Phylogenetic Placement of Species within the Genus Botrychium s.s. (Ophioglossaceae) on the Basis of Plastid Sequences, Amplified Fragment Length Polymorphisms, and Flow Cytometry

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PHYLOGENETIC PLACEMENT OF SPECIES WITHIN THE GENUS *BOTRYCHIUM* S.S. (OPHIOGLOSSACEAE) ON THE BASIS OF PLASTID SEQUENCES, AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS, AND FLOW CYTOMETRY

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Ferns in the genus *Botrychium* s.s. are morphologically simple, and often there are only subtle differences among species that co-occur in ephemeral populations. The concentration of diversity in the Upper Peninsula of Michigan includes both common species and rare species of conservation concern. We used flow cytometry to estimate C-values from 76 specimens dried for up to 36 mo, confirming allopolyploidy in *Botrychium matricariifolium* and two other species. Diploid species averaged 28.12 ± 0.98 pg of DNA, whereas tetraploids averaged 50.62 ± 1.06 pg. We used plastid sequences and amplified fragment length polymorphisms (AFLPs) to determine relationships within the genus and to analyze morphological variation with *B. matricariifolium*. Sequences from the plastid regions trnL-F and rpl16 expanded on information from past studies, allowing us to place more species into new, well-supported sections within the genus. Relationships within the section Lanceolatum, including tetraploid species of confusing morphology, remain unresolved. Adding data on AFLP nuclear loci further resolved relationships in this section, including the placement of *B. matricariifolium*. This structure, however, does not reflect morphology or geography. Adding data from additional plastid regions, neutral markers, and nuclear regions should provide the power needed to further resolve relationships among this complex set of diploid and polyploid *Botrychium* species.

*Keywords:* amplified fragment length polymorphisms, fern, flow cytometry, hybridization, rpl16, trnL-F.

Introduction

The Ophioglossaceae is an early diverging fern family with reduced features that is thought to be sister to Psilotaceae (Pryer et al. 2004). The aboveground leaf has two parts, a defining characteristic of the family: a sterile trophophore and a fertile sporophore. Within the family, numerous generic subdivisions have been proposed (Clausen 1938; Kato 1987). Clausen (1938) described three genera: *Ophioglossum* L., *Helminthostachys* Kaulf., and *Botrychium* Sw. Kato (1987) recognized six genera on the basis of morphological characteristics: *Botrypus*, *Japanobotrychium* Masam., *Sceptridium* Lyon, *Helminthostachys*, *Ophioglossum*, and *Botrychium*. Haufler et al. (2003) recognized four genera following Kato (1987): *Botrychium* s.s., *Botrypus*, *Japanobotrychium*, and *Sceptridium*. Here, we concentrate on the cryptic species in the genus *Botrychium* s.s. (Paris et al. 1989), which includes the most petite (<15 cm) and morphologically simple species, using the nomenclatorial conventions of Hauk (1995).

Species from *Botrychium* s.s. have a nearly global distribution, with areas of high diversity in North America in the Rocky Mountains, the Pacific Northwest, and the Great Lakes regions. This includes the Upper Peninsula (U.P.) of Michigan, where 12 of the 26 traditionally recognized North American species occur (Wagner and Wagner 1993; table 1). Extensive field and laboratory work in the United States from the 1980s to the present revealed many of these species within the genus (Wagner and Wagner 1983, 1986, 1990a; Farrar and Johnson-Groh 1991; Stensvold et al. 2002; Wagner and Grant 2002). Many of these species have subtle morphological differences (Wagner and Wagner 1983) and include species of conservation concern due to limited ranges, scarce and local distributions, and fragile forms.

Hybridization within the Genus

In the field, researchers often recognized fertile hybrids from their intermediate forms and sterile hybrids from spore inviability (Wagner et al. 1986), often confirmed using chromosome counts (Wagner 1993; table 1). A number of scenarios result from hybridization, including transient infertile hybrids or the production of fit and ecologically viable offspring via chromosome doubling, giving rise to new distinct, fertile allotetraploids (Wagner et al. 1985; Wagner 1991). Much of the diversity in the U.P. reflects the latter hybridization scenario, as five of the 12 species are reported to be allotetraploids (Wagner 1993). Hybridization followed by polyploidy has played a large role in the diversity of the genus (Hauk and Haufler 1999) and likely remains an active process in *Botrychium* populations. These fertile allotetraploids can backcross with diploids, forming sterile triploids (Wagner 1980, 1991; Wagner and Wagner 1988). In addition, populations of sterile hybrids, such as *Botrychium × watertonense*. 

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Table 1  
Species and Internal Sections in the Fern Genus *Botrychium*

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. acuminatum</em> W. H. Wagner</td>
<td>4x/90</td>
<td><em>B. lanceolatum × pallidum</em></td>
<td>X (E)</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. boreale</em> Milde</td>
<td>4x/2n=180</td>
<td></td>
<td></td>
<td>Not sampled</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. campestre</em> W. H. Wagner &amp; Farrar</td>
<td>2x/45</td>
<td></td>
<td></td>
<td>Campestre</td>
<td>Campestre</td>
</tr>
<tr>
<td><em>B. echo</em> W. H. Wagner</td>
<td>4x/90</td>
<td><em>B. lanceolatum × B. lanceolatum</em></td>
<td>X (T)</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. gallicomontanum</em> Farrar &amp; Johnson-Groh</td>
<td>4x</td>
<td><em>B. pallidum × B. campestre</em></td>
<td>[SC]</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. hesperium</em> (Maxon &amp; Clausen) W. H. Wagner &amp; Lellinger</td>
<td>4x/90</td>
<td><em>B. lanceolatum × B. lanceolatum</em></td>
<td>X (T)</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. lanceolatum</em> (J. Gmel.) Angstr. ssp. lanceolatum</td>
<td>2x/45</td>
<td></td>
<td></td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. lanceolatum</em> ssp. angustisegmentum (Pease and Moore) Clausen</td>
<td></td>
<td></td>
<td></td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. lunaria</em> (L.) Sw.</td>
<td>2x/45</td>
<td></td>
<td>X (E)</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. mattricariifolium</em> A. Braun</td>
<td>4x/90</td>
<td><em>B. lanceolatum × B. pallidum</em></td>
<td>X</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. minganense</em> Vict.</td>
<td>4x/90</td>
<td><em>B. pallidum</em> × <em>B. neolunaria</em> ined.</td>
<td>X</td>
<td>Lanceolatum</td>
<td>Lanceolatum</td>
</tr>
<tr>
<td><em>B. mormo</em> W. H. Wagner</td>
<td>2x/45</td>
<td></td>
<td>X (T) [SC]</td>
<td>Not sampled</td>
<td>Simplex</td>
</tr>
<tr>
<td><em>B. pallidum</em> W. H. Wagner</td>
<td>2x/45</td>
<td></td>
<td>X (SC) [SC]</td>
<td>Not sampled</td>
<td>Pallidum</td>
</tr>
<tr>
<td><em>B. simplex</em> E. Hitchc.</td>
<td>2x/45</td>
<td></td>
<td>X</td>
<td>Simplex</td>
<td>Simplex</td>
</tr>
<tr>
<td><em>B. spathulatum</em> W. H. Wagner</td>
<td>4x/90</td>
<td><em>B. campestre × B. lunaria</em></td>
<td>X (T)</td>
<td>Campestre</td>
<td>Campestre</td>
</tr>
<tr>
<td><em>B. watertonense</em> W. H. Wagner</td>
<td>4x/2n=180 (noted as “meiosis irreg.”)</td>
<td><em>B. paradoxum × B. hesperium</em></td>
<td></td>
<td>Unresolved</td>
<td>Minganense</td>
</tr>
</tbody>
</table>

