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Assessing Morphological Species and Interspecific Relationships in North American Grapeferns (*Sceptridium*; Ophioglossaceae) Using ISSR Markers

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Abstract—Relationships among North American *Sceptridium* (sensu Škoda) species are often difficult to assess because of few stable distinguishing characters and high intraspecific variability. We used ISSR-PCR to examine relationships among four samples of *Holubiella*, 24 samples representing four diploid North American *Sceptridium* species, and a Russian *Sceptridium* sample. Nine ISSR primers yielded 147 polymorphic loci. We generated three neighbor-joining dendrograms using a Jaccard, Nei & Li, or Dice coefficient. All three coefficients cluster based solely on the presence of bands and not the absence. ISSR data analyses across all three coefficients were consistent with published analyses of DNA sequence data in supporting *Holubiella* as sister to all *Sceptridium* species sampled. Sister group relationships among the four *Sceptridium* species were not consistent across all three neighbor-joining analyses, and bootstrap support was generally low. However, 26 of the 29 samples consistently clustered with other samples of similar morphology, and these clusters generally supported current species concepts. In taxa such as *Sceptridium*, with low DNA sequence variability among species, ISSR-PCR may provide an important tool for evaluating morphologically defined species, but additional data are necessary for establishing robust hypotheses of phylogenetic relationships.

Keywords—Dendrogram, ferns, *Holubiella*, neighbor-joining.

Sceptridium (sensu Škoda 1997) and *Holubiella* Škoda, collectively called the grapeferns, are two of several segregate genera of *Botrychium* s.l. (Zhang et al. 2020). Clausen (1938) recognized 12 grapefern species worldwide. More recent estimates are higher; PPG I (2016) estimated around 25, and Wagner (1990) estimated as many as 30. At least seven grapefern species are found in North America north of Mexico (Wagner and Wagner 1993). *Holubiella* is monotypic and restricted to the southeastern United States, whereas *Sceptridium* is distributed widely through the temperate zones with known centers of diversity in North America and eastern Asia. Members of both genera tend to grow in areas associated with some degree of past disturbance (Lellinger 1985; Wagner 1990) such as second growth woods, old fields, roadsides, pastures, graveyards, or swamps. Putative synapomorphies for the *Holubiella* + *Sceptridium* clade include "evergreen" or "wintergreen" phenology (i.e. leaf generally emerging in summer, persisting through winter, and senescing the next summer), the junction of the fertile and sterile segments near the rhizome (Lyon 1905), and features of early embryology (Lyon 1905). The monophyly of the *Holubiella* + *Sceptridium* clade has been moderately to strongly supported by cpDNA sequence analyses (Hauk et al. 2003; Zhang et al. 2020).

The combination of few stable morphological characters among *Sceptridium* species and high intraspecific variation presents a challenge for assessing the number of species and phylogenetic relationships among species. Some species (e.g. *S. oneidense* and *S. dissectum*) are distinguished by only subtle differences (Wagner 1960, 1961a), and some species (e.g. *S. dissectum*: Figs. 1G, 2E–H) harbor substantial amounts of intraspecific morphological variability (Clausen 1938; Graham and Wagner 1991; Wagner and Wagner 1993), much of which (in the case of *S. dissectum*) is not likely genetically based (Barker and Hauk 2003). Analyses of two plastid regions (*rbcL*, $trnL_{UAA}$-F_{GAA}) failed to robustly resolve relationships among three North American grapefern species because of low levels of DNA sequence variation (Hauk et al. 2003). A broader sampling of ten *Sceptridium* species using analyses of seven plastid regions also revealed low levels of divergence among the same three species (Zhang et al. 2020). However, *Holubiella lunarioides* (Fig. 1A–B) has been well supported as sister to seven *Sceptridium* species (Hauk et al. 2003) or strongly supported as sister to ten other *Sceptridium* species (Zhang et al. 2020).

Dominant DNA markers, also known as arbitrarily amplified DNA (AAD) markers, can be a useful tool for examining both intraspecific genetic variation (e.g. Guicking et al. 2009) and interspecific evolutionary relationships (e.g. Chiron et al. 2009; Akhavan et al. 2015) among closely related species. AAD markers such as inter-simple sequence repeats (ISSRs) have been adapted to a wide range of organisms such as algae (Noormohammadi et al. 2011), ferns (Krattinger 2010), gymnosperms (Trindade et al. 2010), flowering plants (Akhavan et al. 2015), fungi (Park et al. 2008), fish (Antunes et al. 2010), birds (Wink et al. 2008), and rodents (Bugarski-Stanojević et al. 2011). ISSRs target multiple, highly variable regions of nuclear and mitochondrial DNA flanking microsatellite regions and often produce more variability (e.g., Hundsdoerfer et al. 2005) and greater resolution (e.g. Tikunov et al. 2003) than techniques such as allozymes or DNA sequencing. ISSRs typically use longer primers and higher annealing temperatures than RAPDs, and presumably this contributes to higher reproducibility (Nagaoka and Ogihara 1997; Wolfe and Liston 1998; Wolfe et al. 1998). The presence of a 3' nucleotide anchor helps to avoid strand slippage during PCR reactions, and thus non-specific amplification (Archibald et al. 2006). The use of AAD markers (e.g. AFLPs, ISSRs, RAPDs) for establishing phylogenetic relationships is most valid when comparing closely related, non-reticulating taxa (Bussell et al. 2005), when close relationships among taxa evaluated are established by DNA sequencing data (Archibald et al. 2006), and when analyses employ coefficients that consider only the shared presence of bands and not shared absences (Archibald et al. 2006).

We used ISSR markers to: 1) examine sister group relationships among *Holubiella lunarioides* (Fig. 1A–B) and four diploid North American *Sceptridium* species: *S. biternatum* (Fig. 2A–D), *S. dissectum* (Figs. 1G, 2E–H), *S. multifidum* (Fig. 1C–D), and *S. rugulosum* (Fig. 1E–F), 2) test the morphological

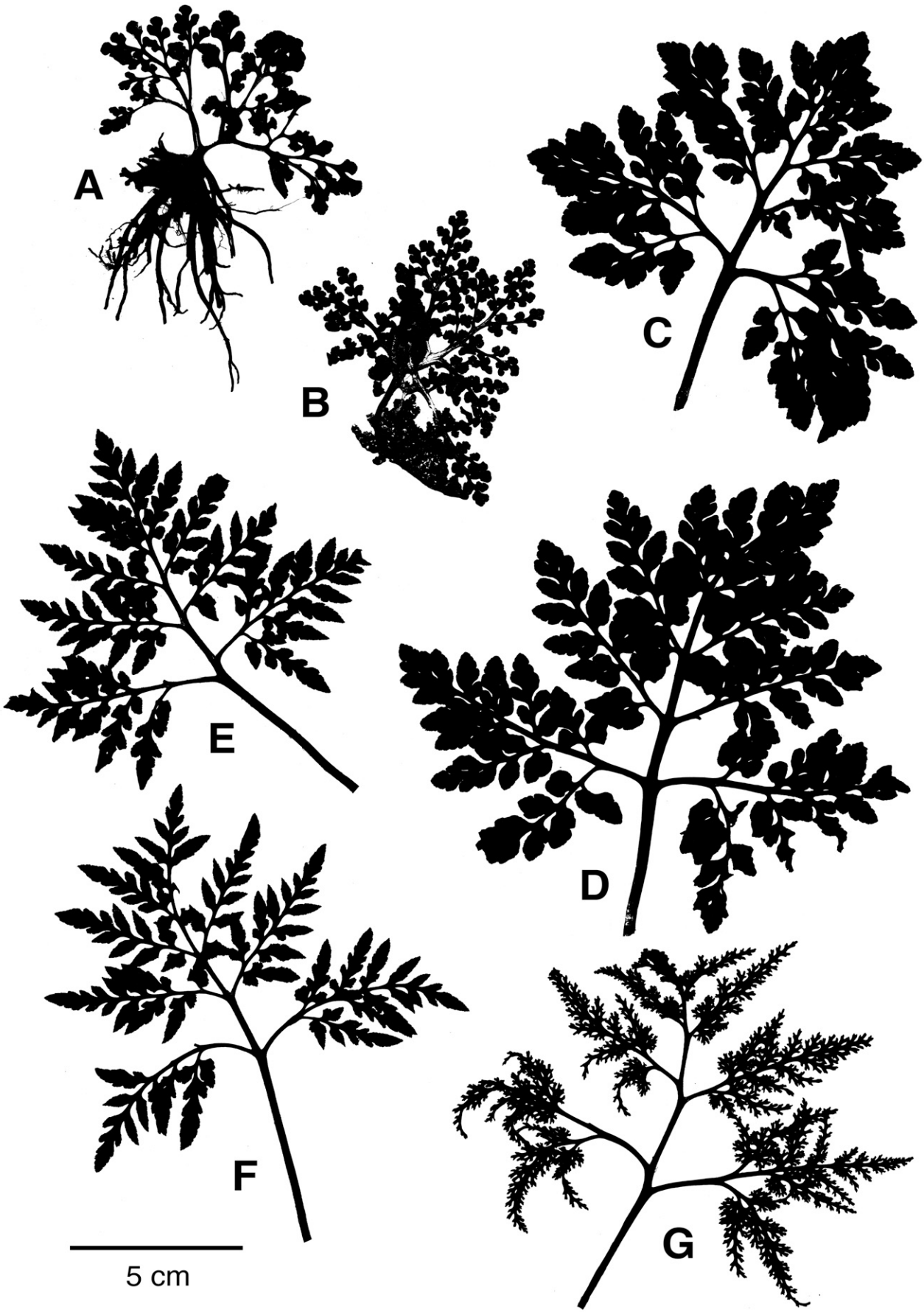

FIG. 1. Silhouettes of four grapefern species. A–B. *Holubiella lunarioides*. C–D. *Sceptridium multifidum*. E–F. *S. rugulosum*. G. *S. dissectum* var. *dissectum*.

