Genetic Diversity in the Worldwide *Botrychium lunaria* (Ophioglossaceae) Complex, with New Species and New Combinations

Mary Clay Stensvold[1] and Donald R. Farrar[2]

[1] P.O. Box 1042, Sitka, AK 99835, USA; e-mail: kruzov@yahoo.com
[2] Department of Ecology, Evolution and Organismal Biology, Iowa State University, Ames, IA 50011, USA; e-mail: dfarrar@iastate.edu

Abstract. The *Botrychium lunaria* (Ophioglossaceae) complex worldwide includes the named species *B. lunaria*, *B. crenulatum*, *B. tunux,* and *B. yaaxudakeit*. These species have been distinguished from each other morphologically and genetically. This study further investigates the genetic diversity and geographic distribution of this complex, examining a large number of plants worldwide. Enzyme electrophoresis was used to examine allelic variation of 22 loci for 1574 plants of putative *B. lunaria*, *B. crenulatum* and *B. tunux* from North America, Eurasia, and New Zealand, and *B. dusenii* from the Falkland Islands. Variation in allelic composition assessed by genetic identity and cluster analysis using the programs PopGene and STRUCTURE as well as morphology and geography indicated that the complex is composed of six distinct entities; two of which warrant recognition as new species, **B. neolunaria**, endemic to North America, and **B. nordicum**, sister to the *B. lunaria* complex, from Iceland and Norway; and a new combination, **B. lunaria var. melzeri**, endemic to Greenland, Iceland, and Norway. The new taxa are described in this paper. Three entities within *B. tunux* are discussed but not proposed for recognition at this time. *Botrychium lanceolatum,* included in this study, is composed of three morphologically and genetically distinct entities warranting taxonomic recognition.

Keywords: Cryptic species, Genetic diversity, Enzyme electrophoresis, Allelic variation, T-axonomic revision, New taxa of *Botrychium*.

Moonwort ferns (*Botrychium* Sw. subgenus *Botrychium* Clausen) are relatively small and morphologically simple, ranging in height from 1 to 20 cm. Moonworts provide many examples of cryptic speciation where distinguishing morphological characters are few and subtle. One such example is the *Botrychium lunaria* (L.) Sw. complex. Its species morphologically resemble *B. lunaria,* being once-pinnate with broadly fan-shaped pinnae. Besides *B. lunaria,* the complex includes *B. crenulatum* W. H. Wagner, *B. tunux* Stensvold & Farrar, and *B. yaaxudakeit* Stensvold & Farrar, all of which have been segregated from *B. lunaria* (Stensvold et al., 2002; Wagner & Wagner, 1981).

The members of the *Botrychium lunaria* complex are variable in size of the plant, length of the common stalk, sporophore length, proportion and angle of branches, trophophore shape and texture, number of pinna pairs, pinnae shape and size, and pinna margin cutting. Species of the *B. lunaria* complex are morphologically distinct from other diploid moonworts, primarily in having a basal pinna span approaching 180 degrees of a circle. Analyses of variation in the plastid gene *rbcL* (Hauk, 1995), allozymes (Farrar, 1998; Hauk & Haufler, 1999; Stensvold et al., 2002) and combined data sets consisting of morphology, *rbcL* and *trnL-F* plastid sequences (Hauk et al., 2003, 2012; Dauphin et al., 2014) support a distinct *B. lunaria* complex. Wagner and Wagner (1990) used morphological characters to group the once-pinnate moonworts resembling *B. lunaria* as the "*Lunaria* Group", comprised of *B. lunaria, B. minganense* Victorin*, B. crenulatum, B. ascendens* W. H. Wagner*, B. pallidum* W. H. Wagner and *B. spathulatum* W. H. Wagner. However, in the same paper, they questioned whether this was a natural group. Subsequent morphological and genetic work concluded that some of the moonworts of the "*Lunaria* Group" were allotetraploid taxa

Brittonia 69(2): 148–175 (2017), DOI 10.1007/s12228-016-9455-z
ISSN: 0007-196X (print) ISSN: 1938-436X (electronic)

Published Online: 13 December 2016

resulting from hybridization between members of the *B. lunaria* complex and diploid species outside the *B. lunaria* complex. Thus *B. ascendens* is putatively derived from *B. crenulatum* × *B. lineare* (Zika & Farrar, 2009); *B. spathulatum* from *B. lunaria* × *B. campestre* (Farrar, 2011); *B. minganense* from a member of the *B. lunaria* complex × *B. farrarii* Legler & Popovich ined., an undescribed diploid genetically similar to *B. pallidum* (Farrar, 2011). South American populations of *B. lunaria* described by Alston (1960) as *B. dusenii* (Christ) Alston are derived from hybridization between a diploid of the *B. lunaria* complex and a diploid of the *B. campestre* complex (Meza-Torrez et al., 2016).

In the late 1990's, populations of *Botrychium lunaria* from southern Alaska were found exhibiting an unusually wide array of morphological variation. Starch-gel enzyme electrophoresis was used to help identify the plants and investigate potential correlations between genetics and morphology. This genetic analysis led to the recognition of the two new species, *B. tunux* and *B. yaaxudakeit* (Stensvold et al., 2002). Electrophoretic results also showed *B. yaaxudakeit* to be an allotetraploid with the common North American genotype of *B. lunaria* as one parent, and a European genotype of *B. lunaria* as the other parent. Subsequently the European genotype was discovered in the Aleutian Islands, inland Alaska and across Canada. The great dissimilarity between European and American genotypes constituted the first recognition that "*B. lunaria*" worldwide, harbored additional cryptic species.

From 2001 to 2006 *Botrychium lunaria* collections were made from across North America, Iceland, southern Greenland, Europe, Japan, Taiwan, New Zealand and the Falkland Islands to search for previously unrecognized taxa and to determine their genetic relationships. As genetic analysis was conducted on an increasingly large number of *B. lunaria* from many locations, it became clear that several distinct genotypes existed within *B. lunaria*. The relationships of these entities are investigated in this study.

Materials and Methods

PLANT COLLECTION AND PROCESSING

Specimens for this study were collected during 1997 to 2006 from North America including Greenland, Iceland, Norway, Sweden, Denmark, Germany, Poland, France, Taiwan, Japan, the Russian Far East, the Falkland Islands and New Zealand. Multiple plants from multiple populations representative of known taxon ranges were sampled. For each collection, leaves were taken by cutting the base of the common stalk at ground level. Collection times varied from late spring through late summer. As soon as possible after collection the living material was placed in plastic bags, kept cool and quickly sent to the electrophoresis laboratory at the Department of Ecology, Evolution and Organismal Biology at Iowa State University.

Upon arrival at the lab, specimens were refrigerated and assigned electrophoresis voucher numbers. To extract enzymes for electrophoresis, a 0.5 to 1 cm segment of plant tissue was cut from the bottom of the common stalk, then ground using the bottom of a chilled test tube on a chilled spot plate in two drops of a phosphate-polyvinylpyrrolidone grinding buffer solution (Cronn et al., 1997). The resulting homogenate was soaked into a 2 cm by 2 cm piece of Kimwipe (manufactured by Kimberly-Clark) and packed into a 0.5 ml microcentrifuge tube and frozen at –80° C until the time of electrophoresis. Extracts were used in electrophoresis one to nine months after freezing. The remainder of each extracted specimen was pressed as a voucher for morphological study. Voucher specimens were deposited at the Ada Hayden Herbarium (ISC) at Iowa State University.

ENZYME ELECTROPHORESIS

Starch gel enzyme electrophoresis was used to reveal genetic diversity by displaying alleles as banding patterns on a horizontal starch gel of 12% potato starch. Just before electrophoresis, tubes containing the homogenate were allowed to thaw during centrifugation for two minutes at 12,000 rpm to 14,000 rpm. The solution from each tube was absorbed into a corresponding wick measuring 3 mm by 7 mm made from Whatman's chromatography paper (Whatman International, Maidstone, UK), and the wick loaded into a slit paralleling the cathodic end of the starch gel. Twenty-six wicks were run in each gel.

We studied the differential migration patterns for 22 loci of 10 enzymes, using three buffer systems from Soltis et al. (1983): buffer system 7 (0.038 M LiOH, 0.188 M boric acid) for resolving enzyme systems, aspartate aminotransferase (AAT), and triose-phosphate isomerase (TPI); buffer system 9 (0.065 M L-Histidine,

0.015 M to 0.016 M citric acid, anhydrous) for resolving enzyme systems, malate dehydrogenase (MDH), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGD), and phospho-glucoisomerase (PGI); and buffer system 11 (0.4 M citric acid, trisodium salt) for resolving enzyme systems, aconitase (ACN), diaphorase (DIA), isocitrate dehydrogenase (IDH), shikimate dehydrogenase (SKDH).

After loading, the gels were covered with an acetate sheet and an ice pack, and placed in electrophoresis trays in a cooler, where constant electrical current was applied. For buffer system 7, 50 volts were applied at an amperage of 40 mA for 5.5 hours; for buffer system 9, 160 volts were applied at an amperage of 40 mA for 4.5 hours; and for buffer system 11, 80 volts were applied at an amperage of 55 mA for 4.5 hours.

Immediately after electrophoresis, gels were sliced horizontally to produce a thin gel slice for each of the ten enzyme systems and stained with stains specific to each enzyme being assayed. Stains were prepared just before staining and followed the recipes of Soltis et al. (1983). To document the stained bands, gels were placed on a light box, which provided backlighting, photographed from above and the images saved as TIFF files.

SCORING

For most loci, allelic band scores were recorded as numerical values, where the most anodally migrating locus (in cases where there is more than one locus per enzyme system) and allele were assigned the lowest number, and the least anodally migrating locus and allele assigned the highest number. Scoring values were entered into a Microsoft Excel spreadsheet for data storage and preparation for data analysis.

Some taxa consistently displayed additional "accessory" bands migrating with the primary band of a known locus. These loci were *Mdh-3t* (indicating the presence of a band trailing the *Mdh-3* band), *Mdh-3p* (indicating a band preceding the *Mdh-3* band), and *Dia-1p* (indicating the presence of a band preceding the *Dia-1* band). Accessory bands were considered post-translational products of the primary bands (Gastony, 1988) rather than true alleles and not included in the PopGene analysis. Because of their consistent association with certain taxa, occurrence of these accessory bands were used to independently test relationships among groups. Distribution frequencies of these bands were calculated manually.

GENETIC ANALYSIS OF DATA

To investigate genetic diversity in the *Botrychium lunaria* complex, we focused on the basic diploid genotypes. Specimens with banding patterns implying polyploidy or hybridization were omitted. For gene frequency analysis, rare alleles with a frequency of less than 5% were eliminated (Hartl & Clark, 1997). The 1624 analyzed specimens were sorted into groups based on existing taxonomy, morphology, geographic location and allelic composition. Fifty specimens of *B. lanceolatum* (S. G. Gmelin) Ångström, represented by three morphotypes, *B. angustisegmentum* (Pease & A.H. Moore) Fernald, and two morphotypes of *B. lanceolatum* labeled herein as green and red morphotypes, were included in the genetic analysis. When *Botrychium* is divided into sections (Clausen, 1938), *B. lanceolatum* represents the section sister to the *B. lunaria* section.

To investigate genetic variation between groups, the application PopGene (Yeh et al., 1997) was used to compute Nei's (1978) unbiased genetic identity (GI) and genetic distance (D) for the multiple groups through a diploid data analysis of the dataset's co-dominant markers. The computation resulted in a matrix of pairwise genetic identities and genetic distances for all groups. Groups with high GI relationships were combined, resulting ultimately in 11 groups, each having a distinct genotype.

For the final dataset of combined groups, genetic variation within groups was calculated as percent polymorphic loci (P) and mean number of alleles per locus (A). Genetic differentiation between groups was calculated as genetic identities (GI) and genetic distances (D). Phylograms were constructed using an UPGMA analysis of Nei's genetic distance; calculations are adapted from NEIGHBOR in PHYLIP version 3.5c (Felsenstein, 1989). Final phylograms were created from the PopGene output using the application MEGA (Kumar et al., 2004).

To estimate the taxonomic relationship of the specimens, we subjected the dataset to an admixture analysis using the program STRUCTURE version 2.3 (Pritchard et al. 2000). The Bayesian

likelihood analysis of the samples being assigned to K anonymous genetic clusters was conducted for the set of K={6:14}, based on previous knowledge of the taxonomic groupings of the collection. Data was collected for 100,000 replications following a 20,000 replication burn in period. The most likely number of genetic clusters was estimated using the method of Earl & vonHoldt (2012), as implemented in the program STRUCTURE HARVESTER. To determine the likely relationships of the specimens, STRUCTURE assigned individuals to genetic clusters as well as estimating the Neighbor Joining relationship of these clusters.