*Botrypus virginianus* (L.) Sw. | 4x/92 | X | | ... | ... |

Note. Sections are based on chloroplast data and reflect maternal lineage relationships between diploid and allotetraploid species. Chromosome number is based on Wagner (1993). Of the putative parents, the hypothesized chloroplast donor is listed first. U.P. = Upper Peninsula.

*Botrychium pallidum* is the closest isoenzyme allelic match, but the parent may be an extinct relative of *B. pallidum* (D. R. Farrar, personal communication).
W. H. Wagner, can persist, perhaps through vegetative propagation (Wagner et al. 1984; Farrar et al. 1986).

**Flow Cytometry**

Given the roles of hybridization and polyploidy in this genus, data on ploidy levels can be useful for separating species, estimating contemporary hybridization, and identifying individuals. Chromosome numbers for *Botrychium* s.s. have already been determined for most species (table 1) by means of chromosome squashes (Wagner and Wagner 1981, 1990b; Wagner 1993) with a base of 45 chromosomes. A newer method for rapidly determining relative DNA content is flow cytometry (FCM). Here, we used FCM to confirm the relative ploidy level of samples and to confirm putative hybrid individuals. To match FCM and genetic results, we took FCM and genetic samples from the same individuals by means of Suda and Travnicke’s (2006) procedure for dried tissue. Pteridophytes are underrepresented in the Royal Botanical Gardens, Kew, DNA C-values database. FCM can thus provide needed estimates of C-values, allowing us to study genome evolution and how this trait evolves within lineages (Obermayer et al. 2002; Bainard et al. 2011a). FCM can also help determine the ploidy of small and/or ambiguous individuals found in the field.

**Past Genetic Work in the Genus**

Isozyme studies first suggested how species relationships and hybridization could lead to high diversity in *Botrychium* (Farrar and Johnson-Groh 1991; Hauk and Haufler 1999). Most of the 17 taxa studied by Hauk and Haufler (1999) had low intraspecific and interspecific isozyme variation. Although isozymes successfully distinguished morphologically confusing species from Greenland (Zika and Farrar 2009), species like *Botrychium matricariifolium* A.Br. and *Botrychium acuminatum* W. H. Wagner show nearly identical isozyme profiles (Hauk and Haufler 1999). Isozymes have also been used to calculate genetic identity scores as a way to quantitatively distinguish species (Farrar 1998). Additional genetic studies in this group have relied mostly on chloroplast genome DNA (cpDNA) data to infer phylogenies (Hauk 1995; Hauk et al. 2003; Small et al. 2005). Of these, Hauk (1995) sampled the most taxa—26 individuals from 20 putative species sequenced for the *rbcL* plastid region. Five of these individuals came from the U.P. of Michigan. The level of variation across sequences was low, with only nine haplotypes among the 20 putative species (Hauk 1995). These data were used to determine the placement of species into “sections” within the genus on the basis of phylogenetic analyses (Hauk 1995; table 1).

Traditional classifications within *Botrychium* s.s. have relied on both ploidy and morphology. Wagner and Wagner in Hauk (1995) proposed the Lunaria, Simplex, and Campestre sections, containing fan-leaved species with linear, ovate, or oblong trophophore. They identified the section Lanceolatum as being composed of a single diploid species with a deltate trophophore. Hauk (1995) updated and revised these sections, notably including the allotetraploid *B. matricariifolium* in section Lanceolatum. Hauk et al. (2003) expanded taxon sampling within the genus to determine family-wide relationships within Ophioglossaceae by adding the plastid spacer sequence *trnL-F*. They sampled 13 species within the subgenus, including one from the U.P. The *trnL-F* sequences provided similar species groupings as *rbcL*, and both sequences strongly supported the monophyly of *Botrychium* s.s. within the family (Hauk et al. 2003). Combined analyses generally supported the sections proposed in Hauk (1995). Expanding on these two plastid regions, Small et al. (2005) used five regions for a family-level phylogeny. *Botrypus virginianus* had high bootstrap support (>90%) as an outgroup sister to *Botrychium* s.s. and *Sceptridium*. The fan-leaved clade of *Botrychium lunaria*, *Botrychium campestre*, and *Botrychium simplex* remained unresolved, however, and species-level sampling did not include *B. matricariifolium* or other allotetraploids, such as *B. acuminatum*.

**The Case for Amplified Fragment Length Polymorphisms (AFLPs)**

Because past isozyme and cpDNA sequence data have been unable to resolve all relationships within *Botrychium*, it may be useful to use AFLPs (Vos et al. 1995) to examine fine-scale relationships among populations and species. As cpDNA is generally uniparentally inherited, it may not be appropriate to use such data to classify hybrid species. AFLPs are biparentally inherited and are useful both for analyzing population-level variation and for reconstructing fine-scale species relationships within complex reticulate phylogenies (Koopman 2005; Meudt and Clarke 2007; Koopman et al. 2008; McKinnon et al. 2008). Among closely related taxa—and particularly among hybrid taxa—AFLPs offer more power than cpDNA to distinguish cryptic species (Bardy et al. 2010). To examine closely related U.P. *Botrychium* s.s. species, we therefore developed AFLP markers. Here, we analyze relationships on the basis of cpDNA data alone and combined with AFLP data.

