SYSTEMATICS OF MOONWORTS
BOTRYCHIUM SUBGENUS BOTRYCHIUM

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SYSTEMATICS OF GENUS *BOTRYCHIUM* SUBGENUS *BOTRYCHIUM*

A. 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 and the first treatment to recognize the current subgenera 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 1993). This order of plants has no close relatives among the ferns. 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 (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 (2000) reported *Botrychium* and *Sceptridium* to be well supported entities, but found *Botrypus* to be paraphyletic.

In 1938 Clausen recognized only six species of moonworts: *Botrychium lunaria*, *B. simplex*, *B. pumicola*, *B. boreale*, *B. matricariifolium* and *B. virginianum*. 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*. 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 subgenus *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 1998 the number of species recognized in subgenus *Botrychium* increased rapidly. Through extensive field studies and chromosome analyses the Wagners described five new diploid species, *B. campestre, B. crenulatum, B. lineare, B. montanum*, and *B. pallidum*, and seven polyploid species, *B. acuminatum, B. ascendens, B. echo, B. paradoxum, B. pedunculosum, B. pseudopinnatum*, and *B. spathulatum*. Three additional species recognized by the Wagners during this period (*B. adnatum, B. alaskense, 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*.

Currently the North American species of subgenus *Botrychium* include 27 species, 11 diploids (n = 45), 15 tetraploids (n = 90) and 1 hexaploid (n = 135) (Table 1, 2). This list does not include *B. boreale*, the only European species not yet recorded in North America (see discussion of *B. pinnatum*). The list also does not segregate varieties of *B. simplex* that may ultimately be raised to species level (see discussion of *B. simplex*).

Table 1. Diploid North American Species of *Botrychium* subgenus *Botrychium*

Species	General Distribution
B. campestre	Great Lakes and northern Great Plains
B. crenulatum	western mountains of US
B. lanceolatum	boreal US and Canada western and eastern mountains of NA
B. lineare	Western mountains of US
B. lunaria	boreal US and Canada western mountains of NA
B. montanum	western mountains of US
B. mormo	boreal regions of Great Lakes states
B. pallidum	Great Lakes and northern Great Plains occasional in Rocky Mts. and New England
B. pumicola	Cascade mountains of western Oregon
B. simplex	boreal US and Canada western and eastern mountains of NA
B. tunux	southeastern Alaska

Table 2. Polyploid North American Species of *Botrychium* subgenus *Botrychium*

Species*	General Distribution	Probable Parentage**
*B. acuminatum****	Great Lakes states	*B. lanceolatum* x *B.* "*pallidum*"
B. ascendens	boreal US and Canada western mountain of NA	*B. crenulatum* x (*B. lineare* or *B. campestre*)
B. alaskense	Alaska	*B. lanceolatum* x European *B. lunaria*
B. echo	Rocky Mountains (Colorado)	*B. lanceolatum* x (*B. lineare* or *B. campestre*)
B. gallicomontanum	western Minnesota northwestern Montana	*B. campestre* x *B. pallidum*
B. hesperium	Rocky Mountains of US and Canada	*B. lanceolatum* x *B.* "*pallidum*"
B. matricariifolium	Great Lakes and north-eastern US and Canada	*B. lanceolatum* x *B.* "*pallidum*"
B. minganense	boreal US and Canada western mountains of NA	*B. lunaria* x *B.* "*pallidum*"
B. paradoxum	Rocky Mountains of US	undetermined
B. pedunculosum	Rocky Mountains of US rare in Quebec and Alaska	*B. lanceolatum* x *B.* "*pallidum*"
B. pseudopinnatum	Northern Great Lakes	*B. lanceolatum* x *B. lunaria* x *B* "*pallidum*"
B. pinnatum	western North America	*B. lanceolatum* x American *B. lunaria*
B. spathulatum	Great Lakes states Alaska and western Canada	European *B. lunaria* x *B. lineare*
B. yaaxudakeit	Alaska and western Canada (Montana)	American *B. lunaria* x European *B. lunaria*
B. 'adnatum'	Northwestern Montana	*B. simplex* x *B. pallidum*
B. 'michiganense'	Great Lakes, Black Hills, US northern Rocky Mountains	*B. lanceolatum* x *B.* "*pallidum*"

