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MOLECULAR PHYLOGENETICS SUPPORTS WIDESPREAD CRYPTIC SPECIES IN MOONWORTS (*BOTRYCHIUM* S.S., OPHIOGLOSSACEAE)[1]

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- *Premise of the study:* Previous phylogenetic studies of moonworts (*Botrychium* sensu stricto (s.s.)) included few taxa from outside of North America. This low geographical representation limited interpretations of relationships of this group rich in cryptic species. With 18 out of 30 species in the genus being polyploid, understanding their evolutionary history remains a major challenge.
- *Methods:* A new molecular phylogeny was reconstructed using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses based on multiple accessions of the most wide-ranging Arctic taxa of *Botrychium* in North America and Europe using three noncoding plastid DNA regions (*psbA-trnH*^{GUG}, *trnL*^{UAA}*-trnF*^{GAA} intergenic spacer, and *rpL16* intron).
- *Key results:* The new phylogeny confirms the identity of several recently described species and proposed new taxa. Nine subclades are newly identified within the two major clades in *Botrychium* s.s.: Lanceolatum and Lunaria. Chloroplast DNA was variable enough to separate morphologically cryptic species in the Lunaria clade. On the contrary, much less variation is seen within the morphologically variable Lanceolatum clade despite sampling over the same broad geographic range. The chloroplast region *psbA-trnH*^{GUG} is identified as an efficient DNA barcode for the identification of cryptic taxa in *Botrychium* s.s.
- *Conclusions:* The combined increase in species representation, samples from throughout the geographic range of each species, and sequencing of multiple plastid DNA regions supports morphologically cryptic species in *Botrychium* s.s.

Key words: *Botrychium*; cryptic species; ferns; moonworts; Ophioglossaceae; phylogeny; polyploidy; *psbA-trnH*^{GUG} intergenic spacer; *rpL16* intron; *trnL*^{UAA}*-trnF*^{GAA} intergenic spacer.

Despite the large number of molecular phylogenies produced in the past two decades, relatively few have focused on the ferns, the sister clade to the seed plants. With 45 families, ferns are the second most diverse group of land plants after the angiosperms (Smith et al., 2006; Christenhusz et al., 2011). The Ophioglossaceae and Psilotaceae sister clades are the most basal ferns, which diverged from the other ferns approximately 300 mya (Pryer et al., 2004).

Botrychium sensu lato belongs to the Ophioglossaceae, the taxonomy of which has been revised several times (Clausen, 1938; Kato, 1987). Currently, five segregate genera are accepted based on both morphological and molecular data, *Botrychium* s.s. (*Bo.*), *Botrypus* (*Bp.*), *Japanobotrychium*, *Osmundopteris*, and *Sceptridium* (*Sc.*) (these abbreviations to genera are used throughout below) (Kato, 1987; Hauk, 1995; Hauk et al., 2003; Shinohara et al., 2013). Two macrofossils indicate an ancient divergence between these genera, with *Botrychium wightonii* Rothwell and Stockey and *Sceptridium underwoodianum* (Maxon) Lyon dating to 57 and 23 mya respectively (Rothwell and Stockey, 1989; Vladimir et al., 2010).

Botrychium s.s., commonly known as the moonworts, has 30 recognized species that generally occur in open natural grasslands (Farrar, 2011). Species distributional ranges vary in size and connectivity, with some exhibiting long-range disjunctions (e.g., *Bo. lunaria* (L.) Swartz and *Bo. pedunculosum* W.H. Wagner), whereas others are common throughout a relatively broad range (e.g., *Bo. lanceolatum* (S.G. Gmelin) Ångström and *Bo. minganense* M. Victorin) or locally common in a very restricted range (e.g., *Bo. gallicomontanum* D. Farrar & Johnson-Groh and *Bo. pseudopinnatum* W.H. Wagner) (Farrar and Johnson-Groh, 1991; Wagner and Wagner, 1990). Some species are considered rare or endangered and may be targeted for conservation (e.g., *Bo. gallicomontanum* and *Bo. mormo* W.H. Wagner) (Johnson-Groh and Lee, 2002; Casson et al., 2002). The greatest species diversity seems to be in North America, especially in the Rocky Mountains and the Great Lakes region where the genus has been most studied. However, *Botrychium* s.s. also occurs in Africa (Atlas Mountains) Asia, Australia, Europe, Pacific islands, New Zealand, and Patagonia in South America (Wagner and Wagner, 1993), but distributional ranges and relationships outside of North America and Europe remain poorly investigated.

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The subterranean gametophytes of these ferns are completely dependent on arbuscular mycorrhizal fungal symbionts (Clausen, 1938). The obligatory close relationship has been characterized in *Botrychium lunaria* var. "*crenulatum* (W.H. Wagner) Stensvold" ined. (as *Bo. crenulatum* W.H. Wagner) and *Bo. lanceolatum* (Winther and Friedman, 2007), and *Bp. virginianus* (L.) Michx. (Kovacs et al., 2007) where the symbionts have been identified as members of the generalist genus *Glomus*. Another characteristic of the moonwort lifestyle is the predominance of intragametophytic selfing as the major reproductive mechanism (Hauk and Haufler, 1999).

Species of *Botrychium* s.s. have a simple organization with two distinct parts, a sterile trophophore (frond) and a fertile sporophore. The trophophore is most often used in identification and its morphological characters support the monophyly of the genus (Hauk, 1995). Morphological differences among species may be subtle, especially in the *Bo. lunaria* complex (Stensvold, 2008). According to Paris et al., (1989), the three criteria that define cryptic species in homosporous ferns including *Botrychium* s.s. are: "1) poor morphological differentiation, 2) reproductive isolation, and 3) misinterpretation of taxa as members of a single broader species." For instance, *Bo. minganense* was described as a new species by Victorin (1927) but reduced to a variety of *Bo. lunaria* by Clausen (1938) due to the lack of discriminating morphological criteria. But finally, *Bo. minganense* was reinstated as a species after 14 morphological characters were identified to separate it from *Bo. lunaria*, including an unambiguous distinction of ploidy level (n = 45 in *Bo. lunaria* against n = 90 in *Bo. minganense*) (Wagner and Lord, 1956). Likewise, other species have been morphologically confused as in the three northern allotetraploids, *Bo. alaskense* W.H. Wagner & J.R. Grant, *Bo. boreale* J. Milde, and *Bo. pinnatum* H. St. John. To resolve the problematic identification among taxa in *Bo. lunaria* complex, Stensvold and coauthors (2002; 2008) extensively sampled the group in North America and Europe, covering the entire known distributional range. Based on over 1000 accessions analyzed with codominant nuclear alleles from 20 independent coding genes, two new species were identified (*Bo. tunux* M. Stensvold & D. Farrar and *Bo. yaaxudakeit* M. Stensvold & D. Farrar) and three more taxa are still under investigation (*Bo. lunaria* var. "melzeri M. Stensvold" ined., *Bo.* "neolunaria M. Stensvold" ined., and *Bo.* "nordicum D. Farrar" ined.) (Stensvold et al., 2002; Stensvold, 2008). Stensvold's study also reveals introgressions between *Bo. lunaria* var. *lunaria* and *Bo.* "neolunaria" ined.

Another factor complicating the study of *Botrychium* species relationships is the high number of polyploids. It is a rare case study where 18 of the 30 recognized species are polyploids; 17 allotetraploids (*Bo. alaskense*, *Bo. ascendens* W.H. Wagner, *Bo. boreale*, *Bo. dusenii* (Christ) Alston, *Bo. echo* W.H. Wagner, *Bo.* "furculatum D. Farrar" ined., *Bo. gallicomontanum*, *Bo. hesperium* (Maxon & R.T. Clausen) W.H. Wagner & Lellinger, *Bo. matricariifolium* (Döll) A. Braun, *Bo.* "michiganense D. Farrar" ined., *Bo. minganense*, *Bo. paradoxum* W.H. Wagner, *Bo. pedunculosum, Bo. pinnatum* H. St. John, *Bo. spathulatum* W.H. Wagner, *Bo. watertonense* W.H. Wagner, *and Bo. yaaxudakeit*); and a single hexaploid (*Bo. pseudopinnatum*). These polyploids have resulted from various hybridizations between members of the twelve diploids (*Bo. campestre* W.H. Wagner & D. Farrar, *Bo. lanceolatum, Bo. lineare* W.H. Wagner, *Bo. lunaria, Bo. montanum* W.H. Wagner, *Bo. mormo, Bo.* "neolunaria" ined., *Bo.* "nordicum" ined., *Bo. pallidum* W.H. Wagner, *Bo. pumicola* Coville, *Bo. simplex* E. Hitchc., and *Bo. tunux*). These parental lineages have been identified by combined analyses of morphology (Wagner and Lord, 1956; Wagner and Grant, 2002), karyology (Wagner, 1955; Wagner and Lord, 1956; Wagner and Wagner, 1986, 1990; Wagner, 1993), molecular phylogenetics (Hauk, 1995; Hauk et al., 2003, 2012; Williams and Waller, 2012), and isozyme analyses (Hauk and Haufler, 1999; Zika and Farrar, 2009; Farrar, 2011).

