Systematics and Taxonomy of Genus *Botrychium*

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Historical Review of Taxonomy and Species Recognition

The first description of a *Botrychium* species was of *B. lunaria*, described in 1542 by Fuchs as *Lunaria minor*. Linnaeus recognized two species of *Botrychium* in his 1753 *Species Plantarum, B. lunaria* and *B. virginiana*. He placed both in the genus *Osmunda*. Presl (1845) was the first to use the name *Botrychium*, recognizing 17 species in his treatment of the genus. The first modern comprehensive treatment of the family was that of Clausen in his 1938 *Monograph of the Ophioglossaceae*. This publication provides the best reference point from which to discuss more recent taxonomic assessments and recognition of new species.

Clausen (1938) recognized three genera within the Ophioglossaceae: *Botrychium*, *Ophioglossum* and *Helminthostachys*. These three genera plus *Cheiroglossa*, a segregate from *Ophioglossum*, continue to be recognized by most botanists as constituting the family Ophioglossaceae and the order Ophioglossales (Wagner and Wagner 1993). This order of plants has no close relatives among the remainder of ferns (Smith et al. 2006). Cladistic analyses based on DNA sequences consistently place the Ophioglossales as sister to the Psilotales (*Psilotum* and *Tmesipteris*) (Manhart 1995, Pryer et al. 2001).

Within the genus *Botrychium*, Clausen (1938) described three subgenera: *Eubotrychium* (= *Botrychium*), *Sceptridium* and *Osmundopteris*. The first two groups continue to be recognized as the moonworts (subgenus *Botrychium*) and the grapeferns (subgenus Sceptridium). The third of Clausen's subgenera remains controversial. Wagner and Wagner (1993) continued to recognize *Osmundopteris* as the subgenus containing the North American rattlesnake fern, *B. virginianum*. Kato (1987) split *Osmundopteris* into two subgenera, *Botrypus* (containing *B. virginianum*) and *Japanobotrychium*. Using two molecular data sets plus morphological/anatomical characters, Hauk et al. (2003) reported *Botrychium* and *Sceptridium* to be well supported entities, but found *Botrypus* to be paraphyletic.

In this assessment and in future treatments of the Ophioglossaceae, including the Second Edition of The Jepson Manual, Farrar (2012) recognizes the moonwort ferns as constituting the genus *Botrychium*, the grapeferns as genus *Sceptridium*, and the rattlesnake fern as genus *Botrypus*, following the recommendations of Hauk et al. (2003) and Kato (1987).

In 1938 Clausen recognized only six species of moonworts: *Botrychium lunaria, B. simplex, B. pumicola, B. boreale, B. matricariifolium* and *B. lanceolatum*. All of these

except *B. pumicola* were known from Europe as well as North America. While this seems over simplified compared to the current list of species, we must also credit Clausen with recognizing some varieties and subspecies that would later be defined as species. He recognized *B. minganense* as a variety of *B. lunaria, B. pinnatum* as *B. boreale* subspecies *obtusilobum* and *B. hesperium* as a variety of *B. matricariifolium*. A. A. Eaton, in 1989, described *B. tenebrosum*, which Clausen reduced to a variety of *B. simplex*. Clausen also retained *B. angustisegmentum* as a subspecies of *B. lanceolatum* although it had earlier been classified as a distinct species by Fernald (1916). Clausen undoubtedly saw herbarium collections of other western U.S. moonworts but took a conservative approach in attributing these to variation within the species he recognized. His work was based on morphology without the knowledge of chromosome numbers and the role of allopolyploidy in speciation. He probably did not see some of the less common species now recognized.

Current recognition of North American species of *Botrychium* traces primarily to the work of W. H. and F. S. Wagner. Prior to Clausen's monograph, Victorin (1927) had described *B. minganense* as a new species. In 1956 Wagner and Lord confirmed the species status of that taxon listing a suite of morphological characters as well as chromosome number differentiating *B. minganense* from *B. lunaria*. Also prior to Clausen's (1938) description of *B. boreale* var. *obtusilobum*, Harold St. John (1929) had described this North American taxon as *B. pinnatum*. W. H. and F. S. Wagner (1983) agreed that it was a species distinct from the European *B. boreale*. In the same publication they raised Clausen's *B. matricariifolium* var. *hesperium* to species level as *B. hesperium*.

From 1981 through 2002 the number of species recognized in subgenus *Botrychium* increased rapidly. Through extensive field studies and chromosome analyses the Wagners described six new diploid species, *B. campestre*, *B. crenulatum*, *B. lineare*, *B. montanum, B. mormo,* and *B. pallidum*, and eight polyploid species, *B. acuminatum, B. alaskense, B. ascendens, B. echo, B. paradoxum, B. pedunculosum, B. pseudopinnatum*, and *B. spathulatum* (Wagner and Grant, 2002; Wagner and Wagner 1981, 1983a, 1886, 1990a, 1990b, 1994). Two additional species recognized by the Wagners during this period (*B. "adnatum"* and *B. " michiganense"*) are currently being described.

Recent work by Farrar, Johnson-Groh and Stensvold (Farrar and Johnson-Groh 1991, Farrar 2001, Stensvold et al. 2002) has resulted in recognition of three new species (*B. gallicomontanum, B. tunux, and B. yaaxudakeit*). In his treatment of northeastern moonworts, Farrar (2005), based on morphological and genetic distinctions, recognizes *B. tenebrosum* as a species distinct from *B. simplex* and *B. angustisegmentum* as a species distinct from *B. lanceolatum*.

A recent study of the *Botrychium lunaria* complex worldwide by M. Stensvold (2008) has revealed additional taxonomic complexity in this group. She has shown that plants of the contiguous US and of coastal Alaska historically referred to as *B. lunaria* are, in fact, sufficiently different genetically to warrant recognition as a distinct species. Stensvold has proposed recognizing these North American plants as *B. neolunaria ined.*.

She has also demonstrated the presence of true *B. lunaria* in inland Alaska and Canada. Her results also show that *B. crenulatum* is genetically very similar to true *B. lunaria*, sufficiently so to rank *crenulatum* as a variety of *B. lunaria*. Stensvold will also recognize an additional variety of *B. lunaria* and an additional new species in Europe.

More recently, Farrar and co-workers have discovered four additional undescribed taxa. Rocky Mountain plants resembling *B. pallidum* have for many years been recognized as that species. However, Farrar's genetic analysis has shown all Rocky Mountain plants to be allotetraploid, with *B. pallidum* as one parent (Farrar and Popovich 2012). These plants must therefore receive a new name, and, since all tested plants of this type in the Rocky Mountains have proven to be of the new tetraploid taxon, *B. pallidum* can no longer be recognized as occurring west of the Black Hills of South Dakota and eastern Wyoming and near Edmonton, Alberta. Formal publication of this species as *B. furculatum* is in preparation. A second undescribed allotetraploid taxon appears to have originated from hybridization of *B. campestre* or *B. lineare* with *B. tunux*. This taxon has been found in Colorado and in Montana. It should be sought in areas where the parent taxa co-occur, including California.

Genetic analysis has also revealed a third taxon in the *B. lanceolatum* complex. This complex has long been considered to consist of two subspecies, *B. lanceolatum* subsp. *angustisegmentum* in eastern North America and *B. lanceolatum* subsp. *lanceolatum* in western N.A. In fact, the western plants segregate genetically and morphologically into two taxa, each of which is equally distinct from subspecies *angustisegmentum* and from each other (Stensvold 2008). Publication of data justifying recognizing these three entities as separate species, *B. angustisegmentum*, *B. lanceolatum* and *B. viride* is in preparation. *B. viride* ined., the "green" taxon, occurs in southern Oregon (Crater Lake) and should be sought in northern California.

B. lineare and *B. campestre* are "sister" species that are highly similar both genetically and morphologically. Populations of plants belonging to this complex recently discovered in Colorado contain morphological and genetic variability inclusive of both *B. lineare* and *B. campestre*. They differ from both *B. lineare* and *B. campestre* in possessing gene alleles not present in either, and most importantly, in exhibiting an outcrossing breeding behavior. On the basis of these data, and publication dates of the two species, Farrar (2009) has recommended reducing *B. lineare* to a variety of *B. campestre*.