*Species in single quotes are undescribed. Names are provisional pending official publication. Two additional tetraploid taxa are under study.
**Determined by allelic comparison to known diploid species (see next section). *B.* "*pallidum*" is a species that among known diploid species is most similar to extant *B. pallidum* but containing alleles not present in that species. Possibly *B.* "*pallidum*" is extinct.
***B. acuminatum* is genetically undifferentiated from *B. matricariifolium* and morphological intermediates exist.

B. 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, genus and subgenus as outlined below (see Figure 1).

Botrychium species 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.

Moonworts vs Grapeferns and Rattlesnake Fern.—Moonworts belong to subgenus *Botrychium*, one of three subgenera of genus *Botrychium* in North America. Species of subgenus *Sceptridium* are known as the evergreen grapeferns. In addition to being everggreen, they differ from moonworts (subgenus *Botrychium*), in their generally larger size (trophophore width usually greater than 2") leathery texture, trophophore attitude (blade held ± parallel to the ground) and position of the trophophophore – sporophore union (at or below ground level). Although the trophophore of large moonworts may equal or exceed that of grapeferns in length, it is seldom greater than 2" in width and usually much narrower. Moonworts have a fleshy or delicate texture with the trophophore angled distinctly upward. With the exception of *B. simplex*, the sporophore – trophophore union of moonwort species is well above ground level. In *B. simplex*, pinnae above the first pair are simple and fan-shaped whereas those of grapeferns are elongate and often lobed or pinnately divided.
Small plants with no sporophore present are almost always grapeferns or rattlesnake fern.

Plants of subgenus *Osmundopteris*, the rattlesnake fern (*B. virginianum*), are usually much larger than moonworts, but in some habitats they may be of equal size, have a blade texture similar to that of many moonworts, and have a high sporophore – trophophore junction. Small plants of *B. virginianum* can be differentiated from all moonworts except *B. lanceolatum* by their distinctly triangular and glossy surfaced trophophore. They can be differentiated from *B. lanceolatum* by the long stalk of the sporophore as compared to the very short stalk (shorter than or equal in length to the spore-bearing portion) of the sporophore of *B. lanceolatum*.

Moonwort characteristics.—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-dissected (pinnate), fan-leaflet form of most diploid species (Figure 1d), the triangular, twice-dissected (bipinnate) form of *B. lanceolatum* (Figure 1f), and the intermediate

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(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 strong central veins, 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.

Unusually large plants of the fan-leaved, 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 5c). Convenient dividing points are: less than 60°, between 60° and 120°, between 120° and 180°, and greater than 180°. **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 ($30-90^o$), obtuse ($>90^o$), truncate (180°) 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, broadest and most highly dissected.