**AFLP Case Study: Botrychium matricariifolium**

*Botrychium matricariifolium* is an allotetraploid with a range of morphology that makes identification difficult. This range of morphology makes it a species complex that may in fact represent multiple morphologically and genetically unique lineages (Paris et al. 1989). In addition, it grows in sympatry with morphologically similar species, such as *B. acuminatum* (Wagner 1993). Genetic techniques are thus needed to separate *B. matricariifolium* into distinct subspecies or varieties. In this case, AFLPs may prove to be more useful than cpDNA because of increased variation within species and biparental inheritance. We utilized multiple accessions of *B. matricariifolium* to evaluate the amount of genetic variation and relative utility of AFLPs within this morphologically variable group.

**Goals and Approaches**

We first use the C-value data from FCM to confirm the existence of polyploids in the U.P., evaluating how well dried material works for providing accurate estimates of C-values. We then build on past work on *Botrychium* systematics by sampling an expanded set of taxa across two cpDNA regions...
used by Small et al. (2005): \textit{trnL}-\textit{F} and \textit{rpl}16. We also incorporate published sequences of \textit{trnL}-\textit{F} and \textit{rpl}16 from Hauk et al. (2003), Small et al. (2005), and Hauk et al. (2012). We analyzed these chloroplast data sets using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) techniques. We present separate analyses of AFLP variation, both alone and combined with cpDNA, using MP and BI. We also densely sampled many individuals of \textit{B. matricariifolium}, noting morphologies of individual accessions to assess how variation in cpDNA sequences and/or AFLPs correspond to patterns of morphological variation seen across the U.P. These expanded data sets allow us to determine the level of support for the sections proposed by Wagner and Wagner and revised by Hauk (1995).

Thus, our goals are to determine where U.P. species fall within \textit{Botrychium} s.s. and specifically whether sampling additional species alters the sections proposed by Wagner and Wagner in Hauk (1995). We also sought to learn whether AFLP data produce phylogenetic trees consistent with those produced by the plastid data but at higher resolution for species within the \textit{B. matricariifolium} complex and whether variation in these new cpDNA sequences could resolve any differences within \textit{B. matricariifolium}.

**Material and Methods**

**Specimen Collection and DNA Extraction**

We collected specimens during the summers of 2007 and 2008 from seven locations distributed across the U.P. of Michigan (fig. 1). We assigned these samples provisional names on the basis of morphology and currently described species (Wagner and Wagner 1993) with help from local expert Don Henson. After identification, we pressed each fern and took a small tissue sample from the stem to dry using silica gel. Vouchers were deposited in the Wisconsin State Herbarium (WIS). We also collected outgroup samples of \textit{Botrypus virginianus} from Itasca State Park, Minnesota, in 2007. Donald Farrar provided additional identified specimens from outside the U.P., including the western United States and Greenland.

**Plastid Region Sequencing**

Using the protocol of Small et al. (2005), we amplified the \textit{trnL}-\textit{F} spacer using 25-\textit{µL} reactions (13.75 \textit{µL} of water, 3 \textit{µL} of 10x buffer, 1.25 \textit{µL} of DMSO, 3.5 \textit{µL} of 2.5 mM dNTP, 0.5 \textit{µL} of 100 ng/100 \textit{µL} BSA, 0.5 \textit{µL} of each 10 mM primer, 0.25 \textit{µL} of Ex Taq DNA polymerase (TaKaRa, Otsu, Shiga, Japan), and 1 \textit{µL} of 5 ng/\textit{µL} DNA) and followed their PCR program (30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min). Primers used were \textit{trnL}UPA--\textit{trnFGAA} \textit{e} and \textit{f} (Taberlet et al. 1991), \textit{rpl}16-F-\textit{ferm}, and \textit{rpl}16-R-\textit{ferm} (Small et al. 2005). To amplify the \textit{rpl}16 intron region, we followed Small et al. (2005) using the same 25-\textit{µL} reactions as above, cycled at 30 cycles of 95°C for 1 min, 50°C for 1 min, a ramp of 1°C/8 s to 65°C, and then 65°C for 4 min. We prepared samples for se-

**Flow Cytometry**

Our methods follow Johnson et al. (1999), Dolezel et al. (2007), and Bainard et al. (2010). A total of 76 individuals (table 2) were analyzed using two different standards, \textit{Allium cepa} var. “Copa” and \textit{Zea mays}. These were chosen on the basis of estimated C-values of \textit{Ophioglossum} species (Bennett and Leitch 2001) and the standards recommended by Johnston et al. (1999). We compared dried tissue in \textit{B. virginianus} with live samples collected and analyzed on the same day. All dried samples were 16–39 mo old. One individual was compared using air-dried and silica-dried material as well. All samples were processed using LB01 lysis buffer following Bainard et al. (2011a), prepared using 15 mM (363.4 mg) Tris, 2 mM (148.9 mg) Na2EDTA, 0.5 mM (34.8 mg) spermine tetrahydrochloride, 80 mM (1.193 g) KCl, 20 mM (233.8 mg) NaCl, and 1% by volume (200 \textit{µL}) Triton X-100, adjusted to 200 mL with distilled water and to pH 7.5 with 1 M HCl. After β-mercaptoethanol (15 mM, 220 \textit{µL}) was added, the buffer was filtered through 30-µm filters (Spectrum Laboratories, Rancho Dominguez, CA) and stored at −20°C.