Few nuclear DNA regions have been used in ferns to reconstruct biparental patterns of inheritance (Sessa et al., 2012), although both specific (Tsutsumi et al., 2011; Chen et al., 2012) and universal primers (Ishikawa et al., 2002; Schuettpelz et al., 2008) are available. Unfortunately, nuclear DNA markers have proven difficult to use and interpret in polyploid ferns (Shepherd et al., 2008). A previous molecular study assessed the variability and phylogenetic utility of nine plastid DNA regions in ferns including the intergenic spacer *psbA-trnH*^{GUG}, but with few representatives of *Botrychium* s.s. (Small et al., 2005). Interestingly, this marker appears to have evolved more rapidly in basal clades than in more recently diverged fern groups (Ebihara et al., 2010). Several phylogenetic studies presented relationships between diploid and polyploid species of *Botrychium* s.s. using plastid DNA regions and nuclear dominant markers (Amplified Fragment-Length Polymorphism) (Hauk, 1995; Hauk et al., 2003, 2012; Williams and Waller, 2012). However, with the exception of the latter paper, few accessions per species were presented.

In this study, we focused on acquiring material from the most wide-ranging Arctic taxa, *Botrychium lanceolatum* and *Bo. lunaria*, as well as from three formerly confused northern allotetraploids, *Bo. alaskense*, *Bo. boreale*, and *Bo. pinnatum*. We also included material from species that had not been included in previous phylogenies (*Bo. alaskense*, *Bo. tunux*, and *Bo. yaaxudakeit*) (Wagner and Grant, 2002; Stensvold et al., 2002), as well as five potentially new taxa from various regions (*Bo. alaskense* var. "salchaketense J.R. Grant" ined., *Bo.* "michiganense" ined., *Bo. lunaria* var. "melzeri" ined., *Bo.* "neolunaria" ined., and *Bo.* "nordicum" ined.). We analyzed three noncoding plastid DNA regions (*psbA-trnH*^{GUG}, *trnL*^{UAA}*-trnF*^{GAA} intergenic spacers, and *rpL16* intron) for 25 of 30 species from North America and Europe (only five species are missing, *Bo. ascendens*, *Bo. dusenii*, *Bo.* "furculatum" ined., *Bo. hesperium*, *and Bo. pseudopinnatum*), including some previously published sequences from GenBank (Hauk et al., 2003, 2012). The aims were: (1) to reconstruct a molecular phylogeny of *Botrychium* s.s. based on extensive geographic and taxonomic sampling; (2) to test the validity of new taxa proposed by other authors; and (3) to find a molecular region for DNA barcoding allowing identification of cryptic taxa in *Botrychium* s.s.

MATERIALS AND METHODS

***Plant material*—**Fieldwork to collect material for this study took place in Switzerland, Sweden, and south central and interior Alaska during the summer of 2012. Additional material was sent from colleagues in Alaska and Washington State, but most importantly from Don Farrar who sent verified key collections from Canada, Greenland, Iceland, Norway, and USA without which our study could not have been completed. All taxa sampled and analyzed are listed in Appendix 1. Leaf material was dried in silica gel and vouchers deposited in the herbarium of University of Neuchâtel (NEU) (Appendix 1).

***DNA extraction*—**Total DNA was extracted from leaves dried in silica gel or herbarium specimens using the cetyltrimethylammonium bromide (CTAB) buffer protocol (Doyle and Doyle, 1987). CTAB and other residues were removed

by washing with 70% ethanol and 10 mM ammonium acetate and a final wash of just 70% ethanol.

***PCR amplification*—**The *psbA-trnH*^{GUG} intergenic spacer was amplified with the *trnH*^{GUG} primer (Tate and Simpson, 2003) and *psbA* primer (Sang et al., 1997) in 25 µL reactions (17.4 µL of ddH_2O, 5 µL of buffer, 0.5 µL of dNTP mix at 10 mM, 0.5 µL of each 20 mM primer, 0.1 µL of GoTaq Hot Start DNA polymerase (Promega, Madison, Wisconsin, USA), and 1 µL of 5 ng/µL of DNA) and using the PCR program of Shaw et al., (2005) including the initial denaturation at 94°C for 3 min.

The *trnL*^{UAA}*-trnF*^{GAA} intergenic spacer was amplified with e and f universal primers (Taberlet et al., 1991) in 25 µL of reaction (16.4 µL of ddH_2O, 5 µL of buffer, 1 µL $MgCl_2$ at 25 mM, 0.5 µL of dNTP mix at 10 mM, 0.5 µL of each 20 mM primer, 0.1 µL of GoTaq Hot Start DNA polymerase (Promega), and 1 µL of 5 ng/µL of DNA). Amplification was performed using the PCR program of Hauk, (1995) with minor modifications (3 min of initial denaturation at 94°C; 40 cycles of 94°C for 1 min, 55°C for 0.45 min, and 72°C for 1.30; with a final extension 72°C for 10 min).

The *rpL16* intron region was amplified with rpL-16F71 and rpL16R1516 primers (Small et al., 1998) in 25 µL of reaction (17.4 µL of ddH_2O, 5 µL of buffer, 0.5 µL of dNTP mix at 10 mM, 0.5 µL of each 20 mM primer, 0.1 µL of GoTaq Hot Start DNA polymerase (Promega), and 1 µL of 5 ng/µL of DNA) and using the PCR program of Hauk et al., (2012) including few modifications (3 min of initial denaturation at 94°C; 36 cycles of 94°C for 1 min, 50°C for 0.45 min, and 72°C for 1.30; with a final extension 72°C for 10 min).

***DNA sequencing, alignment, sequence acquisition*—**Sequencing was done on an ABI3730 XL Automated Sequencer by Macrogen Europe (Amsterdam, Netherlands) on 5 µL of PCR product with 5 µL of primer at 10 µM. All markers employed for PCR amplification are the same as used for sequencing. Each accession was sequenced with both forward and reverse reactions and a consensus was assembled with Geneious software V.5.5.3 (http://www.geneious.com). All *rpL16*, *trnL*^{UAA}*-trnF*^{GAA}, and *psbA-trnH*^{GUG} sequences were aligned with MAFFT online (http://mafft.cbrc.jp/alignment/ server/) G-INS-i setting. Some adjustments by eye were necessary in the three alignments. The 25 *trnL*^{UAA}*-trnF*^{GAA} and 26 *rpL16* sequences (Appendix 1) already published (Hauk, 1995; Hauk et al., 2012), were imported from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and included in the dataset for geographical areas or species missing from our own sampling and collaborations.

***Phylogenetic analysis*—**Phylogenetic analysis was simultaneously conducted with Maximum Likelihood (ML) and Bayesian Inferences (BI). The best evolutionary change model (GTR+G+Γ for each regions separately) was estimated with the program jModelTest (Posada, 2008) V.0.1.1 according to Akaike information criterion (Akaike, 1973) and in view of Bayesian information criterion (Posada and Buckley, 2004). Plastid regions sequenced were concatenated with the software SequenceMatrix V.1.7.8, after having checked the similarity of topologies between each. ML was implemented in RAxML V.7.3.2 (Stamatakis, 2006; Stamatakis et al., 2008) with one partition for each marker. The RAxML program was run through the Cyber-Infrastructure for Phylogenetic Research (CIPRES) (Miller et al., 2010) web portal (http://www.phylo.org/) with RAxML-HPC BlackBox tool. Halt ML bootstrapping automatically as recommended on CIPRES was executed. Independent analysis was performed with 500 bootstraps. BI was run with MrBayes program V.3.2.1 (Ronquist et al., 2012) with ten million generations, including two independent runs, each containing three heated and one cold chain and uniform priors. Each plastid region was settled with an unlinked evolutionary model. The bootstrap values and posterior probabilities of branches are reported in trees and both are rooted with four out-groups.

RESULTS

***Plastid sequences analysis*—**The combined data set provided 1751 aligned nucleotides of which 144 (8.2%) were variable and 112 (6.4%) were parsimony-informative. The *psbA-trnH*^{GUG} intergenic spacer had intermediate variability sites (6.9%) and parsimony-informative sites (6.2%), the *trnL*^{UAA}*-trnF*^{GAA} intergenic spacer is the highest with 9.4% and 8.3%, and lowest for the *rpL16* intron with 8.7% and 5.6%, respectively. All details are summarized in Table 1. Despite several attempts, amplification of the *matK* coding region was unsuccessful.