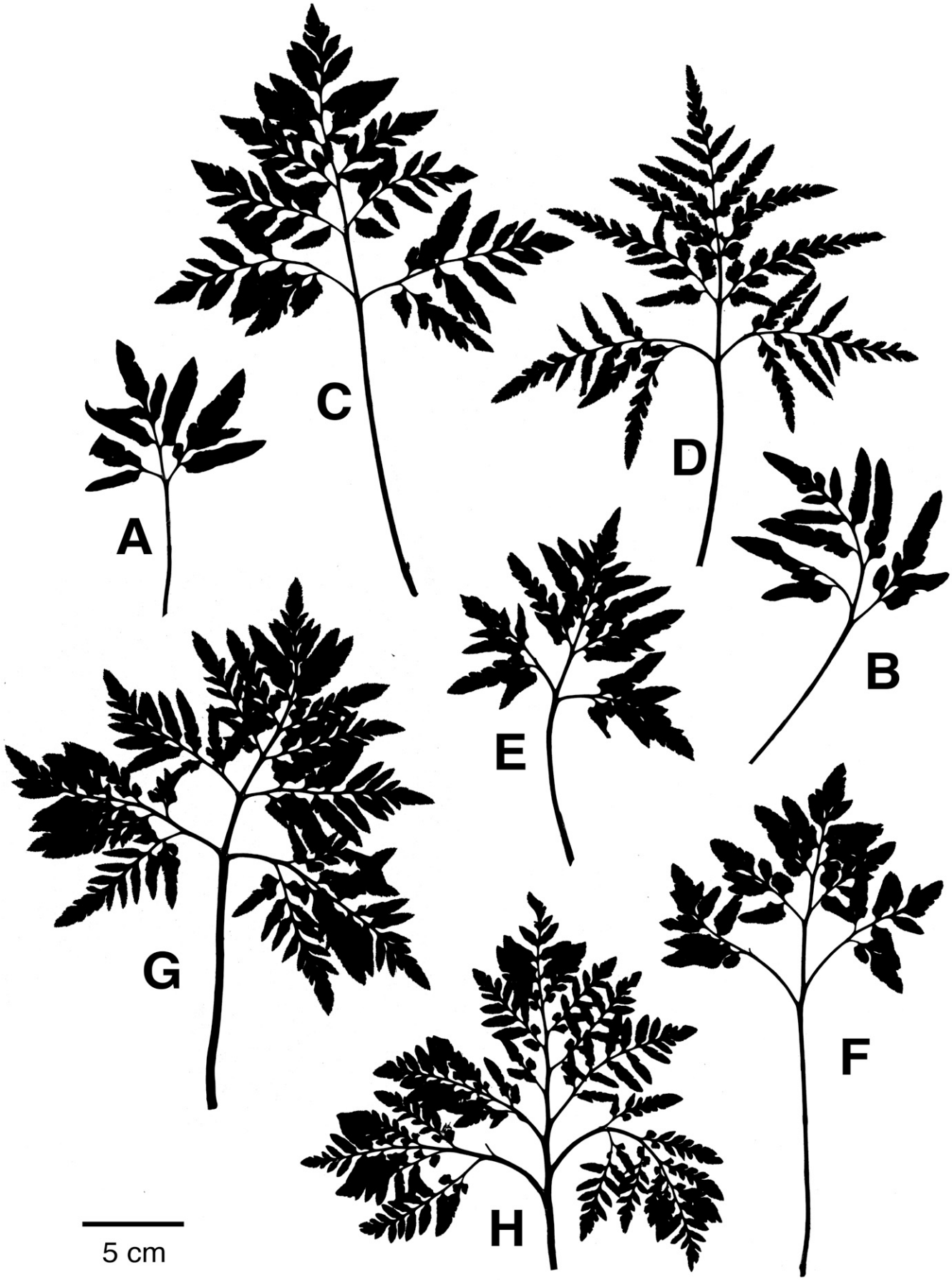

FIG. 2. Silhouettes of two North American grapefern species. A–D. *Sceptridium biternatum*, with C–D representing the "bayou" morphotype. E–H. *S. dissectum* var. *obliquum*, with G–H representing the "johnsonii" morphotype.

species concept in four North American *Sceptridium* species, 3) evaluate the status of two morphological variants of currently recognized North American *Sceptridium* species, and 4) evaluate the consistency of neighbor-joining dendrograms produced from three different similarity coefficients.

Materials and Methods

Sampling—We selected 2–12 samples of each of five diploid North American grapefern species (N = 28 sporophytes). See Table 1 for vouchers, localities, and sample abbreviations. For four species (*S. biternatum*, *S. dissectum*, *S. multifidum*, and *H. lunarioides*) these collections sampled a representative portion of the geographic distribution of the species (Table 1). From a larger pool of individuals, we selected exemplars that: 1) represented "typical" morphology of each species (e.g. features used in keys), and 2) were within the known geographic range of the species. We excluded individuals that: 1) had ambiguous and/or intermediate morphology, or 2) were collected from geographic areas and/or habitats not typically attributed to the species. For three species (*S. dissectum*, *S. rugulosum,* and *H. lunarioides*) our sampling included at least two individuals from the same population. Samples of *S. oneidense* were not available for this study.

As a point of comparison to the North American samples, we obtained a single sample from the Russian Far East (Sakhalin Island, Russia). Clausen (1938) treated *Sceptridium robustum* as a subspecies of *S. multifidum* (i.e. *Botrychium multifidum* subsp. *robustum* (Rupr. ex Milde) R.T. Clausen), but Zhang et al. (2013) recognized it at the level of species within *Botrychium* s.l. Here we provisionally recognize it as a species within the more narrowly defined *Sceptridium* sensu Škoda. Clausen (1938) reported *S. robustum* (as *Botrychium multifidum* subsp. *robustum*) from south and west Alaska, as well as Russia, China, Japan, and Korea.

In addition, we sampled two putative morphotypes of recognized North American *Sceptridium* species: 1) presumably based on the account of Johnson (1960), Wagner and Wagner (1993) noted a morphotype of *Botrychium dissectum* var. *obliquum* (Muhl.) Clute (=*Sceptridium dissectum*) that grows in eastern Kentucky (KY) and central Tennessee (TN) and possesses "narrowly linear, somewhat blunt-tipped segments with a more or less whitish gray central line above the veins." We included two samples of this "johnsonii" morphotype from a single TN population (Table 1); 2) in the University of Florida Herbarium, Warren H. Wagner annotated some specimens of *Botrychium biternatum* (Savigny) Underw. (=*Sceptridium biternatum*) with the moniker "bayou" and placed them in a folder separate from other *S. biternatum* collections. We included two individuals of this morphotype, each from a different county in central Florida (FL; Table 1). Based on a phylogeny developed from analyses of *rbcL* and $trnL_{UAA}$-F_{GAA} sequences and morphology (Hauk et al. 2003), we selected *Botrypus virginianus* as the outgroup and included two Ohio (OH) samples (Table 1).

Pressed specimens representing the *Sceptridium* populations sampled were scanned, and Adobe Photoshop CC (Adobe, Inc., San Jose, California) was used to convert the scanned images into silhouettes. Fertile segments, including stalks, were trimmed from the images. Unaltered, color scans are available from the corresponding author (WDH) upon request.