Results

ENZYME ELECTROPHORESIS

Twenty loci were resolved from 10 enzyme systems in the three buffer systems. Four loci resolved in system 7, two loci for *Aat*, and two for *Tpi*. In system 9 eight loci resolved, two for *Pgm*, one for *6-Pgd*, four for *Mdh* and one for *Pgi*. Eight loci resolved for system 11, four for *Dia*, two for *Acn*, one for *Skdh* and one for *Idh*. After specimens displaying polyploidy, hybridization, or insufficient clarity of banding were removed from the dataset, 1574 specimens of the *Botrychium lunaria* complex remained for further analysis. Through morphological, geographic, and genetic analysis these specimens were assigned to 46 groups in the *B. lunaria* complex and three groups in the *B. lanceolatum* complex (Table 1).

GENETIC ANALYSIS

Twenty loci were resolved among the 49 groups. Within the *Botrychium lunaria* complex 19 loci were polymorphic, with *Dia-2* being monomorphic. A small number of specimens (3 plants) from Norway (group 33) was the single monomorphic group, the remaining 45 groups being polymorphic for at least one locus. For the entire *B. lunaria* complex there were 84 alleles among the 20 loci, with a mean of 4.2 alleles per locus. The most polymorphic loci were *Pgi* and *Dia-1*, each with seven alleles, and *Acn-1* and *Dia-3* each with five alleles. The least polymorphic loci were *Tpi-1* and *Mdh-4* with two alleles each, and *Tpi-1*, *Acn-2*, *Aat-2* and *Skdh* each with three alleles.

A matrix of Nei's (1978) unbiased GI and D for the 49 groups, calculated from allele frequencies, revealed patterns of cohesion and distinction among the 49 groups. From this analysis we recognized 11 supergroups, each possessing high GI values within the supergroup and much lower GI values with members of other supergroups:

1) LUNLUN combined 23 groups (60 to 91) of circumpolar *Botrychium lunaria* var. *lunaria*
2) LUNMEL combined three groups (31 to 33) from Greenland, Iceland and Norway that we propose recognizing as *Botrychium lunaria* var. *melzeri*.
3) NEOLUN combined nine North American groups that we propose recognizing as a new species *Botrychium neolunaria*.
4) CRENUL was recognized as a single group (40) representing plants from throughout the range of North American *Botrychium crenulatum*.

5 to 7) TUNTUN, TUNNEV, and TUNNOR combined six groups (50 to 56) representing northwestern North American *Botrychium tunux*, plus southernmost North American populations (group 58) in Nevada, and Norwegian populations (group 57) of this species.

8) NORDIC combined two groups (92 and 93) from Iceland and Norway of a new taxon that we propose recognizing as *Botrychium nordicum*.

9 to 11) LANANG, LANRED, and LANGRN represent three groups of *Botrychium* section Lanceolatae (Clausen, 1938) that we recognize as *Botrychium angustisegmentum* (from eastern NA), *B. lanceolatum* (red morphotype/genotype from western NA and Greenland) and *B. lanceolatum* (green morphotype/genotype from western NA and Iceland).

A summary of the supergroup's genetic variability as the percentage of polymorphic loci (P) and the mean number of alleles per locus (A), along with the sample size is shown in Table 2. Genetic variability of taxa within the *Botrychium lunaria* complex, as shown by percent polymorphic loci, ranged from low, 5% in TUNNEV, to moderate, 35% in CRENUL. The three *B. lanceolatum* taxa each had low genetic variability, with the percent polymorphic loci ranging from 5% to 15%. Table 3 compares genetic variability in the *B. lunaria* complex with other *Botrychium* species, ferns in general and seed plants. Although high for *Botrychium*, the *B. lunaria* complex displayed a low

Table 1 Forty-six groups of the *Botrychium lunaria* complex, and three groups of *B. lanceolatum*, totaling 1624 specimens and 49 groups.

Group number	Initial group name, general location	Final group name	Number of specimens
7	*B. angustisegmentum*, Alberta, Ohio, Michigan	LANANG	15
8	*B. lanceolatum* red morph Yukon, British Columbia, Colorado, Greenland	LANRED	15
9	*B. lanceolatum* green morph Alaska, Yukon, Oregon, Iceland to Norway	LANGRN	20
20	*B. lunaria* Amer.[a], Michigan, South Dakota	NEOLUN	4
21	*B. lunaria* Amer., Newfoundland New Brunswick	NEOLUN	21
22	*B. lunaria* Amer., Washington State	NEOLUN	6
23	*B. lunaria* Amer., Colorado	NEOLUN	29
24	*B. lunaria* Amer., Alaska (south coast)	NEOLUN	295
25	*B. lunaria* Amer., Manitoba (Churchill)	NEOLUN	10
26	*B. lunaria* Amer., British Columbia, Alberta	NEOLUN	31
27	*B. lunaria* Amer., Yukon (southwest)	NEOLUN	62
28	*B. lunaria* Amer., Alaska (interior & mountains)	NEOLUN	72
31	*B. lunaria* circum., Melzer, Iceland	LUNMEL	11
32	*B. lunaria* circum., Melzer, Greenland	LUNMEL	56
33	*B. lunaria* circum., Melzer, Norway	LUNMEL	3
40	*B. crenulatum,* North America (western)	CRENUL	115
50	*B. tunux*, Alaska (Yakutat)	TUNTUN	150
51	*B. tunux*, Canada, Yukon (southwest)	TUNTUN	69
52	*B. tunux*, Alaska (Wrangell-St. Elias Mountains)	TUNTUN	21
53	*B. tunux*, Alberta, Montana	TUNTUN	13
55	*B. tunux*, Alaska (Chichagof Island)	TUNTUN	4
56	*B. tunux*, Alaska (Pribilof Islands, Dall Island)	TUNTUN	22
57	*B. tunux*, Norway (Finmark, Lofoten Islands)	TUNTUN	28
58	*B. tunux*, Nevada (Spring Mountains)	TUNTUN	27
60	*B. lunaria* circum.[b], Greenland (Narsarsuaq)	LUNLUN	110
62	*B. lunaria* circum., Quebec, Newfoundland	LUNLUN	5
64	*B. lunaria* circum., Yukon (southwest)	LUNLUN	21
65	*B. lunaria* circum., Alaska (south coast & mts.)	LUNLUN	9
70	*B. lunaria* circum., Iceland (no location)	LUNLUN	14
71	*B. lunaria* circum., Iceland (Vogar)	LUNLUN	9
72	*B. lunaria* circum., Iceland (Grindavik)	LUNLUN	28
73	*B. lunaria* circum., Iceland (northwest)	LUNLUN	9
74	*B. lunaria* circum., Iceland (Jokulsarlon)	LUNLUN	42
75	*B. lunaria* circum., Iceland (Landbrot)	LUNLUN	26
77	*B. lunaria* circum., Iceland (Myvatn)	LUNLUN	51
78	*B. lunaria* circum., Iceland (Vogar)	LUNLUN	27
80	*B. lunaria* circum., Norway (Finmark)	LUNLUN	5
81	*B. lunaria* circum., Norway (Lofoten)	LUNLUN	17
82	*B. lunaria* circum., Norway (Rogaland coast)	LUNLUN	20
83	*B. lunaria* circum., Norway (Hordaland)	LUNLUN	8
84	*B. lunaria* circum., Norway (More og Romsdal)	LUNLUN	13
85	*B. lunaria* circum., Norway (Buskarud)	LUNLUN	6
86	*B. lunaria* circum., Norway (M. og R., Sundal)	LUNLUN	18
87	*B. lunaria* circum., Norway (Oppland)	LUNLUN	20
88	*B. lunaria* circum., Sweden (no location)	LUNLUN	5
90	*B. lunaria* circum., Denmark, Scotland	LUNLUN	7
91	*B. lunaria* circum., Poland, Austria, France	LUNLUN	6
92	*B. lunaria* circum., Incised, Iceland (northwest)	NORDIC	40
93	*B. lunaria* circum., Incised, Norway	NORDIC	8

Plants were assigned to groups based on genotype, location and morphology. Group numbers range from 7 to 93 and are a scheme of specimen category rather than a count of groups. The initial group name is the name currently applied to the plants, and final group designation indicates a name based on the results of the electrophoretic analysis.

[a] American genotype, [b] Circumpolar genotype.

TABLE 2 Genetic variability at 20 allozyme loci for eleven taxa, eight in the *Botrychium lunaria* complex and three in *B. lanceolatum* as shown by percentage of polymorphic loci (P) and the mean number of alleles per locus (A).

Taxon name abbreviation	Number of specimens	Number of polymorphic loci	P	A
NEOLUN	536	4	20	1.20
LUNLUN	471	6	30	1.45
CRENUL	115	7	35	1.40
LUNMEL	71	2	10	1.10
NORDIC	48	3	15	1.15
TUNTUN	279	3	15	1.20
TUNNEV	27	1	5	1.05
TUNNOR	27	6	30	1.36
LANANG	15	2	10	1.10
LANRED	15	3	15	1.15
LANGRN	20	1	5	1.05

number of polymorphic loci and alleles per locus relative to other genera.

The supergoups were further analyzed for exclusion of rare alleles (those with frequencies less than 5%) from the dataset, leaving 20 loci resolved for 51 alleles. On this basis, *Mdh-4* and *Dia-2* were found to be invariable and excluded from further analysis within the *Botrychium lunaria* complex as uninformative. Distribution of alleles by taxon is presented in Table 4.

A matrix of Nei's genetic identity and genetic distance (Table 5) was calculated from allele frequencies for the eleven supergroups. Pairwise genetic identity values between supergroups within the *Botrychium lunaria* complex were generally below 0.8 with exceptions of LUNMEL which had higher genetic identity with its sister variety LUNLUN, and CRENUL which displayed high identity with North American groups of LUNLUN. Relationships within and among groups are explored in greater detail in the discussion.

An UPGMA analysis of Nei's genetic distance was performed to display genetic similarities among the 11 supergroups (Fig. 1). NORDIC and NEOLUN are well separated from the remaining groups, which form two clusters: the LUNLUN, CRENUL and LUNMEL cluster; and TUNTUN, TUNNEV and TUNNOR. The three *Botrychium lanceolatum* groups (LANANG, LANRED and LANGRN) are well separated from all of the groups in the *B. lunaria* complex.

The distribution of accessory bands occurring at >5% among the supergroups are shown in Fig. 1. The presence and absence of accessory bands is similar for distantly related NEOLUN and NORDIC, and similar for the more closely related *Botrychium lunaria* and the *B. tunux* clusters. LUNCRN was distinctive in being the only taxon having both states of the *Mdh-3* accessory bands

TABLE 3 Comparison of genetic distinction between seed plants, ferns and *Botrychium* at the taxonomic levels of infraspecific groups, varieties, subspecies and interspecific taxa as measured by genetic identity (GI). A GI value of 1.00 means the plants are allelically identical.

Taxonomic level	Seed plants	Ferns	*Botrychium*
Conspecific	0.80 – 1.00, mean 0.95[a]	0.83 – 1.0, mean 0.94[b] 0.92 – 1.0, mean 0.96[d]	0.67 – 1.00, mean 0.91[c]
Variety	0.71 – 0.99, mean 0.91[a]		0.67 – 1.00, mean 0.85[c]
Subspecies		0.88 – 0.94, mean 0.91[e]	0.74 – 0.79, mean 0.77[f]
Congeneric	0.35 – 0.97, mean 0.67[g]	0.09 – 0.98 mean 0.57[b] 0.19 – 0.92, mean 0.55[c]	0.18 – 0. 80, mean 0.48[b] 0.05 – 0. 84, mean 0.41[c]

[a] Gottleib 1981; Crawford 1983, 1985; [b] Farrar, 2005; [c] Hauk & Haufler, 1999 (*B. simplex*); [d] Suter et al., 2000 (European *Asplenium*); [e] Kelloff et al., 2002 (*Athyrium*); [f] This study (*B. angustisegmentum*, (green form), *B lanceolatum* (red form); [g] Crawford 1983.

TABLE 4 Allele frequencies of 20 isozyme loci for the eight diploid taxa identified in the *Botrychium lunaria* complex, and three taxa of *B. lanceolatum*.