The 81 *psbA-trnH*^{GUG} intergenic spacer accessions including 609 base pairs (bp) aligned nucleotides have the highest variable sites in Lunaria clade (2.5%). Two of our total accessions (86) are missing (2.3%) (BD1203721 and BD1206330). One insertion is shared by *Botrychium* "nordicum" ined. and *Bo. lunaria* var. *lunaria* by all individuals from Switzerland and southern Sweden, and two from Iceland (BD1203721 and BD1203721). For *trnL*^{UAA}*-trnF*^{GAA} intergenic spacer, the 372 nucleotides aligned of our 82 accessions contain 6.5% of variable sites with 6.2% of parsimony-informative sites, and 9.4% and 8.3% respectively combined with sequences of prior studies. Four accessions (4.6%) are missing (DF19337, DF19171, BD1205343, and BD1206209). In our analysis, the highest variable sites were recorded in the Lunaria clade (1.1%). However, in the combined data set they are less than in the Simplex-Campestre clade (1.9% vs. 3.2%) due to the low number of taxa sampled. A deletion of 57 nucleotides (223-280) is shared by all *Bo.* "neolunaria" ined., *Bo*. "nordicum" ined., and *Bo. yaaxudakeit* as well as several *Bo. lunaria* var. *lunaria*. With 770 aligned bp, the *rpL16* intron provided 37 variable sites (4.8%) with 30 parsimony-informative sites (3.9%) and 67 (8.7%) and 43 (5.6%), respectively, for both datasets. Only two accessions (2.3%) are missing in analysis (DF15097 and outgroup BD1205503). One insertion of three nucleotides (416-418) is shared by all taxa of Lunaria clade.

TABLE 1. Characteristics of data sets for noncoding plastid DNA regions following three major clades.

Plastid region	Sequence provenance	Total No. accession	Aligned* length (bp)	No.–% of variable sites total	No.–% of variable sites Simplex-Campestre–clade	No.–% of variable sites Lanceolatum clade	No.–% of variable sites Lunaria clade	No.–% of parsimony informative total sites
psbA–trnH^{GUG}	This study	81 (84)	609	42–6.9 (129–21.2)	2–0.3	3–0.5	15–2.5	38–6.2 (81–13.3)
trnL^{UAA}*–trnF*^{GAA}	This study	78 (82)	372	24–6.5 (66–17.8)	1–0.3	2–0.5	4–1.1	23–6.2 (64–17.2)
	Hauk et al., 2003 & 2012; this study	100 (104)	372	33–8.9 (74–19.9)	12–3.2	5–1.3	7–1.9	29–7.8 (69–18.5)
rpL16	This study	81 (84)	770	37–4.8 (153–19.8)	2–0.3	6–0.8	8–1.0	30–3.9 (79–10.2)
	Hauk et al., 2003 & 2012; this study	105 (107)	770	67–8.7 (178–23.0)	28–3.6	13–1.7	13–1.7	43–5.6 (92–11.9)
Three Plastid regions	Hauk et al., 2003 & 2012; this study	106 (110)	1751	144–8.2 (388–22.1)	42–2.4	21–1.2	37–2.1	112–6.4 (245–14.0)

Note: Numbers in parentheses include outgroups.

*Alignment with four outgroups (two *Botrypus virginianus* and two *Sceptridium multifidum*).

Maximum Likelihood and Bayesian Inference analysis— Topologies of the three independent plastid DNA regions were congruent before concatenation. The topology and support values generated from the concatenated dataset by ML and BI analysis (Fig. 1) were concordant without major differences. The BI tree generated is shown in Fig. 1 with labeled posterior probabilities (PP) associate and ML bootstrap values BV (BV/PP). In both, the Simplex-Campestre, Lanceolatum, and Lunaria clades are strongly supported as monophyletic, with 88/0.97, 100/1.0 and 100/1.0, respectively, for ML and BI. Each is included within a monophyletic *Botrychium* s.s. clade (100/1.0) and are consistent with prior molecular phylogenies (Hauk et al., 2003, 2012).

Although little genetic distance appears within the Lanceolatum clade, three subclades can be identified, Matricariifolium (60/0.65) (for *Botrychium matricariifolium* and *Bo. pedunculosum*), Pinnatum (50/0.87) (for *Bo. pinnatum*), and Alaskense (73/0.99) (for *Bo. alaskense* and *Bo. alaskense* var. "salchaketense" ined.), the latter two subclades being well-supported with a posterior probability above to 0.85. Two other subclades are represented as variations of *Bo. lanceolatum* from western Alaska (61/0.88) and Iceland (60/0.93). Due to the short branches in the tree, a zoom of the Lanceolatum and Lunaria clades is presented (Fig. 2). In the Lunaria clade, five of eight subclades are well supported (PP > 0.85, Figs. 1 and 2): Crenulatum (100/1.0), Tunux (73/1.0), Lunaria SWE (–/–), Melzeri (–/0.82), Nordicum (88/1.0), Lunaria CH (77/1.0), Lunaria ISL&GRL (96/1.0) and Neolunaria (–/0.83) subclades. The Lunaria CH subclade appears sister (80/0.90) to the Nordicum subclade and several *Bo. lunaria* taxa from Iceland and Sweden, and all are sister to the Melzeri subclade with the node poorly supported by the posterior probability (–/0.82). The node between Lunaria ISL & GRL and Neolunaria subclades is not supported.

DISCUSSION

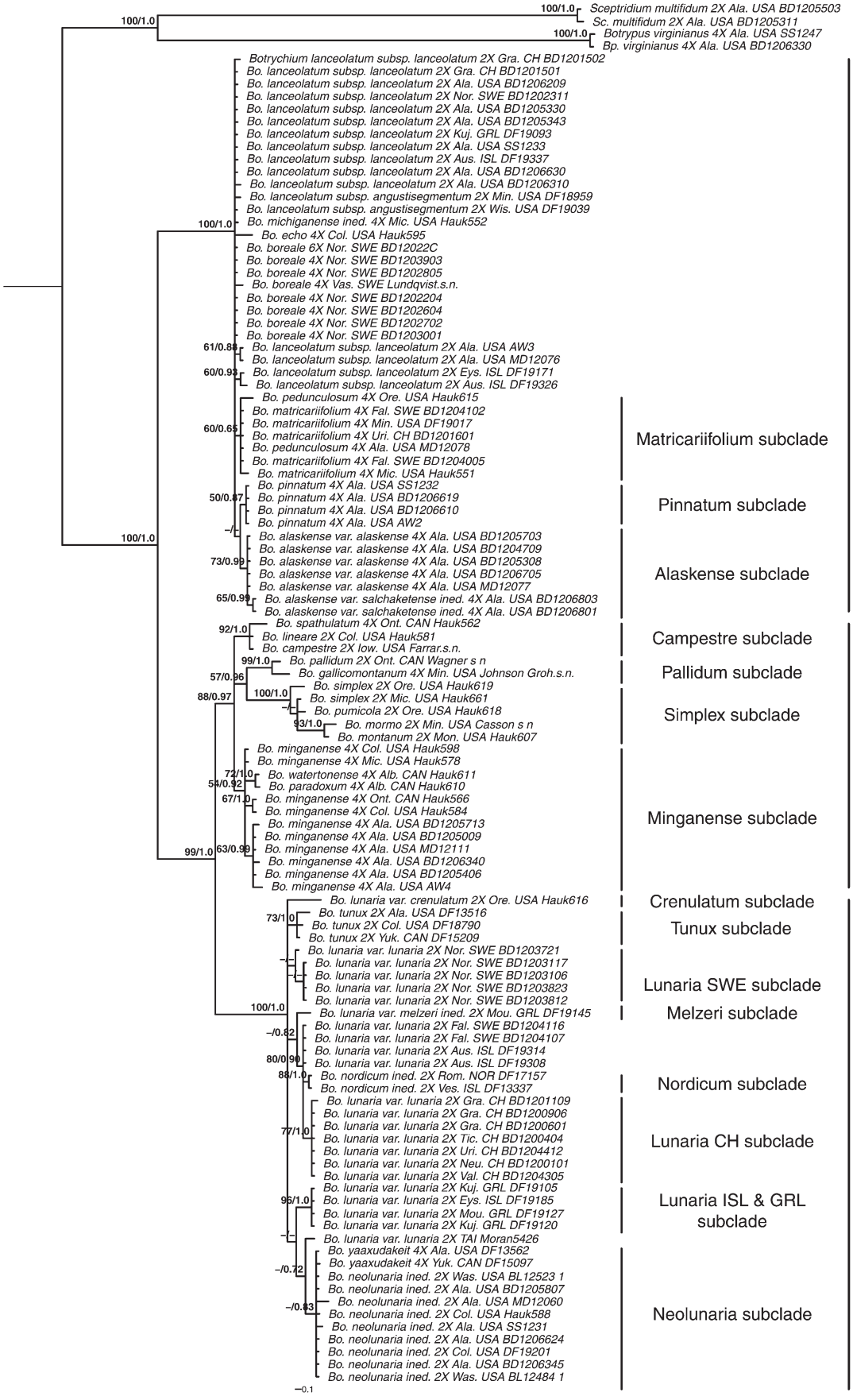
***Chloroplast DNA barcoding for cryptic species in* Botrychium *s.s*—** Since identification based on morphological traits is difficult in numerous groups of *Botrychium* s.s., and other molecular tools (e.g., RAPDs) have been used on these species with limited success (Swartz and Brunsfeld, 2002), a plastid DNA barcode could be a useful aid in species identification. In our analysis, the universal region *psbA-trnH*^{GUG} improved the phylogenetic resolution in the Lunaria clade (Table 1) better than any other previously tested plastid marker (Small et al., 2005). For the same number of taxa analyzed in Lunaria clade, the variability of *psbA-trnH*^{GUG} region was more than twice as variable as the *trnL*^{UAA}*-trnF*^{GAA}, which was previously the most variable region known. However, this is not the case for the Lanceolatum clade where little variation is observed. Although *matK* has recently been used with success in closely related Ophioglossaceae and Psilotaceae (Kuo et al., 2011; Li et al., 2011; Shinohara et al., 2013), we were unsuccessful in amplifying it in *Botrychium*. Therefore, the barcode *psbA-trnH*^{GUG} is a good candidate to identify taxa of *Botrychium* at the fine scale in the Lunaria clade, as was found in the sister genus *Sceptridium* (Ebihara et al., 2010).