DNA Methods—Total genomic DNA was extracted from approximately 100 mg of silica gel dried leaf material using Qiagen DNeasy columns (Qiagen, Inc., Valencia, California). Genomic DNA from each sample was quantitated fluorometrically using the PicoGreen dsDNA reagent (Molecular Probes, Inc., Eugene, Oregon) and a TD-360 mini-fluorometer (Turner Designs, Sunnyvale, California) according to the manufacturer's protocol. ISSR primers were selected from Barker and Hauk (2003) and/or from screening of the University of British Columbia Biotechnology Laboratory (UBC) primer set #9. We selected nine primers (Table 2) that generated reproducible banding patterns and chose those primers that produced maximal variation in the screening gels: UBC-815, UBC-818, UBC-824, UBC-835, UBC-842, UBC-846, UBC-848, UBC-850, and UBC-880. In a total volume of 25 μl, 10–20 ng of genomic DNA was amplified in 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM of a single ISSR primer, and 0.625 units HS *Taq* (Takara Bio Inc., Shiga, Japan). PCR reactions were performed in an Eppendorf Mastercycler Thermocycler (Eppendorf AG, Hamburg, Germany) using the following regime: 60 s at 94°C, followed by

Table 1. Samples of *Holubiella*, *Sceptridium*, and *Botrypus* species and collector and collection number of voucher, locality, and sample abbreviation used in the text and in Figs. 3–5. All Hauk vouchers are deposited at OS. Co. = county; Pa. = parish. AL = Alabama; AK = Alaska; AR = Arkansas; FL = Florida; GA = Georgia; KY = Kentucky; LA = Louisiana; MI = Michigan; MN = Minnesota; MS = Mississippi; NC = North Carolina; OH = Ohio; TN = Tennessee; UT = Utah; VA = Virginia; RU = Russia. Unless otherwise indicated, all collections made within the USA.

Species	Collector, Collection number Voucher/Herbarium	Locality	Sample Abbreviation
Botrypus virginianus (L.) Michx.	Hauk 2008–01–1	Licking Co., OH	Bvir OH1
B. virginianus	Hauk 2008–01–2	Licking Co., OH	Bvir OH2
Holubiella lunarioides (Michx.) Škoda	Hauk 2003–02–2	Claiborne Pa., LA	Hlun LA
H. lunarioides	Hauk 2003–16–2	Coffee Co., GA	Hlun GA
H. lunarioides	Hauk 2003–09–6	Crenshaw Co., AL	Hlun AL
H. lunarioides	Hauk 2003–03–4	Jones Co., MS	Hlun MS
Sceptridium biternatum (Savigny) Lyon	Hauk 2002–19–7	Garland Co., AR	Sbit AR
S. biternatum	Hauk 2002–24–8	St. Landry Pa., LA	Sbit LA
S. biternatum	Hauk 2002–25–1	Macon Co., AL	Sbit AL
S. biternatum	Hauk 2002–30–1	Baldwin Co., GA	Sbit GA
S. biternatum "bayou"	Hauk 2002–26–3	Leon Co., FL	Sbit bay FL1
S. biternatum "bayou"	Hauk 2002–29–5	Alachua Co., FL	Sbit bay FL2
S. dissectum (Spreng.) Lyon [var. *dissectum*]	Hauk 2002–40–5	Buncombe Co., NC	Sdis dis NC
S. dissectum [var. *dissectum*]	Hauk 2002–12–4	Preble, Co., OH	Sdis dis OH1
S. dissectum [var. *dissectum*]	Hauk 2002–12–6	Preble, Co., OH	Sdis dis OH2
S. dissectum [var. *dissectum*]	Hauk 2002–43–6	Roanoke Co., VA	Sdis dis VA
S. dissectum [var. *obliquum*]	Hauk 2002–13–7	Pulaski Co., KY	Sdis obl KY
S. dissectum [var. *obliquum*]	Hauk 2002–41–9	Yancey Co., NC	Sdis obl NC1
S. dissectum [var. *obliquum*]	Hauk 2002–11–1	Calhoun Co., MI	Sdis obl MI
S. dissectum [var. *obliquum*]	Hauk 2002–38–3	Iredell Co., NC	Sdis obl NC2
S. dissectum [var. *obliquum*]	Hauk 2002–12–1	Preble, Co., OH	Sdis obl OH1
S. dissectum [var. *obliquum*]	Hauk 2002–12–9	Preble, Co., OH	Sdis obl OH2
S. dissectum [var. *obliquum*] ("johnsonii")	Hauk 2002–17–1	Davidson Co., TN	Sdis obl john TN1
S. dissectum [var. *obliquum*] ("johnsonii")	Hauk 2002–17–2	Davidson Co., TN	Sdis obl john TN2
S. multifidum (S.G.Gmel.) M.Nishida ex Tagawa	Hauk 2002–02–1	Aitkin Co., MN	Smul MN
S. multifidum	Hauk 2002–05–4	Alger Co., MI	Smul MI
S. multifidum	Hauk 2008–02–1	Uinta Co., UT	Smul UT
S. multifidum	Stensvold *s.n.*	Yakutat Co., AK	Smul AK
S. robustum (Rupr. ex Milde) Lyon	Joneson 3284 WTU	Sakhalinski Bay, Russia	Srob RU
S. rugulosum (W.H.Wagner) Škoda & Holub	Hauk 2002–09–2	Saginaw Co., MI	Srug MI1
S. rugulosum	Hauk 2002–09–7	Saginaw Co., MI	Srug MI2

TABLE 2. ISSR primers, primer sequence, number of scored bands per primer, percent of polymorphic bands, total number of scored bands, and mean number of scored bands per primer. Y = C or T; R = A or G.

Primer	Primer Sequence 5'-3'	Number of Scored Bands per Primer	Percent of Polymorphic Bands
UBC-815	CTCTCTCTCTCTCTCTG	23	100
UBC-818	CACACACACACACACAG	18	100
UBC-824	TCTCTCTCTCTCTCTCG	14	100
UBC-835	AGAGAGAGAGAGAGAGYC	26	100
UBC-842	GAGAGAGAGAGAGAGAYG	8	100
UBC-846	CACACACACACACACART	15	100
UBC-848	CACACACACACACACARG	9	100
UBC-850	GTGTGTGTGTGTGTGTYC	20	100
UBC-880	GGAGAGGAGAGGAGA	14	100
Total		147	
Mean number of scored bands per primer		16.33	

35 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C followed by a final 5 min 72°C extension. Each set of PCR reactions contained a negative control and was repeated twice to verify reproducibility. PCR products were electrophoresed through a 1.5% agarose gel containing 0.2 ng/ml EtBr in 1 × TAE buffer and four lanes of a 1 kb Plus DNA Ladder size standard (Gibco BRL, Life Technologies, Inc., Rockville, Maryland). Bands were visualized on a UV transilluminator and photographed using a FluorChem HD2 imager (Alpha Innotech, San Leandro, California). We scored only reproducible bands from gel photographs. Bands of indistinguishable mobility were scored as orthologous, and, among closely related species, this may be a reasonable assumption (Bussell et al. 2005). The low levels of cpDNA sequence divergence (~0.04%) among North American *Sceptridium* species (Hauk et al. 2003) are consistent with the hypothesis that these species are recently diverged. For every sample, we coded each locus as "1" if present and "0" if absent, and assembled bands from all primers into a single dataset of multilocus profiles.

Data Analyses—Using PAST 3.13 (Hammer et al. 2001), we conducted neighbor-joining (NJ) analyses using a Jaccard (1908) (henceforth "Jaccard") coefficient with 500 bootstrap replicates and a Dice (1945) (henceforth "Dice") coefficient with 500 replicates. We reported all bootstrap values as percentages (BP). A third NJ analysis using the Nei & Li (1979) coefficient (henceforth "Nei & Li") was performed using NTSYSpc 2.21n (Rohlf 2011). According to Archibald et al. (2006), the Nei & Li coefficient may be tailored for either restriction site data (e.g. PHYLIP, PAUP*) or for hypervariable DNA markers (e.g. NTSYSpc). However, unlike PAST, NTSYSpc does not perform bootstrap analyses. We used FreeTree (Pavlicek et al. 1999) to replicate Dice and Jaccard analyses and generate BP (500 bootstrap replicates). We used TreeView X version 0.5.0 (Page 1996) to visualize the dendrograms. The Dice, Jaccard, and Nei & Li coefficients are similar in that they consider only the shared presence of bands to assemble clusters (Archibald et al. 2006). To interpret all analyses, we assumed that ISSR locus variation was representative of overall genetic variation.

RESULTS

Nine ISSR primers produced a total of 147 distinct and reproducible loci (mean of 16.3 loci/primer) ranging from around 150–2000 bp with 100% of loci polymorphic (Table 2). The UBC-842 primer yielded the smallest number of loci (N = 8), whereas UBC-835 produced the most loci (N = 26) (Table 2). No two samples had identical multilocus ISSR profiles. A small number (~3) of the 147 loci identified had co-migrating bands across the four *Sceptridium* species (*S. biternatum, S. dissectum, S. multifidum*, and *S. rugulosum*) included in our ISSR analyses. The ISSR dataset is available through Dryad (Cao and Hauk 2022).