Both Hauk (in press) and Farrar (1998) have predicted the occurrence, or former occurrence, of an unknown diploid species based on the occurrence of orphan alleles and DNA sequences in several allotetroploid species. This predicted species is represented in Table 2 as *B.* "X". Preliminary data from plants recently discovered in Wyoming suggest that these plants may be of the predicted species.

The formally described *Botrychium* taxa in North America currently recognized by Farrar (this website) includes 14 diploid species (n = 45), 16 tetraploid species (n = 90)

and 1 hexaploid species (n = 135) (Table 2, 3) plus 2 varieties each of *B. campestre*, *B. lunaria* and *B. simplex* for a total of 34 *Botrychium* taxa in North America.

Problematic taxa—The above list of North American taxa does not include *B. acuminatum* described by Wagner and Wagner (1990b). Genetic investigation of this tetraploid revealed no differences from *B. matricariifolium* despite the distinctive morphology of plants of the type locality (Farrar 1997), and broader of sampling of B. matricariifolium revealed a continuum of morphological variation from the "acuminatum" type to typical *B. matricariifolium* (Farrar 1997).

The allopolyploid, *B. paradoxum*, is appropriately named because of mysteries of its origin, its parent species, and its morphology (having two nearly identical sporophores and no trophophore). Another mystery is the frequent occurrence of two distinctive genotypes within populations throughout its range. Possibly the simple morphology of this species may be disguising the presence of more than a single taxon.

Genetic and morphologicl evidence support the origin of *B. xwatertonense* as a sterile hybrid between allotetraploids *B. paradoxum* and *B. hesperium* as described by Wagner et al. (1984). This named hybrid is of interest because of its morphological expression of intermediacy between trophophore and sporophore., but occurrence of sterile hybrids between co-occurring species in *Botrychium* is not uncommon. *B. xwatertonense* is also of interest because of its greater than expected abundance (for a sterile hybrid) in its type locality. Possibly this could be due to underground vegetative propagules (gemmae) as has been demonstrated for many *Botrychium* species (Farrar and Johnson-Groh 1990, Johnson-Groh *et al*. 2002).

Another hybrid taxon is surprisingly common in the southern Rocky Mountains. It appears to have a parentage of *B. echo* X *B. lunaria* (or possibly *B. echo* X *B. minganense*). It is frequently identified as *B. minganense* with pinnatifid basal pinnae.

Although a number of North American species of moonworts range beyond North America, only two species known outside the continent have not yet been found in continental North America. *Botrychium boreale* is a European tetraploid that occurs westward to southern Greenland. *Botrychium nordicum* ined. is a European diploid resembling *B. lunaria* var. *lunaria* recently discovered in Iceland and Norway (Stensvold 2008). It seems probable that Europe and Asia harbor additional undiscovered species and that some of these have contributed to the high diversity of species in North America. The southern hemisphere appears to be genuinely without a center of *Botrychium* evolution. Tetraploid *B. dusenii* of extreme South America (Meza Torres et al. 2011) and *B. lunaria*-like plants of New Zealand and Austrialia mirror nearly identical genotypes on the northern end of transpolar bird migration routes and likely result from long range dispersal via bird transport.

Table 1. Diploid North American Species of *Botrychium*

Species	General Distribution
B. angustisegmentum	Northeastern US and boreal Canada [= *B. lanceolatum* subsp. *angustisegmentum*]
B. campestre var. *campestre ined.*	Great Lakes and northern Great Plains, Vermont
B. campestre var. *lineare ined.*	Western mountains of NA, Black Hills Great Lakes area [= *B. lineare*]
B. lanceolatum	western mountains of NA, coastal mts. south to Oregon, Rocky Mts. south to New Mexico
B. lunaria var. *crenulatum ined.*	western mountains of US and couthern Canada east to Minnesota and James Bay [= *B. crenulatum*]
B. lunaria var. lunaria	inland Alaska and across Canada [= European genotype of *B. lunaria*]
B. montanum	western mountains of US and southern Canada south to California, absent from southern Rocky Mts.
B. mormo	boreal regions of Great Lakes states
B. neolunaria ined.	contiguous US and coastal Alaska [= NA genotype of *B. lunaria*]
B. pallidum	Great Lakes and northern Great Plains Black Hills
B. pumicola	Cascade mountains of western Oregon and Mt. Shasta in California
B. simplex var. *simplex*	boreal US and Canada south to North Carolina, west to southern Rocky Mts, rare in coastal western mountains.
B. simplex var. *compositum*	Western NA from southern Canada to southern California, rare in southern Rocky Mts.
B. tenebrosum	Great Lakes area and northeastern NA [= *B. simplex* var. *tenebrosum*]
B. tunux	Western mountains from southeastern Alaska to Nevada and New Mexico
B. viride ined.	Western NA south to Oregon, rare in southern Rocky Mts. [= green phenotype of *B. lanceolatum*]
B. "X"	[Undescribed species predicted by "orphan" alleles in several NA allotetraploid species.]

Species followed by "*ined.*" to be described by D. R. Farrar and M. S. Stensvold based on genetic and morphological evidence.

Table 2. Polyploid North American Species of *Botrychium*

Species*	General Distribution	Probable Parentage**
B. ascendens	boreal US and Canada western mountain of NA	*B. lunaria* var. *crenulatum* x *B. campestre*
B. alaskense	Alaska and Yukon	*B. lanceolatum* x *B. lunaria*
B. echo	Rocky Mountains (Colorado)	*B. lanceolatum* x *B. campestre* var. *lineare*
B. gallicomontanum	western Minnesota, Black Hills, Glacier NP	*B. campestre* x *B. pallidum*
B. hesperium	Rocky Mountains of NA	*B. lanceolatum* x *B.* "X"
B. matricariifolium	Great Lakes and north-eastern US and Canada	*B. angustisegmentum* x *B.* "X"
B. minganense	boreal US and Canada western mountains of NA	*B. neolunaria ined.* x *B.* "X"
B. paradoxum	western Mountains of US and southern Canada	*B.* "X" *x B. ?*
B. pedunculosum	northwestern US and Canada, eastern Quebec	*B. lanceolatum* x *B.* "X"
B. pseudopinnatum	northern and southern Ontario	*B. lanceolatum* x *B. neolunaria* x *B. "X"*
B. pinnatum	western North America	*B. lanceolatum* x *B. neolunaria ined.*
B. spathulatum	northeastern NA, Rocky Mts. from Alaska to Colorado	*B. lunaria* x *B. campestre*
B. yaaxudakeit	Alaska and western Canada, Montana, Oregon, California	*B. lunaria* x *B. neolunaria ined.*
B. adnatum ined.	northwestern Montana	*B. simplex* x *B. pallidum*
B. michiganense ined.	Great Lakes, Black Hills, US northwest, Canada southwest	*B. lanceolatum* x *B.* "X"
B. furculatum ined.	Black Hills, RM from southern Canada to New Mexico	*B. pallidum* x *B. "X"*
B. tunux x *lineare*	Montana, Colorado	*B. tunux* x *B. campestre var. lineare*

*Species followed by *ined*. are not formally described. Names are provisional pending official publication.
**Determined by allelic comparison to known diploid species (see next section). *B. "X"* is one (or more) undiscovered diploid species possessing orphan alleles (alleles not known in any described diploid species) found in the allotetraploid under consideration.

Morphology and Identification of *Botrychium*

Members of the Ophioglossaceae have a peculiar morphology, unlike any other ferns. They are described and differentiated using terms and concepts specific to the family as outlined below (see Figure 1). Moonworts, like other members of the family, typically produce one leaf per year from an underground upright stem with a single apical meristem. The above-ground portion of a mature leaf is divided into two axes. One axis, bearing an expanded, usually photosynthetic lamina or blade, is called the **trophophore** or sterile segment. The other axis, bearing numerous globose sporangia, is called the **sporophore** or fertile segment. The trophophore and sporophore are joined into a **common stalk** or petiole, usually near the base of the expanded lamina. The common stalk extends underground to the stem apex where its base encloses the apical bud. Species of *Botrychium* are differentiated from species of the other genera in having trophophores that are at most twice pinnate and generally much smaller than the large, three or more times divided trophophores of genera *Sceptridium* and *Botrypus*.