		Taxon name abbreviation										
Locus name	Allele	NEOLUN	LUNLUN	CRENUL	LUNMEL	NORDIC	TUNTUN	TUNNEV	TUNNOR	LANANG	LANRED	LANGRN
Tpi-1	3	0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	4	1.000	0	0	0	0	0	0	0	0	0	0
N		531	467	110	68	39	247	27	27	14	15	20
Tpi-2	3	0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	4	1.000	0	0	0	0	0	0	0	0	0	0
N		536	471	115	68	39	270	27	27	14	15	20
Pgi	1.5	0	0	0	0	0	0.097	0	0	0	0	0
	2	1.000	1.000	0.892	1.000	0	0.629	0	1.000	0	0	0
	4	0	0	0.108	0	1.000	0.273	1.000	0	1.000	1.000	1.000
N		521	435	111	71	48	267	27	20	15	15	20
6-Pgd	3.8	0.094	0	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	1.000	1.000	0.926	1.000	1.000	1.000
	5	0.906	1.000	1.000	1.000	0	0	0	0.074	0	0	0
	7	0	0	0	0	1.000	0	0	0	0	0	0
N		505	437	115	69	42	274	21	27	15	15	20
Mdh-1	1.5	0	1.000	1.000	1.000	0	1.000	1.000	1.000	0	0	0
	1.8	0	0	0	0	1.000	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	1.000	1.000	1.000
	3	1.000	0	0	0	0	0	0	0	0	0	0
N		532	471	111	69	48	275	54	25	15	15	20
Mdh-2	2	0	0	1.000	0	0	0	0	0	0	0	0
	3	0	1.000	0	1.000	1.000	1.000	1.000	1.000	1.000	0	1.000
	5	1.000	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0	1.000	0
N		534	466	115	71	48	276	54	27	15	15	20
Mdh-3	1	0	0	0.07	0	0	0	0	0	0	0	0
	2	1.000	1.000	0.93	1.000	0.468	1.000	1.000	1.000	0	0	0
	2.5	0	0	0	0	0	0	0	0	0.267	0.533	0
	3	0	0	0	0	0.531	0	0	0	0.733	0.467	1.000
N		524	465	115	71	47	274	54	27	15	15	20
Mdh-4	2	0	0	0	0	0	0	0	0	1.000	1.000	1.000
	Null	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0	0	0
N		523	471	115	71	45	279	54	27	14	13	20
Pgm-1	0.5	0	0	0.174	0	0	0	0	0	0	0	0
	1	1.000	1.000	0.826	1.000	1.000	1.000	0	1.000	0	0	0
	3	0	0	0	0	0	0	1.000	0	1.000	1.000	1.000
N		531	467	115	71	48	274	25	27	14	15	20

TABLE 4 Continued

		Taxon name abbreviation										
Locus name	Allele	NEOLUN	LUNLUN	CRENUL	LUNMEL	NORDIC	TUNTUN	TUNNEV	TUNNOR	LANANG	LANRED	LANGRN
Pgm-2	1	0	0	0	0	0	1.000	1.000	1.000	0	0	0
	1.5	0	0	0	0	0	0	0	0	1.000	1.000	1.000
	2	1.000	1.000	1.000	1.000	1.000	0	0	0	0	0	0
N		499	409	96	69	48	210	23	13	12	13	20
Acn-1	0.5	0	0.301	0	0	1.000	0	0	0	0	0	0
	0.8	0	0	0	0.062	0	0	0	0	0	0	0
	1	1.000	0.699	1.000	0.936	0	1.000	1.000	1.000	0	0	0
	3	0	0	0	0	0	0	0	0	1.000	1.000	1.000
N		201	209	52	48	44	136	17	3	9	6	10
	2	0	0	0	0	0	0	0	0.2	0	0	0
	3	1.000	1.000	0.904	1.000	0	1.000	1.000	0.8	1.000	1.000	1.000
N		378	338	83	66	47	223	19	10	14	15	20
Aat-2	2	0	0	0	0	0	0	0	0.074	0	0	0
	3	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.926	1.000	1.000	1.000
N		520	446	111	70	48	275	26	27	15	15	20
Aat-4	0.2	0	0	0	0	0	0	0	0	1.000	0	0
	0.5	0	0.133	0	1.000	0	0.748	0	0	0	0.429	0
	1	0	0	0	0	0	0	0	0	0	0.571	1.000
	2	0.089	0.060	0.245	0	0	0	0	0.143	0	0	0
	3	0.912	0.807	0.755	0	1.000	0.252	1.000	0.857	0	0	0
N		486	399	98	69	48	246	25	14	14	14	16
Dia-1	1	0	0	0	0	0	0	0	0	1.000	1.000	1.000
	2	0.904	0.763	1.000	0	1.000	0.356	0.173	0.308	0	0	0
	4	0.096	0.050		1.000	0	0	0	0.539	0	0	0
	5	0	0.114	0	0	0	0	0	0	0	0	0
	Null	0	0.074	0	0	0	0.644	0.827	0.154	0	0	0
N		479	379	111	64	48	261	26	13	15	15	20
Dia-2	0.5	0	0	0	0	0	0	0	0	0	0.867	1.000
	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.133	0
N		499	407	111	68	48	236	27	16	15	15	20
Dia-3	2.5	0	0	0	0	0	0	0	0	0.6	0	0.941
	3	1.000	1.000	1.000	0.5	1.000	1.000	1.000	1.000	0.4	1.000	0.059
	4	0	0	0	0.5	0	0	0	0	0	0	0
N		403	213	107	6	48	199	23	12	5	13	17
Dia-4	4	0.228	0.185	0	0	0.4	0	0	-	0	0	0
	5	0	0	0	0	0	0	0	-	1.000	0	0
	6	0	0	0	0	0	0	0	-	0	1.000	1.000
	8	0.772	0.815	1.000	1.00	0.6	1.000	1.000	-	0	0	0
N		241	178	12	23	40	118	7	0	13	15	20
Idh	1									1.000	1.000	

Table 4 Continued

Locus name	Allele	Taxon name abbreviation NEOLUN	LUNLUN	CRENUL	LUNMEL	NORDIC	TUNTUN	TUNNEV	TUNNOR	LANANG	LANRED	LANGRN
	2	1.000	0.101	0.084	0	0.104	1.000	0	0.938	0	0	0
	3	0	0	0.439	0	0	0	0	0	0	0	0
	4	0	0.899	0.477	1.000	0.896	0	1.000	0.063	0	0	1.000
N		454	414	107	68	48	220	21	16	15	15	20
Skdh	1	0	0.647	0.944	0	0	1.000	1.000	0	0	1.000	0
	2	1.000	0.353	0.056	1.000	1.000	0	0	1.000	1.000	0	1.000
N		483	432	108	68	46	242	26	20	15	15	20

N = sample size

Table 5 Matrix of Nei's (1978) unbiased genetic identity (above the diagonal) and genetic distance (below the diagonal) for the 11 taxa.

GROUP NAME	NEOLUN	LUNLUN	CRENUL	LUNMEL	NORDIC	TUNTUN	TUNNEV	TUNNOR	LANANG	LANRED	LANGRN
NEOLUN	****	0.7110	0.6963	0.6343	0.5237	0.5798	0.4551	0.6491	0.2301	0.1653	0.1568
LUNLUN	0.3411	****	0.9159	0.8809	0.7117	0.7942	0.7482	0.7912	0.3651	0.3184	0.3344
CRENUL	0.3620	0.0879	****	0.7744	0.6117	0.7513	0.7024	0.7041	0.2935	0.3299	0.2422
LUNMEL	0.4552	0.1268	0.2557	****	0.6004	0.7271	0.6367	0.7535	0.3744	0.2656	0.3582
NORDIC	0.6469	0.3400	0.4915	0.5101	****	0.5366	0.5781	0.5914	0.4108	0.2863	0.3869
TUNTUN	0.5451	0.2304	0.2859	0.3186	0.6225	****	0.8409	0.8680	0.4067	0.4132	0.3289
TUNNEV	0.7873	0.2900	0.3532	0.4515	0.5480	0.1733	****	0.7421	0.4845	0.4750	0.4575
TUNNOR	0.4321	0.2342	0.3509	0.2831	0.5252	0.1416	0.2983	****	0.4414	0.3200	0.3639
LANANG	1.4691	1.0075	1.2257	0.9823	0.8896	0.8997	0.7246	0.8178	****	0.7406	0.7862
LANRED	1.7997	1.1443	1.1090	1.3257	1.2506	0.8838	0.7445	1.1395	0.3003	****	0.7747
LANGRN	1.8530	1.0956	1.4181	1.0266	0.9496	1.1121	0.7819	1.0110	0.2406	0.2553	****

Taxon name abbreviations are shown on the top row and left hand column under the group name

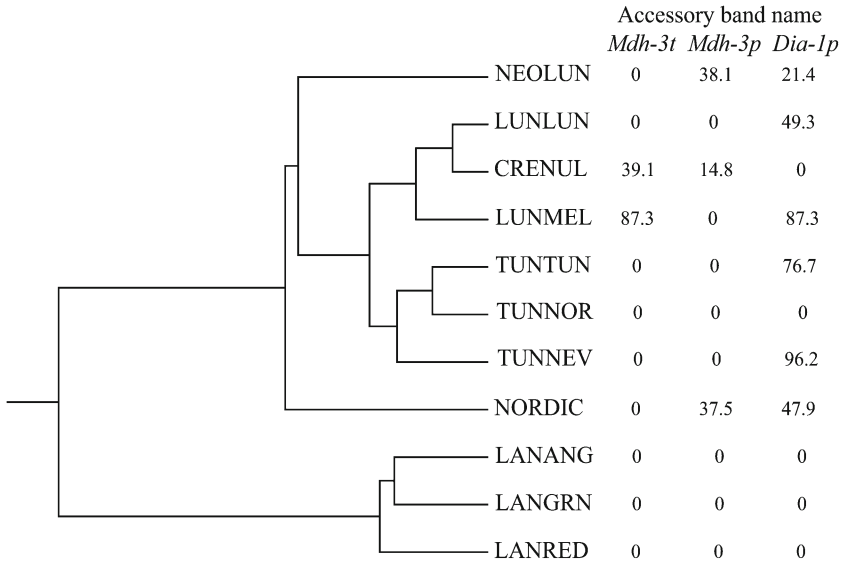

Fig. 1. Phenogram displaying the relationships of the supergroups. The columns to the right of each group summarize the presence and absence of electrophoretic accessory bands by the percent of the plants in each supergroup displaying the accessory bands.

and the only species to not contain *Dia-1p* accessory band. LUNMEL was distinct in being the only group to display only *Mdh-3t* and *Dia-1p*. No samples of *B. lanceolatum* displayed accessory bands.

The admixture (STRUCTURE) analysis identified nine groups as the most likely number of anonymous genetic clusters in the complete data set. The individual assignment tests to the nine

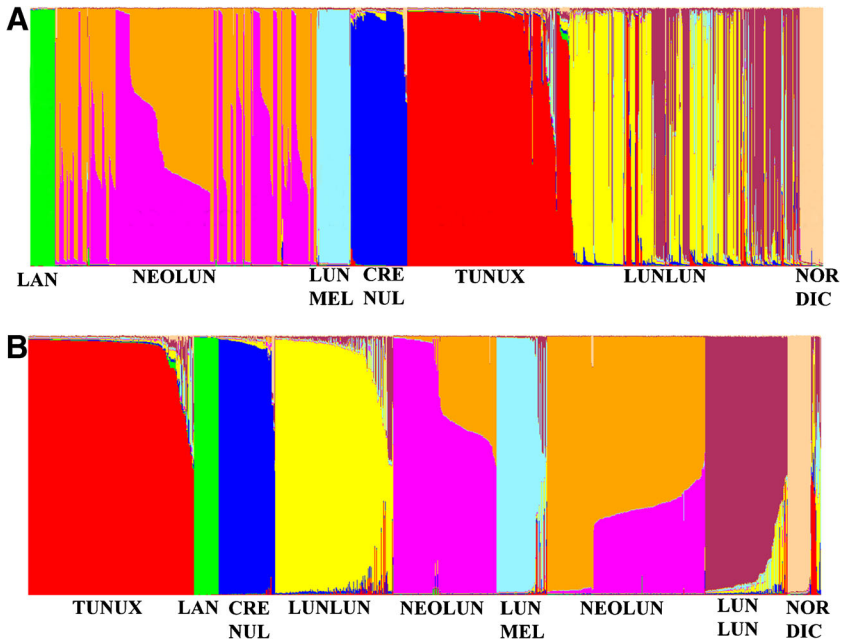

Fig. 2. **A.** Each thin vertical bar represents one individual of 1624 plants, ordered by group number (see Table 1). STRUCTURE analysis identified nine groups as the most likely number of genetic clusters in the dataset. Individuals sharing genetic composition with two or more genotypes are represented by a *multicolored bar*. **B.** Individuals reordered by genotype as reflected by color composition.

groups combined the three *Botrychium tunux* groups, and combined the Lanceolatae group (Fig. 2a). The analysis clearly differentiated the smaller Lunariae groups LUNMEL, CRENUL and NORDIC, as well as the two largest groups, LUNLUN and NEOLUN, which displayed greater variation among individuals within each group. The individual assignment tests assigned LUNLUN and NEOLUN to two groups each. Within the NEOLUN groups there was no apparent correlation of this diversity with geography, but within the LUNLUN groups the diversity was split between two geographic areas, Europe plus a portion of plants from Iceland vs. North America plus Greenland plus a portion of plants from Iceland (Fig. 2b). A neighbor joining tree generated by STRUCTURE (Fig. 3.) displays the relationship between the nine groups.