We chopped ∼20 mg of dried or live tissue with either 20 mg of living \textit{Z. mays} or 100 mg of \textit{A. cepa} var. “Copa” bulb tissue with 1.5 mL of cold buffer with a new razor blade. We filtered samples through 30-µm nylon filters and stained them with propidium iodide, which provides more accurate estimates of C-values relative to other staining techniques, such as DAPI (Johnston et al. 1999). We added 20 \textit{µL} of propidium iodide fluorescent stain and 20 \textit{µL} of RNase and placed the sample on ice. All samples were processed within 2 h (Bainard et al. 2010) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) to measure fluorescence with a blue laser operating at 488 nm. We used the following instrument settings: forward scatter at E6 V and 4.92 A, side scatter at 366 V, FL2 height at 401 V, FL2 area at 1 A, and FL2 width at 2.47 A. A minimum of 2500 (more commonly, 5000) events were recorded and analyzed using FlowJo (True Star, 2010). We recorded the mean and the CV for each specimen and its standard, with an upper limit of 5% allowed for the CV (Dolezel et al. 2007; Bainard et al. 2010). C-values were obtained by comparing the mean \textit{Botrychium} peak to the mean standard peak. The \textit{Botrychium} 2C-value is equal to the \textit{Botrychium} peak divided by the standard peak and then multiplied by the 2C-value of the standard (Bainard et al. 2010).

**Fig. 1** Sites sampled in the Upper Peninsula (U.P.) of Michigan for this study. Symbols below circles correspond to localities in figs. 6 and 7: one asterisk (*) for Grand Sable Dunes, two asterisks [**] for Delta County, three asterisks [***] for Laughing Whitefish Falls, one pound sign (#) for Iron County, and two pound signs (##) for Marquette County.
Table 2

Flow Cytometry Results for Botrychium Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Ploidy</th>
<th>C-values with corn standard</th>
<th>C-values with onion standard</th>
<th>C-values with corn and onion standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. plants</td>
<td>Average C-value (pg)</td>
<td>SE</td>
<td>Average C-value (pg)</td>
</tr>
<tr>
<td>B. lanceolatum</td>
<td>Diploid</td>
<td>11</td>
<td>30.44</td>
<td>.87</td>
</tr>
<tr>
<td>B. lunaria</td>
<td>Diploid</td>
<td>3</td>
<td>27.95</td>
<td>4.52</td>
</tr>
<tr>
<td>B. montanum</td>
<td>Diploid</td>
<td>1</td>
<td>28.19</td>
<td>...</td>
</tr>
<tr>
<td>B. pallidum</td>
<td>Diploid</td>
<td>1</td>
<td>28.19</td>
<td>...</td>
</tr>
<tr>
<td>B. simplex</td>
<td>Diploid</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>All tetraploid Botrychium</td>
<td></td>
<td>16</td>
<td>29.62</td>
<td>.98</td>
</tr>
<tr>
<td>B. matricaristolium</td>
<td>Tetrploid</td>
<td>23</td>
<td>51.97</td>
<td>1.60</td>
</tr>
<tr>
<td>B. michiganense</td>
<td>Tetrploid</td>
<td>3</td>
<td>46.64</td>
<td>2.85</td>
</tr>
<tr>
<td>B. minganense</td>
<td>Tetrploid</td>
<td>3</td>
<td>53.68</td>
<td>5.05</td>
</tr>
<tr>
<td>B. spathulatum</td>
<td>Tetrploid</td>
<td>4</td>
<td>53.07</td>
<td>5.43</td>
</tr>
<tr>
<td>B. echo</td>
<td>Tetrploid</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>B. bespernum</td>
<td>Tetrploid</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>All tetraploid Botrychium</td>
<td></td>
<td>33</td>
<td>51.36</td>
<td>1.38</td>
</tr>
<tr>
<td>B. virginianus (dried)</td>
<td>Tetrploid</td>
<td>2</td>
<td>26.21</td>
<td>4.74</td>
</tr>
<tr>
<td>B. virginianus (fresh)</td>
<td>Tetrploid</td>
<td>4</td>
<td>23.18</td>
<td>2.20</td>
</tr>
<tr>
<td>All B. virginianus</td>
<td></td>
<td>6</td>
<td>22.90</td>
<td>1.82</td>
</tr>
</tbody>
</table>

Note. Samples were evaluated using two different standards (Zea mays and Allium cepa var. Copra), with comparable results.

We generated AFLP data from a library of genomic DNA digested with the restriction enzymes EcoRI and MseI using 0.1 μL of 10,000 U/μL MseI, 0.05 μL of 20,000 U/μL EcoRI, 0.5 of μL EcoRI buffer (New England BioLabs, Beverly, MA), 0.05 μL of 100 ng/100 μL BSA, and 3.7 μL of 5 ng/μL DNA for 2 h at 37°C. Adaptors were ligated immediately using 5 μL of product, 3.6 μL of H2O, 1 μL of 10× ligase buffer, 0.19 μL of 50 μM EcoRI adapter, 0.19 μL of 50 μM MseI adapter, and 0.02 μL of 3U/μL T4 ligase (New England BioLabs) and incubated for 14 h at 16°C. Preamplification reactions used 13 μL of water, 2.5 μL of 5× buffer, 2 μL of 2.5 μM dNTPs, 0.19 μL of both 20 μM primers with one selective base (MseI+C, EcoRI+A), 0.25 μL of e2TAK (TaKaRa), and 5 μL of 1:5-diluted digestion product. The final amplification used 10.75 μL of water, 2.5 μL of buffer, 2 μL of dNTPs, 0.5 μL of deionized formamide, 2.5 μL of 10 μM MseI primer, 0.5 μL of 10 μM EcoRI fluorescently labeled primer, 0.25 μL of e2TAK, and 5 μL of 1:18-diluted preamplification product. We used 8 primer pairs with different restrictive bases: E+AGC with M+CCTC, M+CTTGC, M+CCTG, M+CGCTT, M+CCGT, M+C, M+CAG, and E+AGCA with M+CCCCG. To analyze the DNA fragments, we used the program GeneMarker (SoftGenetics, 2005), creating panels and scoring fragments by hand using conservative criteria following Holland et al. (2008); intensity more than 100, band sizes greater than 100, symmetric peaks, and small bin sizes.

Creating Species Phylogenies

We analyzed each DNA region individually and in combination with published sequences. We also combined the trnl-F and rpl16 data sets and analyzed the data for our sequences in combination with published sequences. We used either B. virginianus or Sceptridium lunariodes as outgroups. We analyzed the AFLP data both separately and combined with cpDNA. The trnl-F sequences downloaded from GenBank were shorter than those we developed (361 versus 433 bases), resulting in fewer characters in the combined analyses. We removed 70 bp from the beginning of our sequences to fit with the downloaded sequences.