Diagnostic characteristics of moonworts are present in both sporophore and trophophore, but more numerous in the latter (Figure 1). Moonworts are of three basic forms, the once-pinnate, fan-leaflet form of most diploid species (Figure 1d), the triangular, twice-pinnate form of *B. lanceolatum* (Figure 1f), and the intermediate, pinnate-pinnatifid form of the allopolyploid species derived from ancestral hybridization between *B. lanceolatum* and species of the fan-leaflet group (Figure 1e). The last two are sometimes referred to as the **midribbed** species because their pinnae have a central vein, whereas those of the fan-leaflet species have multiple parallel veins of equal size. Presence of a midrib in the basal pinnae is a good way to identify plants of the pinnate-pinnatifid group when they are too small to have developed pinna lobing. When the midrib is indistinct, plants of this group can be distinguished by basal and mid pinnae that are elliptic in outline (broadest in the middle) rather then fan-shaped (broadest at the outer margin).

Unusually large plants of the fan-leaflet, once-pinnate species may have lower pinnae that become secondarily divided, more or less repeating the general morphology of the entire trophophore. This is especially true of *B. simplex*, but occasionally it happens in most species. However, this subdivision of pinnae is seldom repeated in non-basal pinnae as it is in the pinnate-pinnatifid species.

Initial segregation of species in the fan-leaflet group is usually made on the basis of **pinna span**. Pinna span refers to that portion of a circle that is "spanned" by the outer circumference of the pinna (Figure 1c). Convenient dividing points are: less than 60^{o}, between 60^{o} and 120^{o}, and greater than 150^{o}. **Pinna bases** may be sessile or short-stalked. Pinna sides may be straight or concave, and converge at angles producing pinna bases that are acuminate ($<30^{o}$), acute ($30\text{-}90^{o}$), obtuse ($>90^{o}$), truncate (180^{o}) or cordate ($>180^{o}$). The **outer pinna margin** may be entire, crenulate, dentate, lacerate or lobed. Unless noted otherwise, when used in a key or species description, pinna characters refer to the basal pinnae which are typically the largest and broadest.

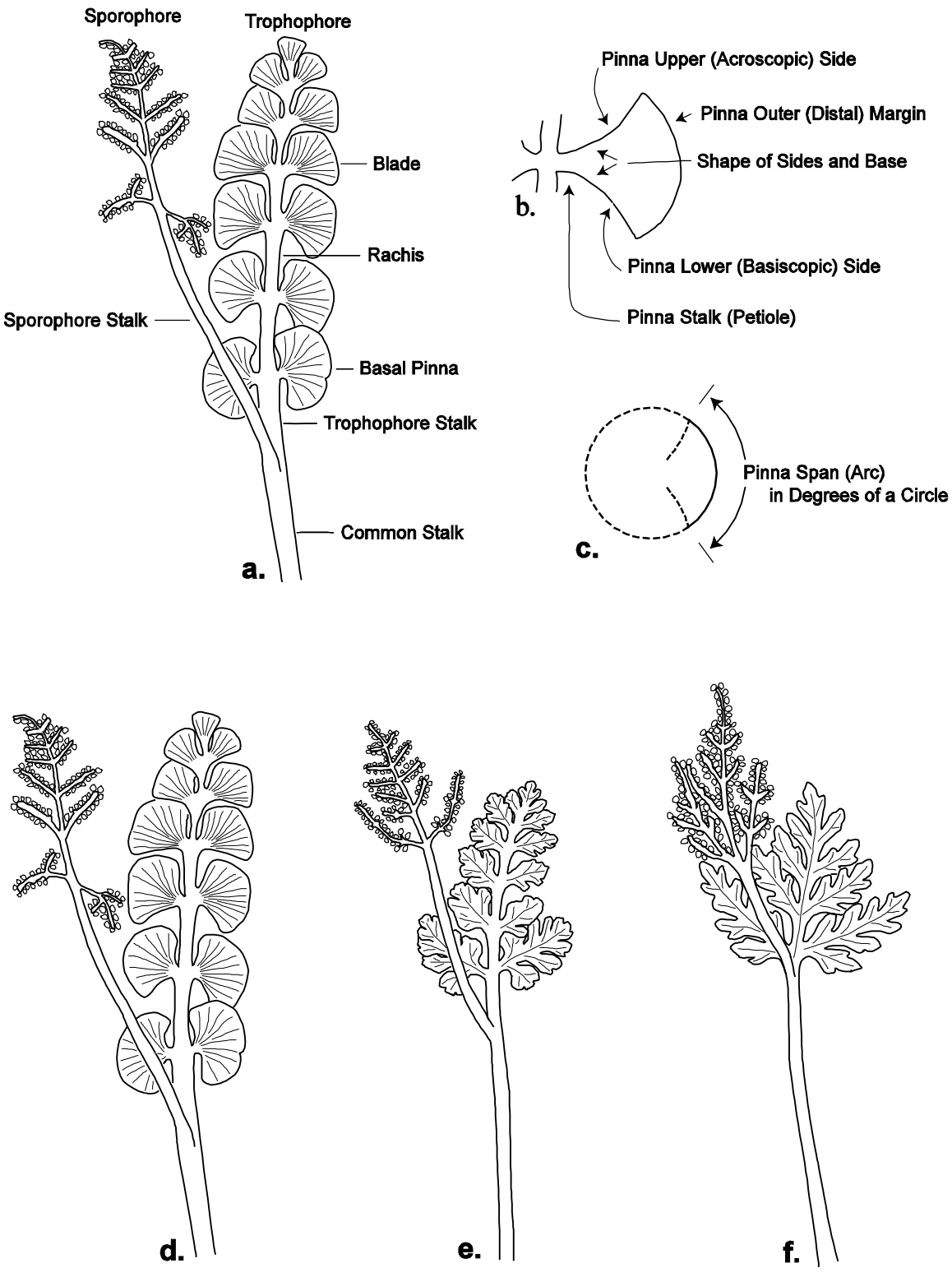

Figure 1. Morphology and terms used in moonwort identification.

The trophophore may be sessile or stalked (petioled) below the basal pair of pinnae. If stalked, the degree of **trophophore stalk** is best measured in relation to the distance between the first two pair of pinnae, i.e. whether the trophophore stalk is longer or shorter than the distance between the first two pair of pinnae.

A number of moonwort species have a glaucous surface giving them a gray or bluish cast that easily distinguishes them from species with a deep green color and lustrous surface.

Plant size varies considerably in most populations and is of limited usefulness in identifying species. Small plants often fail to fully develop the characters of full-sized plants, especially in pinna span and margin dissection. Extremely large plants often develop abnormalities (unusually large and highly divided basal pinnae, often with extra sporangia or small sporophores, and otherwise misshapen pinnae) uncharacteristic of the species.

Sporangia are occasionally produced on the basal trophophore pinnae of all species. Regular occurrence of these extra, or supernumerary, sporangia is limited to three species, *Botrychium ascendens, B. pedunculosum and B. tenebrosum*, but shade-grown plants of these species often do not have supernumerary sporangia. *Botrychium paradoxum* is a special case in which no trophophore is produced. Instead, the trophophore has been converted to a second sporophore. *Botrychium* X*watertonense* is a sterile first-generation hybrid between *B. paradoxum* and *B. hesperium* in which all pinnae of the trophophore produce sporangia around their margins.

The sporophore of *B. lanceolatum* is usually divided into three main branches. This character may or may not be expressed in allopolyploid taxa having *B. lanceolatum* as one parent. When present, a distinctly three parted sporophore is usually a good indicator of ancestral parentage by *B. lanceolatum*.