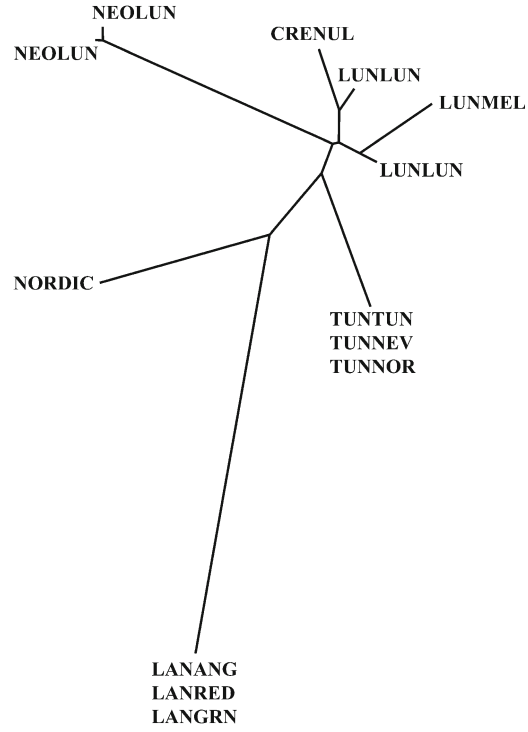

Fig. 3. Neighbor Joining tree displaying the relationships between the supergroups of the *Botrychium lunaria* complex and *B. lanceolatum*.

Discussion

Genetic variation within the *Botrychium lunaria* complex across North America and Eurasia support taxonomic recognition of at least six genetically distinct diploid taxa in addition to the tetraploid *B. yaaxudakeit*. Genetic distinction of these taxa is based on differences in allelic composition, genetic variability within the taxa and overall genetic differentiation between the taxa measured as Nei's (1978) genetic identities.

Earlier genetic analyses using enzyme electrophoresis to assay for allelic variation revealed little genetic diversity within *Botrychium lunaria* (Farrar, 1998; Hauk & Haufler, 1999) due to limited sampling in a small portion of the species' range in the United States excluding Alaska. Thus, those studies sampled only the taxon we recognize as *B. neolunaria*.

Past studies have shown that *Botrychium* has an extremely high level of inbreeding (McCauley et al., 1985; Soltis & Soltis 1986, 1992; Hauk & Haufler, 1999; Farrar, 1998, 2005) through a breeding system known as intragametophytic selfing (Klekowski, 1979). In our study, less than 5% of the individuals analyzed displayed heterozygosity.

The following considerations provide a background for discussions of taxon recognition and taxonomic ranking of the entities we have identified in the *Botrychium lunaria* complex.

1) Intragametophytic selfing has profound effects on genetic variability of individuals. Resulting sporophytes are homozygous and genetically identical to the parent gametophyte. Progeny of such sporophytes are likewise genetic "clones" of their parents, barring rare cases of outcrossing between gametophytes of different genotypes, and progeny of occasional heterozygotes will probably again be homozygous because of the very high likelihood of intragametophytic selfing.

Although reducing genetic variability, intragametophytic selfing also generates genetic and ecological benefits. Successful progeny are purged of recessive lethal alleles, and a single spore of a well-adapted genotype is capable of establishing a new population in a distant location.

2) Usual measures of overall genetic variability are percent polymorphic loci (P), and mean number of alleles per locus (A). In addition to allele distributions, overall levels of genetic variability reflect gene flow and migration within taxa and provide insight into progenitor-derivative relationships. Compared with seed plants and ferns

in general, *Botrychium* species have a lower percent of polymorphic loci and lower average number of alleles per locus. Variability within the *B. lunaria* complex is somewhat lower than average for the genus (Table 6). Taxa and populations derived by limited migration (founder effect) may be expected to maintain a subset of the alleles of the source taxon or population, providing evidence of biogeographic as well as phylogenetic history.

3) Many taxonomists have used genetic identity values (calculated from allele frequencies) as a guide to measuring genetic distinctness between taxa and as an aid in determining taxon rank. Genetic identities between groups can range from a high of 1.0 where groups have identical alleles in identical frequencies down to a low of 0.0 where pairwise groups share no alleles (Crawford, 1985); thus closely related, or non-diverged taxa have on average higher GIs than do less closely related, diverged taxa. Table 3 presents some comparisons of genetic identities between infraspecific populations, varieties, subspecies and species for seed plants, ferns and *Botrychium.* The range of GIs between taxa reflects, in part, different taxonomic concepts among taxonomists. Consequently, mean values reflecting a consensus of taxon concepts form the most appropriate guide. Based on many studies the average GI between populations of a species is near 0.95. This is consistent with Crawford's (1989) suggestion that that GIs for populations within species are generally above 0.90. Cosner and Crawford (1990) observed that the genetic identities between varieties and subspecies of seed plants are often similar to the GIs among populations within species (note Table 3), attributing these high values to a lack of reproductive isolation. Comparisons among varieties and subspecies of ferns indicate GIs intermediate between those among populations within a species and those among congeneric species. At the congeneric level, GIs among species average 0.67. After reviewing numerous taxonomic studies employing GI values, Farrar (1998) suggested that a GI value of 0.70 or less usually indicates differences at the level of species.

4) In addition to variations in general allelic composition, the presence or absence of accessory bands (at *Mdh-3t, Mdh-3p* and *Dia-1p* in *Botrychium* taxa) that associate with particular taxa may provide independent evidence of genetic relationships between the taxa.

TAXON RECOGNITION

Six taxa of the *Botrychium lunaria* complex identified in the genetic analysis are discussed below, and compared to diversity within the *B. lanceolatum* complex. Each possesses a distinctive morphology (Figs. 4, and 5) and distribution, and is genetically characterized by unique alleles (Table 4), a trait considered indicative of distinct taxa (Gottlieb, 1977), and a characteristic complement of accessory bands. Levels of genetic cohesion among subgroups within each taxon, and distinctions among the six taxa are discussed in regard to overall genetic variability, and genetic identities. Combining these indicators of taxon distinction and applying genetic identity guidelines suggested by Crawford (1989), we recommend levels of taxonomic recognition for each taxon. Finally, we briefly discuss the disposition of three entities associated with the *Botrychium*

TABLE 6 Comparison of genetic diversity between seed plants, ferns, genus *Botrychium* and the *B. lunaria* complex analyzed in this study as measured by percent polymorphic loci and average number of alleles per locus.

Taxon group	Percent polymorphic loci	Average number of alleles per locus	Reference
Seed plants	mean 58.9	mean 2.29	Hamrick & Godt, 1990
Ferns	0.00 – 80, mean 36	1.00 – 2.8, mean 1.65	Li & Haufler, 1999 (data for 32 taxa)
Botrychium	0.00 – 16.7, mean 7.44	1.00 – 1.2, mean 1.10	Farrar, 1998
	0.00 – 21, mean 7.30	1.00 – 1.21, mean 1.07	(data for nine diploid species) Hauk & Haufler, 1999 (data for five diploid species)
B. lunaria complex	5 – 35, mean 20	1.05 – 1.45, mean 1.20	This study

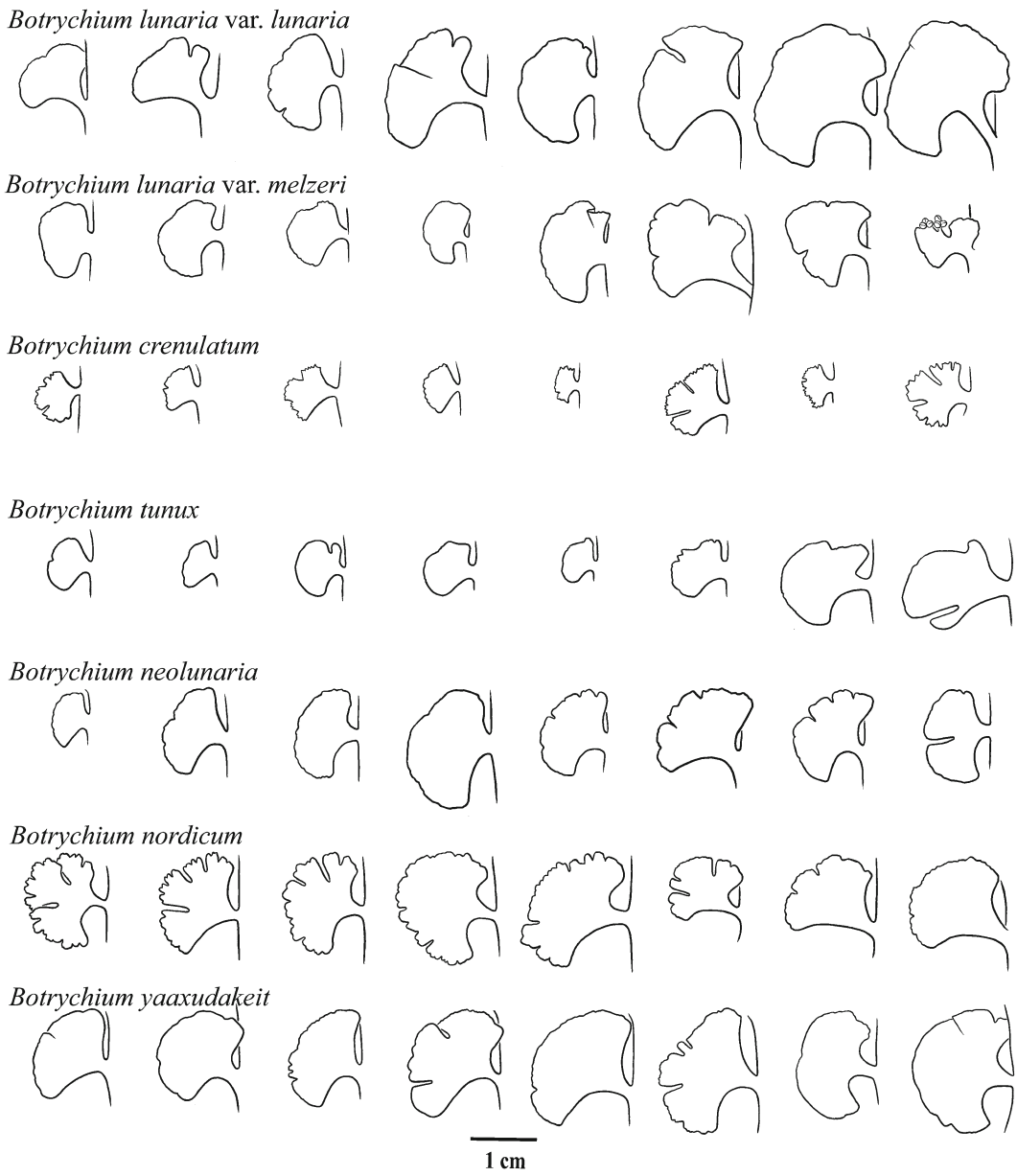

Fig. 4. Comparative basal pinnae outlines of *Botrychium lunaria* var. *lunaria, B. lunaria* var. *melzeri, B. crenulatum, B. tunux, B. neolunaria, B. nordicum,* and *B. yaaxudakeit.* Pinnae selected from plants throughout the range of each taxon to show representative variability.

lunaria complex, but not included in this analysis. Following this section, three new taxa within the *B. lunaria* complex are formally described. Stensvold (2008) presented further details of the genetic analyses summarized herein.