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 deeper green color and often a 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. Exceptionally 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 two species, *Botrychium ascendens* and *B. pedunculosum*, but not all plants of these species have supernumerary sporangia, especially when growing in deep shade. *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
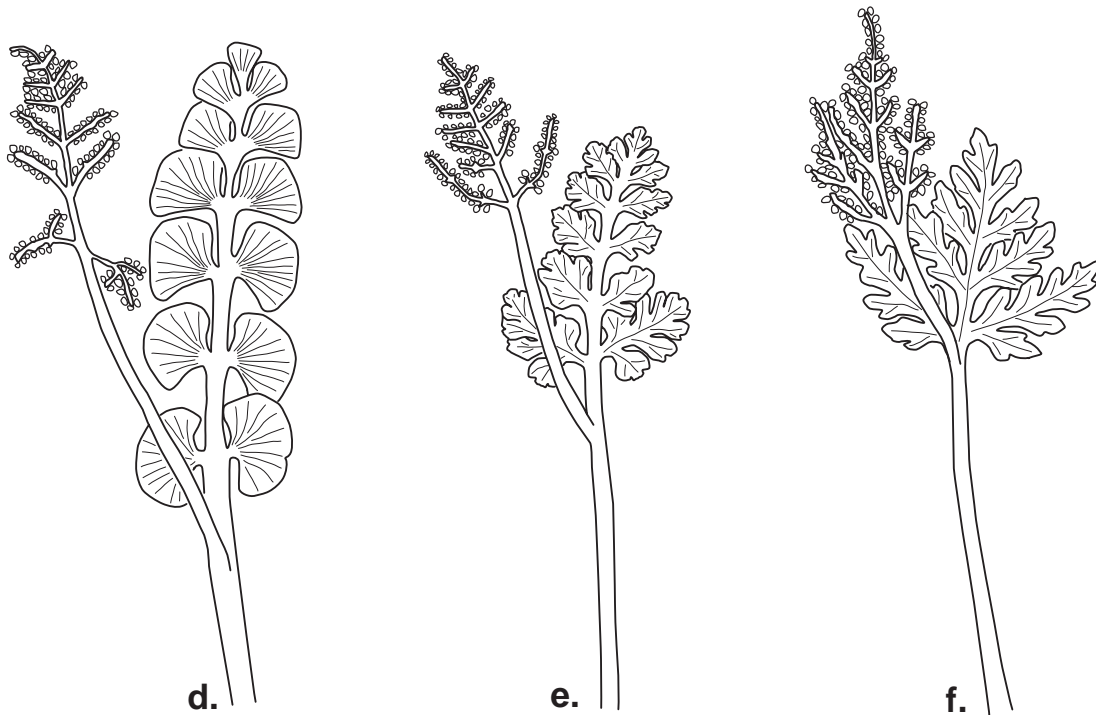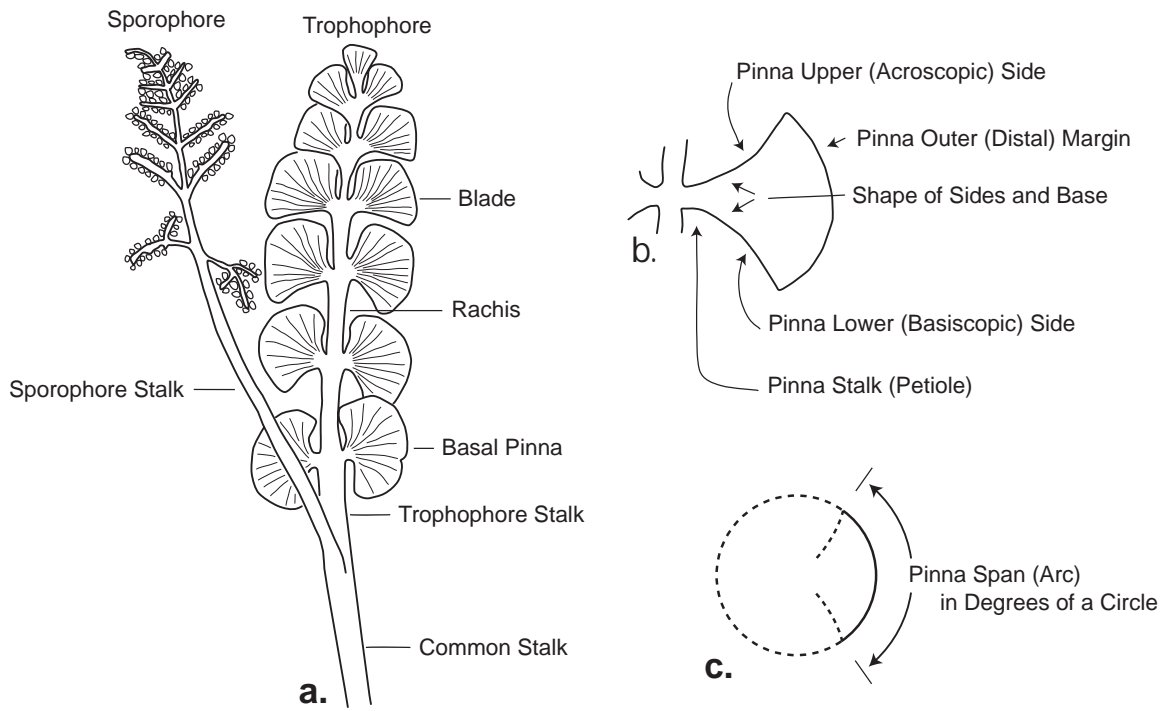
Figure 1. Morphology and terms used in moonwort identification.