One of the most useful sporophore characters is the length of the **sporophore stalk**. This character must be used with caution because the sporophore stalk continues to lengthen until the time of spore release. The most useful comparison is the length of the sporophore stalk relative to the entire length of the trophophore, i.e., whether the sporangia-bearing portion of the sporophore is raised entirely above the trophophore at the time of spore release. The degree of sporophore branching and the length and angle of the branches may also be useful.

The **length** of the common stalk usually serves to distinguish *B. simplex* from other moonworts. It is so short in *B. simplex* that the sporophore and trophophore often appear to be joined at ground level. In all other species the sporophore-trophophore junction is well above ground. In compensation for its short common stalk, sporophore and trophophore stalks in *B. simplex* tend to be unusually long.

Presence of a pink to maroon **color** in the sporophore stalk occurs only in B. lanceolatum and in some allotetraploid species having *B. lanceolatum* as one parent. In B.

lanceolatum (except in the "green" type, see discussion in IIIa) and *B. pinnatum* color gradually increases toward the base of the common stalk. In allotetraploids having *B.* "X" as the second parent, color is expressed as a stripe below the trophophore.

Spore size is a useful character, especially in distinguishing between diploid and polyploid species. Most diploid species have spores that are significantly smaller than those of tetraploids with which they might be confused. For example, the spores of *B. lunaria* average about 36 microns whereas those of the similar tetraploid, *B. yaaxudakeit* average about 45 microns (Stensvold et al. 2002). The spores of *B. simplex* are unusually large for a diploid species, ranging from 40 to 50 microns.

Genetic Distinction of Moonwort Species

With the development of molecular genetic methods it has become possible to directly measure the level of genetic differentiation between taxa and to correlate these levels of genetic difference to other measures of differentiation. In the last decade, studies by Hauk (1995), Hauk and Haufler (1999) and Farrar (1998, 2001; in Ahlenslager and Potash 2007) have demonstrated the utility of starch-gel enzyme electrophoresis in producing diagnostic chemical "fingerprints" for each species. In addition to producing markers unique to individual species, this technique also allows detection of polyploidy and the probable ancestry of polyploid species. Almost without exception these chemical methods have supported the species recognized by the Wagners and earlier workers.

Warren Hauk (1995) compared a number of species of *Botrychium* subgenus *Botrychium* using enzyme electrophoresis . Results from that study closely parallel those presented here, however all genetic data and conclusions presented in this report are from electrophoretic analysis conducted by D. R. Farrar (2001, Zika and Farrar 2009).

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Enzyme electrophoresis yields data on the specific gene alleles present in each species and their frequencies within populations. Populations and species can then be quantitatively compared to one another in allele frequencies (including absence) at a number of gene loci. The result is a set of relative similarity values called **genetic identities** (GI) between populations or species. Farrar (1998, 2001, Zika and Farrar 2009) conducted enzyme analysis on more than 10,000 plants representing all of the North American taxa of moonworts. Calculations of genetic identities among the diploid species based on analysis of 19 gene loci from 10 enzyme systems are presented in Table 4 Popgene (version 1.31, Francis Yeh et al. 1997) was used to calculate Genetic Identity (GI) values according to the formulae of Nei (1978).

Table 4. **Nei's Unbiased Measures of Genetic Identity** (Farrar 2001)*

Species	lineare	pallidum	neolunaria	tunux	crenulatum	simplex	pumicola	montanum	lanceolatum
campestre	0.8019	0.4975	0.3325	0.3817	0.3264	0.4439	0.4020	0.3029	0.3818
lineare		0.6398	0.3060	0.5545	0.4489	0.5465	0.5525	0.4089	0.3613
pallidum			0.4801	0.5372	0.6690	0.7917	0.6970	0.5773	0.2807
neolunaria				0.5102	0.7015	0.4646	0.6227	0.3678	0.1748
tunux					0.6440	0.4512	0.6671	0.4559	0.3437
crenulatum						0.5267	0.6590	0.4722	0.3429
simplex							0.6738	0.6977	0.2042
pumicola								0.4938	0.1989
montanum									0.2339

*These values are being updated to reflect additional sampling. They are not expected to change dramatically and are presented here to facilitate discussion.

In flowering plants, many species have been examined electrophoretically and GI values computed between populations within species and between species within genera. Between different populations of the same species the average GI value is 0.95 with a range of 0.80 to 1.00. The average GI between recognized varieties within a species is 0.91 with a range of 0.71 to 0.99. Between species in the same genus the average GI is 0.73 with a reported range of 0.35 to 0.99 (Gottleib 1981, Crawford 1983, 1985).

In ferns, genetic identities obtained between populations of a species are very close to that of flowering plants (avg. = 0.94; range = 0.83 – 1.00), but the average GI between species in a genus is somewhat lower at 0.57 overall, and much lower when only temperate species are considered (avg. = 0.41; range = 0.09 – 0.85) (Soltis & Soltis 1989, 1990; Ranker 1992; Haufler 1996). The average GI between moonwort species falls between these values at 0.48 (0.18 – 0.80) (Farrar 2001).

Because taxonomic concepts vary among genera and among researchers, the broad ranges obtained in GI values are not surprising and in some cases probably indicate erroneous classification. Most researchers agree that in typical species derived by the gradual process of primary speciation (also called divergent or geographic speciation) genetic identities reflect time and degree of differentiation between taxa. Thus the average values obtained from many studies provide reasonable guidelines for interpretation of GI data in *Botrychium*. Independently of data from other genera, we can also look at the genetic identity of closely related but clearly distinct species pairs such as *B. neolunaria ined.* and *B. crenulatum* as a standard for comparison between other species. Using these guidelines along with commonly accepted morphological criteria we can comfortably accept GI values of 0.7 or lower as indicative of species-level differentiation. Values much greater than 0.7 indicate a genetic similarity more commonly accorded subspecific taxa.

Nearly all of the diploid species currently recognized in the western United States and Canada clearly warrant species designation. A number of close species relationships are indicated by GI values between 0.6 and 0.7, yet these species are easily distinguished morphologically. The most questionable case is the distinction between *B. campestre* and

B. lineare with a GI of 0.8019. This high GI as well as morphological similarity indicates that these two species are very closely related and that *B. lineare* could reasonably be considered a variety of *B. campestre*. In describing *B. lineare*, W. H. Wagner (1994) recognized its close relationship to *B. campestre*.

A second GI that is unexpectedly high is between *B. pallidum* and *B. simplex*. This may be, in part, an artifact of combining the varieties of *B. simplex* into a single taxon. Subspecific differentiation in *B. simplex* and its relationships with *B. pallidum* is being studied. A significant illustration of the species-level genetic distinction between *B. simplex* and *B. pallidum* is the fact that they have combined to produce a fertile tetraploid species, *B. adnatum*. Speciation by allopolyploidy does not generally occur in response to hybridization between two elements of the same species (see discussion of allopolyploidy).

Two distinctions are particularly noteworthy. *Botrychium pumicola* is clearly distinct from *B. simplex*, contrary to some speculation that it might be only a variety of *B. simplex*. A new diploid species discovered in coastal areas of southeastern Alaska, *B. tunux*, is clearly distinct from *B. neolunaria* with which it was formerly confused.

Botrychium lanceolatum has an exceptionally low GI with all other species. This represents a high genetic divergence that is also indicated by its morphology, being the only diploid moonwort species with a twice pinnate trophophore and leaves reflexed in the bud. On the other hand, affinity of *B. lanceolatum* to other moonworts is indicated by repeated hybridization events with fan-leaved species that have produced allotetraploid species. *Botrychium lunaria* and *B. campestre* are also quite distant from all other species except their sister species *B. crenulatum* and *B. lineare* respectively.

In Table 5, plants of all three types of *B. lanceolatum* are combined. When these are separated into subtypes *angustisegmentum, lanceolatum* and *viride*, GI values among the subtypes range from 0.74 to 0.79 (Stensvold 2008). Supporting the reduction of *B. crenulatum* to a variety of *B. lunaria*, the GI value between *B. lunaria* var *lunaria* and *B. lunaria* var. *crenulatum* is 0.92 whereas the GI value between *B. neolunaria* and *B. lunaria* is 0.70 when the two varieties of *B. lunaria* are combined (Stensvold 2008).

