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Patterns of Molecular and Morphological Variation in *Leucobryum albidum*, *L. glaucum*, and *L. juniperoideum* (Bryopsida)

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ABSTRACT. Restriction digest patterns from 18S–26S nuclear ribosomal DNA internal transcribed spacers (ITS) were employed to investigate delineation between the morphologically similar moss species *Leucobryum glaucum*, *L. juniperoideum*, and *L. albidum*. Discriminant analysis allowed assignment of specimens to haplotypes based on their morphological features and supported the recognition of *L. glaucum* and *L. albidum*. In contrast, *L. albidum* and *L. juniperoideum* both corresponded to the same haplotype. Many populations could be readily assigned to either *L. glaucum* or *L. albidum* by their morphological features. However, morphological variation between these two species was continuous and one of the ITS haplotypes could not be unambiguously characterized by its morphology. Genetically and morphologically identifiable specimens of *L. albidum* were sampled in Europe and North America, in contrast to the traditional interpretation of *L. albidum* as a North American endemic. Although *L. albidum* seems to have a more southern-Atlantic distribution pattern than *L. glaucum*, the two species occupy broadly overlapping geographic ranges and were sometimes found intermixed.

The moss floras of Europe and North America possess a comparable diversity of about 1100–1500 species. Sixty-eight genera (approximately 1/5 of the total number) are represented by the same species in Europe and North America and probably belong to an old common stock of Laurasian taxa (Frahm and Vitt 1993). In one such genus, *Leucobryum*, two species, *L. albidum* (Brid. ex P. Beauv.) Lindb. and *L. glaucum* (Hedw.) Ångstr., were reported from Europe and North America until Pilous (1962) interpreted the European plants named *L. albidum* as a third species, *L. juniperoideum* (Brid.) Müll. Hal. This species concept has generally been accepted in Europe (see e.g., Corley et al. 1981). *Leucobryum albidum* was subsequently reported from Macaronesia (Düll 1980), but the record has been questioned (Eggers 1982).

The taxonomic status of these three *Leucobryum* species has been somewhat controversial owing to morphological intergradation. The most reliable features for distinguishing them are sporophytic, and even those authors who argue that the species are distinct acknowledge the difficulties distinguishing them based on gametophytic characters alone (e.g., Smith 1978; Crum and Anderson 1981). Unfortunately, the species are dioicous and rarely produce sporophytes. Hence, species identification relies heavily on gametophytic features, especially the size and structure of the peculiar costa. Costal anatomy consists of several layers of hyaline cells (leucocysts) above and below a central layer of small chloroplyllose cells (chlorocysts). Although some specimens of *Leucobryum* can be readily identified using gametophytic characters, comparable patterns of intergradation have been reported among the larger, widespread species *L. glaucum* and the smaller, vicariant species *L. albidum* and *L. juniperoideum* in North America and in Europe, respectively (Crundwell 1972; Crum and Anderson 1981; Touw and Rubers 1989). Other characters, including the number of laminal cells between the costa and the leaf margin, the shape of the costa in cross-section, and the shape of the leaf apex, have also been proposed to distinguish the species. The taxonomic utility of these gametophytic characters, however, varies from one geographic region to another (Smith 1978; Crum and Anderson 1981; Nyholm 1986; Yamaguchi 1993; Nebel and Philippi 2000). More problematically, these characters do not always co-vary, sometimes conflict with plant size or sporophytic features (Touw and Rubers 1989), or are interpreted differently by various authors. For example, the ratio of leaf base to leaf acumen is one of the key characters used in North American and European treatments to separate *L. glaucum* from *L. albidum* or *L. juniperoideum*, respectively. In North America, a leaf acumen as long as to twice as long as the leaf base is used to distinguish *L. glaucum* from *L. albidum* (Crum and Anderson 1981), whereas the reverse trend has been described in most European floras between *L. glaucum* and *L. juniperoideum* (e.g., Smith 1978; Frey et al. 1995).