Jaccard Coefficient NJ Dendrogram—Figure 3 shows the Jaccard dendrogram in which the Bvir OH1 sample was designated the outgroup. For ease of comparison, seven main clusters were identified. Cluster 1 contained the two Bvir OH samples, and cluster 2 contained all four Hlun samples (BP 100). Cluster 2 was sister to a group comprised of clusters 3–7 (BP 99) and corresponds to *Sceptridium* sensu Škoda (= Wagner and Wagner's (1993) *Botrychium* subg. *Sceptridium* sect. *Sceptridium*). Within cluster 2, the Hlun LA and Hlun MS samples were paired (BP 87), with Hlun AL (BP 83) and Hlun GA samples grouping successively outside that pair. Cluster 3 was comprised of the Russian *S. robustum* sample, and it was sister to the group comprised of clusters 4–7, which included all North American *Sceptridium* samples. Cluster 4 was sister to the group comprised of clusters 5–7 and contained the four samples of *S. multifidum.* Smul AK and Smul UT were paired (BP 56), and Smul MI and Smul MN nested successively outside that pair. Cluster 5 contained the two samples of *S. rugulosum* (MI1 and MI2; BP 60) and was sister to the group comprised of clusters 6 and 7. Cluster 6 contained all six samples of *S. biternatum*. The two *S. biternatum* "bayou" individuals (Sbit bayou FL1 and Sbit bayou FL2) formed a pair (BP 72) that was sister to the group comprised of the remaining four samples (Sbit LA, Sbit GA, Sbit AR, and Sbit AL; BP 53). Cluster 7 contained all 12 samples of *S. dissectum*, including the two samples dubbed "johnsonii" (Sdis obl john TN1 and Sdis obl john TN2). The two "johnsonii" individuals of *S. dissectum* var. *obliquum* formed a pair sister to a group comprised of the other ten *S. dissectum* individuals. The following four pairs were observed in cluster 7: Sdis obl KY + Sdis obl OH1, Sdis obl NC1 + Sdis obl NC2, Sdis dis OH1 + Sdis dis OH2, and Sdis dis NC + Sdis dis VA. Only Sdis dis OH1 and Sdis dis OH2 had a BP above 50 (BP 67). All *S. dissectum* var. *dissectum* samples paired with another *S. dissectum* var. *dissectum* individual, but the two pairs of *S. dissectum* var. *dissectum* samples did not form a cluster separate from a group composed of all *S. dissectum* var. *obliquum* samples. For samples collected from the same population, four paired with another from the same population (e.g. Sdis dis OH1 and Sdis dis OH2 from Preble Co., OH; Sdis obl john TN1 and Sdis obl john TN2 from Davidson Co., TN), whereas two other individuals from the same population did not (e.g. Sdis obl OH1 and Sdis obl OH2 from Preble Co., OH).

Nei & Li Coefficient NJ Dendrogram—The Nei & Li dendrogram (Fig. 4) was more similar to the Jaccard dendrogram (Fig. 3) than to the Dice dendrogram (Fig. 5). Eight main clusters were identified for ease of comparison. Clusters 1–4 in the Nei & Li dendrogram were identical in topology to that of the Jaccard dendrogram (Fig. 3). In Fig. 4, cluster 5 contained the two *S. biternatum* "bayou" samples and was sister to clusters 6–8 instead of sister to the other four individuals of *S. biternatum* as in the Jaccard dendrogram (Fig. 3). The remaining four *S. biternatum* individuals (LA, GA, AR, and AL) formed cluster 6, which was sister to a group comprised of all samples of *S. rugulosum* and *S. dissectum* (clusters 7–8). Cluster 7 contained the two *S. rugulosum* samples and was sister to a group comprised of all 12 *S. dissectum* samples (cluster 8). Relationships among *S. dissectum* samples were similar to those in the Jaccard dendrogram (Fig. 3) except that the two "johnsonii" individuals were embedded inside (instead of sister to) a group comprised of the other ten *S. dissectum* samples.

Dice Coefficient NJ Dendrogram—Figure 5 shows the Dice dendrogram in which the Bvir OH1 sample was designated as the outgroup. For ease of comparison six main clusters were identified, although only clusters 2 and 3 had BP above 50. The Bvir OH1 and Bvir OH2 samples (BP 99) comprised cluster 1, and this cluster was sister to the group comprised of clusters 2–6. Cluster 2 contained all four *H. lunarioides*

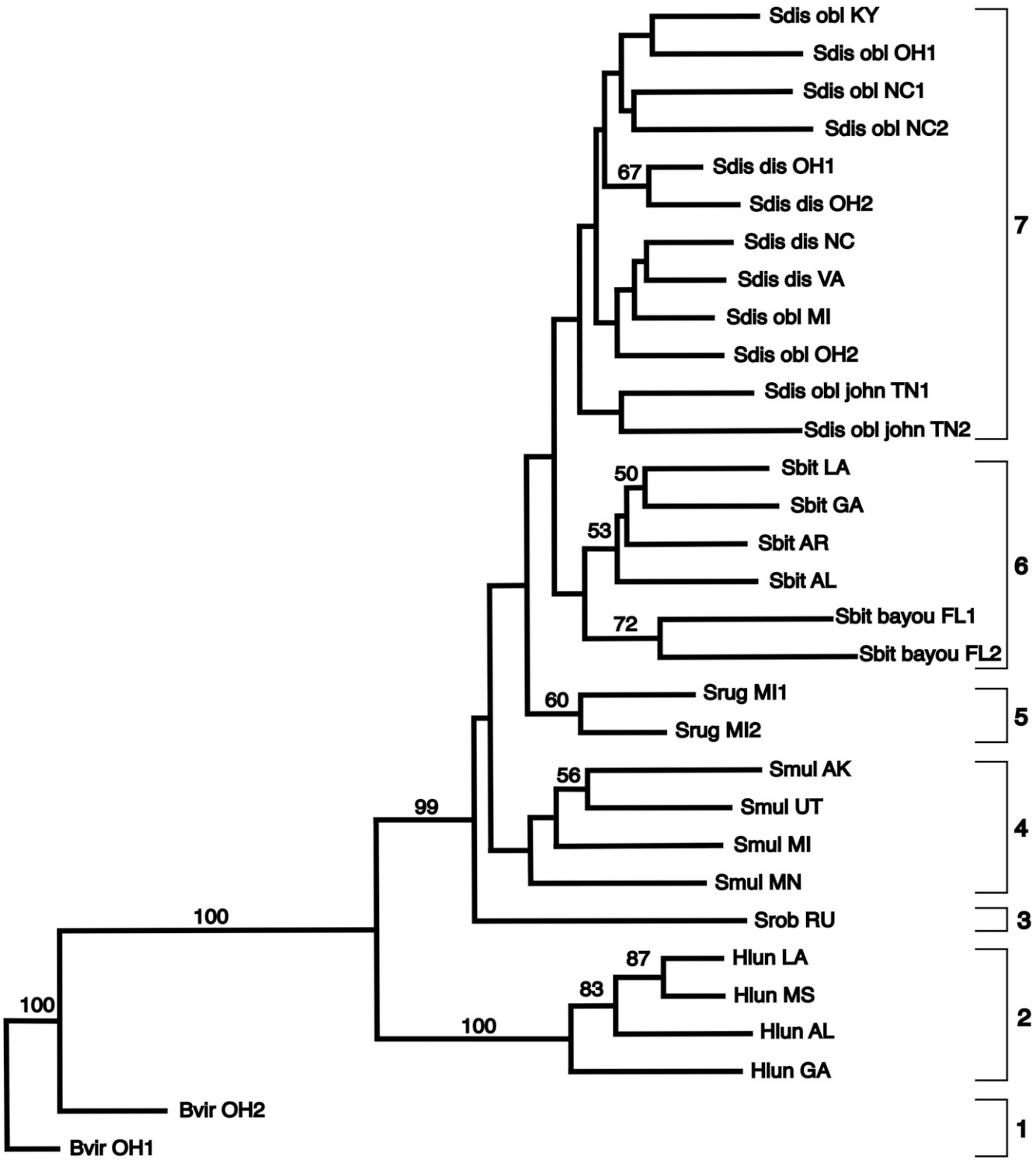

FIG. 3. Jaccard coefficient NJ dendrogram for 28 samples representing *Holubiella lunarioides* and four diploid North American *Sceptridium* species plus one Russian sample. Numbered brackets reference seven main clusters identified for ease of comparison. Values above branches are bootstrap percentages, and only values above 50% are shown. Table 1 lists the scientific name, collection site, and each sample abbreviation. *Botrypus* sample Bvir OH1 served as the outgroup.