Phylogenetic reconstruction from plastid DNA sequences and allopolyploid species— Phylogenetic reconstruction from plastid DNA sequences depicts the phylogeny of the maternally inherited chloroplast. Accordingly, the phylogenetic tree may be inappropriate to resolve the true reticulate evolution of allopolyploid species derived through interclade hybridization (following an allopolyploidization event(s)) (Linder and Rieseberg, 2004). We recognize that we only present a phylogenetic reconstruction of the maternal evolutionary history of the allopolyploid species (Figs. 1 and 2) as we lack appropriate and successful nuclear markers.

The Lanceolatum clade— The Lanceolatum clade comprises ten species, only one of which is diploid, *Botrychium lanceolatum*. This species appears to be the maternal contributor for each of the nine polyploids (Figs. 1 and 2; Hauk et al., 2012): *Bo. alaskense*, *Bo. boreale*, *Bo. echo, Bo. hesperium*, *Bo. matricariifolium*, *Bo* "michiganense" ined., *Bo. pedunculosum*, *Bo. pinnatum*, and *Bo. pseudopinnatum*. A few of the more interesting results are discussed below for the most sampled species: *Bo. alaskense, Bo. boreale, Bo. lanceolatum, Bo. matricariifolium, Bo. pedunculosum,* and *Bo. pinnatum*.

The sequenced plastid regions of *Botrychium lanceolatum* appear genetically uniform from all areas collected without any identifiable divergence between *Bo. lanceolatum* subspecies *angustisegmentum* Pease & A.H. Moore and subspecies *lanceolatum* in either of the latter's 'red' or 'green' forms (Figs. 1 and 2). However, the latter two forms have distinct genotypes identified by isozyme analyses and are morphologically distinct where the 'red' genotype has a red coloration on the stem and at the base of the trophophore, whereas no red coloration occurs on the trophophore of the 'green' genotype (Farrar, 2011). Our results are interesting since Stensvold (2008) used nuclear markers to clearly differentiate these three taxa. This supports the idea that the allotetraploid species in the Lanceolatum clade were formed before differentiation and/or diversification in *Bo. lanceolatum* (D. Farrar, Iowa State University, personal communication in 2012; Williams and Waller, 2012). The most probable explanation of this lack of genetic variation is that the chloroplast genome is more conservative than the nuclear (Wolfe et al., 1987). Due to low heterozygosity between individuals within populations (Hauk and Haufler, 1999; Camacho and Liston, 2001), intragametophytic selfing is considered as a major reproductive process, which excludes the hypothesis of widespread gene flow through sampled geographical areas and supports a recent long-range dispersal of the *Bo. lanceolatum* taxa in North America and Europe (D. Farrar, personal communication). The low genetic variation among the morphologically distinct and diverse polyploids in the Lanceolatum clade supports recent evolutionary polyploidization with the same maternal contributor *Bo. lanceolatum*. However, whether these are of single or multiple origins remains unknown.

Botrychium matricariifolium was sampled from five collections from distinct geographical areas in North America (Michigan and Minnesota) and Europe (Sweden and Switzerland). They have nearly identical sequences, and form a poorly supported subclade (60/0.65) with the two accessions of *Bo. pedunculosum*. The specimen of *Bo. pedunculosum* from the Kotzebue area (MD12078) represents the first identification of this species in northern Alaska and confirms the highly disjunct distribution of the species, ranging from the Aleutian Islands of Alaska to eastern Québec in Canada and southern California, USA. The material is reminiscent of *Bo. alaskense* (with which it is sympatric), *Bo. pinnatum*, and *Bo. matricariifolium*, but is identifiable as *Bo. pedunculosum* by the orange coloration of its stem and by the triangular shaped pinnae. With little genetic variation between