Leucobryum glaucum has been reported in both Eurasia and North America from a variety of habitats ranging from xeric sandy sites to *Sphagnum* bogs (Ireland 1982; Yamaguchi 1993; Dierssen 2001), whereas *L. juniperoideum* tends to occur in the driest part of that range (Crundwell 1972). In addition, *L. glaucum* has a more northern distribution than *L. albidum* in North America or *L. juniperoideum* in Europe. Such observations raise the possibility that morphological differences simply reflect habitat variation. As a result, *L. juniperoideum* and *L. glaucum* have sometimes been synomized (Touw and Rubers 1989) while the status of *L. albidum* has been debated (Crum and Anderson 1981).

However, Patterson et al. (1998) reported in a recent study that restriction fragments of DNA amplified by polymerase chain reaction (PCR) exhibited different patterns of variation between morphologically continuous populations of *L. albidum* and *L. glaucum* from a single North Carolina locality.

The analysis of length polymorphism in restriction fragments of amplified DNA (hereafter, PCR-RFLP) often enables the distinction among closely related species (Grechko et al. 1997) and has therefore been used as a molecular marker to address taxonomic problems (e.g., Isshiki et al. 1998; Vivek and Simon 1999; He et al. 2000; Parani et al. 2001). In bryophytes, the internal transcribed spacers of nuclear ribosomal DNA (ITS) have been increasingly used to address taxonomic problems at the specific and infra-specific levels (Colacino and Mishler 1996; Shaw 2000; Shaw and Allen 2000; Vanderpoorten et al. 2001; Quandt et al. 2001). In this paper, we employ restriction digest patterns from nuclear ribosomal DNA ITS sequences to investigate species delineation between *L. glaucum*, *L. juniperoideum*, and *L. albidum*. Morphological measurements were made on the same plants to assess correspondence between gametophyte structural features and DNA sequence variation.

Material and Methods

Taxon Sampling. Thirty-one collections were selected to cover the range of morphological variation among the three putative species and include a wide geographical sampling from Asia, Europe, and North America (Table 1). Type specimens of all three species were also included in the morphological analyses.

Molecular Protocol. DNA extraction, ITS amplification, digestion of the PCR products using *Hha*I, *Hinf*I, and *Taq*I restriction enzymes, and gel analyses were performed according to the protocols described in Patterson et al. (1998). Two specimens each of four distinguishable PCR-RFLP haplotypes were sequenced subsequently to produce a map of restriction sites and determine if there were any additional nucleotide differences among the haplotypes. The ITS region was sequenced using the primers and following the protocol described in Shaw (2000).

Morphological characters. Taxonomically relevant characters for *Leucobryum* species include plant habit, stem anatomy, leaf morphology and anatomy, with a special emphasis on the peculiar combination of leucocysts and chlorocysts, rhizoids, position of the sexual organs, sporophyte morphology, and production of asexual gemmae (Yamaguchi 1993). In this study focussing on the three similar species, *L. glaucum*, *L. albidum*, and *L. juniperoideum*, a total of 12 characters previously used for species delineation (Pilous 1962; Crundwell 1972; Crum and Anderson 1981; Nyholm 1986; Yamaguchi 1993) were variable in the set of examined specimens. These characters were scored for plants from the 31 populations included in the molecular analyses and for the type specimens of the three species (Table 3). The eight gametophytic characters were scored, for each population, on five leaves chosen at random. In asymmetric leaves, the longest base length was measured (character 3 in table 3). This character could not be scored on all leaves because the base and the acumen were not clearly demarcated in some leaves. Five contiguous leucocyst cells per leaf were measured from the middle of the costa for character 7. Four sporophytic characters were scored for two fertile populations.

Data Analysis. Presence-absence of the restriction fragments for each specimen was scored as binary characters. The matrix was submitted to a UPGMA cluster analysis using PAUP 4.0b10 (Swofford 2001) to classify the populations into *h* different haplotypes. The sequences obtained for each haplotype were aligned manually with inserted gaps to preserve positional homology. Regions including one to several consecutive gaps were scored as indels.