samples (BP 100) and was sister to a group comprised of all *Sceptridium* samples (clusters 3–6). Within cluster 2, the Hlun LA and Hlun MS samples were paired (BP 71), with Hlun AL (BP 79) and Hlun GA samples grouping successively outside that pair. Clusters 3–6 were comprised of the "core" *Sceptridium* cluster that corresponds to Wagner and Wagner's (1993) *Botrychium* subg. *Sceptridium* sect. *Sceptridium*. Cluster 3 contained the two samples of *S. biternatum* dubbed "bayou" from Florida (BP 64) and was sister to the group comprised of clusters 4–6. Cluster 4 contained the other four samples of *S. biternatum*, with the Sbit LA and Sbit GA samples paired and nested successively by Sbit AR and Sbit AL. Cluster 5

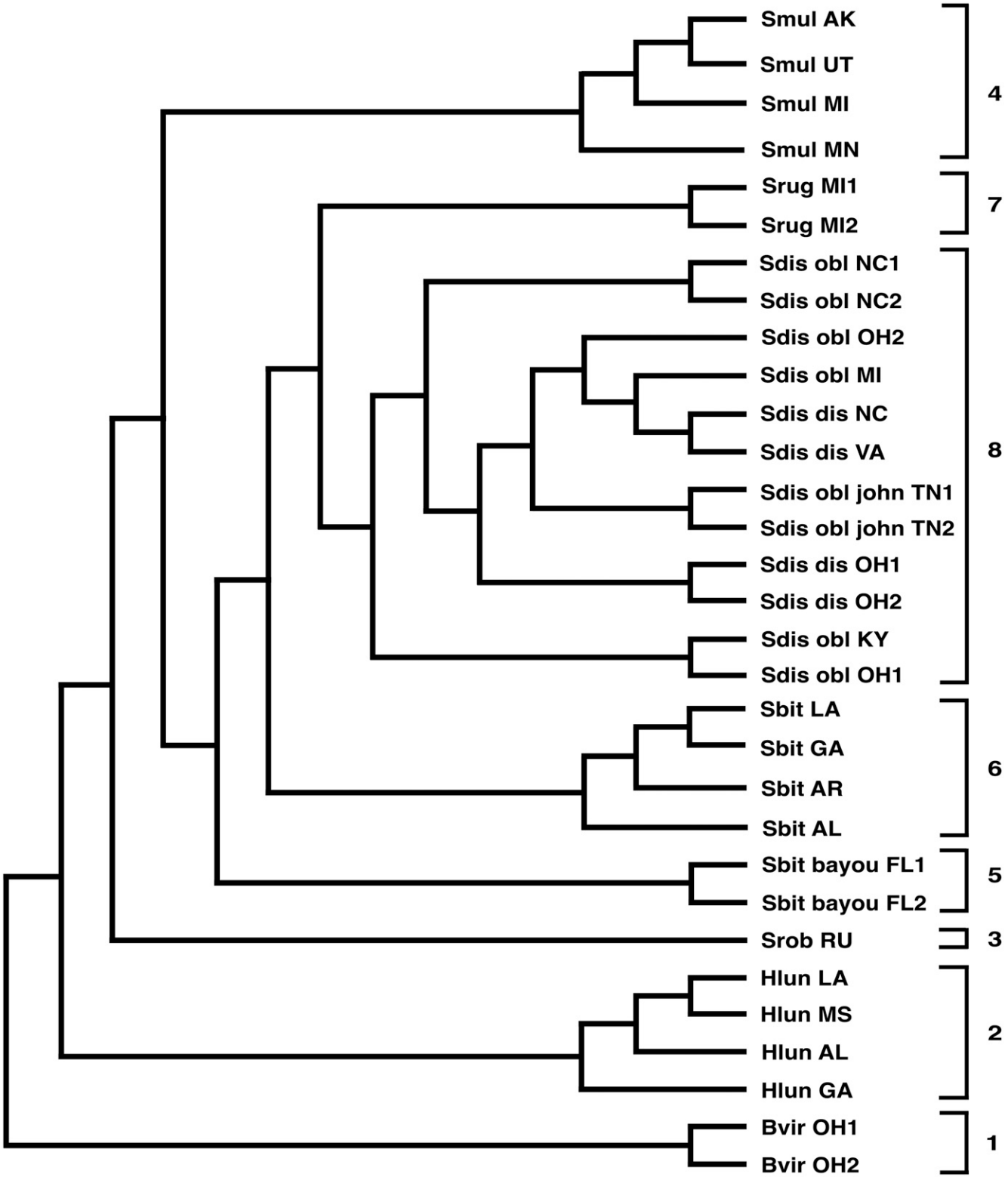

FIG. 4. Nei & Li coefficient NJ dendrogram for 28 samples representing *Holubiella lunarioides* and four diploid North American *Sceptridium* species plus one Russian sample. Numbered brackets reference eight main clusters identified for ease of comparison. Table 1 lists the scientific name, collection site, and each sample abbreviation. *Botrypus* sample Bvir OH1 served as the outgroup.

contained the two *S. rugulosum* samples (Srug MI1 and Srug MI2), the Russian *S. robustum* sample, and the four samples of *S. multifidum*. In cluster 5 the four samples of North American *S. multifidum* formed a group, Srob RU and Srug MI1 formed a pair, and Srug M2 was sister to a group comprised of all other samples in cluster 5. Unlike in Figs. 3 and 4, the single sample from outside of North America (Srob RU) was grouped within a cluster of North American samples of *S. multifidum* and *S. rugulosum*. Cluster 6 contained all 12 samples of *S. dissectum,* including the two samples of var.

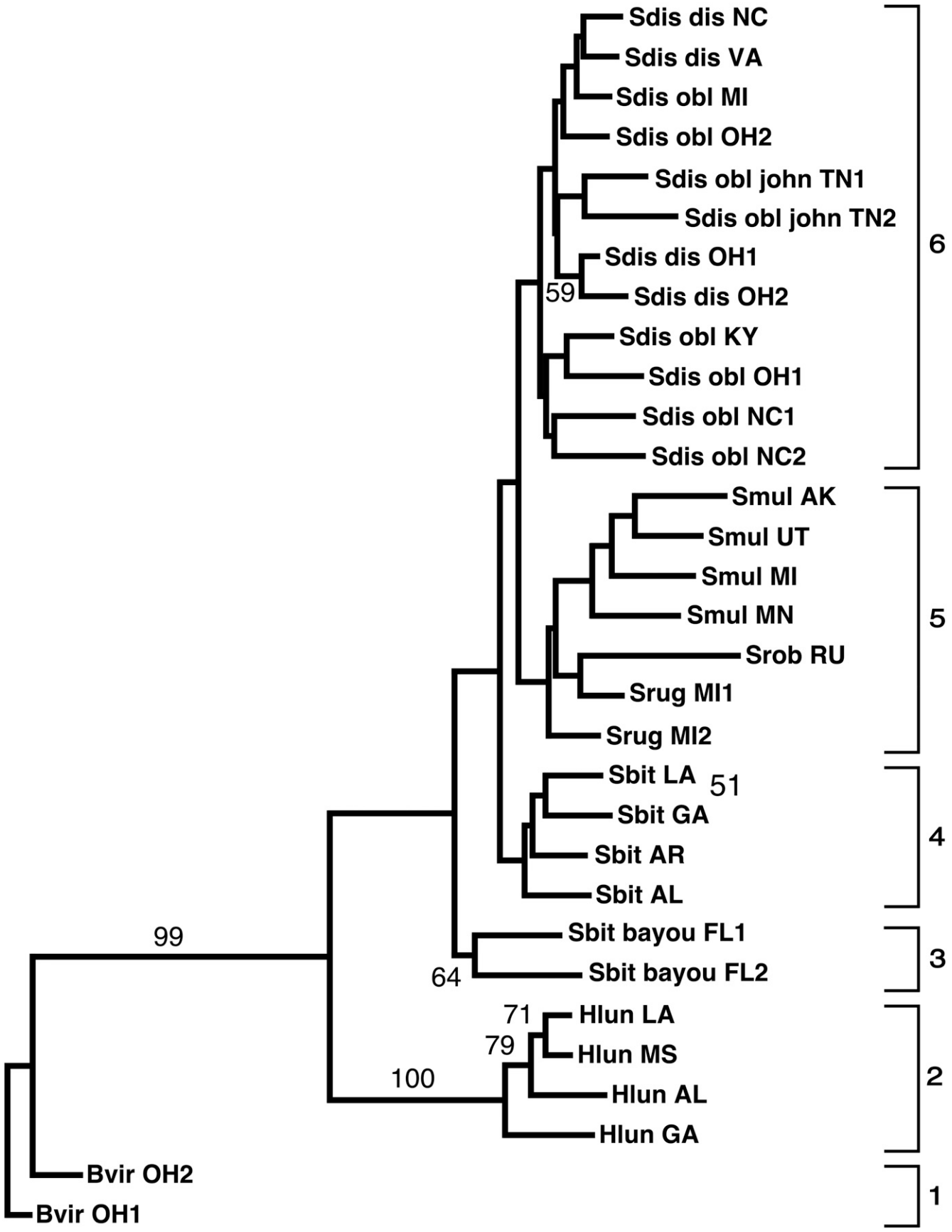

FIG. 5. Dice coefficient NJ dendrogram for 28 samples representing *Holubiella lunarioides* and four diploid North American *Sceptridium* species plus one Russian sample. Numbered brackets reference six main clusters identified for ease of comparison. Values above branches are bootstrap percentages, and only values above 50% are shown. Table 1 lists the scientific name, collection site, and each sample abbreviation. *Botrypus* sample Bvir OH1 served as the outgroup.