We performed MP analyses on plastid and AFLP data using the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal (Miller et al. 2010) with the PAUPRatchet tool (Nixon 1999; Swoford 2001). We ran all analyses with default settings except for the following: 4 repetitions total
for each run, all optimal trees were saved, and the number of trees saved automatically increased by 1000. We calculated bootstrap values from 1000 replicates subject to full heuristic searches using simple taxon addition with 10 trees held at each step, tree bisection and reconnection (TBR) branch swapping, and no more than 100 trees kept per replicate.

We used ML analysis under a number of different models of evolution (Jukes-Cantor, Kimura two parameter, Hasegawa-Kishino-Yano, generalized time reversible [GTR], + gamma, + invariable sites) with likelihood ratio tests (Felsenstein 1988) to determine the best model for these data. GTR + gamma was then used for ML and BI for all loci and data sets. ML searches with the CIPRES Portal RAxML tool (Stamatakis 2006; Stamatakis et al. 2008) used default parameters, allowing the program to determine the number of bootstrap replicates.

To test whether branch lengths were significantly different from zero, we used the likelihood ratio test for zero-length branches in the likelihood settings in PAUP* To make sure these values were similar to setting branch lengths to zero by hand, we created trees with branch lengths set to zero and used the likelihood ratio test to determine whether the trees had significantly higher likelihood scores.

We performed BI using Markov chain Monte Carlo (Yang and Rannala 1997) in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001), with two runs for 5,000,000 generations, sampling every 1000. Each run included 4 linked chains with heating temperature at 0.05 or lower, as determined by examining the chain swap frequencies for good mixing and aiming for mixing above 0.4 between all chains in both runs.

We examined a plot of likelihood versus generation time to determine stationarity and number of burn-in generations (minimum of 10,000 generations). A Bayesian technique for analyzing AFLP was developed by Luo et al. (2007), but the number of taxa made it prohibitive for this data set, and AFLP data were analyzed using the “standard” model.

To determine whether the plastid and AFLP data were drawn from the same distribution, we used an incongruence length difference (ILD) test (Farris et al. 1994) in PAUP*, using 1000 replicates of a heuristic search with simple addition, holding 1 tree, TBR, and max trees = 1000. To examine gene diversity of *B. mattricarfolium* accessions using cpDNA and AFLPs, we estimated percent polymorphic loci and Nei’s gene diversity (the probability that random individuals differ in alleles at a locus; Nei 1978) using Arlequin, version 3.1 (Excoffier et al. 2003).

### Results

#### Flow Cytometry

FCM produced similar estimates of ploidy levels from both dried and live specimens of *Botrypus virginianus* (fig. 2). Dried specimens had 2C-values of 26.21 ± 4.73 pg, whereas live specimens had 23.18 ± 2.2 pg. The dried specimen peak was lower and wider, suggesting lower quality, but the results were otherwise consistent. Dried specimens also showed patterns of relative ploidy levels consistent with their described morphological differences (table 2; fig. 3). With the *Zea mays* standard, *B. virginianus* was estimated to have a 2C-value of 22.9 ± 1.82 pg. Diploid species had 2C-values that ranged from 27.95 to 30.44 pg when *Z. mays* was used as a standard, with an average of 29.62 ± 0.98 pg, compared with a range of 22.05 to 28.16 pg and an average of 25.13 ± 2.02 pg when *Allium cepa* was used as a standard. This difference can be attributed to *Botrychium lanceolatum* specimens, which ranged from 24.93 to 33.54 pg, with an average of 30.44 ± 0.87 pg across standards. The difference likely reflects the subspecies used in the analyses. *Botrychium lanceolatum angustisegmentum* individuals were measured versus *Z. mays*, while *Botrychium lanceolatum lanceolatum* individuals were measured versus *A. cepa*. The same general pattern of agreement between standards holds for tetraploids, with an average of 51.26 ± 1.38 pg with *Z. mays* that drops to 48.32 ± 0.88 pg with the *A. cepa* standard. When averaged across standards, diploid *Botrychium* species had 28.12 ± 0.98 pg of DNA, whereas tetraploids had 50.62 ± 1.06 pg.

#### Species Phylogenies

Analyses using the different outgroups resulted in identical tree topologies that had similar statistical support. In all cases, zero-branch-length trees had significantly higher (P < 0.05) likelihood

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**Fig. 2** Comparison of flow cytometry data derived from dried (A) and live (B) material of *Botrypus virginianus*. Although dried material provided a lower-intensity signal with fewer cells, the ploidy level can still be determined. The first gate on the left marks the *Zea mays* standard; the second gate indicates the *B. virginianus* gate.
scores. Plastid and AFLP phylogenies from MP, ML, and BI analyses provided well-supported clusters (MP and ML bootstrap > 80%, BI posterior probability p > 80%; table 3). Technically, the sister groups in this study cannot be considered clades, as they include both diploid and tetraploid species. We therefore refer to them as “clusters.” In the same manner, the “section” designations include both diploids and tetraploids and are thus not formal clades.

**Plastid Data Sets**

Analysis of the combined trnL-F and rpl16 plastid regions for 34 individuals supports both the fan-leaved cluster and the Lanceolatum section (fig. 4). There is support, although less than 90%, for the Lunaria section at the base of the fan-leaved cluster being sister to the other fan-leaved species (*Botrychium pallidum* and *Botrychium minganense*) and the Simplex section.

Within the Lanceolatum section, *B. lanceolatum angustisegmentum* has strong support as a separate species from *Botrychium matricariifolium*. Although the morphologies of the different *B. matricariifolium* accessions varied, there was little to no variation in either cpDNA region (table 3). Only rpl16 had variable sites, whereas trnL-F sequences were identical.

Including published sequences (Hauk et al. 2003, 2012; Small et al. 2005) for both plastid regions expanded the data set from 34 to 60 individuals (fig. 5). All three analyses confirmed the topology of sister groups showing a primary division between fan-leaved species and members of section Lanceolatum (MP and BI p.p. > 80%). These additions strengthen support for the Lunaria section separate from *B. pallidum*, *B. minganense*, and section Simplex (ML and MP bootstrap > 90%, BI > 90%). The Lunaria section is at the base of this fan-leaved cluster and is sister to the other fan-leaved species. BI provided strong support for differentiation within the fan-leaved clade among *B. minganense*, *B. pallidum*, the Simplex section, and the Campestre section (>90%), but analyses based on MP or ML techniques were somewhat weaker (<90%). This larger enhanced data set, however, provided little resolution of the polytomy within the Lanceolatum section. Accessions H571 and W50 *B. lanceolatum* from the U.P. (presumably *B. lanceolatum angustisegmentum*) were strongly supported as a separate clade from accessions E040 and E042 (MP, ML, BI p.p. > 84%).