Additional comparisons of population genetic structure and differentiation between populations are being analyzed and may be expected to contribute further understanding to the process of evolution and speciation in *Botrychium*.

Nei's genetic identity measure was developed for diploid taxa. Interpretation of GI values between tetraploid species is problematic. Two allotetraploid species with one ancestral parent in common will have a much higher GI than two allotetraploid species with neither parents in common, yet all are equally valid species. Systematists generally agree that allotetraploids differing in their ancestral parentage should be recognized as distinct species.

Speciation and Evolution in Moonworts.

Diploid species of *Botrychium* are assumed to have evolved through gradual differentiation of plant populations growing in and adapting to different habitats. This is a continuing process resulting in levels of differentiation that we recognize as varieties, subspecies and species. An example of two species recently diverged from a common ancestor is *B. campestre* and *B. lineare*. Determining that differentiation has reached the level of species has traditionally been based on morphological discontinuity, especially if such discontinuity is observed between taxa growing together in the same habitat.

A biological test of species-level differentiation is possible if the taxa in question form natural hybrids. If these hybrids are fertile (produce viable spores), then little genetic differentiation has occurred and the taxa cannot be recognized as distinct species. In this case continued production of hybrids may be expected to produce a continuum of morphological intermediates between the two taxa, all of which are equally fertile. If the hybrids between two putative species are sterile (incapable of producing viable spores) then it is assumed that genetic differentiation is so great that homologous chromosomes no longer recognize one another, and cannot pair and segregate perfectly in meiosis (see discussion below). Sterility of hybrids maintains distinction of species.

Allopolyploid speciation—The base nunber of chromosomes in *Botrychium* subgenus *Botrychium* is 45. Diploid species possess two homologous copies of each chromosome for a total of 90 chromosomes in the sporophyte stage. To produce spores, cells in the sporangia under meiosis, a type of cell division in which identical "homologous" chromosomes pair, exchange genetic information, then separate and move into different cells to form haploid spores. For spores to be viable, they must receive a copy of each of the genes on each of the 45 chromosomes. This requires that homologous chromosomes be able to recognize one another so as to produce "perfect pairing". Any imperfection in this pairing process results in spores not receiving a full set of genes and causes them to be unviable.

Hybridization between closely related plant species is not uncommon, and this is true of *Botrychium*. Generally, these hybrids, when first formed, are sterile (Figure 2). Because their genetic material has accumulated so many differences, homologous chromosomes from the two parent species do not pair and separate perfectly during meiosis. This results in spores that receive both copies of some genes and neither copy of other genes, consequently they fail to develop normally and do not germinate. The original hybrid plant fails to produce additional plants and is thus an evolutionary dead end. [Sterile hybrids can often be recognized by examining their spores under a microscope. Spores of great variation in size, some twice the size of others, as well as collapsed and misshapen spores are indicative of sterile hybrids.]

Occasionally plant cells undergo chromosome replication without an accompanying division of the cell and nucleus. This results in a cell with double the base number of chromosomes. That cell may then continue to divide normally and produce a tissue that is tetraploid rather than diploid. If this tissue becomes involved in spore

production, there are two possible results. When this series of events happens in a normal diploid it produces an autotetraploid. This plant fails in spore production because it now has four homologous chromosomes. In attempting to pair, often three or all four homologous chromosomes become bound together making perfect separation impossible. Spores receive all four copies of some genes and none of others. As with diploid hybrids, the spores are non-viable and the autotetraploid is an evolutionary dead end.

When chromosome doubling occurs in a plant that is an interspecies hybrid, a very different result occurs—fertility is instantly restored (Figure 2). Now, in the tetraploid plant, there are two identical sets of chromosomes from each of the two parent species. Instead of attempting to pair with a distantly related chromosome from the other species, identical homologous chromosomes from the same species preferentially pair and separate perfectly in meiosis. Resulting spores receive a complete set of chromosomes from each of the parental species and are completely viable. This allotetraploid is thus fully capable of reproducing, dispersing, and evolving as an independent species with its own distinctive morphology and behavior.

About half of moonwort species are allotetraploids, and one, *B. pseudopinnatum*, is an allohexaploid, having formed from hybridization between an allotetraploid species and a third diploid species. Allotetraploid species are common in bryophytes and seed plants as well as ferns. In the absence of direct genetic evidence or chromosome counts, poplyploids can usually be recognized by having substantially larger spore sizes relative to their diploid parents.

Fixed heterozygosity.—Allotetraploid species are usually first detected in the field by a morphology that is intermediate between two known diploid species. Their doubled number of chromosomes can be confirmed in the laboratory by a direct count, but allopolyploid plants can also be detected through enzyme electrophoresis. Allotetraploid plants possess chromosomes, genes and gene alleles from both of the original diploid parents. If the two ancestral parent species possessed different alleles at a given enzyme locus, their allotetraploid derivative will display both of those different alleles in a heterozygous pattern in enzyme electrophoresis. Because each allotetraploid plant always receives a complete chromosome set from each of the original parent species, this heterozygous condition is "fixed". Fixed heterozygosity is not dependent on recombination through cross-fertilizaion as it is in diploid plants. It is also not eliminated through self-fertilization as it is in diploid plants. Theoretically, tetraploids could display as many as 4 different alleles at a gene locus. However, in *Botrychium* species, because self-fertilization maintains homozygosity within each parental contribution, allotetraploids seldom display at more than two different alleles per locus in an individual plant.