Botrychium angustisegmentum (LANANG) and B. lanceolatum with two morphotypes "red" (LANRED) and "green" (LANGRN).—These taxa are sister to all once-pinnate moonworts, the *Botrychium lanceolatum* complex differs strongly from the *B. lunaria* complex in its twice-pinnate morphology and very low GI values ranging from 0.1568 to 0.4845, these values strongly influenced by presence of

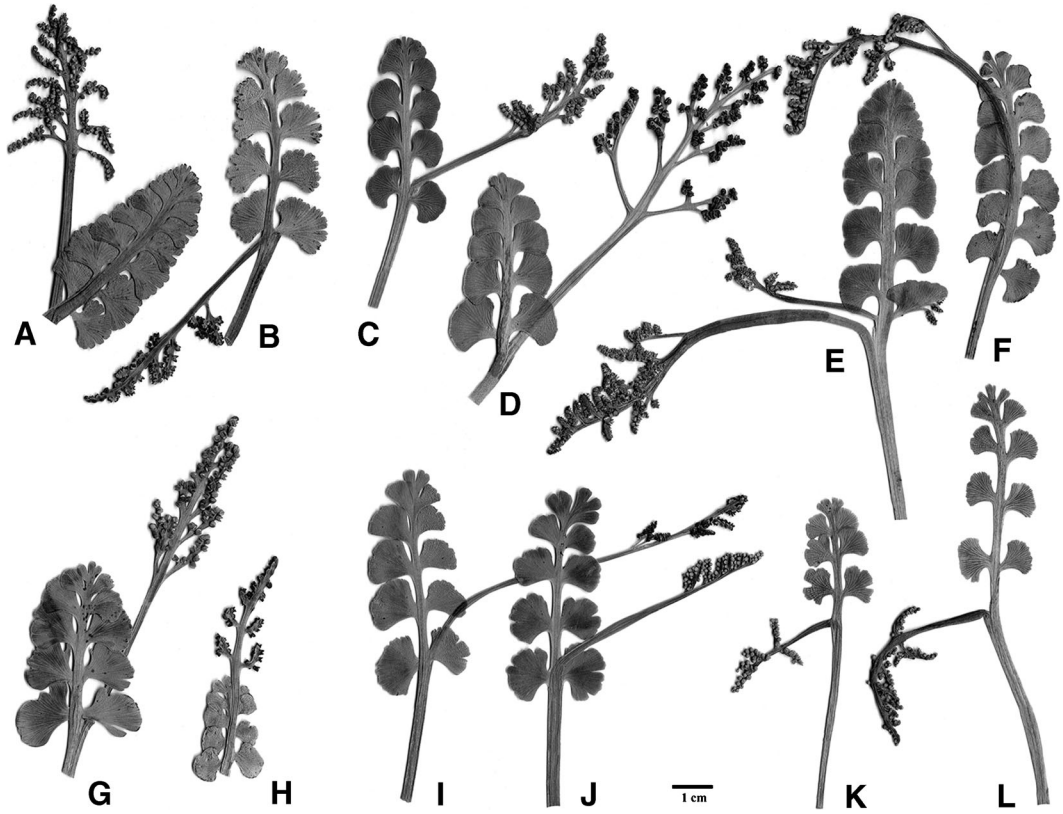

FIG. 5. Morphological comparison of pressed specimens of taxa in the *Botrychium lunaria* complex. Numbers provided below in brackets are electrophoresis voucher numbers. **A.** *Botrychium nordicum*, Norway, More og Romsdal, near Vestnes (*D. Holtan s.n.* [17159], ISC). **B.** *B. nordicum,* Iceland, Isafjord, Kaldalen, (*P. Struck s.n.* [11318], ISC). **C.** *B. lunaria* var. *lunaria,* Canada, Ontario, James Bay (*S. Brinker 2066* [17438], ISC). **D.** *B. lunaria* var. *lunaria*, Canada, Quebec, Bic National Park (*Cayoutte s.n.* [16496], ISC). **E, F.** *B. lunaria* var. *melzeri*, Greenland, Narsarsuaq area (*P. Struck s.n.* [8814, 12808], ISC). **G.** *B. tunux*, USA, Alaska, Yakutat (*M. Stensvold & D. Farrar 7936* [5473], ISC). **H.** *B. tunux*, Norway, Finmark (*P. Zika 16448* [6032], ISC), **I.** *B. neolunaria* USA, Alaska, Delta Junction (*A. Geise s n.* [15286], ISC). **J.** *B. neolunaria,* USA, Michigan, Chippewa County, Trout Lake (*D. Henson s.n.* [1034], ISC). **K.** *B. crenulatum,* USA, Utah, Wasatch County, Silver Meadow (*D. VanKeuren s.n.* [6766], ISC). **L.** *B. crenulatum,* USA, Oregon, Wallowa County, Lostine Canyon (*D. Farrar* [3940], ISC).

12 alleles unique to the *B. lanceolatum* complex. It also displays none of the accessory bands variously characterizing all taxa of the *B. lunaria* complex. Genetic identities among the three taxa within the *B. lanceolatum* provide useful comparisons for determining taxonomic placement of taxa within the *B. lunaria* complex.

In current taxonomy the *Botrychium lanceolatum* complex consists of two named species, *B.angustisegmentum* and *B. lanceolatum*. However, our study found two morphologically and genetically distinct taxa within *B. lanceolatum*. These are a morphotype with a red-colored common stalk and upwardly curved basal pinnae with the basiscopic pinnules twice as long as their acroscopic counterparts, and a morphotype with a green-colored common stalk and straight basal pinnae with basiscopic and acroscopic pinnules of equal length. The GI between these two morphotypes, at 0.7747, was approximately equal to that between either morphotype and *B. angustisegmentum,* 0.7406 (red) and 0.7862 (green). Based on these results, the taxonomy of the *B. lanceolatum* complex is being reassessed.

Botrychium lunaria var. *lunaria (LUNLUN).*—This circumpolar genotype that includes plants from boreal and extreme north temperate North America, southwest Greenland, Iceland, boreal and temperate Europe, and may also extend across boreal and north temperate

Asia. This taxon is morphologically variable but generally characterized as having a narrowly ovate trophophore with fleshy, symmetrical, fan-shaped pinnae with entire to occasionally shallowly cleft outer margins, and a sporophore with a stalk equaling the length of the trophophore and broadly spreading branches occupying ½ of the entire trophophore. *Botrychium lunaria* var. *lunaria* can be effectively circumscribed as lacking the morphological departures from the above description that characterize other taxa in the complex (Figs. 4, and 5).

Botrychium lunaria var. *lunaria* has the allele combination of *Tpi-1*=3, *Tpi-2*=3, *Mdh-1*=1.5 and *Mdh-2*=3 (frequency of 100%), which is distinct from *B. neolunaria*, but similar to all other taxa in the complex. It has a single unique allele, *Dia-1*=5, that occurring with a low frequency of 0.114. Accessory bands *Mdh-3t* and *Mdh-3p* were rare throughout the range of *B. lunaria* var. *lunaria*, especially so in Europe. Overall, frequency of these bands was less than 5% and thus omitted from characterization of the species (Fig. 1).

Genetic variation within *Botrychium lunaria* var. *lunaria* was the highest in the complex, with 30% polymorphic loci and an average of 1.45 alleles per locus. This level of genetic variation can be attributed in part to the broad circumpolar distribution and large number of specimens sampled (471). Despite this variability, *B. lunaria* var. *lunaria* contains only one unique allele, whereas other taxa of the complex display 24 alleles not present in *B. lunaria* var. *lunaria*, indicating diversification beyond subset partitioning of this taxon.

The mean GI among the initial 23 groups that were combined to compose *Botrychium lunaria* var. *lunaria* was 0.9182, with no values between large groups (n >20) less than 0.9111. Its GI values with other members of the complex ranged from a low of 0.7110 with *B. neolunaria*, to a high of 0.8590 between CRENUL and European groups of *B. lunaria* var. *lunaria* (but see discussion of *B. crenulatum*). It has a mean GI of 0.8809 with its sister variety LUNMEL, a mean GI of 0.7779 with the three members of the *B. tunux* group (TUNTUN, TUNNEV and TUNNOR) and a GI of 0.7117 with NORDIC.

Combined data from this study supports recognition of this taxon as the typical variety of *Botrychium lunaria*. The type specimen of *B. lunaria* is northern European, and morphologically characteristic of *B. lunaria* var. *lunaria*.

Botrychium lunaria var. *melzeri (LUNMEL).*—Common in the Narsarsuag area of southwestern Greenland and also found in Iceland. *Botrychium lunaria* var. *melzeri* differs from var. *lunaria* in its relatively narrow, parallel-sided trophophores with five to six pairs of remote pinnae, and with the lowest pair of pinnae not larger than the pair above. Often the lowest three pinna pairs are the same size and shape. Rare in the *B. lunaria* complex, the basal pinnae tend to bear sporangia or support small sporophores in their axils or be replaced entirely by small sporophores.

Genetic variation in *Botrychium lunaria* var. *melzeri* was low with 10% polymorphic loci and an average of 1.1 alleles per locus. *Botrychium lunaria* var. *melzeri* has an allele composition similar to that of *B. lunaria* var. *lunaria*, but has a unique allele, *Dia-3*=4 with a frequency of 0.5, and is fixed for *Aat-4*=0.5, an allele rare in *B. lunaria* var. *lunaria*. The genetic identity between *B. lunaria* var. *melzeri* and *B. lunaria* var. *lunaria* was 0.8809. With other members of the *B. lunaria* complex, its GI values ranged from 0.6004 with *B. nordicum* to 0.7744 with *B. crenulatum*.

Botrychium lunaria var. *melzeri* is a morphologically recognizable entity that maintains its morphological and genetic differences in sympatry with var. *lunaria*. On the basis of these factors we recognize this moonwort as the variety, *B. lunaria* var. *melzeri,* as described herein.

Botrychium neolunaria (NEOLUN).—This North American genotype is distributed across North America from the Commander Islands (Russia) on the extreme western end of the Aleutian Islands southward to California and Arizona and eastward to New England and eastern Canada. Compared with other taxa in the complex it tends to have narrow trophophores; pinnae that are approximate to somewhat overlapping, and generally not overlapping the rachis; basiscopic margins of the basal pinnae that are linear to moderately concave and angles between basiscopic and outer margins that tend to be sharper than in LUNLUN. Its sporophore stalk tends to be longer than in LUNLUN, and it has a shorter branched portion with branches more strongly ascending than in LUNLUN.

Botrychium neolunaria contains four alleles, *Tpi-1*=4, *Tpi-2*=4, *Mdh-1*=3, and *Mdh-2*=5, all

with a frequency of 100%, and none of which were detected in any of the other taxa examined in this study. This set of unique alleles was the major genetic indicator distinguishing this group from other diploids. Common presence (38%) of accessory band *Mdh-3p* further differentiated *B. neolunaria* from LUNLUN, in which this band is absent. Genetic variation within *B. neolunaria* was somewhat lower than the average of *Botrychium* taxa with 20% polymorphic loci and an average of 1.2 alleles per locus.

The amount of genetic differentiation among populations within *Botrychium neolunaria* was low, with the GIs ranging from 0.9199 to 0.9972, with a mean of 0.9723. Genetic identities between *B. neolunaria* and other members of the complex range from a low of 0.4551 with TUNNEV, to a high of 0.7110 with LUNLUN and a mean GI of 0.6070. Its GI of 0.6963 with CRENUL supports Wagner and Wagner's (1981) separation of those two taxa.

Distinctive morphology, presence of unique alleles, low genetic identity with all other members of the complex, endemic North American distribution, and maintenance of distinctions in sympatric occurrences with other taxa of the complex, including LUNLUN (in Alaska and Canada), indicate that *Botrychium neolunaria* warrants recognition as a distinct species, as formally described herein.

Botrychium crenulatum (CRENUL).—This moonwort is currently known from the mountains of western North America ranging from the Rocky Mountains of mid-Alberta south to New Mexico, and in the Cascade, Sierra Nevada and coastal ranges, to southern California and Nevada. East of the Rocky Mountains it is known from disjunct populations in the boreal regions of north-central Alberta, the James Bay region of Ontario, northern Minnesota and Newfoundland. *Botrychium crenulatum* is the only member of the complex that almost always occurs in or near a saturated substrate. It is also morphologically distinct, primarily in its crenulate to denticulate margins and delicate texture not as fleshy as other members of the complex.

Botrychium crenulatum shares a surprisingly high GI with circumpolar *B. lunaria*, but interpretation of this relationship becomes more complex when *B. lunaria* is subdivided into European and North American populations as suggested by STRUCTURE analysis. A high GI (0.8552–0.9202) with the NA populations is lower 0.8579–0.8590 with European populations, the area that includes the type locality for *B. lunaria. Botrychium crenulatum* differs from all *B. lunaria* in possessing unique alleles, *Mdh-2*=2 (100%), *Pgm-1*=0.5 (17%) and *Idh*=3 (44%). Additionally *B. crenulatum* expresses a much higher frequency of accessory band *Mdh-3t*, and 100% difference from *B. lunaria* at accessory bands *Mdh-3p* and *Dia-1p*.

Although *Botrychium crenulatum* appears to have been recently derived from North American genotypes of *B. lunaria*, for reasons of its distinct ecology, morphology and genetic differentiation, as well as its maintenance of these distinctions in populations sympatric with *B. lunaria*, we conservatively retain *B. crenulatum* at the rank of species.

Botrychium tunux (TUNTUN, TUNNEV, TUNNOR).—*Botrychium tunux* differs morphologically from other taxa of the *B. lunaria* complex in its short, stocky stature with rounded to strongly asymmetrical pinnae with expanded lower portions. Most noticeable among the three entities of *B. tunux* is a diminution of genetic variability from Norway, with the highest variability of 30% polymorphic loci and 1.36 alleles per locus, to the Alaska region with 15% P and 1.20 A, to the southernmost mountains of the Sierra Range in Nevada with 10% P and 1.1 A. Six alleles are unique to Norway, whereas one is unique to the Alaska and one to the Nevada group. The simplest hypothesis is that a widespread circumboreal ancestor became isolated in Norway and northwestern North America, the latter migrating southward with gradual loss of genetic diversity.