**AFLP Data Set**

Our separate analysis of 25 individuals with 783 AFLP loci produced sister-group relationships consistent with those identified in analyses of chloroplast sequence data (fig. 6). One major difference between the AFLP and cpDNA data sets is that section Lanceolatum is now nested within the fan-leaved clade. *Botrychium pallidum* and *Botrychium mormo* are sister to section Lanceolatum. In addition, BI results in
Both regions are chloroplast regions.

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<th>No. variable</th>
<th>% variable</th>
<th>No. parsimony informative</th>
<th>% informative</th>
<th>B. matricariifolium % polymorphic loci</th>
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Note. Both regions are chloroplast regions.

* Nei (1978).

Botrychium simplex being sister to the rest of the species and not forming a clade with B. mormo, as it does with cpDNA. Within section Lanceolatum there is increased structure, with support (BI p.p. > 90%), for various clusters of B. matricariifolium individuals. Gene diversity and percent polymorphic loci increased with AFLPs for accessions of B. matricariifolium (table 3).

Plastid and AFLP Data Set

Combining the AFLP data with the trnL-F and rpl16 data further resolves relationships within section Lanceolatum (fig. 7). After performing the ILD test, we failed to reject the null hypothesis that these two data sets were from the same distribution (P = 0.17). With the AFLP data, the Lanceolatum section is nested within the fan-leaved clade. Within the fan-leaved cluster, B. mormo and B. simplex emerge as sister species with high BI support. Some differentiation also exists among the B. pallidum accessions, with E033 separate from the clade with the other accessions. The AFLP data again reveal more structure within the Lanceolatum polynomy. Paired individuals showing strong separation (BI p.p. > 90%) do not reflect a geographic pattern, as accessions E013 and E014 were collected from the same location, whereas E038, E072, E046, and E074 were not (fig. 7).

Discussion

Flow Cytometry

The FCM results from 76 individuals confirm the ploidy levels reported by past researchers (Wagner and Lord 1956; Wagner and Wagner 1981, 1990a; Wagner 1993) and provide estimates of C-values for 11 more species. The 2C-values for allotetraploids were less than twice those for diploids. This reduction in C-value commonly occurs in polyploids and presumably reflects diploidization and the loss of DNA following hybridization (Leitch and Bennett 2004). Bainard et al. (2011a) report a similar 2C-value estimate for Botrypus virginianus. The high SEs reported for some species may result from the use of dried material (Bainard et al. 2011b; Suda and Travnicek 2006). The divergence in C-values of diploid species between the two standards could also reflect this imprecision, or it could reflect species differences. Only one individual of Botrychium mormo was measured using the Zea mays standard, and it had a higher C-value than the single Botrychium simplex measured against Allium cepa (table 2). The largest variation in C-values observed between the two standards occurred in Botrychium lanceolatum. This could reflect either the difference in standards or a real difference between two subspecies (Botrychium lanceolatum angustisegmentum C-values averaged 30.44 ± 0.87 pg, vs. 24.69 ± 0.93 pg in B. lanceolatum lanceolatum).

These 2C-values were lower than those reported for Ophioglossum gramineum (129.6 ± 4.38 pg) and Ophioglossum petiolatum (131.1 ± 3.86 pg; Bennett and Leitch 2001, 2010; Obermayer et al. 2002). These Ophioglossaceae species’ 2C-values are among the highest in ferns surveyed by Obermayer et al. (2002). Our 2C diploid average of 28.12 ± 0.98 pg place Botrychium s.s. species in the middle of C-values reported for ferns generally and close to Equisetum species, such as Equisetum palustre (25 pg) and Equisetum arvense (28.4 pg; Bennett and Leitch 2010).

We found no differences in ploidy between morphologically similar species. FCM succeeded in confirming ploidy levels and identification even in older dried specimens. Botrychium lunaria and Botrychium minganense were long considered the same species (Wagner and Lord 1956) and share general morphological characteristics, but B. minganense is a tetraploid and B. lunaria is a diploid. Although spore size is roughly correlated with ploidy (Wagner and Lord 1956; Zika and Farrar 2009), ranges of spore size can overlap between diploid and tetraploid species. Being able to use dried material to confirm ploidy is a boon for botanists, who often collect dried material. Two caveats should be added here, however. First, the quality of data recorded declines with time and depends on the method of drying (Suda and Travnicek 2006). Using silica-dried material may improve results (Bainard et al. 2011b). Second, Botrychium ferns are relatively free of secondary compounds, which often interfere with the lysis buffer in other plant species. The LB01 buffer has been found to work across plant families, perhaps because of the reducing properties of β-mercaptoethanol (Bainard et al. 2010). Future botanists should experiment further with buffer and staining duration on dried material. It is also important to choose a standard with a C-value close to that of the experimental organism (Johnston et al. 1999).
As hypothesized, increased taxon sampling allowed us to assess within-genus relationships. These data also refine our understanding of relationships between hybrids and their putative parent species by allowing us to compare trees on the basis of either uniparental cpDNA or biparental nuclear (AFLP) data. The combined plastid data set provided suitable power for reevaluating relationships within the genus (fig. 5). Fan-leaved accessions within section Lunaria revealed genetic differences that warrant further taxonomic division. Future work that includes individuals of confirmed Botrychium neolunaria ined. could help to resolve relationships within this section (Stensvold and Farrar 2008).