first-generation hybrid between *B. paradoxum* and *B. michiganense* 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.

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* range from 24 to 32 microns whereas those of *B. minganense* range from 32 to 40 microns (Wagner and Lord 1956). The spores of *B. simplex* are unusually large for a diploid species, ranging from 40 to 50 microns.

Characters of the once-pinnate species of moonworts (*Botrychium* subgenus *Botrychium*)

character \ species	ascendens	campestre	crenulatum	lineare	lunaria	minganense	montanum	pallidum	pumicola	simplex	tunux	yaaxudakeit
stalked trophophore	no	no	yes	no	no (yes)	yes	yes	yes	no (yes)	yes	yes	yes
trophophore stalk length relative to avg. pinna spacing	v	v	< or >	v	v	= or >	^	= or >	v	^	v	v
sporophore stalk length relative to trophophore length	< to =	v	< to =	v	^	< to =	= to >	< to =	v	^	v	^
disproportionate spacing between 1st and 2nd pinna pairs	no	no	no	no	no	no (yes)	yes (no)	yes (no)	yes	yes	no	no
basal pinnae disproportionately enlarged	no	no	no	no	no	no (yes)	no	no	yes	yes	no	no (yes)
basal pinnae pinnately divided	no	no	no	no	no	no	no	no	yes	yes	no	no
basal pinnae with sporangia	often	rarely	rarely	rarely	no	rarely	rarely	rarely	no (may be extra sporophore)	no (may be extra sporophores)	no	no
span of basal pinnae (°)	60 - 90	10 - 45	90 - 160	10 - 45	120 - 180	60 - 120	0 - 60	60 - 100	90 - 120 if simple	60 - 120 if simple	120 - 180	180 - 250
span of third pinnae (°)	45 - 60	10 - 45	60 - 90	10 - 45	60 - 90	60 - 90	0 - 30	60 - 90	90 - 120	60 - 90	60 - 90	90 - 180
basal pinnae cleft	often	often	rarely	often	rarely	rarely	rarely	often	often	often	rarely	rarely
third pinnae cleft	often	often	rarely	often	rarely	rarely	rarely	rarely	rarely	rarely	rarely	no
upper pinnae adnate to rachis	no	rarely	no	no	no	rarely	yes	+ or -	+ or -	yes	no	no
pinna ascending	yes	yes	no	yes	yes	yes	yes	no	yes	yes	no	yes
pinna shape	fan to spatulate	linear to spathulate	fan to lunate	linear to spathulate	fan to lunate	fan to spathulate	blocky	fan to spathulate	fan to spathulate	fan to butterfly	fan to lunate	lunate
pinna margin	dentate to fimbriate	dentate to lobed	finely crenulate	dentate to lobed	entire to dentate	entire to lobed	dentate to fimbriate	entire to dentate	entire to lobed	entire to crenulate	entire to lobed	entire to dentate
basal pinna shape												