Differences in leaf size and anatomical details among haplotypes were investigated using standard statistical analyses (see, e.g., Sosa and De Luna 1998; Sastad et al. 1999; St-Laurent et al. 2000). All computations were performed using Minitab 13.20 (Minitab Inc., PA) and SAS 8.2 (SAS Institute Inc., NC). Ranges of variation, means, and standard deviations for each morphological trait were calculated for each haplotype identified in the molecular analyses. Anderson-Darling and Bartlett's tests (Sokal and Rohlf 1995) showed that morphological characters exhibited significant departures from normality and homoscedacity, with the exception of character 8, the ratio of the thickest to thinnest part of the leaf in transverse section. Differences among haplotypes for the latter were investigated using a one way analysis of variance followed by a Newman & Keuls test (Sokal and Rohlf 1995) to determine which pairs of populations differed significantly. Because the Newman & Keuls test is highly sensitive to departures from the assumptions, non-parametric analyses were performed to search for morphological differences among haplotypes for the other characters. For each character, the Kruskal-Wallis statistic *T* (Conovor 1999) was used to test the hypothesis that at least one of the populations tended to yield larger observations than at least one

Table 1. Voucher information. All specimens are at DUKE. Haplotypes were assigned based on molecular data, which suggest that haplotypes G and H1 correspond to *L. glaucum,* haplotype A to *L. albidum,* and haplotype H2 to a cryptic species, a hybrid, or a molecular variant of the two former species.

H1-1 (Belgium, Bruxelles-Capitale, acidic humus in *Fagus sylvatica* forest; *Vanderpoorten 1040*). **H1-2** (France, Ardennes, acidic humus in forest; *Sotiaux 13900*). **H1-3** (Poland, Poméranie occidentale, mixed humid forest; *Liseweski et al. 138*). **H1-4** (Belgium, Liège, forest slope, under *Calluna vulgaris; Sotiaux 15273*). **H1-5** (Belgium, Antwerpen, in wood, near a floating meadow; *Sotiaux 18857*). **H1-6** (Japan, Hokkaido, moist humus, ca. 30m; *Yamaguchi 18780*). **H1-7** (Japan, Hokkaido, moist humus, ca. 30m; *Yamaguchi 18774*). **H1-8** (Japan, Hokkaido, moist humus, ca. 30m; *Yamaguchi 18773*). **H2-1** (France, Ile de France, acidic humus in *Fagus-Quercus* forest; *Vanderpoorten 2230*). **H2-2** (France, Champagne-Ardennes, base of a tree; *Sotiaux 16473*). **H2-3** (Belgium, Luxembourg, siliceous rocks; *Sotiaux 16671*). **G1** (Canada, Newfoundland, shaded base of outcrops, blackhead highland with outcrops knobs and bogs; *Schofield 101062*). **G2** (Canada, Nova Scotia, humus on forest floor; *Belland & Schofield 16704*). **G3** (USA, Minnesota, soil over rock under *Picea mariana, Pinus banksiana, Abies; Bowers 22343*). **G4** (Denmark, Sjaelland, *Alnus glutinosa* swamp; *McMurray 030M*). **G5** (Hungary, Zemplen Mts, forested slopes; *Schofield 104672*). **G6** (France, Ile de France, acidic *Sphagnum* bog; *Vanderpoorten 2214*). **G7** (USA, North Carolina, mixed pine and hardwood forest; *Patterson B2*). **G8** (idem, idem; *Patterson A4*). **G9** (USA, Alabama, shaded creek bank; *Shaw 10291*). **G10** (France, Champagne-Ardennes, bog; *Sotiaux 18937*). **G11** (Belgium, Namur, creek bank; *Sotiaux 18954*). **A1** (Czechoslovakia, Bohemia; *Pilous s.n.*). **A2** (Spain, Teneriffe, on soil in woodland, ca. 900m; *Townsend 78/271*). **A3** (Germany, Saarland, forest sandstone, ca. 250m; *Frahm 897675*). **A4** (Portugal, Madeira, *Erica-Vaccinium* scrub; *Holmen & Rasmussen 105*). **A5** (USA, North Carolina, mixed pine and hardwood forest; *Patterson B3*). **A6** (idem, idem; *Patterson A2*). **A7** (USA, West Virginia, mixed deciduous *Tsuga/Rhododendron* forest; *Shaw 10436*). **A8** (France, Bretagne, forest humus; *Vanderpoorten 2993*). **A9** (USA, Florida, xeric hammock and associated pond; *Seman 42*).