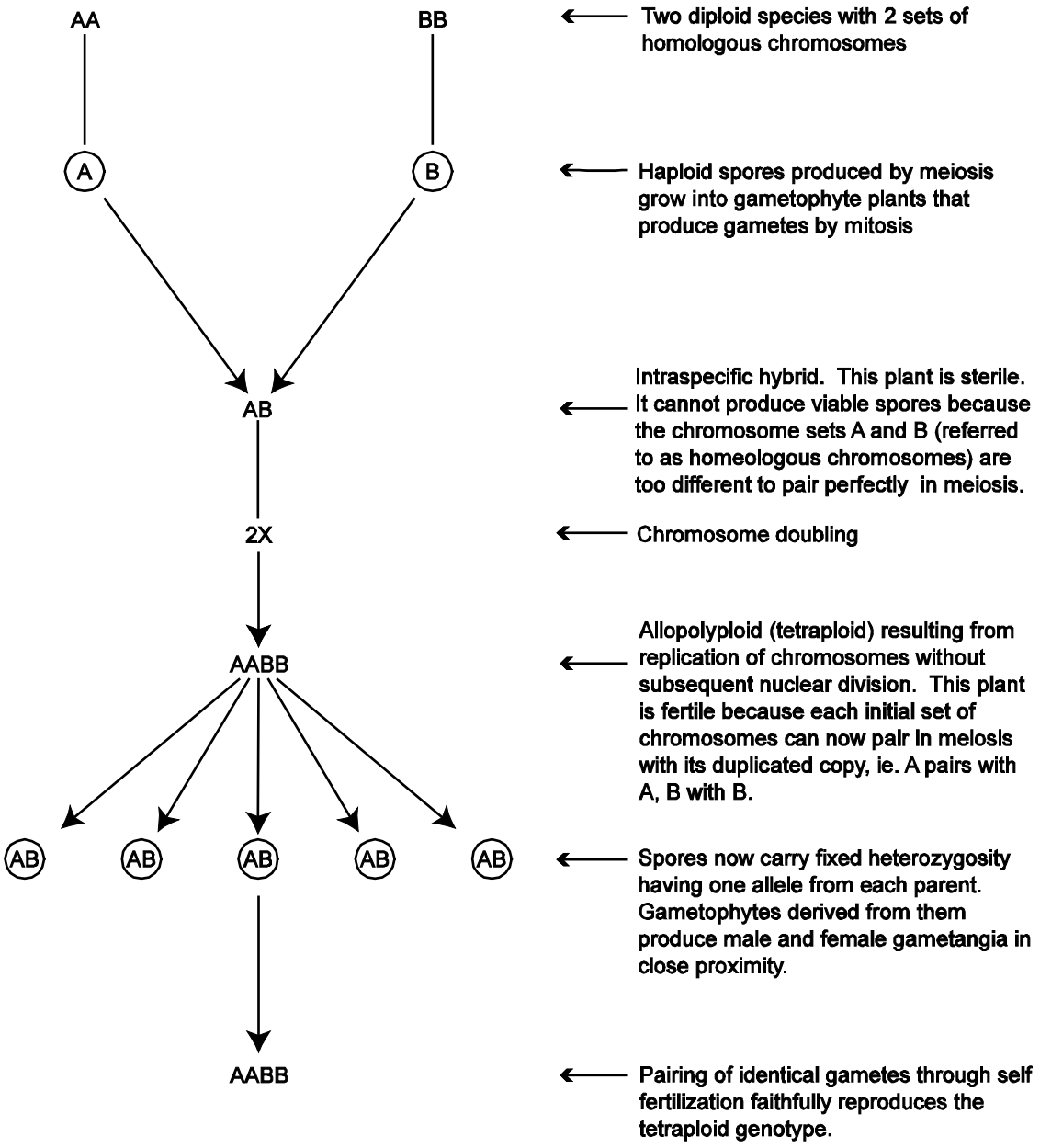

Figure 2. Origin of tetraploid species of *Botrychium*. Each letter represents one set of chromosomes. AA and BB represent two different diploid species.

Detecting ancestral parents of allotetraploid species.—When the typical isozyme patterns of all the diploid species have been determined, it is possible to determine which of these patterns, when combined, produce the pattern observed in a given tetraploid species. The answer is sometimes clear, as in the case of *Botrychium pinnatum* which shows a contribution from each of its parents, *B. lanceolatum* and *B. lunaria*, for nearly every gene tested. Furthermore, *B. lanceolatum* and *B. lunaria* are the only two diploid species which, when paired, can account for all of the patterns observed in *B. pinnatum*.

The parentage of other allotetraploid species is not as straightforward. Often the allotetraploid pattern implicates one set of parents at one gene locus, but a different set of parents at another locus. Occasionally a tetraploid expresses "orphan" alleles, alleles not present in any known diploid. A simple explanation for these anomalies is that the parent diploid species have changed since the ancient hybridization event that led to the formation of the allotetraploid. Because each species continues to evolve, over thousands of years it may be expected that some alleles currently present in the allotetraploid have been lost from the diploid species. It is also possible that one or both of the parental diploids may have become extinct. In cases of imperfect matches, electrophoresis adds to the clues provided by morphology, suggesting "best" matches among existing species. Table 3 lists the most probable parents of each polyploid *Botrychium* species. Figures 1 and 2 illustrate the morphological intermediacy of allotetraploid species between their probable ancestral diploid parents.

Multiple origins of allotetraploids.—Allotetraploid species result from a past hybridization event between two diploid species followed by chromosome doubling. It is reasonable to expect that such an event, involving the same two species happened more than once. That is, the same allotetraploid will have been formed from different hybridizations between different individuals of the same two diploid species, and this will have happened repeatedly. If the two parental diploid species possessed normal genetic and morphological variability among individual plants, combinations of different individuals will have produced slightly different forms of the same allotetraploid species. These different allopolyploid speciation events, occurring at different times and in different places, could result in a confusing array of slightly different genotypes and morphologies in different areas, each propagating its peculiar form through self-fertilization.

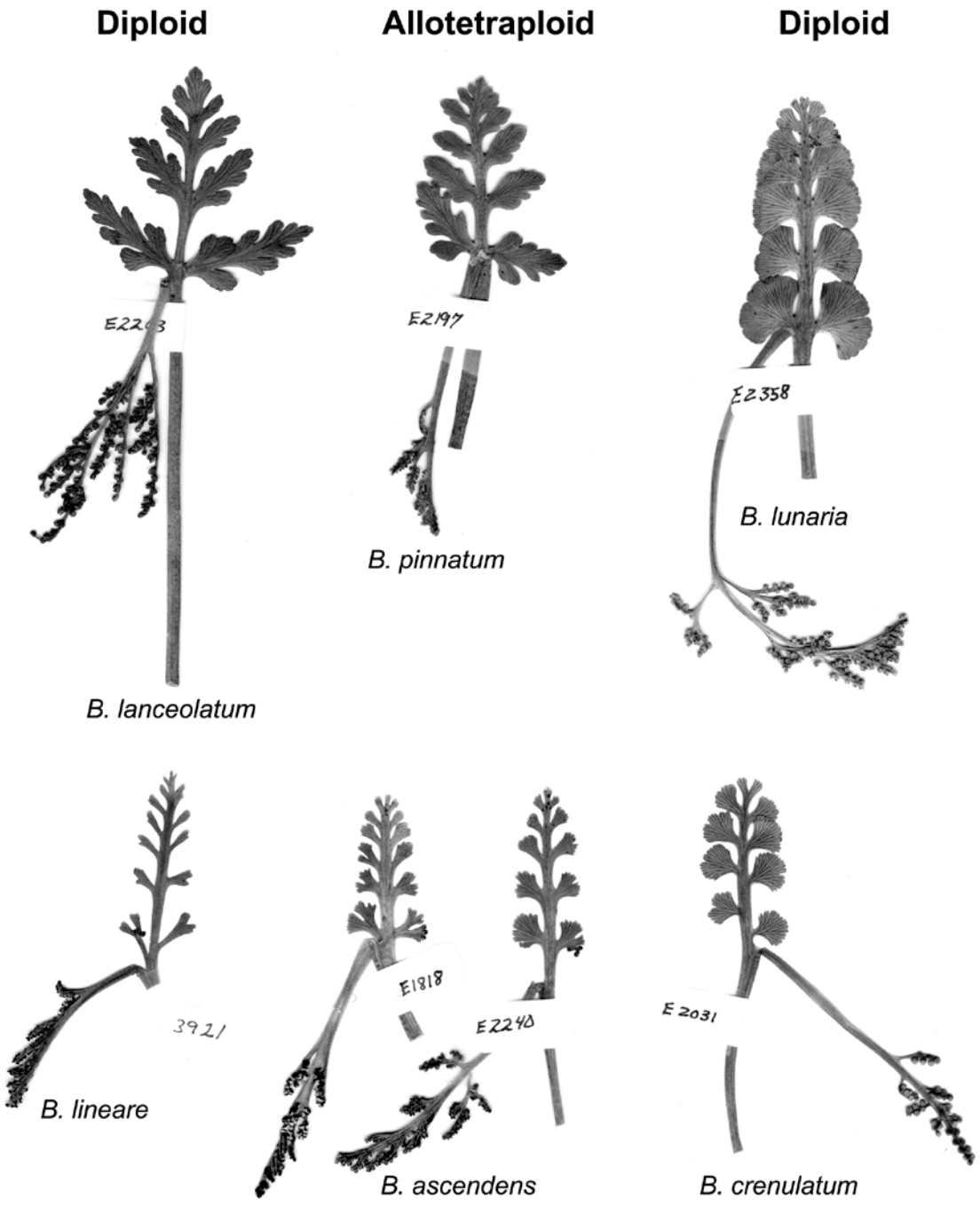

Figure 2. Allotetraploid species of *Botrychium* and their ancestral parents.
[This older diagram does not reflect the new taxonomy recognized elsewhere in this document. American *B. lunaria* is now recognized as *B. neolunaria sp. nov.*, *B. lineare* is now *B. campestre* var. *lineare comb. nov.*, and *B. crenulatum* is now *B. lunaria* var. *crenulatum* comb. *nov*.]

The above scenario seems to be the best explanation for the genotypic and morphological diversity displayed by *Botrychium matricariifolium* in the Great Lakes Region (Farrar and Wendel 1997). In a given site, one or several distinctive genotype/morphotype associations may exist, but when viewed from a regional perspective, all are subtypes of a basic species genotype derived from the same two ancestral diploid species. Furthermore, genotype/morphotype associations observed locally do not hold on a regional scale. The same genotype, as expressed in enzyme electrophoresis, may associate with a different morphology in different areas. A similar occurrence of multiple, subtly different morphotypes, all within the same basic species genotype, is present in western populations of *Botrychium minganense*, and, to a lesser extent, in other tetraploids, and may be the result of multiple origins of the species.