obliquum dubbed "johnsonii." Within cluster 6, the following samples formed pairs: Sdis obl john TN1 + Sdis obl john TN2, Sdis dis NC + Sdis dis VA, Sdis dis OH1 + Sdis dis OH2 (BP 59), Sdis obl NC1 + Sdis obl NC2, and Sdis obl KY + Sdis obl OH1. As in Figs. 3 and 4, the *S. dissectum* var. *dissectum* samples paired with another sample of the same variety. Most of the eight samples of *S. dissectum* var. *obliquum* paired with another sample of *S. dissectum* var. *obliquum.* Each of the two pairs of *S. dissectum* var. *dissectum* samples clustered more closely to samples of *S. dissectum* var. *obliquum* (or to clusters containing samples of *S. dissectum* var. *obliquum*) than to the other pair of *S. dissectum* var. *dissectum* samples. Thus, consistent with Figs. 3 and 4, all samples of *S. dissectum* var. *obliquum* did not cluster together as a group sister to a group comprised of all samples of *S. dissectum* var. *dissectum*. The Sdis obl john TN1 and Sdis obl john TN2 pair clustered most closely with the paired OH1 and OH2 samples of *S. dissectum* var. *dissectum* within a larger cluster comprised of all other *S. dissectum* samples.

Discussion

Analyses of ISSR data from four diploid North American *Sceptridium* species and *Holubiella lunarioides* revealed that 100% of the 147 ISSR loci coded were polymorphic with a mean of 16.3 loci produced per ISSR primer and a range of 8 to 26 loci per primer. No two samples had identical multilocus banding patterns. Pairwise comparisons of *rbcL* and $trnL_{UAA}$-F_{GAA} sequences showed 0.4% mean variation (7 of 1682 bp) between samples of *S. multifidum* and *S. dissectum* (Hauk et al. 2003). In our ISSR dataset, a mean of around 50% of loci were variable in pairwise comparisons of samples from these two species. Thus, on average ISSR data were considerably more variable than the combination of *rbcL* and $trnL_{UAA}$-F_{GAA} cpDNA sequence data.

When using AAD markers, the presence of around 20% co-migrating (i.e. monomorphic) bands across ingroup taxa should signal taxa that have diverged recently enough to justify the assumption that co-migration of bands indicates homology (Bussell et al. 2005). With less than 5% co-migrating bands, our ISSR data did not meet the 20% benchmark. However, these ISSR data were generated solely from primers selected to generate maximal variation within *Sceptridium*. The low number of monomorphic ISSR bands across all *Sceptridium* samples in this dataset may, nevertheless, be consistent with considering these species as recently diverged taxa because data from primers generating invariant or minimally variant bands within the ingroup were not gathered. A few loci had co-migrating bands across *Sceptridium* species (*S. biternatum, S. dissectum, S. multifidum*, and *S. rugulosum*) included in our ISSR analyses, and this is consistent with interpreting co-migrating bands as orthologous (see Bussell et al. 2005). Because we selected only primers that produced the most variable banding patterns in ISSR test gels, we surmise that the low number of monomorphic, co-migrating bands among our samples of North American *Sceptridium* species may well be an underestimate of monomorphic band frequency, at least relative to a hypothetical dataset assembled from a random selection of band-producing primers from the UBC Primer Set #9.

UPGMA and NJ are frequently employed distance methods for analyzing AAD data, but NJ has several advantages over UPGMA. UPGMA assumes that the tree is additive and ultrametric, and NJ does not make these potentially problematic assumptions (Hall 2011). However, NJ trees are not ideal because they "are always fully resolved, even when this resolution is not supported by the data" and because NJ does not guarantee the shortest tree (Bussell et al. 2005). NJ appears to be more accurate than UPGMA across several evolutionary factors and contexts (Kim et al. 1993). Despite some limitations, NJ may make fewer potentially problematic assumptions than UPGMA (Archibald et al. 2006), so we considered only NJ dendrograms.

Distance methods can employ a host of coefficients to construct NJ dendrograms, and each coefficient has advantages and disadvantages. We initially chose Jaccard, Nei & Li, and Dice coefficients because they consider only the shared presence of bands to determine similarity, and this is appropriate for many AAD datasets (Meyer et al. 2004; Bussell et al. 2005; Archibald et al. 2006; Dalirsefat et al. 2009); although see Beharav et al. (2010) and Kosman and Leonard (2005). Because bands can be lost for different, non-homologous reasons, homology is more certain when bands are present (Archibald et al. 2006). For taxa that are recently diverged and exhibit inbreeding, Kosman and Leonard (2005) reasoned that the simple mismatch coefficient, which is equivalent to the normalized squared Euclidian distance, is most appropriate for dominant marker data because it uses both shared presence and shared absence of bands. Among closely related species, shared absences are more likely to be homologous than among more distantly related taxa, and therefore shared absences may contain useful evolutionary signal (Kosman and Leonard 2005). Diploid organisms that primarily inbreed (or in the case of Ophioglossaceae primarily self; McCauley et al. 1985; Soltis and Soltis 1986; Watano and Sahashi 1992; Chung et al. 2013; although see Dauphin et al. 2020) should have relatively low levels of heterozygosity and thus could be treated as haploid for purposes of analysis. If diploid species are more distantly related, the shared absence of bands is not as likely to reflect similarity and the Nei & Li coefficient may be more appropriate than the simple mismatch coefficient (Kosman and Leonard 2005).

Kosman and Leonard (2005) preferred the Jaccard coefficient over the Dice coefficient for closely related haploid taxa because the Jaccard coefficient does not weigh shared bands as much as the Dice coefficient does. Lamboy (1994), however, argued that for RAPD datasets without correction for artifacts, the Nei & Li similarity coefficient may be more appropriate. We employed no correction for artifactual bands because generating such calculations is labor intensive and expensive. The Nei & Li coefficient and the Dice coincident index (1945) are reported to be identical (Archibald et al. 2006), however we found they produced different topologies for our data (Figs. 4, 5) when computed by NTSYSpc 2.21n (Rohlf 2011) and PAST 3.13 (Hammer et al. 2001), respectively. We have no explanation for this outcome.

Across all three analyses, 26 of 29 grapefern samples clustered with other samples identified as the same or similar morphological species, and this is consistent with current hypotheses of species concepts. Of the three samples that did not consistently cluster with other samples of the same or similar morphological species, one (Srob RU) was a sample from outside of North America and may represent a Russian or Asian species clade, and two (Sbit bayou FL1 and FL2) represented an infraspecific morphotype that may merit further investigation (see below).

All three coefficients we employed, Jaccard (Fig. 3), Nei & Li (Fig. 4), and Dice (Fig. 5), produced different topologies. We interpreted points of concordance across the three NJ dendrograms as a more robust signal, whereas we regarded points of disagreement among the three dendrograms as indications of more equivocal support, with emphasis on the Jaccard and Nei & Li dendrograms over the Dice dendrogram. The mixture of concordance and disagreement in topology across different dendrograms may be in part a consequence of selecting only ISSR primers that produced the most highly variable banding patterns in test gels. For future investigations it may be desirable to select a set of ISSR primers that produce a broader range of variability in screening gels. Inclusion of data from primers that generated less than maximal variation may produce data more suitable for consistently resolving *Sceptridium* species-level relationships in NJ analyses.

Patterns consistent across the Jaccard (Fig. 3), Nei & Li (Fig. 4), and Dice (Fig. 5) dendrograms were: 1) the two samples of *Botrypus virginianus* (Bvir OH1 and OH2) were sister to a group comprised of all *Sceptridium* + *Holubiella* samples, 2) all four *H. lunarioides* samples (Hlun LA, MS, AL, GA) formed a cluster that was sister to a group comprised of all samples of *Sceptridium*, 3) all four samples of *S. multifidum* (Smul AK, UT, MI, and MN) clustered together, 4) four of the six *S. biternatum* samples (Sbit LA, GA, AR, and LA) clustered together, 5) all 12 *S. dissectum* samples clustered together, 6) the two *S. dissectum* var. *obliquum* "johnsonii" samples clustered together (Sdis obl john TN1 and TN2), and 7) four other pairs of *S. dissectum* (Sdis dis VA + Sdis dis NC, Sdis dis OH1 + Sdis dis OH2, Sdis obl OH1 + Sdis obl KY, Sdis obl NC1 + Sdis obl NC2) were observed.