Considering only the larger groups (n >20), genetic identities among the northern NA groups ranged from 0.8909 to 0.9836, and between these groups and the Nevada populations, from 0.7984 to 0.8614, the latter reflecting fixation for fewer alleles in TUNNEV. GI values between the northern NA groups and the Norway group ranged from 0.8442 to 0.8801. A further difference between NA groups and the Norway group was strong presence of *Dia-1p* in the North America plants and absence of this accessory band from plants in Norway.

Botrychium tunux as a species contains unique alleles at 20% of its loci, split equally among North America and Norway groups. Both groups also differ from all other taxa of the *B. lunaria* complex in lacking expression of either of the

Mdh-3 accessory bands. In GI relationships *B. tunux* has its closest relationships with *B. lunaria* and *B. crenulatum*, ranging from 0.6367 to 0.7942, and lower similarities with *B. neolunaria* and *B. nordicum*, ranging from 0.4551 to 0.6491.

The lower than expected GI relationship between Norway and North American plants of *Botrychium tunux* warrants further study of this taxon in Europe. Pending such studies, and in consideration of shared morphological and genetic characteristics, we retain *B. tunux* as a distinct species without varietal segregation.

Botrychium nordicum (NORDIC).—This distinct moonwort, known from rare populations in Iceland and Norway, differs morphologically from other taxa in the *B. lunaria* complex, principally by its deeply incised pinna margins.

Genetic variation within *Botrychium nordicum* displayed in the limited number of populations and individuals (48) was relatively low, with 15% polymorphic loci and a correspondingly low number of alleles per locus (1.15). However, within its limited variability are fixed unique alleles at five loci and a dominant allele at a 6th locus not present in LUNLUN, three of which are unique in the *B. lunaria* complex to *B. nordicum*. It also displays the accessory band at *Mdh-3p* that is absent in LUNLUN.

Cohesion among the Iceland and Norway populations of *Botrychium nordicum* is indicated by a high genetic identity of 0.9734. From larger groups (n >16) of LUNLUN, its distinction is indicated by GI values ranges from 0.6301 to 0.7200, and from combined groups of other taxa in the complex, by GI values from a low of 0.5237 with *B. neolunaria*, to a high of 0.6117 with *B. crenulatum.*

Since the genetic analysis was completed, a population of *Botrychium nordicum* was located in Iceland near Höfn on the southeastern coast and another population was found near the Vestnes population in More og Romsdal, Norway. The identity of these plants was confirmed through enzyme electrophoresis.

Morphological and genetic characteristics of *Botrychium nordicum,* maintained in sympatry with *B. lunaria* var. *lunaria*, support recognition of this taxon as a distinct species as described herein.

OTHER TAXA RELEVANT TO THIS STUDY

Botrychium yaaxudakeit.—In Alaska and Canada where the ranges of *Botrychium lunaria* var. *lunaria* and *B. neolunaria* overlap, the two species are generally separated by habitat, with *B. lunaria* occupying well-drained, sloping uplands and *B. neolunaria* occurring in less well-drained flatlands. We encountered very few instances of intermixed populations, these usually in association with hilltops flattened for installations of communications towers, where we detected F1 hybrids identifiable by heterozygous combinations of alleles otherwise unique to the two species. A presumed product of these hybrids is tetraploid *Botrychium yaaxudakeit,* described by Stensvold et al. (2002). Known then from a very restricted distribution in the Yakutat and Glacier Bay areas of southeastern Alaska. *Botrychium yaaxudakeit* has since been found in the Wrangell-St. Elias Mountains of Alaska, Yukon and British Columbia, central British Columbia, Alberta, Montana, Oregon and California (Farrar, 2012). The allelic composition of *B. yaaxudakeit* shows the high level of fixed intra-locus heterozygosity expected in a recently formed allotetraploid. Heterozygous banding patterns are consistently expressed at *Mdh-1*=1.5 3, *Mdh-2*=35, *Idh*=24 and *Skdh*=12. Heterozygosity is also frequent at *Tpi-1*=34 and *Tpi-2*=34. The alleles expressed at these loci indicate *B. yaaxudakiet*'s diploid ancestors to be *B. neolunaria* and *B. lunaria.*

The morphology of robust *Botrychium yaaxudakeit* overlaps with large *B. lunaria*, having overlapping pinnae that tend to also overlap the rachis, and basal pinnae with strongly recurved inner margins (Fig. 4). Smaller plants in the southern part of its range lack these characters and overlap the morphology of *B. neolunaria*. Because of these similarities, *B. yaaxudakeit* cannot always be distinguished from *B. lunaria* and *B. neolunaria* on the basis of leaf morphology alone. It can be distinguished from both diploids by its significantly larger spores.

Botrychium dusenii.—*Botrychium dusenii*, occurring in southernmost South America, has been considered by many authors to be part of the *B. lunaria* complex. Specimens for this study were obtained from the Falkland Islands. As with *B. yaaxudakeit*, plants of *B. dusenii* exhibited fixed heterozygosity at several loci indicative of allotetraploidy, with the allele composition

suggesting origin through hybridization between *B. lunaria* and a North American diploid not a part of the *B. lunaria* complex. *Botrychium dusenii* is retained as a distinct taxon pending further investigation of its relationship to North American allotetraploid species (Meza-Torrez et al., 2016).

Botrychium neolunaria/lunaria introgressed.—A number of individuals and populations, especially in the areas of overlap between *Botrychium neolunaria* and *B. lunaria* var. *lunaria* expressed alleles of both taxa, but not as intra-locus heterozygosity as in F1 hybrids and allopolyploid species (e.g., *B. yaaxudakeit*). These are most easily explained as segregated genotypes from F1 hybrids carrying a mix of alleles from both taxa, and stabilizing this mix through intragametophytic inbreeding. Most of these recombined and stabilized F2 hybrids are local in distribution in northern NA, but a few have become more widespread, occurring in Europe and Asia. The most notable is an introgressed genotype occurring along the coast of eastern Asia and south into New Zealand and Australia. Plants of identical genotype occur in Alaska, supporting a hypothesis of long-distance dispersal, possibly by transpolar bird migrants. These plants are morphologically indistinguishable from *B. lunaria* var. *lunaria*, and we do not recommend formal taxonomic treatment beyond recognition of their origin as introgressed hybrids.

TAXONOMIC TREATMENT

Botrychium lunaria (L.) Sw. var. **melzeri** Stensvold & Farrar, **var. nov.** Type: Greenland, Vestgrønland, Narsarsuaq area, ridgetop about 3.5 km NE of the Narsarsuaq airport terminal, 90 m, 5 Jul 2005, *Peter Struck s.n.* (holotype: ISC; isotypes: AMNH, BG, BM, ICEL, MO, NY, O, US) (Fig. 6).

This variety morphologically resembles *Botrychium lunaria* var. *lunaria*, from which it can be distinguished by its long and narrow, somewhat glaucous trophophores and remote pinnae. Basal pinnae are equal to or smaller than the adjacent pair, are often rhombic or lobed and often are replaced by or support small sporophores in their axils. *Botrychium lunaria* var. *melzeri* tends to mature earlier in the summer than var. *lunaria*. *Botrychium lunaria* var. *lunaria* has narrowly ovate to narrowly triangular trophophores with lunate basal pinnae that are generally larger than the adjacent pair, and pinnae that often overlap one another.

Rhizomes erect, unbranched, their apex 2–4 cm below the soil surface, bearing fleshy roots. Aboveground plants 12 (6–21) cm long, with a common stalk 4 (1.5–6.5) cm long. Trophophores green, somewhat leathery and glaucous; stalks 0.4 (0.2–1) cm long; blades 5.5 (1.5–8.3) cm long, and 2.5 (1.4–3.8) cm wide at the base, narrowly oblong, once pinnate. Pinna pairs 3–8, spreading to somewhat upswept, remote. Basal pinnae pair approximately equal in size or slightly smaller than the adjacent pair, 10 (5–20) mm long, 13 (8–20) mm wide, stalked, broadly fan-shaped to lunate to rhombic, spanning an arc of 120° to nearly 180°, often asymmetrical; outer pinna margins entire to undulate, occasionally denticulate, often having 3–4 lobes with shallow sinuses; veins dichotomous. One basal pinna often replaced by a small sporophore. Sporophores 10 (4.4–15.5) cm long; sporophore stalks 5 (2.2–8.5) cm long, fertile portion 5 (1.5–8) cm long, broadly ovate to triangular in outline; sporangia-bearing branches 2–8 pairs, spreading to upswept, 1–2 pinnate. Sporophore longer at maturity than the length of the trophophore. Spores 38 (36–43) μm in longest diameter. Diploid, *2n*=90. A range of *B. lunaria* var. *melzeri* morphologies are shown in Fig. 7.

Distribution.—Greenland and Iceland. Relatively common throughout the Narsarsuaq area of southwestern Greenland, and expected in other areas of southern Greenland. Because var. *melzeri* was found at three sites in Iceland quite distant from each other, it is expected to occur elsewhere in Iceland.

Habitat.—*Botrychium lunaria* var. *melzeri* grows on well drained, sandy to gravelly soil in open heaths, areas dominated by grasses, bryophytes and lichens, as well as open sandy areas. *Botrychium boreale* and *B. lanceolatum* as well as *B. lunaria* var. *lunaria* are often associated with var. *melzeri*.

Etymology.—This variety was discovered in Iceland by Peter Struck. At the suggestion of Dr. Struck, the specific epithet *melzeri* honors the late Helmut Melzer (1922–2011), a noted Austrian botanist.

Additional specimens examined. GREENLAND. Vestgrønland: Narsarsuaq, Signal Hill, just SE of the Narsarsuaq airport, 90 m, 7 Jul 2003, *P. Struck s.n.* (ISC); Narsarsuaq, between the north end of the Narsarsuaq Airport

Fig. 6. *Botrychium lunaria* var. *melzeri*, pressed type specimens from Greenland, Vestgrønland, Narsarsuaq area, ridgetop about 3.5 km NE of the Narsarsuaq airport terminal, 90 m, 5 Jul 2005, (*P. Struck s.n.* [12802, 12803, 12807, 12808, 12810, 12811, 12813, 12814, 12816, 12817], ISC). *Numbers in brackets* are electrophoresis voucher numbers.

FIG. 7. *Botrychium lunaria* var. *melzeri*, pressed specimens from across distributional and morphological ranges. Numbers provided below in parentheses after the collector name are electrophoresis voucher numbers. **a–d.** Greenland, Narsarsuaq (8814, 11178, 10718, 10825). **e. f.** Greenland, Blomsterdalen (12912, 12904). **g, h, k.** Iceland, Mývatn (lake) area (11308, 11306, 12940). **I, J.** Iceland, Grindavik (13363, 13368). **l.** Iceland, Jökulsárlón outlet (12986). **a–l.** (*P. Struck s.n.,* ISC).

runway and river, 30 m, 7 Jul 2004, *P. Struck s.n.* (ISC); Narsarsuaq area, Blomsterdalen, about 6 km NE of the Narsarsuaq Airport terminal, 50 m, 6 Jul 2005, *P. Struck s.n.* (ISC).

ICELAND. Austur-Skaftafellssýsla: about 800 m east of the bridge crossing Jökulsárlón's outlet stream, 10 m, 13 Jul 2005, *P. Struck s.n.* (ISC).

Botrychium neolunaria Stensvold & Farrar, **sp. nov.** Type: U.S.A. Colorado: Boulder County, ca. 14 km W of Nederland on 4th of July Road, near 4th of July trailhead, 39.994°N, 105.631°W, 3082 m, 19 Jul 2009, *M. C. Stensvold & D. R. Farrar 8388.* (holotype: ISC; isotypes: ALA, BM, CS, COLO, DAO, JEPS, MO, NY, UBC, US, WTU) (Fig. 8).

This species resembles *Botrychium lunaria* var. *lunaria* in morphology but can generally be distinguished by a combination of characters. Its trophophore is oblong to narrowly ovate, and its pinnae are more remote to somewhat overlapping. The basal pinnae are generally not longer than the adjacent pair, usually not stalked, the basiscopic side margins are straight to moderately recurved and the junction between the basiscopic side margin and outer pinna margin is generally angled. The sporophore stalk exceeds the fertile portion of the sporophore in length and the fertile portion generally comprises about one-third of the entire sporophore length and has strongly ascending branches.

Botrychium lunaria var. *lunaria* has narrowly ovate to narrowly triangular trophophores with pinnae that often overlap one another. Basal pinnae are usually larger than the adjacent pair and usually stalked; the basiscopic side margins are slightly to strongly recurved and the junction between the basiscopic side margin and outer pinna margin is generally rounded. The length of the sporophore stalk is equal to or less than the fertile portion in which at least the basal branches are more strongly spreading.