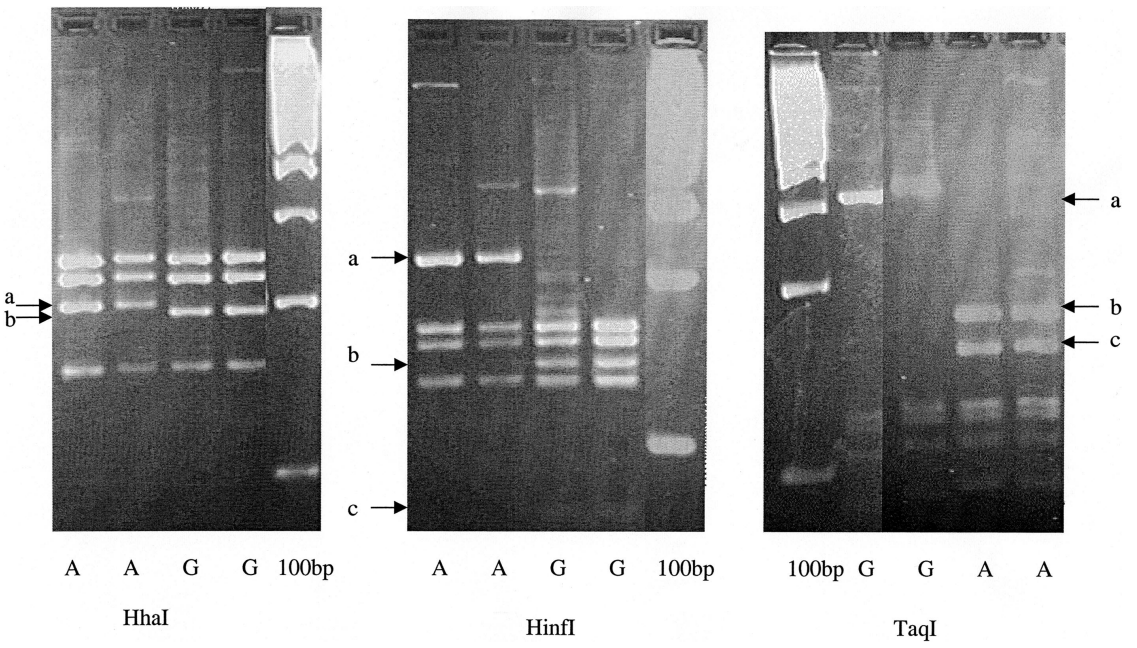

FIG. 1. Agarose gel showing polymorphism in restriction fragments for the three restriction enzymes *Hinf*I, *Taq*I, and *Hha*I. Scoring polymorphism as presence/absence of the fragments of different molecular weights (labels a, b, c) allows recognition, for each enzyme, of two different band patterns labeled A and G. 100bp is the 100 base pair ladder.

of the other populations. A χ^2 distribution with h-1 degrees of freedom was used as an approximation to the null distribution of T. The following procedure was used to determine which pairs of populations tended to differ:

$$\left|\frac{R_i}{n_i} - \frac{R_j}{n_j}\right| > t_{1-\alpha/2}\left[\frac{N(N+1)}{12}\frac{N-1-T}{N-h}\right]^{1/2}\left(\frac{1}{n_i}+\frac{1}{n_j}\right)^{1/2}$$

where N is the total number of observations, n_i and n_j are the number of observations of samples i and j, R_i and R_j are the rank sums of the two samples, and $t_{1-\alpha/2}$ is the $(1-\alpha/2)$ quantile of the t distribution with N-h degrees of freedom (Conovor 1999).