Sceptridium multifidum 2X Ala. USA BD1205503
Sc. multifidum 2X Ala. USA BD1205311
Botrypus virginianus 4X Ala. USA SS1247
Bp. virginianus 4X Ala. USA BD1206330
Botrychium lanceolatum subsp. lanceolatum 2X Gra. CH BD1201502
Bo. lanceolatum subsp. lanceolatum 2X Gra. CH BD1201501
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA BD1206209
Bo. lanceolatum subsp. lanceolatum 2X Nor. SWE BD1202311
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA BD1205330
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA BD1205343
Bo. lanceolatum subsp. lanceolatum 2X Kuj. GRL DF19093
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA SS1233
Bo. lanceolatum subsp. lanceolatum 2X Aus. ISL DF19337
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA BD1206630
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA BD1206310
Bo. lanceolatum subsp. angustisegmentum 2X Min. USA DF18959
Bo. lanceolatum subsp. angustisegmentum 2X Wis. USA DF19039
Bo. michiganense ined. 4X Mic. USA Hauk552
Bo. echo 4X Col. USA Hauk595
Bo. boreale 6X Nor. SWE BD12022C
Bo. boreale 4X Nor. SWE BD1203903
Bo. boreale 4X Nor. SWE BD1202805
Bo. boreale 4X Vas. SWE Lundqvist.s.n.
Bo. boreale 4X Nor. SWE BD1202204
Bo. boreale 4X Nor. SWE BD1202604
Bo. boreale 4X Nor. SWE BD1202702
Bo. boreale 4X Nor. SWE BD1203001
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA AW3
Bo. lanceolatum subsp. lanceolatum 2X Ala. USA MD12076
Bo. lanceolatum subsp. lanceolatum 2X Eys. ISL DF19171
Bo. lanceolatum subsp. lanceolatum 2X Aus. ISL DF19326
Bo. pedunculosum 4X Ore. USA Hauk615
Bo. matricariifolium 4X Fal. SWE BD1204102
Bo. matricariifolium 4X Min. USA DF19017
Bo. matricariifolium 4X Uri. CH BD1201601
Bo. pedunculosum 4X Ala. USA MD12078
Bo. matricariifolium 4X Fal. SWE BD1204005
Bo. matricariifolium 4X Mic. USA Hauk551
Bo. pinnatum 4X Ala. USA SS1232
Bo. pinnatum 4X Ala. USA BD1206619
Bo. pinnatum 4X Ala. USA BD1206610
Bo. pinnatum 4X Ala. USA AW2
Bo. alaskense var. alaskense 4X Ala. USA BD1205703
Bo. alaskense var. alaskense 4X Ala. USA BD1204709
Bo. alaskense var. alaskense 4X Ala. USA BD1205308
Bo. alaskense var. alaskense 4X Ala. USA BD1206705
Bo. alaskense var. alaskense 4X Ala. USA MD12077
Bo. alaskense var. salchaketense ined. 4X Ala. USA BD1206803
Bo. alaskense var. salchaketense ined. 4X Ala. USA BD1206801
Bo. spathulatum 4X Ont. CAN Hauk562
Bo. lineare 2X Col. USA Hauk581
Bo. campestre 2X low. USA Farrar.s.n.
Bo. pallidum 2X Ont. CAN Wagner s n
Bo. gallicomontanum 4X Min. USA Johnson Groh.s.n.
Bo. simplex 2X Ore. USA Hauk619
Bo. simplex 2X Mic. USA Hauk661
Bo. pumicola 2X Ore. USA Hauk618
Bo. mormo 2X Min. USA Casson s n
Bo. montanum 2X Mon. USA Hauk607
Bo. minganense 4X Col. USA Hauk598
Bo. minganense 4X Mic. USA Hauk578
Bo. watertonense 4X Alb. CAN Hauk611
Bo. paradoxum 4X Alb. CAN Hauk610
Bo. minganense 4X Ont. CAN Hauk566
Bo. minganense 4X Col. USA Hauk584
Bo. minganense 4X Ala. USA BD1205713
Bo. minganense 4X Ala. USA BD1205009
Bo. minganense 4X Ala. USA MD12111
Bo. minganense 4X Ala. USA BD1206340
Bo. minganense 4X Ala. USA BD1205406
Bo. minganense 4X Ala. USA AW4
Bo. lunaria var. crenulatum 2X Ore. USA Hauk616
Bo. tunux 2X Ala. USA DF13516
Bo. tunux 2X Col. USA DF18790
Bo. tunux 2X Yuk. CAN DF15209
Bo. lunaria var. lunaria 2X Nor. SWE BD1203721
Bo. lunaria var. lunaria 2X Nor. SWE BD1203117
Bo. lunaria var. lunaria 2X Nor. SWE BD1203106
Bo. lunaria var. lunaria 2X Nor. SWE BD1203823
Bo. lunaria var. lunaria 2X Nor. SWE BD1203812
Bo. lunaria var. melzeri ined. 2X Mou. GRL DF19145
Bo. lunaria var. lunaria 2X Fal. SWE BD1204116
Bo. lunaria var. lunaria 2X Fal. SWE BD1204107
Bo. lunaria var. lunaria 2X Aus. ISL DF19314
Bo. lunaria var. lunaria 2X Aus. ISL DF19308
Bo. nordicum ined. 2X Rom. NOR DF17157
Bo. nordicum ined. 2X Ves. ISL DF13337
Bo. lunaria var. lunaria 2X Gra. CH BD1201109
Bo. lunaria var. lunaria 2X Gra. CH BD1200906
Bo. lunaria var. lunaria 2X Gra. CH BD1200601
Bo. lunaria var. lunaria 2X Tic. CH BD1200404
Bo. lunaria var. lunaria 2X Uri. CH BD1204412
Bo. lunaria var. lunaria 2X Neu. CH BD1200101
Bo. lunaria var. lunaria 2X Val. CH BD1204305
Bo. lunaria var. lunaria 2X Kuj. GRL DF19105
Bo. lunaria var. lunaria 2X Eys. ISL DF19185
Bo. lunaria var. lunaria 2X Mou. GRL DF19127
Bo. lunaria var. lunaria 2X Kuj. GRL DF19120
Bo. lunaria var. lunaria 2X TAI Moran5426
Bo. yaaxudakeit 4X Ala. USA DF13562
Bo. yaaxudakeit 4X Yuk. CAN DF15097
Bo. neolunaria ined. 2X Was. USA BL12523 1
Bo. neolunaria ined. 2X Ala. USA BD1205807
Bo. neolunaria ined. 2X Ala. USA MD12060
Bo. neolunaria ined. 2X Col. USA Hauk588
Bo. neolunaria ined. 2X Ala. USA SS1231
Bo. neolunaria ined. 2X Ala. USA BD1206624
Bo. neolunaria ined. 2X Col. USA DF19201
Bo. neolunaria ined. 2X Ala. USA BD1206345
Bo. neolunaria ined. 2X Was. USA BL12484 1
0.1
Matricariifolium subclade
Pinnatum subclade
Alaskense subclade
Campestre subclade
Pallidum subclade
Simplex subclade
Minganense subclade
Crenulatum subclade
Tunux subclade
Lunaria SWE subclade
Melzeri subclade
Nordicum subclade
Lunaria CH subclade
Lunaria ISL & GRL subclade
Neolunaria subclade
Lanceolatum clade
Simplex–Campestre clade
Lunaria clade

Bo. matricariifolium and Bo. pedunculosum, morphological characters may be the best way to identify these taxa.

Botrychium boreale (*Bo. lanceolatum* × *Bo. lunaria* var. *lunaria*) has often been confused in older literature, and many North American specimens have been incorrectly identified as this species (Hultén, 1968; Welsh, 1974; Cody, 1996). It is actually restricted to northern Europe and for our analyses was sampled from eight localities in Sweden. Our results indicate that it is clearly morphologically and genetically distinct from both *Bo. alaskense* and *Bo. pinnatum.* A single hexaploid individual of *Bo. boreale* (BD12022C) was identified by flow cytometry analysis (Dauphin et al., in preparation). Its origin is through an unknown pair, but perhaps simply through backcrossing to one of the genetic contributors in tetraploid *Bo. boreale*. This is only the second record of a hexaploid taxon in *Botrychium* s.s. after *Bo. pseudopinnatum* (Wagner, 1990).

Botrychium alaskense (*Bo. lanceolatum* × *Bo. lunaria* var. *lunaria*) appears as a distinct, well-supported subclade (73/0.99). Typical forms have a triangular grass-green trophophore and stem that is heavily infused with red coloration at the base (Wagner and Grant, 2002). A potentially new variety "salchaketense" has a pale light-green trophophore and stem with basal pinnae that are much further developed, sometimes almost the same length as the main pinna itself. The two samples of "salchaketense" included in our study have one SNP that groups them together (65/0.99). Further morphological and genetic studies are required to determine whether this warrants separate taxonomic recognition, and to determine relationships of both to the recently described (but unseen by us) *Bo. alaskense* var. *pavlovii* Tzvelev from Mongolia (Tzvelev, 2004).

Botrychium pinnatum (*Bo. lanceolatum* × *Bo. "*neolunaria" ined.) was sampled from coastal areas in Alaska but occurs as far south as Arizona. Despite the lack of genetic variation in the Pinnatum subclade (50/0.85), we speculate there may be variation in plastid haplotypes from specimens from these distant areas. *Botrychium pinnatum* has often confused with *Bo. alaskense* since they share the same maternal contributor (*Bo. lanceolatum*) but two closely related paternal species (*Bo. "*neolunaria" ined. for *Bo. pinnatum*, and *Bo. lunaria* var. *lunaria* for *Bo. alaskense*) (Farrar, 2011; Stensvold, 2008). Interestingly, the known distributions of these two allotetraploids in Alaska do not overlap, where *Bo. pinnatum* is found on the rainy coast from the Aleutians to Kodiak Island, the Kenai Peninsula and Anchorage area, while *Bo. alaskense* is found in the much dryer interior and north (Fairbanks, Wrangell-St. Elias, Denali National Park, and Kotzebue).

The Simplex-Campestre clade—The Simplex-Campestre clade has 14 species including seven diploids (*Botrychium campestre, Bo. lineare, Bo. montanum, Bo. mormo, Bo. pallidum, Bo. pumicola,* and *Bo. simplex*) and seven allotetraploids (*Bo.* "furculatum" ined., *Bo. ascendens, Bo. gallicomontanum, Bo. minganense, Bo. paradoxum, Bo. spathulatum,* and *Bo. watertonense*). The clade is strongly supported as monophyletic (88/0.97), and has four subclades including Campestre (92/1.0), Pallidum (99/1.0), Simplex (100/1.0), and Minganense (54/0.92) as best described by Hauk et al. (2012).

In the Minganense subclade, material tentatively identified by morphology as *Botrychium minganense* (63/0.99) from six specimens from distant localities in Alaska (Dutch Harbor, Girdwood, Kotzebue, and the Fairbanks area [Elliott Hwy., Richardson Hwy., and Steese Hwy.]) appears genetically distinct from material from central North America (Michigan and Colorado, USA,and Ontario, Canada). *Botrychium minganense* has putatively been formed from hybridization between *Bo. "*neolunaria" ined. and an unknown taxon of the Simplex-Campestre clade, displays subclustering in the Minganense subclade that may be indicative of multiple independent origins as suggested by Farrar (2011). Additional specimens from throughout the range of *Bo. minganense* need to be analyzed before any taxonomic considerations can be made.

