequilibrium estimates were about 3× higher when clones were included in the computation than when only distinct multilocus genotypes were included shows that clonality contributes significantly to genetic structure at the site. Linkage disequilibrium is still significantly greater than zero at Occoneechee Mountain when distinct genets only are included, which shows that sexual recombination and spore dispersal within the forest site are insufficient to homogenize subpopulations.

The genetic patterns we observed are consistent with the model of spore dispersal Sundberg (2005) developed for peat mosses (*Sphagnum*), another group of spore-reproducing plants. From empirical estimates of spore dispersal patterns, Sundberg made the prediction that a signal of isolation-by-distance should be detectable over local scales but that over increasing distances the spore rain would have a higher and higher representation from more distant populations. Our results, with no evidence of geographic structure at regional scales but strong evidence of structure at local scales, fit those predictions well.

Few genera of mosses and liverworts are endemic to single continents, and even most individual temperate or boreal species have intercontinental ranges (Crum, 1972; Schofield, 1985; Shaw, 2001). Some have interpreted intercontinental ranges as evidence of ancient vicariance associated with continental drift and slow morphological evolution within species (e.g., Anderson, 1963; Crum, 1972; Frey et al., 1999), but there is a growing consensus, based on phylogenetic analyses, that most disjunctions more likely reflect long-distance dispersal (Shaw, 2001; Hartmann et al., 2006; Heinrichs et al., 2006, 2009a; Feldberg et al., 2007). On the other hand, infraspecific genetic structure at local and regional scales frequently show evidence of isolation-by-distance (Cronberg, 2000; Snäll et al., 2004a; Pohjamo et al., 2006). Ecological studies similarly suggest dispersal limitations on patch occupancy in fragmented habitats (Snäll et al., 2004b; Pharo and Zartman, 2007; Löbel et al., 2009).

Some studies of genetic variation in widespread species have failed to detect population structure (e.g., Cronberg et al., 1997; Akiyama, 1994; Shaw et al., 2009) and imply near-panmixis at the landscape and regional scales. Anthropogenic substrates are often colonized by species not present in the local environment (Abts and Heinrichs, 1997; Soro et al., 1999; Miller and McDaniel, 2005), demonstrating effective dispersal and establishment over regional scales. Of course, the bryophyte floras of oceanic islands attest to the dispersal abilities of species. Repeated colonization of islands is a likely explanation for low levels of island endemism compared with seed plants (Vanderpoorten et al., 2008). Clearly, there is tremendous interspecific variation among mosses and liverworts in regional and local genetic structure and in the extent to which species exhibit dispersal limitations. Divergent observations raise interesting questions about species-specific metapopulation processes such as dispersal and establishment abilities and local extinction rates.

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APPENDIX 1. Voucher list of specimens included in genetic analyses of *Frullania asagrayana*. Herbarium acronyms: DUKE = Duke University, TENN = University of Tennessee, and UNAF = University of Northern Alabama.

Laboratory DNA number, cluster (S = Southern group, N = Northern group, O = Occoneechee Mountain, and T = *F. tamarisci* outgroup), collector and collection number, state/province: county/district, herbarium, GenBank accessions for selected specimens (*trnG*, *trnL*, ITS).