A canonical discriminant analysis was performed to search for the best combinations of morphological characters that discriminate the haplotypes. The analysis was performed only on the gametophytic characters, as sporophytes were present in just two of the collections. The procedure derived linear combinations of morphological characters that had the highest possible multiple correlation coefficient with the haplotypes. Significance of the canonical correlation coefficients was assessed using an F approximation, assuming that the variables had an approximate multivariate normal distribution within each class with a common covariance matrix. Data from the type specimens of *L. albidum*, *L. glaucum*, and *L. juniperoideum* were then plotted against the significant canonical axes to assign type specimens to one of the haplotypes.

TABLE 2. *Leucobryum glaucum-albidum* species complex shown as four haplotypes (A, H1, H2, G) with restriction enzymes *Hinf*I, *Taq*I, and *Hha*I. Polymorphic fragments (a, b, c) for each restriction enzyme are indicated as being present (1) or absent (0).

Enzyme	Fragment label	Haplotype			
		A	G	H1	H2
*Hinf*I	a	1	0	1	1
	b	0	1	0	0
	c	0	1	0	0
*Taq*I	a	0	1	1	0
	b	1	0	0	1
	c	1	0	0	1
*Hha*I	a	1	0	0	0
	b	0	1	1	1

RESULTS

Representative restriction patterns for each of the three enzymes are presented in Fig. 1, and these polymorphic ''loci'' yielded four multilocus haplotypes (A, H1, H2, and G) (Table 2). Haplotype A differed from haplotypes G, H1, and H2 by three, two, and one restriction sites, respectively. Haplotypes A and G had congruent haplotypes for all three enzymes, which differed with respect to all eight polymorphic fragments. The two other haplotypes, H1 and H2, exhibited the G restriction pattern for *Hha*I and the A pattern for *Hinf*I. Haplotype H1 had the G pattern for *Taq*I, whereas H2 had the A pattern (Table 2).

The two plants of each haplotype that were sequenced were identical across ITS1:5.8S:ITS2 (GenBank accession numbers AY062893, AF464062, AY062892, and AY062894 for haplotypes H1, H2, G, and A, respectively). Haplotype G differed from haplotype H1 by one indel, from haplotype A by eight indels and three substitutions, and from haplotype H2 by nine indels and three substitutions. Haplotype A differed

TABLE 3. Morphological features of the four *Leucobryum* haplotypes. For gametophytic characters (1–8), minimum and maximum values are above and means (standard deviations) are below. Boldface characters indicate significantly different mean values among haplotypes. The standardized canonical coefficients of the first canonical function giving the best weighted combination of gametophytic characters to separate the haplotypes are given in the column labeled 'coeffs'. Sporophytic characters (9–12) could be scored from only two collections and were not included in the discriminant analysis.

Character	Haplotype G	A	H1	H2	coeffs
1. leaf length (mm)	3.750–8.750	1.500–7.500	3.250–7.750	2.875–9.625	0.92
	5.786 (1.122)	**4.147** (1.472)	5.613 (1.270)	6.058 (2.244)	
2. leaf width (mm)	0.825–1.750	0.475–1.450	0.700–1.755	0.775–1.750	0.23
	1.222 ± 0.221	**0.859** (0.235)	1.219 (0.292)	1.111 (0.308)	
3. length of leaf base (expressed in % of leaf length)	30–81	25–69	33–93	30–53	0.83
	49 ± 11	**43** (11)	55 (13)	**41** (7)	
4. number of laminal cells (costa to leaf margin)	4–13	5–14	4–15	6–15	−0.29
	8 (2)	9 (2)	9 (2)	10 (2)	
5. minimum number of leucocyst layers	2–5	2–4	2–6	2–3	0.49
	3 (1)	2 (0)	2 (1)	2 (0)	
6. maximum number of leucocyst layers	3–6	3–6	3–7	4–6	−0.43
	5 (1)	**4** (1)	5 (1)	5 (1)	
7. width of leucocysts (μm)	15–62	12–50	15–55	17–68	−0.33
	33 (9)	**27** (8)	34 (8)	33 (1) 1	
8. thickest/thinnest part of leaf (%)	50–100	45–92	43–100	47–77	1.03
	81 (10)	70 (11)	73 (12)	**62** (9)	
9. capsule erect/curved	curved	not seen	curved	not seen	—
10. capsule strumose/not strumose	strumose	not seen	strumose	not seen	—
11. capsule length (mm)	<2	not seen	<2	not seen	—
12. seta length (cm)	1–1.5	not seen	1–1.5	not seen	—