Rhizomes erect, unbranched, their apex 2–4 cm below the soil surface, bearing fleshy roots. Aboveground leaves 12 (3–22) cm tall, with a common stalk 4 (2–7) cm long. Trophophores green, somewhat leathery; stalks generally lacking, when present 0.3 (0.2–3) cm long; blades 5 (1.5–10) cm long, and 2.5 (1.5–4.5) cm wide at the base, narrowly oblong to narrowly ovate, once pinnate. Pinna pairs 3–8, those above the basal pair ascending, remote to somewhat overlapping one another. Basal pinnae pair broader but approximately equal in length and cutting to adjacent pair, 12 (3–20) mm long, 15 (5–28) mm wide, generally sessile, fan-shaped to lunate, spanning an arc of 110° to slightly less than 180°, usually symmetrical; basiscopic side margin straight to recurved; junction between the basiscopic side margin and outer pinna margin generally angled, rather than rounded; outer pinna margins entire to undulate, occasionally denticulate or occasionally shallowly cleft; veins dichotomous. Sporophores 10 (1.5–17) cm long; sporophore stalks 6 (0.8–8.2) cm long, fertile portion 4 (0.5–10.5) cm long, with branches strongly ascending. Sporangia-bearing branches 2–8 pairs, 1–2 pinnate, lanceolate to narrowly ovate in shape. Sporophore stalk longer at maturity than the length of the trophophore. Spores 36 (33–39) μm in longest diameter. Diploid, $2n$=90. A range of *B. neolunaria* morphologies are shown in Fig. 9.

Distribution.—Botrychium neolunaria is known only from North America, ranging from the Commander Islands (Russia) on the extreme western end of the Aleutian Islands to eastern Canada and New England, south in the western mountains to California and New Mexico. *Botrychium neolunaria* is included in Wagner and Wagner's (1993) *B. lunaria*. The plants mapped on the southern part of the Wagner's distribution map generally reflect the distribution of *B. neolunaria*, not *B. lunaria.* Plants in the northern part of that map include *B. lunaria* var. *lunaria*. Furthermore, the moonworts shown as *B. lunaria* in our 2002 paper (Stensvold et al., 2002) describing *B. tunux* and *B. yaaxudakeit* are *B. neolunaria*, not *B. lunaria*.

Habitat —Poorly to moderately well drained open areas dominated by perennial, herbaceous vegetation. At the type locality in Colorado, *Botrychium neolunaria* grows in somewhat sparsely vegetated subalpine meadows supporting a mosaic of low herbs, lichens and some open, gravelly soil, often adjacent to boulders or outcrops. In other montane situations *B. neolunaria* grows on scree slopes as well as lush subalpine meadows. In Alaska this moonwort often occupies areas of natural disturbance such as river bars, the vicinity of melting glaciers and well-drained areas near beaches. Habitats in the vicinity of beaches can range from bare sand on stabilized dunes, to lightly vegetated upper beaches to herbaceous upper beach meadows. In northeastern NA *B. neolunaria* grows in mesic meadows, sand dunes and beach meadows. Throughout its range *B. neolunaria* has also been

Fig. 8. *Botrychium neolunaria*, pressed type specimens from U.S.A. Colorado, Boulder County, dry subalpine meadows, ca. 14 km W of Nederland on 4th of July Road, near 4th of July trailhead, 39.994°N, 105.631°W, 3082 m, 19 July 2009, (*M. Stensvold & D. Farrar 8388,* ISC). The electrophoresis voucher (13029) from this site is shown in Fig. 9F.

documented in mid-successional meadow-like vegetation associated with recovery from human-caused disturbance. Human activities such as mowing and grazing may maintain this type of vegetation by preventing succession to woody vegetation that is less supportive of moonworts. By this process, old (>20 years) roadsides, landing strips, power lines, abandoned roads, etc. mimic natural meadows.

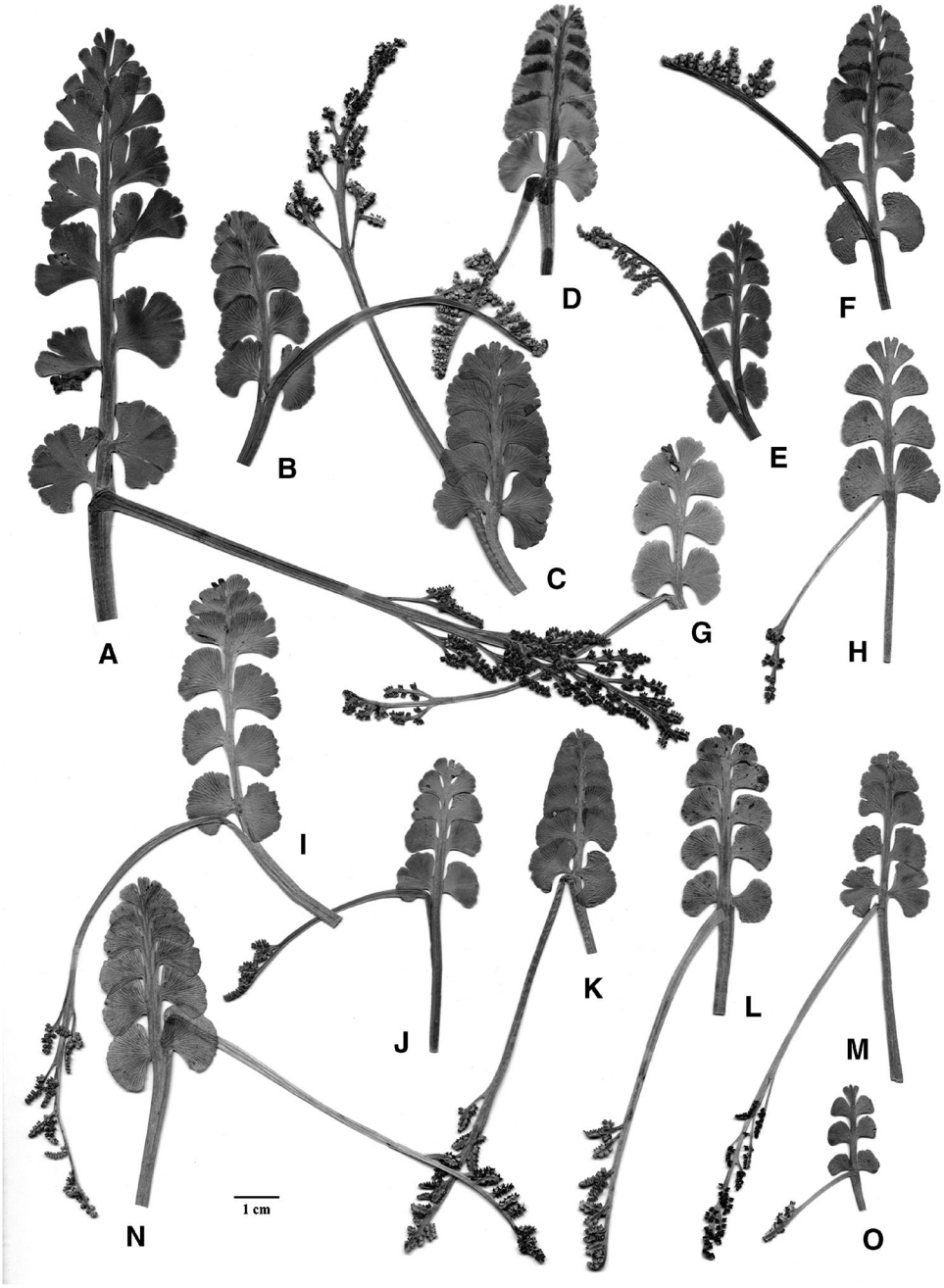

Fig. 9. *Botrychium neolunaria*, pressed specimens from across distributional and morphological ranges. Numbers provided below in brackets are electrophoresis voucher numbers. **a.** USA, Michigan, Chippewa County, (*M. Jaunzems s.n.* [8277], ISC). **b.** Canada, Newfoundland (*P. Zika 16319* [5038], ISC). **C.** Canada, Quebec, Bic National Park (*Cayoutte s.n.* [16464], ISC). **d.** USA, South Dakota, Black Hills (*D. Farrar* [10482], ISC). **e.** Canada, Alberta, Birch Lake (*G. Griffith s. n* [12082], ISC). **f.** USA, Colorado, Boulder County (*D. Farrar* [13029], ISC). **g.** USA, Montana, Jefferson County (*R. Ferriel s. n.* [2606], ISC). **h.** USA, Washington, Okanogan County (*H. Loftis s.n.* [2352], ISC). **I.** Canada, Ontario, James Bay (*S. Brinker SB 139* [18287], ISC). **J.** Canada, Yukon, Haines Junction area (*D. Farrar* [15221], ISC). **k.** Canada, Yukon, Haines Junction area (*M. Stensvold 8232* [10898], ISC). **l.** USA, Alaska, Yakutat area, Dry Bay (*D. Farrar* [4187], ISC). **m.** USA, Alaska, Delta Junction (*A. Geise s.n.* [15298], ISC). **N.** USA, Alaska, Yakutat, Ankau beach (*D. Farrar* [5465], ISC). **o.** USA, Alaska, Yakutat, Black Sand beach (*M. Stensvold 8128* [9622], ISC).

Fig. 10. *Botrychium nordicum*, pressed type specimens from Norway, More og Romsdal, about 12 km SW of Vestnes by road, in Skorgedalen, growing on the road verge, 62.559° N, 9.984° E, 203, 15 Jun 2009, (*D. Holtan s.n.* [17171, 17176, 17177, 17159, 17158, 17160, 17161, 17162, 17163, 17164, 17165], ISC). *Numbers in brackets* are electrophoresis tracking numbers.

Etymology.—The specific epithet, neolunaria, alludes to this species similarity to *Botrychium lunaria* var. *lunaria* and its being a moonwort of the New World.

Selected specimens examined. CANADA. Alberta: Birch Island in Lac la Biche, 54.864°N, 111.979°W, 542 m, 11 Jun 2005, *G. Griffith s.n.* (ISC). **British Columbia**: Kootenay National Park, west side of Highway 93, about 20 km west (by road) of the British Columbia Alberta border; 51.097°N, 116.0700°W, 1357 m, 24 Jun 2005, *D. R. Farrar s.n.* (ISC). **Newfoundland and Labrador**: West coast of Newfoundland at Cow Head beach, 49.917° N, 57.783° W, 5 m, 24 Jun 2001, *P. Zika 16,319* (ISC). **Québec**: Parc National du Bic, south side of the St. Lawrence estuary, about 6 km W of Le Bic village, by road, 48.3580°N, 68.778°W, just above sea level, 27 Jun 2008, *D.R. Farrar s.n.* (ISC). **Yukon Territory**: near the abandoned Wade Lake Road, about 4.6 km WSW of Dalton Post and about 1 km

Fig. 11. *Botrychium nordicum*, pressed specimens from across distributional and morphological ranges. Numbers provided below in brackets are electrophoresis voucher numbers. **a, b.** Norway, More og Romsdal, Vestnes area (*D. Holtan s.n.* [7156, 7155], ISC). **c**–**g.** Iceland, Isafjord, Kaldalen (*P. Struck s.n.* [11310, 11313, 11315, 11318, 11327], ISC). **h**–**j.** Iceland, Isafjord, Melgraseyri area (*P. Struck s.n.* [13333, 13335, 13342],ISC. **k**–**m.** Iceland, Höfn (*P. Zika 42574* [17809, 17818, 17776], ISC).

northwest of the Tatshenshini River, 60.106°N, 137.122°W, 811 m elev., 7 Jul 2004, *P. Caswell PPC-2004-203* (ISC).

U.S.A.: **Alaska**: Aleutians East Borough, Korovin Island, S side, near the head of Korovin Bay, 55.417°N, 160.255°W, 12 M elev., 30 Jun 2002, *P. Zika 16988* (ISC); Kenai Peninsula Borough, about 14 km up the Nuka R. from Beauty Bay, 59.666°N, 150.662°W, 600 m, 29 Jul 2003, *R. Lipkin & M. Carlson 03-274* (ISC); Lake and Peninsula Borough, Aghiyuk Island, E side, 56.192°N, 156.792°W, 25 m, 1 Jul 2002, *P. Zika 16992* (ALA, ISC, MO, US, WTU); Juneau City and Borough, Eagle Beach, about 30 km NNW of the Juneau Airport, 58.530°N, 134.825°W, just above sea level, 18 Jul 1998, *M. Stensvold 7323* (ISC); Southeast Fairbanks Borough, about 6 km E of Delta Junction on the N side of Nistler Road, , 64.042°N, 145.612°W, 372 m, 6 Jul 2007, *A. Geise s.n.* (ISC); Yakutat City and Borough, Blacksand Spit, off the mouth of the Situk River, 59.551°N, -139.812°W, just above sea level., 19 Jul 2002, *M. C. Stensvold 8049* (ISC). **Michigan**: Chippewa Co., 4.25 km NE of Trout Lake via Highway 123 and W Bobby Gay Road, roadside, 46.216°N, 85.000°W, 262 m, 19 Jun 2003, *M. Jaunzems s.n.* (ISC). **South Dakota**: Lawrence Co. about 16.3 km SW of Spearfish on Tinton Rd., 44.386°N, 103.967°W, 1680 m, 22 Jun 2004, *D. R. Farrar s.n.* (ISC).