Accession W67 Botrychium × watertonense emerged as a distinct tetraploid within the B. mingenense cluster, distinguished by its morphology and chromosome number. Yet these two species lack a common putative parent species, as the parents of B. mingenense are presumed to be Botrychium pallidum and B. lunaria, whereas those of B. × watertonense are inferred to be Botrychium hesperium and Botrychium paradoxum (Hauk and Haufler 1999; Farrar 2011; table 1). These hypotheses of parentage may be wrong, however, as both B. hesperium and B. paradoxum are tetraploids, and we can trace a B. pallidum–like chloroplast to B. × watertonense through various hybridizations. B. lanceolatum and B. pallidum are putative parents of B. hesperium, although B. lanceolatum has been shown to be the chloroplast donor and not B. pallidum (fig. 4). The origins of B. paradoxum are unresolved, but B. pallidum is a putative parent (Farrar 2011). This analysis provides support for B. pallidum’s role as the chloroplast donor for B. paradoxum and B. × watertonense,
leading to the conclusion that *B. pallidum* was also the chloroplast donor for *B. minganense*. This pattern of chloroplast inheritance would explain how *B. minganense* could cluster with *B. watertonense*.

There is solid support from ML and BI analyses (>90%) that the Lunaria section is sister to *B. pallidum* and the Simplex and Campestre sections. Our analyses also support a cluster containing the diploid *Botrychium campestre* and the tetraploid *Botrychium spathulatum*. As *B. campestre* is one of the putative parental species of *B. spathulatum*, we conclude that *B. campestre* is the plastid donor parent of *B. spathulatum*.

The well-supported (MP, ML, BI p.p. > 90%) placement of the tetraploid *Botrychium gallicomontanum* as sister to the diploid *B. pallidum* suggests a hybrid origin. Although Farrar and Johnson-Groh (1991) proposed that *B. simplex* and *B. campestre* were the probable parents of *B. gallicomontanum*, Farrar’s results (2011) and our analysis suggest instead that the chloroplast donor is *B. pallidum*.

The final division within the fan-leaved cluster is the Simplex section. This section contains *B. simplex* accessions from both the U.P. (E107 and W54) and the western United States (H619) as well as *B. mormo* accessions (E034 and W117) as a well-supported clade (MP, ML, BI p.p. > 90%). Thus, these inferred
relationships supersede geography. However, there are multiple varieties of B. simplex (Clausen 1938; Farrar 2011), and the differences between E107 and H619/W54 could reflect varieties.

Within section Lanceolatum, there are a number of interesting genetic differences that may reflect real species differences, laboratory artifacts, or differences in identification. The polytomy that includes Botrychium matricariifolium, Botrychium lanceolatum lanceolatum, Botrychium boreale, Botrychium acuminatum, and Botrychium hesperium persists in the combined plastid analysis, although a couple of clades are resolved (fig. 5). Curiously, our B. lanceolatum angustisegmentum accessions from the U.P. (E040 and E042) differ from the two accessions of B. lanceolatum (H571 and W50) from other researchers. Accession H571 differs from E040 and E042 at four nucleotides in the trnL-F sequence, whereas accession W50 shares two of these with H571 but otherwise matches E040. In addition, W50 shares two synapomorphies with H571 in the rpL16 sequences. These differences may reflect laboratory artifacts, although increased sampling may reveal a geographic signal.

In general, our plastid tree topologies from the MP, ML, and BI analyses agreed with Hauk et al.’s (2003) distinction between fan-leaved species and section Lanceolatum. Within the Lanceolatum section, the plastid trees reflected few to no sequence differences and thus a broad polytomy. This could reflect multiple hybridizations resulting in a series of different tetraploid morphs from a consistent cpDNA donor, B. lanceolatum. Farrar (personal communication) used isoenzymes to hypothesize that two different B. lanceolatum varieties hybridized with the same fan-leaved species, leading to a series of genetically distinct tetraploid species. However, if this were the case we would expect a clear split in the uniparentally inherited cpDNA from two distinct maternal parent subspecies.
Analyses, and the medium lines represent *Botrypus matricariifolium* samples are from the Upper Peninsula of Michigan or Minnesota. Support for the other analyses is reported near the branch. All expanded AFLP data set might further resolve relationships these accessions (fig. 7). These results suggest that an expanded AFLP data set might further resolve relationships within section Lanceolatum, particularly among the bipinnate allotetraploids. Genetically similar individuals within this section can have rather distinct leaf forms (e.g., E046 and E074). Again, this variation appears to be unrelated to geographic distance. AFLPs have proved useful for resolving relationships in other polyploid species complexes (Burnier et al. 2009; Emshwiller et al. 2009), including Rosa, a highly reticulate group that has also experienced multiple polyploid events (Koopman et al. 2008). Ploidy levels also help to distinguish taxa within *Botrychium* s.s. On their own, the plastid data do not support *B. lanceolatum* as a monophyletic group (fig. 5). The FCM results confirm that both *B. lanceolatum* lanceolatum and *B. lanceolatum angustisegmentum* are diploids. Many of the other species hypothesized to be hybrids between *B. lanceolatum* and a fan-leaved species emerge here as indeed being tetraploid. Nevertheless, they retain a polytomy even after AFLP data are included. This might be expected if the polytomy reflects how hybridization interferes with the assumption of monophyletic clades implicit in phylogenetic methods. Past work using isozymes (Hauk and Haufler 1999) further supports the hypothesis that *B. matricarifolium*, *Botrychium echo*, and *B. acuminatum* all represent tetraploid hybrids.

We also combined cpDNA and AFLP data to examine fine-scale relationships within these sections. The presence of hybrids complicates our analyses, as the data from two different lineages are being analyzed together. Our justification for combining the cpDNA and AFLP data into one analysis was that the ILD test showed no significant difference between these trees. Nevertheless, there may be problems with this test (Yoder et al. 2001). Given their independence, we found surprisingly few differences in the nodes and topology of the cpDNA tree (fig. 4) compared with the AFLP tree (fig. 6). Future analyses could use multivariate techniques such as ordination (Emshwiller et al. 2009) as an alternate method of visualizing and analyzing AFLP data. Here, we would expect that hybrids would fall between putative parents in genetic space. An alternate method is reverse successive weighting (Trueman 1998), which identifies secondary phylogenetic signals by downweighting nonhomoplasious characters.

Haplotype networks (Clement et al. 2000) from cpDNA can also be used in combination with AFLP data to resolve relationships among hybrid taxa (Bardy et al. 2010; Oberle and Schaal 2011). However, the lack of variation in our cpDNA data made these networks uninformative here. Future work should include additional sequence data from nuclear regions to supplement the inferences made from cpDNA data. Differences between multiple nuclear ribosomal DNA and cpDNA trees could improve our ability to resolve hybrid relationships within the genus (e.g., Hughes et al. 2002). Data sets that include hybrids can be analyzed by examining trees from independent data sets, combing DNA sequences into a single analysis while searching for signals that indicate separate histories, and examining associations between linked markers (Linder and Rieseberg 2004).