Characters of the twice-pinnate species of moonworts (*Botrychium* subgenus *Botrychium*)

species / character	alaskense	echo	hesperium	lanceolatum	michiganense	pedunculosum	pinnatum
trophophore dissection	pinnate	pinnate	pinnate	ternate	pinnate	pinnate	pinnate
trophophore surface	lustrous	glaucous	glaucous	lustrous	glaucous	glaucous	lustrous
middle pinnae base	acute	acute	obtuse	acute	acute	obtuse	obtuse
pinna apices	acute	acute	rounded	acute	rounded to acute	rounded to acute	rounded
pinna lobes	spreading	spreading	parallel to converging	spreading to parallel	parallel	parallel	parallel
basal pinnae disproportionately enlarged	no	no	yes (no)	yes (no)	yes	no (yes)	no
trophophore stalk shorter than average pinna spacing	yes	yes	no	yes	yes	no	yes
sporangia on basal pinnae	no	no	no	no	no	yes	no
sporophore branching	ternate	pinnate	ternate	ternate	ternate or pinnate	ternate	pinnate
sporophore stalk much shorter than trophophore length	yes	no	no	yes	no	yes	no
common stalk color	red or green	green	green or 1/2 brown	green or red	green	1/2 red, 1/2 green	red or green
pinna outline upper / mid / basal							

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Key to Eastern Species of Moonwort Ferns
(*Botrychium* subgenus *Botrychium*)

1. Pinnae above the basal pair fan-shaped, rhombic or linear, sometimes cleft into segments, but not pinnately lobed or divided, veins equal and evenly distributed. . . **2** *(the once dissected, pinnate species)*
1. Pinnae above the basal pair ovate or elliptic, often pinnately lobed or divided, middle veins larger and more crowded. . . **17** *(the twice dissected, bipinnate and pinnate-pinnatifid species)*

2. Basal pinna pair often conspicuously enlarged and/or more distinctly stalked than others, in large plants pinnately divided and replicating the central rachis and pinnae. . . **3**
2. Basal pinna pair not conspicuously larger or more stalked than second pinna pair and not pinnately divided. . . **4**

3. Middle pinnae broadly attached to rachis and strongly decurrent. . . *B. simplex* var. *simplex*
3. Middle pinnae narrowly attached to rachis and slightly or not at all decurrent. . *B. simplex* var. *compositum*

4. Middle (and sometimes basal) pinnae wider than long, often rhombic, broadly attached and strongly decurrent to rachis. . . **5**
4. Middle pinnae longer than wide, not rhombic, narrowly attached to rachis, not strongly decurrent. . . **7**

5. Plants stout; pinnae fleshy, white to pale green to green, with more or less straight outer margins; basal pinnae not elongated. . . *B. mormo*
5. Plants slender; pinnae thin, yellow-green, with outer margins wavy to round; basal pinnae often elongated anteriorly. . . **6**

6. Sporophore stalk at spore release longer than common stalk and longer than the trophophore. . . *B. simplex* var. **simplex**
6. Sporophore stalk at spore release shorter than common stalk and shorter than or equal to the length of the trophophore. . *B. simplex* var. *tenebrosum*

7. Basal pinnae narrow, with a span (degrees of a circle spanned by the outer margin) of less than 45°. . . **8**
7. Basal pinnae wedge-shaped to half-moon shaped, with a span of $> 60^o$. . . **9**

8. Pinna width increasing toward outer margin that is lobed to shallowly cleft; largest pinnae in middle of trophophore. . . *B. campestre*
8. Pinna width not increasing toward outer margin that is often cleft into two or four linear segments; basal pinnae the largest. . . *B. lineare*

9. Basal pinnae wedge-shaped to broadly spathulate (a wedge with rounded margins) with a pinna span of 60 – 120°. . . **10**
9. Basal pinnae half-moon-shaped, with a pinna span of 120 – 180°. . . **15**