The main points of incongruence among the three dendrograms include: 1) the *S. robustum* sample clustered outside of a group comprised of all North American samples of *Sceptridium* in the Jaccard (Fig. 3) and Nei & Li (Fig. 4) dendrograms but *S. robustum* was paired to the *S. rugulosum* MI1 sample in the Dice (Fig. 5) dendrogram, 2) the pair of *S. biternatum* "bayou" samples was sister to the group comprised of the LA, GA, AR and AL *S. biternatum* samples in the Jaccard (Fig. 3) dendrogram but well outside (i.e. not sister) to the LA, GA, AR, and AL *S. biternatum* samples in the Nei & Li (Fig. 4) and Dice (Fig. 5) dendrograms, 3) the pair of *S. rugulosum* samples was sister to a group comprised of the 12 samples of *S. dissectum* in the Nei & Li (Fig. 4) dendrogram and as sister to a group comprised of all samples of *S. dissectum* and *S. biternatum* in the Jaccard dendrogram (Fig. 3), whereas in the Dice dendrogram (Fig. 5) the two were not paired with each other but were instead embedded in a group comprised of the AK, UT, MN, and MI *S. multifidum* samples plus the *S. robustum* sample, 4) in the Jaccard dendrogram (Fig. 3) the cluster of LA, GA, AR, and AL *S. biternatum* samples was sister to the two *S. biternatum* "bayou" samples, sister to a group comprised of *S. dissectum* + *S. rugulosum* samples in the Nei & Li dendrogram (Fig. 4), and sister to a group comprised of *S. dissectum* + *S. multifidum* + *S. rugulosum* + *S. robustum* samples in the Dice dendrogram (Fig. 5), 5) the cluster of 12 *S. dissectum* samples was sister to all samples of *S. biternatum* in the Jaccard dendrogram (Fig. 3), sister to the pair of *S. rugulosum* samples in the Nei & Li dendrogram (Fig. 4), and sister to a group comprised of samples of *S. multifidum*, *S. robustum*, and *S. rugulosum* in the Dice dendrogram (Fig. 5), 6) within the *S. dissectum* cluster, the pair of var. *obliquum* "johnsonii" samples was embedded within the ten other samples in the Nei & Li (Fig. 4) and Dice (Fig. 5) dendrograms and sister to the other ten samples in the Jaccard dendrogram (Fig. 3).

Some patterns produced from these ISSR data were consistent with sister group relationships established by DNA sequence analyses. Figures 3–5 show that both *Botrypus virginianus* samples consistently formed a pair separate from a group comprised of all *Holubiella* + *Sceptridium* samples. The distinction between *Botrypus* and *Holubiella* + *Sceptridium* was consistent with DNA sequence-based phylogenies of Ophioglossaceae that showed *Botrypus* sister to a clade comprised of *Holubiella* + *Sceptridium* + *Botrychium* s.s. (Hauk et al. 2003) or to *Sceptridium* alone when *Botrychium* s.s. was not sampled (Shinohara et al. 2013). In Figs. 3–5 the four samples of *H. lunarioides* clustered separately from *Sceptridium* samples (with BP 100 in the Jaccard and Dice analyses; Figs. 3 and 5, respectively), which is consistent with the position of *H. lunarioides* as sister to a clade comprised of all *Sceptridium* species sampled in a cpDNA plus morphology-based phylogeny (Hauk et al. 2003). The congruence of sister group relationships of *B. virginianus* and *H. lunarioides* between ISSR dendrograms and a cpDNA (+ morphology) phylogeny supports the hypothesis that AAD markers can provide useful estimates of phylogenetic relationships (see Bugarski-Stanojevič et al. 2011). However, poor bootstrap support for most relationships among *Sceptridium* species (Figs. 3, 5) and inconsistent sister group relationships across the three NJ analyses limit our confidence that the sister group relationships presented here among *S. dissectum, S. biternatum, S. rugulosum*, and *S. multifidum* are robust. Archibald et al. (2006) noted that AAD data often do not provide high bootstrap values at many nodes, particularly among taxa that may be recently diverged.

Species-Level Evaluations—*Holubiella lunarioides* (Fig. 1A–B) is restricted to North America and extends from southeastern Arkansas south to southeastern Texas, east to the Florida panhandle, and north to southern South Carolina (Wagner and Wagner 1993). Our sampling represented a substantial proportion of this distribution (LA, MS, AL, and GA). *Holubiella lunarioide*s is unique in possessing tracheoidal idioblasts (Arnott 1960), having prostrate trophophores (Wagner and Wagner 1993), and having a tendency for the sporophores to remain curled through the fall and winter and expand in early spring (Wagner and Wagner 1993). Based on plastid DNA sequences and morphology, Hauk et al. (2003) reported that *H. lunarioide*s (as *S. lunarioides*) was sister to a group comprised of all *Sceptridium* species sampled (three North American species plus four Asian species). The four samples of *H. lunarioides* sampled here formed a cluster with 100% bootstrap support (Fig. 3 cluster 2, Fig. 5 cluster 2); and in all three NJ analyses the *H. lunarioides* cluster was sister to the group comprised of all *Sceptridium* samples. In phylograms Figs. 3 and 5, the *H. lunarioides* cluster had the longest branch of the grapefern species sampled, suggesting a long separation from the *Sceptridium* species sampled.

Sceptridium multifidum (Fig. 1C–D) may be circumboreal and, within North America, is likely the most widely ranging of the grapeferns. In North America, it extends from southern Alaska south to central California, east to New England, and north to Labrador and southern Greenland (Wagner and Wagner 1993). We sampled broadly within this range (AK, UT, MN, and MI). *Sceptridium multifidum* harbors considerable intraspecific morphological variation; Clausen (1938) recognized five subspecies of *Botrychium multifidum*, each

intergrading morphologically with at least one other subspecies. Clausen's *Botrychium multifidum* ssp. *robustum* is represented in our analyses by *S. robustum*. The four North American samples of *S. multifidum* (AK, UT, MI, and MN) clustered together in all three NJ dendrograms (Figs. 3–5). In the Jaccard (Fig. 3) and Nei & Li (Fig. 4) dendrograms, the Russian *S. robustum* sample was sister to a cluster comprised of all North American samples of *S. multifidum*, *S. rugulosum*, *S. biternatum*, and *S. dissectum*. The placement of the Russian *S. robustum* sample sister to all four North American species of section *Sceptridium* suggests that *S. robustum* could belong to a Russian or Asian clade. Because the Jaccard or Nei & Li coefficients appear most appropriate for these ISSR data (Lamboy 1994; Kosman and Leonard 2005) and because these two coefficients produced similar dendrograms, we lean toward regarding *S. robustum* as a Russian or Asian species that may be sister to a North American group composed of *S. biternatum*, *S. dissectum*, *S. multifidum*, and *S. rugulosum*. However, because in the Dice dendrogram (Fig. 5) the *S. robustum* sample clustered among samples of *S. multifidum* and *S. rugulosum,* we cannot discount the possibility of alternative hypotheses. The pairing of the *S. robustum* sample with one of the *S. rugulosum* samples (Srug MI1) in the Dice (Fig. 5) dendrogram did not support the distinction of *S. robustum* and *S. rugulosum*. Furthermore, the position of the second *S. rugulosum* sample (Srug MI2; Fig. 5) sister to a cluster comprised of the four North American samples of *S. multifidum* plus the *S. robustum* + Srug MI1 pair did not support distinctions among *S. robustum*, *S. rugulosum*, and *S. multifidum*. None of the NJ analyses supported Clausen's (1938) hypothesis that North American *S. multifidum* and Russian *S. robustum* are conspecific. Additional data and broad sampling of *S. rugulosum,* northern hemisphere populations of *S. multifidum* inclusive of Clausen's (1938) other three subspecies, and Russian/Asian species including *S. robustum* are necessary before firm conclusions can be drawn.