**Revised Within-Genus Sections**

On the basis of these analyses, we propose updating sectional classification within *Botrychium* s.s. (fig. 5; table 1). Major

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**AFLP and Plastid Region Data Sets**

The polytomy evident in the plastid phylogeny for section Lanceolatum (fig. 4) is somewhat resolved by adding the data on AFLP variation that are available for a subset of these accessions (fig. 7). These results suggest that an expanded AFLP data set might further resolve relationships...
changes to past section assignments include adding a “Minganense” section that includes the allotetraploid *B. minganense*. We also suggest placement of *B. echo* and *B. hesperium* in the Lanceolatum section. *Botrychium pallidum* constitutes a well-supported monophyletic section that includes *B. galli-comtanaum* but remains separate from other Simplex species. These designations are based solely on chloroplast data, however, which trace only one side in the hybrid species. These designations may therefore come to be seen as artificial groupings given the genetic contribution of multiple clades to a hybrid species with a cpDNA *B. lanceolatum* parent.

**Conclusion**

The charismatic fern *Botrychium* presents several problems in delineating species, including reticulate evolution within the section Lanceolatum. Including more species and individuals from the U.P. of Michigan in a data set of widely sampled species supports past work by multiple authors (Hauk 1995; Hauk et al. 2003; Small et al. 2005; Stensvold and Farrar 2008; table 1). This includes support for sections Lunaria, Simplex, Campestre, and Lanceolatum. We propose adding a section Minganense within the fan-leaved species to accommodate tetraploid species that form a separate monophyletic cluster in addition to adding section Pallidum. Additional work within section Lanceolatum could help to determine relationships between hybrid tetraploids and their putative parent species. The use of nuclear sequence data, in particular, would supplement the cpDNA and AFLP markers analyzed here in a way that could resolve relationships. We also found FCM to be a useful tool for identifying polyploids and hybrids even when only dried samples are available.

**Acknowledgments**

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**Appendix**

**Voucher Information and GenBank Accession Numbers**

Provided here are voucher information and GenBank accession numbers, with the following information for each sequence: species, collector, collection location, trnL-F GenBank number (publication source), rp16 GenBank number (publication source), and laboratory number/collection number. All sequences with a laboratory number starting with E were developed for this study and were deposited in the Wisconsin State Herbarium (WIS).

*Botrychium lunaria* (L.) Sw., D. Henson, Schoolcraft Co., Michigan, USA, JN389449, JN389482, E001/EW0094; *Botrychium minganense* Vict., E. Williams, Mark Jaunzems, Chippewa Co., Michigan, USA, JN389459, JN389483, E009/EW0083; *Botrychium lunaria* (L.) Sw., E. Williams, Mark Jaunzems, Chippewa Co., Michigan, USA, JN389447, JN389484, E010/EW0090; *Botrychium pallidum* W. H. Wagner, E. Williams, Mark Jaunzems, Chippewa Co., Michigan, USA, JN389456, JN389485, E012/EW0114; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Delta Co., Michigan, USA, JN389463, JN389486, E013/EW0131; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Delta Co., Michigan, USA, JN389471, JN389487, E014/EW0145; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Delta Co., Michigan, USA, JN389464, JN389488, E016/EW0150; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Grand Sable Dunes, Michigan, USA, JN389465, JN389489, E020/EW0215; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Grand Sable Dunes, Michigan, USA, JN389472, JN389490, E023/EW0249; *Botrychium spathulatum* W. H. Wagner, E. Williams, Grand Sable Dunes, Michigan, USA, JN389475, JN389491, E026/EW0277; *B. acuminatum* W. H. Wagner, E. Williams, Grand Sable Dunes, Michigan, USA, JN389479, JN389492, E028/EW0287; *Botrychium matricariifolium* A. Braun, D. and J. Henson, Grand Sable Dunes, Michigan, USA, JN389474, JN389493, E031/EW0300; *Botrychium simplex* E. Hitchc., D. and J. Henson, Grand Sable Dunes, Michigan, USA, JN389452, JN389494, E033/EW0313; *Botrychium mormo* W. H. Wagner, D. and J. Henson, Grand Sable Dunes, Michigan, USA, JN389453, JN389495, E034/EW0315; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Iron Co., Michigan, USA, JN389473, JN389496, E037/EW0341; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Iron Co., Michigan, USA, JN389460, JN389497, E038/EW0346; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Iron Co., Michigan, USA, JN389468, JN389498, E039/EW0359; *Botrychium lanceolatum* ssp. angustisegmentum (Pease and Moore) Clausen, E. Williams, D. Henson, Iron Co., Michigan, USA, JN389461, JN389499, E040/EW0371; *Botrychium lanceolatum* ssp. angustisegmentum (Pease and Moore) Clausen, E. Williams, D. Henson, Marquette Co., Michigan, USA, JN389462, JN389500, E042/EW0394; *Botrychium matricariifolium* A. Braun, E. Williams, D. Henson, Marquette Co., Michigan, USA, JN389466, JN389501, E046/EW0418; *Botrychium pallidum* W. H. Wagner, E. Williams, D. Henson, Alger Co., Michigan, USA, JN389455, JN389502, E052/EW0002; *Botrychium pallidum* W. H. Wagner, E. Williams, D. Henson, Alger Co., Michigan, USA, JN389457, JN389503, E063/EW0003; *Botrychium matricariifolium* A. Braun, D. and J. Henson, Whitefish Falls, Michigan, USA, JN389469, JN389504, E072/EW0751; *Botrychium matricariifolium* A. Braun, D. and J. Henson, Whitefish Falls, Michigan, USA, JN389470, JN389505, E074/EW0607; *Botrypus virginianus* (L.) Michx., E. Williams, Itasca State Park, Minnesota, USA, JN389481, JN389506, E076/EW0631; *Botrypus virginianus* (L.) Michx., E. Williams, Itasca State Park, Minnesota, USA, JN389480, JN389507, E077/EW0632; *Botrychium simplex* v. simplex E. Hitchc., D. Henson, Delta Co., Michigan, USA, JN389454, JN389508, E107/EW0957; *Botrychium

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