The Lunaria clade—Until recently, the Lunaria clade was comprised of *Botrychium lunaria* alone. However, recent studies on this morphologically cryptic group have identified several additional taxa such that the clade now comprises at least six species including four diploids (*Bo. lunaria*, *Bo. "*neolunaria" ined., *Bo.* "nordicum" ined., and *Bo. tunux*), and two tetraploids (*Bo. dusenii*, and *Bo. yaaxudakeit*) (Stensvold et al., 2002; Stensvold, 2008; Meza Torres et al., 2011). There are also three known varieties within *Bo. lunaria*, i.e., var. *lunaria*, var. *"crenulatum* (W.H. Wagner) Stensvold" ined., and var. "melzeri" ined. (Stensvold, 2008). We have identified eight distinct subclades corresponding to these and possibly additional taxa within this clade. While these taxa are morphologically distinguishable only by subtle traits, genetic variation between them is substantial, thus presenting a classic demonstration of cryptic differentiation.

In terms of the geographic distribution of these taxa, *Botrychium "*neolunaria" ined. is located in North America, *Bo.* "nordicum" ined. in northwestern Europe, *Bo. tunux* in northern and southwestern North America as well as Norway, and the allotetraploid *Bo. yaaxudakeit* in northwestern America. *Bo. yaaxudakeit* (*Bo. lunaria* var. *lunaria* × *Bo. "*neolunaria" ined.) is identical to *Bo. "*neolunaria" ined. in plastid DNA sequences, thus confirming *Bo. "*neolunaria" ined. as the maternal contributor. *Botrychium lunaria* itself has a broad distribution in Europe and northern Asia (Stensvold, 2008), and may also be referable to material found in Australia, Pacific islands, and New Zealand (Wagner & Wagner, 1993) having arrived through long-distance dispersal, probably by birds. *Botrychium dusenii* from Patagonia in the southern cone of South America was previously considered as a variation of *Bo. lunaria*, but has recently been identified as a distinct allotetraploid with *Bo. lunaria* var. *lunaria* as one of its parents (Meza Torres et al., 2011).

All subclades in the Lunaria clade form geographical clusters (Figs. 1 and 2) contrasting with *Botrychium lanceolatum* from the same areas. Surprisingly, no plastid DNA variation was recorded among the five populations of *Bo. lunaria* var. *lunaria* sampled in the Swiss Alps, which exhibit extensive morphological variability (especially shape, size, and margin of the

←

Fig. 1. Bayesian tree inferred using MrBayes on three noncoding plastid DNA regions (*psbA-trnH*^{GUG}, *trnL*^{UAA}-*trnF*^{GAA} intergenic spacer, and *rpL16* intron). The tree topology is congruent with the Maximum Likelihood (ML) tree with exception to *Botrychium lunaria* var. "*crenulatum* (W.H. Wagner) Stensvold" ined. that is poorly supported as sister to the Lunaria clade. Bootstrap values BV and ML and posterior probabilities (PP) are represented above left nodes (BV/PP). Accession names include species or taxa/subspecies or varieties/ploidy/locality/country/voucher number. Clades and subclades are illustrated on the right. Abbreviations refer to Greenland (GRL), Iceland (ISL), Sweden (SWE), and Switzerland (CH). Taxa not yet published are indicated by "ined."

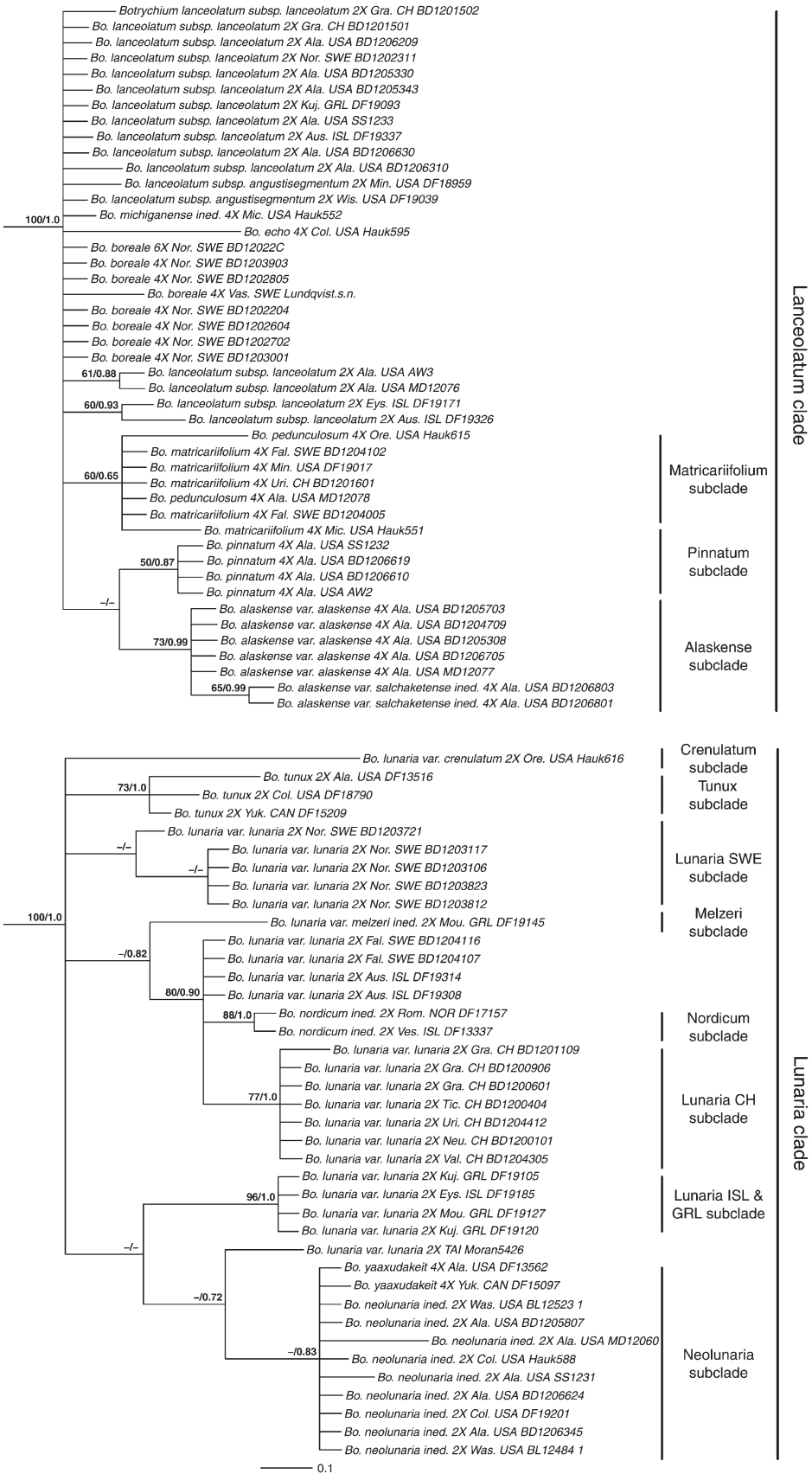


Fig. 2. Details of Lanceolatum and Lunaria clades. Legends as in Fig. 1.

pinnae) in populations separated by mountains up to 3000 m high. With the predominance of the intragametophyte selfing as found in the closely related genera *Botrypus* and *Sceptridium* (McCauley et al., 1985; Soltis and Soltis, 1986; Watano and Sahashi, 1992), we expected reproductive isolation and associated differentiation visible in plastid haplotypes. Because the whole Lunaria clade forms a polytomy in a tree with short branches, and sampling remains incomplete, it is difficult to trace its common ancestor, the geographical origins or constitutive migrations of these species.

CONCLUSION AND PERSPECTIVES

This molecular phylogenetic reconstruction has expanded the geographic representation and number of moonwort taxa investigated. In the most morphologically cryptic group of species in Lunaria clade, plastid DNA is quite diverse, while paradoxically less molecular variation is observed among species of the morphologically variable Lanceolatum clade. New species have been confirmed, and several geographical clusters in the Lunaria clade have been revealed. Our data from plastid DNA regions complements the results from the nuclear markers of the groundbreaking studies of Stensvold and Farrar, such that we can confirm that cryptic species do exist and provide a classic demonstration of cryptic differentiation.

Some important questions persist. Do we have intraspecific variation or distinct species? Have the allotetraploids evolved through single or multiple polyploid events? Can these events be dated? To answer this, it is absolutely necessary to design new molecular tools to more finely diagnose polyploid species, their ancestral lineages and thus better understand their formation and dispersal over time. Therefore, a dated phylogeny would provide valuable information. By covering the broadest geographic and taxonomic representation (filling in sampling gaps and sampling in underrepresented geographic areas, especially in North Africa (Atlas Mountains), northern Asia (Russia, China, Mongolia, and Japan), the Himalayan region, Australia, Pacific islands, New Zealand, and Patagonia in South America), future studies will even better understand within-genus relationships and evolution of this fascinating and important group.