Gene silencing.—Another process that must be taken into account in explaining the genotypic and morphological diversity of allotetraploid species is gene silencing. In sexually reproducing species, it is imperative that at least one copy of each vital gene is maintained on each of two homologous chromosomes. (Although a single copy of a gene may suffice for production of the gene product, two copies on homologous chromosomes are necessary to assure that each haploid spore receives a copy.) It is not necessary that a plant have more than two copies of most genes. The "extra" gene copies in tetraploids are redundant and superfluous. These extra genes can become disfunctional without consequences that are lethal to the plant. Such disfunctional genes are said to have been silenced; they do not produce functional enzymes and thus are not detectable in electrophoresis procedures (Werth and Windham 1991).

When an expected allele from one of the allotetraploid's parent species is not present, it may be due to gene silencing. For example, because of the many unique alleles in *B. lunaria* and their presence in *B. minganense*, we can be fairly certain that *B. lunaria* was one of the diploid species involved in the hybridization leading to *B. minganense*. Yet, there are many populations of *B. minganense* in which the expected allelic contribution from *B. lunaria* is missing (has been silenced) in some enzymes (e.g. in triose phosphate isomerase (TPI)). Interestingly, the particular pattern of silencing is often different in different populations of the same allotetraploid species. In *B. minganense* either one, both or neither of the *B. lunaria* alleles for TPI-1 and TPI-2 may be silenced in a given population (see discussion under *Botrychium minganense*). The degree to which gene silencing can produce morphological or physiological change is unknown, but some researchers have proposed that extensive differentiation between populations via gene silencing could lead to differentiation and even the evolution of new species (Werth and Windham 1991).

Population Genetics of *Botrychium*

Breeding system.—In order to understand the distribution of genetic and morphological variation within and between species, it is necessary to understand the reproductive biology of moonwort ferns (see Life History section for a more complete description). Being pteridophytes, they have two separate life stages. The relatively large above-ground sporophyte produces spores that have half the number of chromosomes of the parent sporophyte. These spores germinate underground and grow into the gametophyte stage. Each gametophyte produces both male and female gametangia containing sperm and eggs, respectively. When a sperm is released from a mature antheridium, it swims to an open archegonium, then down the archegonial neck to an egg with which it fuses to form a diploid zygote, the initial cell of the next sporophyte generation. These acts of sexual reproduction take place underground. Travel through soil by swimming sperm must be considerably hindered relative to sperm swimming in liquid on the soil surface as is the case for most ferns. In the underground environment, sperm from one gametophyte plant may be unable to reach another gametophyte more than a few millimeters distant. They are quite capable though of swimming to archegonia and fertilizing eggs on the same gametophyte less than one millimeter away. This union of gametes from the same gametophyte constitutes intragametophytic self-fertilization.

Enzyme electrophoresis allows recognition of heterozygous individuals, those containing two different alleles at a given gene locus. Because heterozygous individuals of diploid species can be produced only by cross-fertilization between different gametophytes, electrophoretic determination of the number of heterozygous individuals in a population of a diploid species allows estimation of the amount of cross-fertilization that is occurring. Of thousands of individual *Botrychium* plants examined electrophoretically in several studies (Soltis and Soltis 1986, Hauk and Haufler 1999, Farrar 1998, 2001), less than 10% have shown heterozygosity from out-crossing. This observation provides strong support for the hypothesis that sexual reproduction in *Botrychium* is predominantly by intragametophytic self-fertilization.

Intragametophytic self-fertilization in pteridophytes has several important genetic consequences. Because all cells of an individual gametophyte are derived from a single initial cell (the spore), sperm and eggs produced by that gametophyte are genetically identical. Fertilization of an egg by sperm from the same gametophyte unites identical genotypes. The resulting sporophyte has exactly the genotype of the gametophyte from which it was produced. When that sporophyte produces spores, those too will be all be genetically identical and identical to the original gametophyte. Gametophytes growing from those spores will likewise be of the same genotype, and so on as long as intragametophytic selfing occurs. With no means of generating genetic variability (except by rare mutations) sexual reproduction in *Botrychium* through intragametophytic self-fertilization becomes equivalent genetically to vegetative reproduction.

Distribution of variability.—The sexual life cycle of *Botrychium* species, although it fails to generate genetic variability through recombination when accomplished through

intragametophytec selfing, still conveys distinct advantages. First, it facilitates wide distribution of plants through production of spores. Second, since all alleles are expressed when in a homozygous condition, this inbreeding system of *Botrychium* prevents accumulation of deleterious recessive alleles. This is important in considering the potential of a single isolated spore to initiate a new population.

Regularly out-breeding species accumulate a "genetic load" of deleterious recessive genes that are shielded from selection because they are not expressed in heterozygous individuals. If such individuals are forced to undergo self-fertilization, this genetic load is expressed in the homozygous offspring causing them to be inviable. [This is often referred to as inbreeding depression, a potential problem for small populations of out-breeding species.] For successful reproduction, out-crossing species require two genetically different gametophyte plants growing close enough to allow sperm of one to swim to the egg of the other. The farther apart the gametophytes, the less likely is fertilization. The farther spores travel from an established population, the less likely it becomes that two spores will land sufficiently close to allow cross fertilization. Thus, out-crossing species are hindered in colonizing new sites when these sites are at some distance (miles) from existing populations (Peck et al. 1990, Dassler and Farrar 2001, Farrar et al. 2008).

Species that regularly reproduce by intragametophytic selfing carry no genetic load. Inbreeding depression does not occur in such species because there are no shielded deleterious alleles. These species have a distinct advantage in long-distance dispersal and establishment (Crist and Farrar 1983, Peck et al. 1990). A single spore dispersed a long distance from the parent plant is capable of producing an isolated gametophyte which can successfully reproduce by self fertilization. The resulting sporophyte then can produce spores and a new population. However, each plant of this new population will be genetically identical, carrying the genotype of the original spore. This explains why the genetic variability found in *Botrychium* species is often partitioned among populations rather than among individuals within populations (Farrar 2001). That is, all individuals at a given site are often of one genotype, whereas all those at another site may be of a different genotype. [Different genotypes may exist within the species due to differentiation through mutation and, in tetraploids, gene silencing and multiple origins (see Species and Evolution).]

Often an individual genotype is associated with subtle but distinctive morphological traits. In *Botrychium* populations produced from a single "founding" spore, all members will maintain that "phenotype" of distinctive traits. Such a population is essentially a clone, the same as if it had been produced by vegetative reproduction. It is similar to a clone of aspens, all of which often maintain a distinctive appearance.

If a second spore from a different source lands in the vicinity of the first population it may bring a new and distinctive genotype and phenotype to the area. Because members of each phenotype reproduce only by self-fertilization, the two phenotypes may exist side-by-side without blending. It is thus possible to perceive distinctive populations of the same species which are adjacent or co-mingled, much the

same as it is often possible to perceive distinctive adjacent or co-mingling clones of aspens.

It is important to appreciate the difference between adjacent but non-blending populations of the same *Botrychium* species and adjacent but non-blending populations of two different Botrychium species. In the first case, plants of the two types remain distinct because of intragametophytic selfing as described above. In the second case, the two types remain distinct because they are genetically incompatible—interbreeding produces only sterile plants. Maintenance of non-blending phenotypes within the same area is often cited as an indication that genetic differentiation between the two types has reached the level of species distinction, but because of intragametophytic selfing, maintenance of morphological distinctiveness between adjacent "clones" in *Botrychium* is not always an indication of different species.

Migration.—Because of the small size of their propagules (spores), migration of fern species through spore dispersal, on average, is greater than that of most seed plants (Smith 1972). That long-distance migration does occur in some species is undisputable (Crist and Farrar 1983, Ranker et al. 1994), however, as pointed out by W. H. Wagner (1972), most fern species show the same types of range restrictions as seed plants.