from haplotype H1 by eight indels and three substitutions and from haplotype H2 by six indels. Haplotype H1 differed from H2 by nine indels and three substitutions.

Morphological characteristics of the four haplotypes are summarized in Table 3. Haplotype A had the shortest and narrowest leaves. Leaf dimensions in the three remaining haplotypes were substantially larger and had broadly overlapping ranges of variation. Other characters were also broadly overlapping. The sporophytes of the two fertile collections, G6 and H1–2, were identical with curved, strumose capsules slightly less than 2 mm long and 1–1.5 cm long setae.

A canonical discriminant analysis of morphological data confirmed the lack of clear phenotypic discontinuities among the four haplotypes, even in a multivariate context (Fig. 2). Only the first discriminant function *D*1 had a significant canonical correlation coefficient ($r=0.82$, $p<0.05$) and accounted for 72% of the total morphological variation among haplotypes. *D*1 was mostly loaded with leaf length, ratio base to acumen, ratio of the thickest part to thinnest part of the leaf in cross-section, and minimum leucocyst number in costal cross-section (Table 3). The type of *L. glaucum* (Hedw.) Ångstr. [Basionym: *Dicranum glaucum* Hedw., Spec. Musc.: 135 (1801). TYPE: without date, locality, and collector name (holotype: G!)] was assigned to haplotype H1 based on its score on the first canonical component (Fig. 2). The types of *L. juniperoideum* (Brid.) Müll. Hal. [Basionym: *Dicranum juniperoideum* Brid., Bryol. Univ. 1: 409 (1826). TYPE: Teneriffe, without date and collector name (holotype: B!] and of *L. albi-*

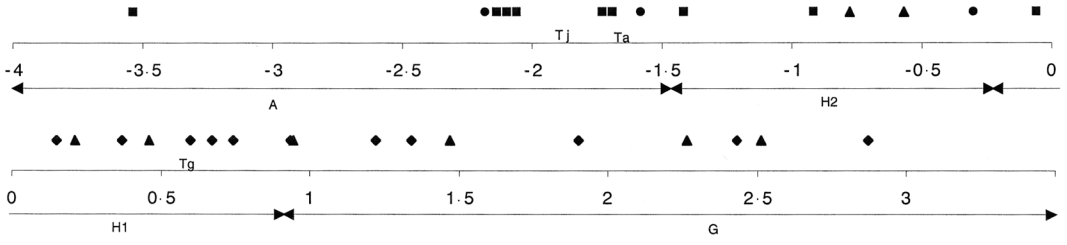

FIG. 2. Representation of the 31 investigated populations along the first canonical component ($r=0.82$, $p<0.05$) accounting for 72% of the total morphological variation among haplotypes. ■, ●, ◆, ▲ represent specimens of haplotypes A, H2, G, and H1, respectively. Horizontal arrows represent the theoretical range (central point of two consecutive class means) of each haplotype along the first canonical component. Tg, Tj, Ta represent the positions of the types of *L. glaucum*, *L. juniperoideum*, and *L. albidum* along the first canonical component, respectively.

dum (Brid. ex P. Beauv.) Lindb. [Basionym: *Dicranum albidum* Brid. ex P. Beauv., Prodr. Aetheogam. 52 (1805)] were assigned to haplotype A based on their score on the first canonical component (Fig. 2). A new lectotype is designated for *L. albidum* because the Bridel collection designated by Pilous (1962) as lectotype from the Bridel herbarium [Bosc. Carolina, 1803, *Paris s.n.* (B)], could not be found. The following collection of the Torrey herbarium: without date and locality, *Schweinitz s.n.* (NY!) was selected as lectotype because it was explicitly examined by Bridel [see Bridel (1826) p. 409].