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Appendix 1. Genbank accessions with corresponding voucher number. Vouchers for this study are deposited at the herbarium of University of Neuchâtel (NEU).

Taxa	Country	State/Province	Locality	Voucher accession	GenBank psbA-trnH	GenBank trnL-trnF	GenBank rpL16	Reference
Bo. alaskense W.H. Wagner & J.R. Grant var. *alaskense*	USA	Alaska	Salcha River	BD1204709	KF700546	KF700380	KF700462	This study
Bo. alaskense W.H. Wagner & J.R. Grant var. *alaskense*	USA	Alaska	Salcha River	BD1205308	KF700545	KF700379	KF700461	This study
Bo. alaskense W.H. Wagner & J.R. Grant var. *alaskense*	USA	Alaska	Steese Hwy.	BD1205703	KF700547	KF700381	—	This study
Bo. alaskense W.H. Wagner & J.R. Grant var. *alaskense*	USA	Alaska	Parks Hwy.	BD1206705	KF700544	KF700378	KF700460	This study
Bo. alaskense W.H. Wagner & J.R. Grant var. *alaskense*	USA	Alaska	Buckland	MD12-077	KF700543	KF700377	KF700459	This study
Bo. alaskense var. "salchaketense J.R. Grant" ined.	USA	Alaska	Salcha River	BD1206801	KF700548	KF700382	KF700464	This study
Bo. alaskense var."salchaketense J.R. Grant" ined.	USA	Alaska	Salcha River	BD1206803	KF700549	KF700383	KF700465	This study
Bo. boreale (6x) J. Milde	SWE	Norrbotten	Siknas	BD12022C	KF700556	KF700390	KF700472	This study
Bo. boreale J. Milde	SWE	Västerbotten	Storberget	Lundqvist s.n. /OSU	—	DQ849127	DQ849128	Hauk et al., 2003 & 2012
Bo. boreale J. Milde	SWE	Norrbotten	Siknas	BD1202204	KF700553	KF700387	KF700469	This study
Bo. boreale J. Milde	SWE	Norrbotten	Melderstein-Herrgard	BD1202604	KF700552	KF700386	KF700468	This study
Bo. boreale J. Milde	SWE	Norrbotten	Finskaret-island	BD1202702	KF700551	KF700385	KF700467	This study
Bo. boreale J. Milde	SWE	Norrbotten	Uddskaet-island	BD1202805	KF700554	KF700388	KF700470	This study
Bo. boreale J. Milde	SWE	Norrbotten	Esterson-island	BD1203001	KF700550	KF700384	KF700466	This study
Bo. boreale J. Milde	SWE	Norrbotten	Vittaniemi	BD1203903	KF700555	KF700389	KF700471	This study
Bo. campestre W.H. Wagner & D. Farrar	USA	Iowa	BeamisCreek	Farrar s.n. /ISC	—	AY138426	DQ849129	Hauk et al., 2003 & 2012
Bo. echo W.H. Wagner	USA	Colorado	Grand	Hauk 595/NCU	—	DQ849131	DQ849132	Hauk et al., 2003 & 2012
Bo. gallicomontanum D. Farrar & Johnson-Groh	USA	Minnesota	Norman	Johnson-Groh	—	DQ849134	DQ849135	Hauk et al., 2003 & 2012
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *angustisegmentum* Pease & A. H. Moore	USA	Minnesota	Itasca	DF18959	KF700558	KF700392	KF700474	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *angustisegmentum* Pease & A. H. Moore	USA	Wisconsin	Baraga	DF19039	KF700557	KF700391	KF700473	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	CH	Graubunden	Val Roseg	BD1201501	KF700572	KF700402	KF700488	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	CH	Graubunden	Val Roseg	BD1201502	KF700573	KF700403	KF700489	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	GRL	Kujalleq	Narsarsuag	DF19093	KF700567	KF700399	KF700483	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	ISL	Norourland	Myvatn	DF19171	KF700566	—	KF700482	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	ISL	Austurland	Jokulsarlon	DF19326	KF700563	KF700397	KF700479	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	ISL	Austurland	Jokulsarlon	DF19337	KF700564	—	KF700480	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	SWE	Norrbotten	Siknas	BD1202311	KF700570	—	KF700486	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Dutch Harbor	AW3	KF700560	KF700394	KF700476	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Salcha River	BD1205330	KF700569	KF700401	KF700485	This study

Appendix 1. Continued.

Taxa	Country	State/Province	Locality	Voucher accession	GenBank psbA-trnH	GenBank trnL-trnF	GenBank rpL16	Reference
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Richardson Hwy.	BD1205343	KF700568	KF700400	KF700484	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Turnagain Pass	BD1206209	KF700571	—	KF700487	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Girdwood	BD1206310	KF700561	KF700395	KF700477	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Hatcher Pass	BD1206630	KF700562	KF700396	KF700478	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Buckland	MD12-076	KF700559	KF700393	KF700475	This study
Bo. lanceolatum (S.G. Gmelin) Ångström subsp. *lanceolatum*	USA	Alaska	Kodiak Island	SS1233	KF700565	KF700398	KF700481	This study
Bo. lineare W.H. Wagner	USA	Colorado	El Paso	Hauk 581/NCU	—	AY138425	DQ849141	Hauk et al., 2003 & 2012
Bo. lunaria (L.) Swartz	CH	Neuchâtel	Creux-du-van	BD1200101	KF700575	KF700405	KF700491	This study
Bo. lunaria (L.) Swartz	CH	Ticino	Val-Di-Campo	BD1200404	KF700590	KF700421	KF700507	This study
Bo. lunaria (L.) Swartz	CH	Graubunden	Val Roseg	BD1200601	KF700591	KF700422	KF700508	This study
Bo. lunaria (L.) Swartz	CH	Graubunden	Val Roseg	BD1200906	KF700592	KF700423	KF700509	This study
Bo. lunaria (L.) Swartz	CH	Graubunden	Val Roseg	BD1201109	KF700593	KF700424	KF700510	This study
Bo. lunaria (L.) Swartz	CH	Valais	Chandolin	BD1204305	KF700574	KF700404	KF700490	This study
Bo. lunaria (L.) Swartz	CH	Uri	Gurtnellen	BD1204412	KF700576	KF700406	KF700492	This study
Bo. lunaria (L.) Swartz	GRL	Kujalleq	Narsarsuag	DF19105	KF700584	KF700415	KF700501	This study
Bo. lunaria (L.) Swartz	GRL	Kujalleq	Narsarsuag	DF19120	KF700585	KF700416	KF700502	This study
Bo. lunaria (L.) Swartz	GRL	—	Mountain 163	DF19127	KF700581	KF700412	KF700498	This study
Bo. lunaria (L.) Swartz	ISL	Norourland	Myvatn	DF19185	KF700583	KF700414	KF700500	This study
Bo. lunaria (L.) Swartz	ISL	Austurland	Hofn	DF19308	KF700577	KF700407	KF700493	This study
Bo. lunaria (L.) Swartz	ISL	Austurland	Jokulsarlon	DF19314	KF700578	KF700408	KF700494	This study
Bo. lunaria (L.) Swartz	SWE	Norrbotten	Tvaran	BD1203106	KF700588	KF700419	KF700505	This study
Bo. lunaria (L.) Swartz	SWE	Norrbotten	Tvaran	BD1203117	KF700589	KF700420	KF700506	This study
Bo. lunaria (L.) Swartz	SWE	Norrbotten	Kitkiojarvi	BD1203721	—	KF700409	KF700495	This study
Bo. lunaria (L.) Swartz	SWE	Norrbotten	Tarendo	BD1203812	KF700586	KF700417	KF700503	This study
Bo. lunaria (L.) Swartz	SWE	Norrbotten	Tarendo	BD1203823	KF700587	KF700418	KF700504	This study
Bo. lunaria (L.) Swartz	SWE	Falköping	Langjum	BD1204107	KF700579	KF700410	KF700496	This study
Bo. lunaria (L.) Swartz	SWE	Falköping	Langjum	BD1204116	KF700580	KF700411	KF700497	This study
Bo. lunaria (L.) Swartz	TAI	—	—	Moran 5426/MO	—	DQ849142	DQ849143	Hauk et al., 2003 & 2012
Bo. lunaria var. "*crenulatum* (W.H. Wagner) Stensvold" ined.	USA	Oregon	Wallowa	Hauk 616/NCU	—	AY138431	DQ849130	Hauk et al., 2003 & 2012
Bo. lunaria var."melzeri M. Stensvold" ined.	GRL	—	Mountain 163	DF19145	KF700582	KF700413	KF700499	This study
Bo. matricariifolium (Döll) A. Braun	CH	Uri	Gurtnellen	BD1201601	KF700596	KF700427	KF700513	This study
Bo. matricariifolium (Döll) A. Braun	SWE	Falköping	Brandstrorp	BD1204005	KF700594	KF700425	KF700511	This study
Bo. matricariifolium (Döll) A. Braun	SWE	Falköping	Langjum	BD1204102	KF700598	KF700429	KF700515	This study
Bo. matricariifolium (Döll) A. Braun	USA	Minnesota	Itasca	DF19017	KF700597	KF700428	KF700514	This study
Bo. matricariifolium (Döll) A. Braun	USA	Michigan	Alger	Hauk 551/NCU	—	DQ849155	DQ849156	Hauk et al., 2003 & 2012
Bo. "michiganense D. Farrar" ined.	USA	Michigan	Alger	Hauk 552/NCU	—	DQ849158	DQ849159	Hauk et al., 2003 & 2012
Bo. minganense M. Victorin	CAN	Ontario	Thunder Bay	Hauk 566/NCU	—	DQ849162	DQ849163	Hauk et al., 2003 & 2012
Bo. minganense M. Victorin	USA	Alaska	Dutch Harbor	AW4	KF700599	KF700430	KF700516	This study
Bo. minganense M. Victorin	USA	Alaska	Richardson Hwy.	BD1205009	KF700603	KF700434	KF700520	This study
Bo. minganense M. Victorin	USA	Alaska	Elliott Hwy.	BD1205406	KF700600	KF700431	KF700517	This study