SB3327, N, *Shaw 6988*, Vermont: Essex Co., DUKE. SB3330, N, *Shaw 8018*, Maine: Washington Co., DUKE. SB3331, N, *Shaw 8019*, Maine: Washington Co., DUKE. SB3332, N, *Shaw 8022*, Maine: Washington Co., DUKE. HM746937, HM746945, HM746926, SB3333, N, *Shaw 8040*, Maine: Hancock Co., DUKE. SB3335, N, *Shaw 8037*, Maine: Hancock Co., DUKE. HM746941, HM746951, HM746916, SB3340, N, *Schofield 95421*, Nova Scotia: Digby Co., DUKE. SB3342, N, *Belland 16761*, Nova Scotia: Kejimikujik NP, DUKE. SB3343, N, *Belland 16786*, Nova Scotia: Kejimikujik NP, DUKE. SB3345, N, *Belland 16945*, Nova Scotia: Kejimikujik NP, DUKE. SB3347, N, *Schofield 97014*, Nova Scotia: Kejimikujik NP, DUKE. SB3348, N, *Belland 97361*, Nova Scotia: Kejimikujik NP, DUKE. SB3349, N, *Schofield 97047*, Nova Scotia: Kejimikujik NP, DUKE. SB3369, N, *Breil 93-5846*, Tennessee: Carter Co., DUKE. SB3370, N, *Risk 591*, Tennessee: Carter Co., DUKE. SB3372, N, *Breil s.n.*, Virginia: Highland Co., DUKE. SB3374, N, *Shaw 10397*, West Virginia: Pocahontas Co., DUKE. SB3375, N, *Shaw 10657*, West Virginia: Pocahontas Co., DUKE. SB3376, N, *Shaw 10404*, West Virginia: Pocahontas Co., DUKE. SB3377, N, *Schofield 17627*, New Brunswick: Fundy NP, DUKE. SB3378, N, *Belland 14134*, New Brunswick: Kouchibouguac NP, DUKE. SB3379, N, *Belland 14119*, New Brunswick: Kouchibouguac NP, DUKE. SB3380, N, *Belland 18463*, New Brunswick: Fundy NP, DUKE. SB3382, N, *Belland 17839*, New Brunswick: Fundy NP, DUKE. HM746931, HM746944, HM746925, SB3383, N, *Weber 17775*, New Brunswick: Fundy NP, DUKE. SB3384, N, *Schofield 17782*, New Brunswick: Fundy NP, DUKE. SB3385, N, *Belland 96899*, Nova Scotia: Kejimikujik NP, DUKE. SB3388, N, *Schofield 17355*, Nova Scotia: Kejimikujik NP, DUKE. SB3398, N, *Belland 16415*, Nova Scotia: Cape Breton Highlands NP, DUKE. HM746929, HM746947, HM746927, SB3400, N, *Risk 596b*, Tennessee: Carter Co., DUKE. SB3401, N, *Risk 546*, Tennessee: Carter Co., DUKE. SB3404, N, *Zartman 627*, North Carolina: Macon Co., DUKE. SB3406, N, *Thornton 48*, North Carolina: Yancey Co., DUKE. SB3661, N, *Hyatt 11544*, Georgia: Union Co., DUKE. HM746930, HM746953, HM746928, SB3837, N, *Davison 2690*, Alabama: Rabun Co., UNAF. SB3881, N, *Briscoe 798*, Maine: Hancock Co., DUKE. HM746933, HM746949, HM746917, SB3336, S, *Shaw 6083*, North Carolina: Orange Co., DUKE. HM746940, HM746952, HM746918, SB3366, S, *Risk 972*, Kentucky:

Letcher Co., DUKE. SB3367, S, *Risk 11469*, Kentucky: Morgan Co., DUKE. HM746939, HM746946, HM746920, SB3368, S, *Bowe s.n.*, South Carolina: Spartanburg Co., DUKE. SB3371, S, *Breil s.n.*, Virginia: Buckingham Co., DUKE. SB3373, S, *Solga 350*, Virginia: Lunenburg Co., DUKE. SB3391, S, *Horn 1831*, North Carolina: Orange Co., DUKE. SB3392, S, *Shaw s.n.*, North Carolina: Durham Co., DUKE. SB3393, S, *Buck 15783*, Arkansas: Franklin Co., DUKE. SB3394, S, *Majestyk 5162*, Arkansas: Conway Co., DUKE. SB3395, S, *Zartman 1396*, Alabama: Jackson Co., DUKE. SB3399, S, *Shaw 5274*, North Carolina: Orange Co., DUKE. SB3403, S, *Shaw 4503*, North Carolina: Watauga Co., DUKE. SB3405, S, *Zartman 642*, North Carolina: Macon Co., DUKE. SB3646, S, *Davison & Smith 113*, Tennessee: Coffee Co., TENN. SB3648, S, *Davison & Smith 361*, Tennessee: Coffee Co., TENN. HM746932, HM746942, HM746922, SB3650, S, *Risk, Davison & Richardson 7745*, Tennessee: Fentress Co., DUKE. SB3651, S, *Davison 5840*, Tennessee: Fentress Co., DUKE. SB3652, S, *Davison & Nordman 5382*, Tennessee: Grundy Co., DUKE. SB3653, S, *Hyatt 11552*, Georgia: Fannin Co., DUKE. SB3657, S, *Davison 5718*, Tennessee: Van Buren Co., UNAF. SB3658, S, *Hyatt 11545*, Georgia: Fannin Co., DUKE. SB3662, S, *Davison & Risk 2520*, Kentucky: Bell Co., UNAF. SB3663, S, *Hyatt 8935*, North Carolina: Macon Co., DUKE. HM746935, HM746943, HM746924, SB3664, S, *Hyatt 8936*, North Carolina: Macon Co., DUKE. SB3665, S, *Davison & Risk 2613*, Tennessee: Campbell Co., UNAF. SB3666, S, *Hyatt 9211*, North Carolina: Macon Co., DUKE. SB3667, S, *Davison 4733*, Alabama: Franklin Co., UNAF. SB3668, S, *Davison 1950*, Alabama: Lawrence Co., UNAF. SB3669, S, *Davison 3140*, Alabama: Lawrence Co., UNAF. SB3670, S, *Davison 4624*, Alabama: Marion Co., UNAF. SB3671, S, *Kittle s.n.*, Alabama: St. Clair Co., UNAF. SB3672, S, *Kittle s.n.*, Alabama: St. Clair Co., UNAF. SB3673, HM746936, HM746954, HM746921, S, *Davison 6704*, Alabama: Winston Co., UNAF. SB3674, S, *Davison 4683*, Alabama: Winston Co., UNAF. SB3677, S, *Davison 3965*, Alabama: Colbert Co., UNAF. SB3678, S, *Davison 6912*, Alabama: Jackson Co., UNAF. HM746938, HM746948, HM746919, SB3679, S, *Davison 5060*, Alabama: Jackson Co., UNAF. SB3680, S, *Davison 5080*, Alabama: Jackson Co., UNAF. SB3681, S, *Davison 5107*, Alabama: Jackson Co., UNAF. SB3683, S, *Davison 4888*, Alabama: Jackson Co., UNAF. SB3684, S, *Davison 4945*, Alabama: Jackson Co., UNAF. SB3685, S, *Davison 4983*, Alabama: Jackson Co., UNAF. SB3828, S, *Kittle s.n.*, Alabama: St. Clair Co., UNAF. SB3835, S, *Davison 4883*, Alabama: Jackson Co., UNAF. SB3836, S, *Davison 6647*, Alabama: Winston Co., UNAF. HM746934, HM746950, HM746923, SB3838, S, *Hyatt 11553*, Georgia: Union Co., DUKE. SB3839, S, *Davison & Nordman 5444*,

Tennessee: Marion Co., UNAF. SB3840, **S**, Davison & Nordman 5399, Tennessee: Marion Co., UNAF. SB3841, **S**, Risk, Davison & Richardson 14860, Tennessee: Fentress Co., TENN. SB3842, **S**, Davison & Smith 120, Tennessee: Coffee Co., TENN. SB3843, **S**, Davison & Smith 397, Tennessee: Coffee Co., TENN. SB3924, **O**, Shaw, Shaw & Ramaiya 151, North Carolina: Orange Co., DUKE. SB3928, **O**, Shaw, Shaw & Ramaiya 155, North Carolina: Orange Co., DUKE. SB3929, **O**, Shaw, Shaw & Ramaiya 211, North Carolina: Orange Co., DUKE. SB3940, **O**, Shaw, Shaw & Ramaiya 132, North Carolina: Orange Co., DUKE. SB3941, **O**, Shaw, Shaw & Ramaiya 136, North Carolina: Orange Co., DUKE. SB3942, **O**, Shaw, Shaw & Ramaiya 231, North Carolina: Orange

Co., DUKE. SB3943, **O**, Shaw, Shaw & Ramaiya 232, North Carolina: Orange Co., DUKE. SB3960, **O**, Shaw, Shaw & Ramaiya 343a, North Carolina: Orange Co., DUKE. SB3969, **O**, Shaw, Shaw & Ramaiya 411, North Carolina: Orange Co., DUKE. SB3973, **O**, Shaw, Shaw & Ramaiya 415, North Carolina: Orange Co., DUKE. SB3979, **O**, Shaw, Shaw & Ramaiya 431, North Carolina: Orange Co., DUKE. SB3983, **O**, Shaw, Shaw & Ramaiya 441, North Carolina: Orange Co., DUKE. SB3989, **O**, Shaw, Shaw & Ramaiya 451, North Carolina: Orange Co., DUKE. SB3991, **O**, Shaw, Shaw & Ramaiya 453, North Carolina: Orange Co., DUKE. SB3994, **O**, Shaw, Shaw & Ramaiya 321, North Carolina: Orange Co., DUKE.