Sceptridium rugulosum (Fig. 1E–F) is the most geographically restricted of the North American grapeferns, typically occurring from central eastern Minnesota through Wisconsin and Michigan to southern Ontario and Quebec, and south into northern New York and Vermont (Wagner and Wagner 1993). Unique among North American grapeferns, it possesses segments that are wrinkled and convex (Wagner and Wagner 1982, 1993). Specimens of *S. rugulosum* were initially identified by Wagner (1959, 1961b) as the Asian species *S. ternatum*; but were later determined to be more closely related to North American *S. dissectum* and *S. multifidum* (Wagner and Wagner 1982). *Sceptridium rugulosum* differs from *S. multifidum*, *S. dissectum,* and *S. oneidense* in phenology, with *S. multifidum* emerging earliest, followed by *S. oneidense*, *S. rugulosum*, and *S. dissectum,* respectively (Wagner and Wagner 1982). *Sceptridium multifidum* and *S. rugulosum* share terminal pinnules that are similar in size to or slightly larger than the lateral pinnules, with pinnules generally divided to the tip (Wagner and Wagner 1993). Because of these morphological similarities, we hypothesized that *S. multifidum* and *S. rugulosum* are closely related. As noted above, the clustering of *S. rugulosum*, *S. robustum,* and *S. multifidum* samples in the Dice analysis (Fig. 5) suggested that these three morphological species may represent a single taxon. However, in the Jaccard analysis (Fig. 3) the two *S. rugulosum* samples clustered sister to the group comprised of all samples of *S. dissectum* and *S. biternatum.* In the Nei & Li analysis (Fig. 4) the two *S. rugulosum* samples clustered sister to the group comprised of all *S. dissectum* samples. None of the NJ analyses of these ISSR data strongly supported a specific sister group for *S. rugulosum*; but the pairing of the two *S. rugulosum* samples in the Jaccard and Nei & Li analyses lends support to the hypothesis that *S. rugulosum* represents a unique evolutionary lineage within *Sceptridium*. However, we collected both samples of *S. rugulosum* included here from a single population in Saginaw Co., Michigan (MI), so these samples represented only a small portion of the geographic distribution of the species. Further studies that include both additional data and additional populations representing a greater proportion of the geographic range of *S. rugulosum* are necessary before robust conclusions can be made about the status of the species and its sister group relationships.

Sceptridium biternatum (Fig. 2A–D) typically occurs from southeastern Texas to Florida, and north to southeastern New York and Connecticut, and west through southern Ohio, Indiana, Illinois, and Missouri (Wagner and Wagner 1993). Clausen (1938) recognized *S. biternatum* as a variety of *S. dissectum* (= *B. dissectum* var. *tenuifolium*). Others have recognized it at the species level, albeit in *Botrychium* s.l. (Underwood 1896; Lellinger 1985; Wagner and Wagner 1993). *Sceptridium biternatum* is distinguished in part by having long, terminal pinnules with sides that are nearly parallel (Wagner and Wagner 1993). Four *S. biternatum* samples (AL, AR, GA, and LA; henceforth the "core" samples) clustered together in all three analyses, and the relationships among the four were consistent across the three analyses. These four core *S. biternatum* samples clustered sister to a group comprised of samples from four other species (*S. dissectum*, *S. multifidum*, *S. rugulosum*, and *S. robustum* in Dice, Fig. 5) or two other species (*S. dissectum* and *S. rugulosum* in Nei & Li, Fig. 4). In the Jaccard dendrogram (Fig. 3), the core cluster was sister to the two *S. biternatum* "bayou" samples (Sbit bayou FL1 and Sbit bayou FL2). Thus, all three NJ analyses clustered the six *S. biternatum* samples differently, and two of the three analyses (Dice and Nei & Li) showed that *S. biternatum* as currently circumscribed is comprised of two separate clusters, the four core samples and the two *S. biternatum* "bayou" samples. The two Florida samples of *S. biternatum* informally dubbed "bayou" by W. H. Wagner also showed incongruent placement across the three NJ dendrograms; but across all three analyses they consistently clustered more closely to each other than to any other sample. The placement of the *S. biternatum* "bayou" samples as sister to either three or five other species in the Nei & Li (Fig. 4) and Dice (Fig. 5) dendrograms is consistent with the informal Wagner hypothesis that the "bayou" morphotype may represent an evolutionary unit distinct from *S. biternatum*. In the Jaccard (Fig. 3) dendrogram the position of the two *S. biternatum* "bayou" samples sister to the other four samples of *S. biternatum* was consistent with at least two hypotheses. First, as the Dice and Nei & Li dendrograms indicated, the two *S. biternatum* "bayou" samples represent a distinct taxon. Second, that all six samples represent one taxon. We strongly recommend corroboration of the status of the "bayou" morphotype through rigorous analyses of independent data sets based on broad geographic sampling, and that such independent datasets possess strong internal support before formal taxonomic recognition of *S. biternatum* "bayou" is conferred.

Sceptridium dissectum (Figs. 1G, 2E–H) is distributed throughout the eastern half of North America from Florida to eastern

Texas, north to the Great Lakes region, and east to Nova Scotia. It is likely the most common *Sceptridium* species in the eastern half of North America. *Sceptridium dissectum* is the most morphologically variable North American *Sceptridium* species and contains two common morphologies (Figs. 1G, 2E–H). We sampled 12 *S. dissectum* from six states, including one morphotype of *S. dissectum* var. *obliquum* dubbed "johnsonii" from TN. All three analyses showed the 12 samples of *S. dissectum* to be more closely clustered to each other than to any other sample. In the Nei & Li (Fig. 4) and the Dice (Fig. 5) dendrograms, the two samples of *S. dissectum* "johnsonii" formed a pair nested within a cluster comprised of the other ten *S. dissectum* samples. In the Jaccard analysis (Fig. 3) the two *S. dissectum* "johnsonii" samples formed a pair that was sister to the other ten samples of *S. dissectum*. Thus, although the two "johnsonii" samples consistently clustered together across the three NJ dendrograms, the "johnsonii" morphotype was not consistently supported as a group distinct from *S. dissectum*.

Barker and Hauk (2003) reported that among 17 Ohio populations (N = 62) there was no evidence from analyses of data generated from three ISSR primers that the var. *dissectum* (Fig. 1G) and var. *obliquum* (Fig. 2E–H) morphologies of *S. dissectum* are genetically distinct. Analyses of our data (generated from nine individual ISSR primers across 12 samples of *S. dissectum* from six states) were consistent with the findings of Barker and Hauk (2003). In the Dice dendrogram (Fig. 5) three pairs of var. *obliquum* morphology were identified (Sdis obl KY + Sdis obl OH1, Sdis obl NC1 + Sdis obl NC2, Sdis obl john TN1 + Sdis obl john TN2) and two pairs of var. *dissectum* morphology (Sdis dis NC + Sdis dis VA, Sdis dis OH1 + Sdis dis OH2) were identified. All five pairs occurred in the Jaccard dendrogram (Fig. 3) and in the Nei & Li dendrogram (Fig. 4). In all analyses, pairs of var. *obliquum* and var. *dissectum* morphology did not group most closely to other pairs (or even singles) of the same morphology. Thus, our smaller but more geographically representative sample provides additional support for the hypothesis that the var. *dissectum* and var. *obliquum* morphologies are not distinct evolutionary units.

One important caveat of this ISSR investigation is that we excluded individuals of ambiguous or intermediate morphology for which North American *Sceptridium* species are notorious. Because a large portion of the available evidence on the breeding system(s) of species of Ophioglossaceae indicates predominant selfing (McCauley et al. 1985; Soltis and Soltis 1986; Watano and Sahashi 1992; Hauk and Haufler 1999; Camacho and Liston 2001; Barker and Hauk 2003; Chung et al. 2013; but see Dauphin et al. 2020), frequent hybridization among diploid species in *Sceptridium* seems unlikely. Wagner (1960) asserted that hybridization might not play a major role in blurring species boundaries in *Sceptridium*; and dense soils may inhibit sperm transfer (Dauphin et al. 2020). The presence of numerous, cryptic allopolyploids in closely related *Botrychium* s.s. (Dauphin et al. 2014, 2017, 2018) indicates that despite predominant selfing of underground, non-photosynthetic gametophytes, hybridization (followed by polyploidy) is an evolutionarily important mechanism in Ophioglossaceae. Putative *Sceptridium* hybrids have been reported (Sahashi 1979, 1983a; Watano and Sahashi 1992) that produce abortive spores (Sahashi 1979, 1983b). With the single, notable exception of *S. jenmanii* (Underw.) Lyon, the absence of North American *Sceptridium* allopolyploids suggests that hybridization among *Sceptridium* species is either not as frequent, not as prone to result in polyploidy as in closely related *Botrychium* s.s., or simply not yet identified. Alternatively, phenotypic plasticity may provide a more likely explanation for *Sceptridium* specimens that do not fit clearly into currently employed morphologically-based species concepts. If phenotypic plasticity is responsible for much of the morphological variability of North American grapeferns, ISSR markers may provide a tool useful for distinguishing specimens of uncertain affinity.

Conclusions—Assessing systematic questions in North American grapeferns has long been challenging at nearly every level of classification. Our comparison of three NJ analyses of ISSR data provide an effective approach for evaluating morphology-based species concepts and putative infraspecific taxa of these morphologically plastic ferns. However, analyses of these ISSR data did not prove useful for determining sister group relationships among *Sceptridium* species. Importantly, no single NJ analysis would have provided as complete of an assessment of the strengths and weaknesses of these ISSR data as a comparative approach did. Based on these analyses we recommend comparisons among more than one coefficient when performing cluster analyses, especially when taxa are thought to be recently diverged and show minimal DNA sequence variation.

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Author Contributions

DC generated and coded data. WDH collected specimens, designed the study, coded data, performed data analyses, wrote and edited text, and constructed tables and figures.

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