Appendix 1. Continued.

Taxa	Country	State/Province	Locality	Voucher accession	GenBank psbA-trnH	GenBank trnL-trnF	GenBank rpL16	Reference
Bo. minganense M. Victorin	USA	Alaska	Steese Hwy.	BD1205713	KF700604	KF700435	KF700521	This study
Bo. minganense M. Victorin	USA	Alaska	Girdwood	BD1206340	KF700601	KF700432	KF700518	This study
Bo. minganense M. Victorin	USA	Michigan	Alger	Hauk 578/NCU	—	DQ849160	DQ849161	Hauk et al., 2003 & 2012
Bo. minganense M. Victorin	USA	Colorado	Lake	Hauk 584/NCU	—	DQ849166	DQ849167	Hauk et al., 2003 & 2012
Bo. minganense M. Victorin	USA	Colorado	Boulder	Hauk 598/NCU	—	DQ849164	DQ849165	Hauk et al., 2003 & 2012
Bo. minganense M. Victorin	USA	Alaska	Kotzebue	MD12-111	KF700602	KF700433	KF700519	This study
Bo. montanum W.H. Wagner	USA	Montana	Lake	Hauk 607/NCU	—	AY138429	DQ849168	Hauk et al., 2003 & 2012
Bo. mormo W.H. Wagner	USA	Minnesota	Cass	Casson s.n.	—	DQ849170	DQ849171	Hauk et al., 2003 & 2012
Bo. "neolunaria M. Stensvold" ined.	USA	Colorado	Guanella Pass	DF19201	KF700607	KF700438	KF700524	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Alaska	Steese Hwy.	BD1205807	KF700611	KF700442	KF700528	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Alaska	Girdwood	BD1206345	KF700606	KF700437	KF700523	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Alaska	Hatcher Pass	BD1206624	KF700608	KF700439	KF700525	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Washington	Big Horn	BL12484-1	KF700605	KF700436	KF700522	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Washington	Whatcom	BL12523-1	KF700612	KF700443	KF700529	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Colorado	Park	Hauk 588/NCU	—	DQ849147	DQ849148	Hauk et al., 2003 & 2012
Bo. "neolunaria M. Stensvold" ined.	USA	Alaska	Selawik	MD12-060	KF700610	KF700441	KF700527	This study
Bo. "neolunaria M. Stensvold" ined.	USA	Alaska	Kodiak Island	SS1231	KF700609	KF700440	KF700526	This study
Bo. "nordicum D. Farrar" ined.	ISL	Vestfiroir	Isafjord	DF13337	KF700613	KF700444	KF700530	This study
Bo. "nordicum D. Farrar" ined.	NOR	More-og-R.	Vestnes	DF17157	KF700614	KF700445	KF700531	This study
Bo. pallidum W.H. Wagner	CAN	Ontario	Algoma	Wagner /MICH	—	DQ849173	DQ849174	Hauk et al., 2003 & 2012
Bo. paradoxum W.H. Wagner	CAN	Alberta	Imp.-district 4	Hauk 610/NCU	—	—	DQ849175	Hauk et al., 2003 & 2012
Bo. pedunculosum W.H. Wagner	USA	Oregon	Wallowa	Hauk 615/NCU	—	AY138434	DQ849176	Hauk et al., 2003 & 2012
Bo. pedunculosum W.H. Wagner	USA	Alaska	Buckland	MD12-078	KF700595	KF700426	KF700512	This study
Bo. pinnatum H. St. John	USA	Alaska	Dutch Harbor	AW2	KF700615	KF700446	KF700532	This study
Bo. pinnatum H. St. John	USA	Alaska	Hatcher Pass	BD1206610	KF700616	KF700447	KF700533	This study
Bo. pinnatum H. St. John	USA	Alaska	Hatcher Pass	BD1206619	KF700617	KF700448	KF700534	This study
Bo. pinnatum H. St. John	USA	Alaska	Kodiak Island	SS1232	KF700618	KF700449	KF700535	This study
Bo. pumicola Coville	USA	Oregon	Deschutes	Hauk 618/NCU	—	AY138428	DQ849178	Hauk et al., 2003 & 2012
Bo. simplex E. Hitchc.	USA	Oregon	Jackson	Hauk 619/NCU	—	AY138427	DQ849179	Hauk et al., 2003 & 2012
Bo. simplex E. Hitchc.	USA	Michigan	Alger	Hauk 661/NCU	—	DQ849180	DQ849181	Hauk et al., 2003 & 2012
Bo. spathulatum W.H. Wagner	CAN	Ontario	Thunder Bay	Hauk 562/NCU	—	DQ849182	DQ849183	Hauk et al., 2003 & 2012
Bo. tunux M. Stensvold & D. Farrar	CAN	Yukon	Kluane NP	DF15209	KF700619	KF700450	KF700536	This study
Bo. tunux M. Stensvold & D. Farrar	USA	Alaska	Yakutat	DF13516	KF700621	KF700452	KF700538	This study
Bo. tunux M. Stensvold & D. Farrar	USA	Colorado	Weston Pass	DF18790	KF700620	KF700451	KF700537	This study
Bo. watertonense W.H. Wagner	CAN	Alberta	Imp.-district 4	Hauk 611/NCU	—	DQ849184	DQ849185	Hauk et al., 2003 & 2012
Bo. yaaxudakeit M. Stensvold & D. Farrar	CAN	Yukon	Kluane NP	DF15097	KF700622	KF700453	KF700539	This study
Bo. yaaxudakeit M. Stensvold & D. Farrar	USA	Alaska	Yakutat	DF13562	KF700623	KF700454	—	This study

Appendix 1. Continued.

Taxa	Country	State/Province	Locality	Voucher accession	GenBank psbA-trnH	GenBank trnL-trnF	GenBank rpL16	Reference
Bp. virginianus (L.) Michx.	USA	Alaska	Girdwood	BD1206330	—	KF700455	KF700540	This study
Sc. multifidum (S.G. Gmelin) M. Nishida ex Tagawa	USA	Alaska	Salcha River	BD1205311	KF700625	KF700457	KF700542	This study
Sc. multifidum (S.G. Gmelin) M. Nishida ex Tagawa	USA	Alaska	Steese Hwy.	BD1205503	KF700626	KF700458	KF700463	This study

Abbreviations: *Botrychium* (*Bo.*); *Botrypus* (*Bp.*); *Sceptridium* (*Sc.*); variety (var.); subspecies (subsp.); Canada (CAN); Iceland (ISL); Greenland (GRL); Norway (NOR); Sweden (SWE), Switzerland (CH); United States (USA); Benjamin Dauphin (BD), Mike Duffy (MD), Don Farrar (DF), Ben Legler (BL), Stacy Studebaker (SS), Abi Woodbridge (AW); Herbarium, University of North Carolina (NCU) Herbarium, Oregon State University (OSU); Herbarium, University of Michigan (MICH); Ada Hayden Herbarium, Iowa State University (ISC); Herbarium, Missouri Botanical Garden (MO).