As discussed above under Breeding Systems, for many fern species migration is restricted to short distances by the requirement for two spores, and the gametophyte plants growing from them, to be sufficiently close (a few centimeters) to permit cross-fertilization. In such species failure to attain bisexuality and/or genetic load (recessive lethal alleles) prevent successful reproduction by isolated gametophytes (Peck et al. 1990).

However, in *Botrychium* species, individual gametophytes regularly become bisexual and all studies examining these plants genetically indicate that most sporophyte plants are produced through intragametophytic selfing, that is, fertilization of the egg by sperm from the same gametophyte. This being the case, successful migration by *Botrychium* species should be limited only by the distance of spore travel and the probability of a still viable spore landing in a suitable habitat. [Suitable habitat, discussed elsewhere, may include access to mineral soil, appropriate soil chemistry and moisture, presence of mycorrhizal fungi, etc.]

Peck et al. (1990) determined that more than 90% of spores released by *Botrychium virginianum* were deposited within five meters of the source plant, with that number increasing with the degree to which the source plant was immersed within surrounding vegetation. This sharply curtailed dispersal pattern is typical of the leptokurtic pattern obtained by other studies of spore dispersal (Ingold 1971). It must be recognized however that even 1% of the spores produced by a typical moonwort is a very large number (thousands) of potential propagules. Despite this seemingly large potential for migration, recent genetic studies on *Botrychium simplex* (Farrar unpublished) further document surprising restriction of migration over small distances.

In populations in the Sierra Nevada range of California, populations of *B. simplex* display unusually high variability in allelic composition, with several alleles restricted to one or a few populations. Such differences could not be maintained among populations with unrestricted inter-populational migration. On a smaller scale, 14 samples of a presumed metapopulation of *B. simplex* within a 2 x 0.5 mile meadow display a similar pattern. Extreme differentiation among populations is present, including unique alleles, between populations less than 100 M apart, even though the plants grow in short meadow vegetation with sporophores elevated above the vegetation. Analysis of allele distributions among populations across the meadow by spatial genetic structure analysis indicated that effective migration via spore dispersal was limited to 500 meters. This evidence indicates restriction of migration such that populations more than a few miles apart may be effectively isolated and that suitable uncolonized habitat at these distances have a low probability of receiving sufficient spore rain to assure colonization.

Genetic variability.—It is important to keep in mind that the overall genetic variability within *Botrychium* species is remarkably low relative to other ferns and vascular plants. The average number of alleles per gene locus in diploid moonwort species is 1.36, the average for all ferns is 2.3 and the average for seed plants is 1.96. In moonworts only 28.8% of gene loci have more than one allele. For all ferns this average is 60.2% and for flowering plants it is greater than 50%. Even self-pollinating flowering plants maintain a higher level of genetic variability (1.69 and 41.8%) than do moonwort *Botrychium* species (Hamrick and Godt 1990, Li and Haufler 1999, Farrar 1998).

The low genetic variability in *Botrychium* is due in large part to its reproductive mode of intragametophytic selfing that causes all alleles to be expressed. Deleterious alleles are not shielded through heterozygosity as is the case in out-breeding species. It is likely also that metapopulation dynamics have contributed to loss of genetic diversity through multiple founder effects where few or only a single genotype is transferred through successive short-lived populations.

Allopolyploid variability.—Low genetic variability and its causes are somewhat ameliorated in allopolyploids. These species can maintain fixed heterozygosity despite intragametophytic selfing. In meiosis, each spore always receives one chromosome from each pair of homologs contributed from each of the diploid “parents”. Each of the two chromosome sets forming the initial hybrid, after doubling, pair and separate independently of the other. Intragametophytic selfing assures that each set remains homozygous, but any differences between the sets remains “fixed”, unless altered by gene silencing or other mutations.

Many more species of *Botrychium*, both diploid and polyploid, have evolved than are now present. We can think of these as evolutionary experiments, some have failed (gone extinct), others have remained, leaving us with our current suite of species. Because such a high proportion (56%) of the extant species are allopolyploids, we can speculate that there are some advantages to being allopolyploid. Possibly allopolyploids have a greater adaptability than diploids because of their ability to retain higher levels of

genetic variability through fixed heterozygosity and gene silencing (Soltis & Rieseberg 1986, Werth and Windham 1991).

Population Dynamics in Relation to Conservation

A number of *Botrychium* populations have been rediscovered in what aare almost certainly their historic sites after periods exceeding a half century. Not surprisingly, plants are no longer seen at many other monitored or carefully described sites. From this evidence it seems prudent for conservation purposes to assume a metapopulation model for *Botrychium* population dynamics. In this model, individual populations have a finite lifespan but their extinction is continuously balanced by the establishment of new populations. Possible causes of individual population extinction include physical habitat alteration, community succession, competition, predation and disease. Although individual populations may become extinct despite conservation efforts, it remains important to maintain their viability for as long as possible because they are the source of spores that will found new populations in appropriate habitat. It is equally important to maintain unoccupied suitable habitat as sites for new populations.

A metapopulation approach assumes some degree of migration among subpopulations within a given metapopulation and restricted migration between metapopulations. Metapopulation dynamics and the potential size of metapopulations of *Botrychium* is dependent upon the frequency and distance of spore dispersal.

Genetic differences between distant populations of the same species indicates that effective migration by long-distance spore dispersal is much more limited than generally assumed. Although spores undoubtedly enter atmospheric air streams, apparently survival of atmospheric conditions and/or deposition in suitable habitats appears to be a rare occurrence. For example, a unique allele of *B. neolunaria* has been recorded in the Black Hills of South Dakota. Genetic analysis of hundreds of *B. neolunaria* plants in the Rocky Mountains, Canada, and the Great Lakes area has not yet detected that allele outside the Black Hills. On a smaller scale, analysis of allele distributions in populations of *B. simplex* var. *compositum* in the Sierra Mountains of California indicate and effective dispersal distance of less than one mile, despite this being the most abundant species in the area (see discussion under Migration). With these limitations to effective dispersal, proximity of suitable habitat is highly important to maintenance of metapopulations

b. Genetic vulnerability to environmental change.

Underground bisexual gametophytes are characteristic of all Ophioglossaceae and of their closest relatives, the Psilotaceae. If low genetic variability in *Botrychium* is due to intragametophytic selfing which, in turn, is imposed by the underground environment, then we can reasonably assume that *Botrychium* species have typically maintained relatively low genetic variability.

Two concerns are often raised regarding the vulnerability of species with low levels of genetic variability, especially those in small populations. First, it is inevitable that small populations of typically out-breeding species experience an increased rate of inbreeding. Such populations can suffer inbreeding depression caused by the expression of recessive deleterious alleles in the homozygous state. Second, low genetic variability can reduce a species' ability to adapt to a change in environment or to a range of environments.

Because of regular intragametophytic selfing, most *Botrychium* species are not subject to inbreeding depression. They do not carry a genetic load of deleterious alleles sheltered in heterozygous individuals. All of their gene alleles have already been exposed to environmental selection, only non-deleterious alleles remain in their genome. Because of their immunity to inbreeding depression, genetic fitness does not vary with population size in *Botrychium*.

How *Botrychium* species cope with environmental variability and change is not clear. On the whole, *Botrychium* species do not seem to be any more habitat specific or any less widespread geographically than are other ferns or seed plants, despite their low genetic variability. A possible answer to this conundrum lies in the mycorrhizal association maintained by *Botrychium* species. A number of observations strongly suggest that moonwort *Botrychium* rely heavily, if not entirely, on their mycorrhizal partner for photosynthates, mineral nutrients and water. With mycorrhizal fungi as an intermediary, *Botrychium* have greatly reduced direct interaction with their environment. They likely have less need for genetic tracking of environmental change than do most plants. Their greater need is for genetic stability in maintaining their mycorrhizal association.

Regardless of the means by which *Botrychium* species cope with reduced genetic variability, we can feel confident that they have done so effectively for thousands if not millions of years. <u>This lack of genetic variability in *Botrychium* should not be a concern in assessing species or population viability</u>.

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