Botrychium nordicum Stensvold & Farrar, **sp. nov.** Type: Norway. More og Romsdal: about 12 km SW of Vestnes by road, in Skorgedalen, growing on the road verge, 62.559°N, 9.984°E, 203, 15 Jun 2009, *Dag Holtan s.n.* (holotype: ISC; isotypes AAU, BG, BM, ICEL, ISC, NY, O, US) Fig. 10.

This species morphologically resembles *Botrychium lunaria* var. *lunaria*, from which it is distinguished by its incised pinna margins and shorter common stalk. *Botrychium lunaria* var. *lunaria* usually does not have deeply incised pinna margins.

Rhizomes erect, unbranched, their apex 2–4 cm below the soil surface, bearing fleshy roots. Aboveground plants 13 (3.5–22) cm long, with a common stalk 3.5 (1–5.5) cm long. Trophophores green, somewhat leathery; stalk 0.4 (0–2) cm long; blades 5 (2–9) cm long, and 2.8 (1.5–3.5) cm wide at the base, narrowly to broadly ovate, once pinnate. Pinna pairs 3–7, spreading, remote to somewhat overlapping one another. Basal pinnae pair larger than the adjacent pair, 13 (7–15) mm long, 14 (8–20) mm wide, stalked, lunate, spanning an arc of 80° to 140°, usually symmetrical; basiscopic and acroscopic margins recurved; outer pinna margins dentate to deeply incised with sinuses to various depths; veins dichotomous. Sporophores 9 (1.1–17) cm long; sporophore stalks 5 (1–8.5) cm long, fertile portion 4 (0.6–8.5) cm long, triangular to lanceolate in outline; sporangia-bearing branches 2–8 pairs, spreading, 1–2 pinnate. Sporophore longer at maturity than the length of the trophophore. Spores 37 (32–43) μm in longest diameter. Diploid, $2n$=90. A range of *B. nordicum* morphologies are shown in Fig. 11.

Distribution.—Botrychium nordicum is known from northwestern and southeastern Iceland, and in Norway, from the Vestnes area on the western central coast and from the mountains of Telemark in the south.

Habitat.—Habitats include open, well-drained areas with a sandy or gravelly substrate. At the roadside site near Vestnes, Norway, the plants grow in roadside gravel with low herbs; this habitat is maintained by infrequent mowing. We expect that *Botrychium nordicum* will be found in similar habitats elsewhere in Scandinavia.

Etymology.—The specific epithet refers to this moonwort's distribution in the Nordic countries.

Additional specimens examined. ICELAND. Austur-Skaftafellssýsla: Höfn, near harbor, 64.25°N, 15.20°W, 5 m, 26 Jul 2009, *P. Zika 24574* (ISC). **Norður-Ísafjarðarsýsla**: between Melgraseyri and the southern mouth of Kaldalon Fjord along the road, 20 m, 15 Jul 2005, *P. Struck s.n.* (ISC); near the southern mouth of Kaldalon Fjord near the road, 10 m, 15 Jul 2005, *P. Struck s.n.* (ISC).

NORWAY. More og Romsdal: about 12 km SW of Vestnes by road, in Skorgedalen, growing on the road verge about 400 m east of the type locality, 195 m, 26 Jun 2002, *John Bjarne Jordal s.n.* (ISC). **Telemark**: Hjartdal, Måråstølsdalen, 59.82°N, 8.48°E, 1040 m, 1 Aug 2001, Aug 5 2004, *J. I. Johnson s.n.* (ISC).

Acknowledgments

We especially thank Peter Struck for making large collections of *Botrychium* in Iceland and Greenland, Peter Zika for collections in Iceland, Newfoundland and Alaska, John Bjarne Jordal, John Inge Johnsen, Dag Holtan and Tor Tønsberg

for very helpful collections as well as working with Stensvold in Norway. Chanda Riedel Skelton for wonderful work in the lab, processing specimens, assisting with setting up and running the electrophoresis. Jennifer DeWoody for valuable assistance with the admixture (STRUCTURE) analysis. Iowa State University, Department of Ecology, Evolution and Organismal Biology, U. S. Fish and Wildlife Service, National Park Service, Forest Service and Parks Canada provided financial and logistical support for this work.

Literature cited

Alston, A. H. 1960. Some new species of ferns from South America. Lilloa 30: 107–112.

Clausen, R. T. 1938. A monograph of the Ophioglossaceae. Memoirs of the Torrey Botanical Club. 19: 1–177.

Cosner, M. B. & D. J. Crawford. 1990. Allozyme variation in *Coreopsis* sect. *Coreopsis* (Compositae). Systematic Botany 15: 256–265.

Crawford, D. J. 1983. Phylogenetic and systematic inferences from electrophoretic studies. *In:* S. D. Tanksley & T. J. Orton (eds.), Isozymes in plant genetics and breeding, part A. Elsevier Science Publishers B. V., Amsterdam.

Crawford, D. J. 1985. Electrophoretic data and plant speciation. Systematic Botany 10: 405–416.

Crawford, D. J. 1989. Enzyme electrophoresis and plant systematics. Pp. 146–164 *In:* D. E. Soltis & P. S. Soltis (eds.), Isozymes in plant biology. Dioscorides Press, Portland.

Cronn, R., R. M. Brothers, K. Klier, P. K Bretting & J. F. Wendel. 1997. Allozyme variation in domesticated annual sunflower and its wild relatives. Theoretical and Applied Genetics 95: 532–545.

Dauphin, B., J. Vieu & J. R. Grant. 2014. Molecular phylogenetics supports widespread cryptic species in moonworts (*Botrychium* s.s. Ophioglossaceae). American Journal of Botany 101: 128–140.

Earl D.A., B. M. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359–361.

Farrar, D. R. 1998. Population genetics of moonwort *Botrychium.* Pp. 109–113 *In:* N. Berlin, P. Miller, J. Borovansky, U. S. Seal & O. Byers (eds.), Population and habitat viability assessment for the goblin fern (*Botrychium mormo*), Final Report. The Conservation Breeding Specialist Group, Apple Valley, Minnesota.

Farrar, D. R. 2005. Systematics of western moonworts *Botrychium* subgenus *Botrychium. In*: Popovich, S. J. (ed.), United States Forest Service moonwort workshop. Arapaho-Roosevelt National Forests and Pawnee National Grassland. Fort Collins, Colorado, July 13–15.

Farrar, D. R. 2011. Systematics and taxonomy of genus *Botrychium.* Ada Hayden Herbarium, Iowa State University, Ames Iowa. www.public.iastate.edu/~herbarium/botrychium/Moonwort-Systematics.pdf.

Farrar, D. R. 2012. Ophioglossaceae and *Botrychium*. Pp. 119–123. *In:* B. G. Baldwin, D. H. Goldman, D. J. Keil, R. Patterson, T. J. Rosatti & D. H. Wilken (eds.), The Jepson manual: vascular plants of California, second edition. University of California Press, Berkeley.

Felsenstein, J. 1989. PHYLIP —Phylogeny Inference Package, Version 3.2. Cladistics 5: 164–166.

Gastony, G. J. 1988. The *Pellaea glabella* complex: electrophoretic evidence for the derivations of the agamosporous taxa and a revised taxonomy. American Fern Journal 78: 44–67.

Gottleib, L. D. 1977. Electrophoretic evidence and plant systematics. Annals of the Missouri Botanical Garden 64: 161–180.

Gottleib, L. D. 1981. Electrophoretic evidence and plant populations. Progress in Phytochemistry 7: 1—46.

Hamrick, J. L. & M. J. Godt. 1990. Allozyme diversity in plant species. Pp 43-63. *In*: A. H. D. Brown, M. T. Clegg, A. L. Kahler, and B. S. Weir (eds.), Plant population genetics, breeding, and genetic resources. Sinauer, Sunderland, Massachusetts.

Hartl, D. L. & A. G. Clark. 1997. Principles of population genetics. Sinauer Associates, Sunderland, Massachusetts.

Hauk, W. D. 1995. A molecular assessment of relationships among cryptic species of *Botrychium* subgenus *Botrychium* (Ophioglossaceae). American Fern Journal 85: 375–394.

Hauk, W. D & C. H. Haufler. 1999. Isozyme variability among cryptic species of *Botrychium* subgenus *Botrychium* (Ophioglossaceae). American Journal of Botany 86: 614–633.

Hauk, W. D, C. R. Parks & M. W. Chase. 2003. Phylogenetic studies of Ophioglossaceae: evidence from *rbcL* and *trnL-F* plastid DNA sequences and morphology. Molecular Phylogenetics and Evolution 28: 131–151.

Hauk, W. D, L. Kennedy & H. M. Hawke. 2012. A phylogenetic investigation of *Botrychium* s.s. (Ophioglossaceae): evidence from three plastid DNA sequence datasets. Systematic Botany 37: 320–330.

Klekowski, E. J. 1979. The genetic and reproductive biology of ferns. Pp. 133–170 *In:* A. F. Dyer (ed.), The experimental biology of ferns, Academic Press, London.

Kelloff, C. L., J. Skog, L. Adamkewicz & C. R. Werth. 2002. Differentiation of eastern North American *Athyrium filix-femina* taxa: evidence from allozymes and spores. American Fern Journal 92: 185–213.

Kumar, S., K. Tamura & M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary analysis and sequence alignment. Briefings in Bioinformatics 5: 150–163.

Li, J. & C. Haufler. 1999. Genetic variation, breeding systems, and patterns of diversification in Hawaiian *Polypodium* (Polypodiaceae). Systematic Botany 24: 339–355.

Meza-Torrez, E. I., M. C. Stensvold, D. R. Farrar & M. S. Ferrucci. 2016. Circumscription of the South American moonwort *Botrychium* (Ophioglossaceae). Plant Biosystems. http://dx.doi.org/10.1080/11263504.2016.1165752

McCauley, D. E., D. P. Whittier & L. M. Reilly. 1985. Inbreeding and the rate of self-fertilization in a grape fern, *Botrychium dissectum*. American Journal of Botany 72: 1978–1981.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590.

Pritchard, J. K, Stephens, M., & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

Soltis, D. E., C. H. Haufler, D. C. Darrow & G. J. Gastony. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. American Fern Journal 73: 9–27.

Soltis, D. E., C. H. Haufler, D. C. Darrow, G. J. Gastony & P. S. Soltis. 1986. Electrophoretic evidence for inbreeding in the fern *Botrychium virginianum* (Ophioglossaceae). American Journal of Botany 73: 588–592.

Soltis, D. E., C. H. Haufler, D. C. Darrow, G. J. Gastony & P. S. Soltis. 1992. The distribution of selfing rates in homosporous ferns. American Journal of Botany 79: 97–100.

Stensvold, M. C. 2008. A taxonomic and phylogeographic study of the *Botrychium lunaria* complex. Ph.D. dissertation. Iowa State University, Ames, Iowa.

Stensvold, M. C, D. R. Farrar & C. Johnson-Groh. 2002. Two new species of moonworts (*Botrychium* subgenus *Botrychium*) from Alaska. American Fern Journal 92: 150–160.

Suter, M., J. J. Schneller & J. C. Vogel. 2000. Investigations into the genetic variation, population structure, and breeding systems of the fern *Asplenium trichomanes* subsp. *quadrivalens*. International Journal of Plant Sciences 161: 233–244.

Wagner, W. H. Jr. & F. S. Wagner. 1981. New species of moonworts, *Botrychium* subg. *Botrychium* (Ophioglossaceae), from North America. American Fern Journal 71: 20–30.

Wagner, W. H. Jr. & F. S. Wagner. 1990. Notes on the fan-leaflet group of moonworts in North America with descriptions of two new members. American Fern Journal 80: 73–81.

Wagner, W. H. Jr. & F. S. Wagner. 1993. Ophioglossaceae C. Agardg: Adder's-tongue family. Pp. 85–106 *In:* Flora of North America Editorial Committee (eds.), Flora of North America, north of Mexico, Part 2: Pteridophytes and gymnosperms. Oxford University Press, New York.

Yeh, F. C., Yang, R-C., Boyle, T., Ye, Z-H. & Mao, J. X. 1997. PopGene, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton.

Zika, P. F. & Farrar, D. R. 2009. *Botrychium ascendens* WH Wagner (Ophioglossaceae) in Newfoundland and notes on its origin. American Fern Journal, 99: 249–259.