Discussion

Four haplotypes (G, A, H1, and H2) were found in a sample of the three traditionally recognized species *Leucobryum glaucum*, *L. albidum*, and *L. juniperoideum*. The discriminant analysis, which allowed the assignment of a specimen to a haplotype based on its morphological features, suggested that the types of *L. albidum* and *L. juniperoideum* both corresponded to haplotype A, whereas the type of *L. glaucum* corresponded to haplotype H1.

Haplotype A was most distinctive morphologically and exhibited significant differences from the other haplotypes, based mostly on the size of gametophytic structures. Typical specimens of haplotype A were characterized by a small size, unambiguously fitting the circumscription of *L. albidum*, and were similar to the type specimen.

The assignment of the type of *L. juniperoideum* to the same haplotype as *L. albidum* based on morphology suggests that the two names have been applied to the same morphological expressions. The characters traditionally used to circumscribe *L. juniperoideum* (Pilous 1962) either did not vary significantly among haplotypes (e.g., number of laminal cells between the costa and the leaf margin) or fell within the range of variability of *L. albidum* [e.g., 4–7 mm long leaves; a ratio leaf base to acumen of (1) 2:3 (4)]. Pilous (1962) further stated that the capsules of *L. juniperoideum* are arcuate and strumose, thus identical to those of *L. glaucum*. This contrasts with other descriptions of straight to slightly curved, non-strumose capsule (thus identical to that of *L. albidum*) for *L. juniperoideum* (e.g., Smith 1978; Nyholm 1986).

The type specimen of *L. juniperoideum* is unfortunately sterile. Its gametophytic features are similar to those of large specimens of *L. albidum*. Confusion regarding *L. juniperoideum* arose from the lack of a well defined circumscription, and apparently the name *L. juniperoideum* has been applied to large specimens of *L. albidum* that do not correspond to a well defined genetical entity. Our results suggest that *L. juniperoideum* is synonymous with *L. albidum*.

Haplotypes G and H1 differed by one indel in the ITS and were morphologically indistinguishable. In addition, the sporophytes found in one collection of haplotype G and H1 were identical and matched the description of the sporophyte of *L. glaucum*. The gametophytes, characterized by a long leaf base and regularly thickened costae, also fit the traditional circumscription of *L. glaucum*. Hence, haplotypes G and H1 were interpreted as molecular variants of *L. glaucum*.

The fourth haplotype, H2, could not be clearly characterized by morphological features. For example, leaf length in haplotype H2 ranged from the smallest (about 1.5 mm) to the largest sizes (about 9 mm) of the 31 specimens. Haplotype H2 may correspond to a European endemic cryptic species. Alternatively, it may reflect retention of ancestral polymorphic ITS in Eurasian populations of *L. albidum* and *L. glaucum*. A third possibility is that haplotype H2 may correspond to hybrids between *L. albidum* and *L. glaucum*. Additional molecular data are necessary to test these hypotheses.

The geographical origins of specimens included in this study suggest that *L. albidum* occurs in North America and Europe, in contrast to the traditional interpretation of the species as a North American endemic. On both continents, samples of *L. albidum* originated from southern and, in most cases, Atlantic areas, from Macaronesia to Germany on the one hand, and from Florida to West Virginia on the other. Conversely, samples of *L. glaucum* originated from more northern localities, from Alabama to Canada in North America and from France to Scandinavia in Europe. However, the two species seem to occupy broadly overlapping geographic ranges and occasionally grow intermixed (Patterson et al. 1998).

The molecular markers presented in this study may be used to further document the distribution and ecology of these taxa in Europe and North America. These markers may also help in resolving similar taxonomic issues across the large genus *Leucobryum*, whose 130 species (Smith 1978) include a number of other problematic taxa, including *L. acutifolium* (Mitt.) Card. and *L. rehmannii* Müll. Hal. in southern Africa (Magill 1981), which only differ in size characters and geographical distribution patterns.

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