10. Trophophore sessile or short-stalked. . . **11**
10. Trophophore distinctly stalked. . . **12**

11. Trophophore sessile; pinna outer margins entire to coarsely dull-toothed to shallowly cleft into non-spreading lobes; basal sporophore branches long, often branched and twisted; basal pinnae seldom bearing sporangia*. . .
                                                            B. spathulatum
11. Trophophore sessile to short-stalked; pinna outer margins sharply toothed, sometimes deeply cleft into spreading lobes with toothed outer margins; basal sporophore branches short, unbranched and straight; basal pinnae often bearing sporangia*. . .   ***B*. *ascendens***

12. Sporophore tall, its stalk (at time of spore release) equal to or exceeding the total length of the trophophore; sporophore branches spreading, not overlapping; pinnae fan-shaped and entire to shallowly lobed. . .
                                                            B. minganense
12. Sporophore short, its stalk (at time of spore release) 3/4 or less the entire length of the trophophore; sporophore branches ascending and overlapping; pinnae entire to deeply cleft into 2 (4) lobes. . . **13**

13. Pinnae entire or symmetrically cleft, outer margins regularly dentate; basal pinnae often bearing supernumerary sporangia*. . .   ***B. ascendens***
13. Pinnae entire or asymmetrically cleft into a larger upper and smaller lower lobe; basal pinnae seldom bearing supernumerary sporangia. . . **14**

14. Plants in the field with a whitish-green appearance (changing to green following collection and storage); outer pinna margins entire to crenulate; pinnae evenly spaced. . .   ***B. pallidum***
14. Plants dull green; outer pinna margins entire to irregularly toothed; space between 1st and 2nd pinna pair disproportionately large. . .
                                        `   ***B. gallicomontanum***

15. Plants in the field with a whitish-green appearance, medial pinnae broadly attached to rachis; usually 4 or fewer pinna pairs; often cleft into larger upper and smaller lower lobes. . .   ***B. pallidum***
15. Plants in the field deep green to yellow-green, medial pinnae narrowly attached to rachis; usually 5 or more pinna pairs; if lobed, lobes more or less equal. . . **16**

16. Trophophore clearly stalked; basal pinnae similar in shape to medial pinnae, green to yellow green. . . ***B. minganense***
16. Trophophore stalkless or nearly so; basal pinnae broader than medial pinnae; dark green to green. . . ***B. lunaria***

17. Trophophore ternate (divided nearly equally into three parts) due to large size of basal pinnae (each more or less mimicing the remainder of the main rachis and pinnae. . . ***B. lanceolatum*** subsp *angustisegmentum*
17. Trophophore with a single central axis although the basal pinnae may be disproportionately elongated (exceeding the length expected in a gradual increase from the apex to the base of the leaf). . . **18**

18. Sporophore pinnate, even in large specimens; trophophore lustrous deep green. . . ***B. pseudopinnatum***
18. Sporophore ternately divided in robust specimens; trophophore dull pea green. . . **19**

19. Trophophore sessile or nearly so; 2nd pinna pair usually much reduced in size and lobing relative to basal pinnae, often nearly entire across anterior side margin. . . ***B. michiganense***
19. Trophophore distinctly stalked; pinna size and lobing gradually reduced from basal to upper pinnae. . . **20**

20. Pinnae remotely and irregularly lobed; pinna bases acute; basal pinnae often bearing supernumerary sporangia*. . . ***B. pedunculosum***
20. Pinnae regularly multilobed; pinna bases obtuse to truncate; basal pinnae seldom bearing supernumerary sporangia*. . . ***B. matricariifolium***

All species occasionally produce sporangia on the lowermost pinnae.

Key to Western Species of Moonwort Ferns
(*Botrychium* subgenus *Botrychium*)

1. Leaf with a trophophore (photosynthetic segment) and a sporophore (spore-bearing segment). . . **2**
1. Leaf with two more or less equal sporophore segments and no trophophore.
   B. paradoxum

2. Pinnae above the basal pair fan-shaped, rhombic or linear, sometimes cleft into segments, but not pinnately lobed or divided, pinna veins equal and evenly distributed. . . **3** *(the once dissected, pinnate species)*
2. Pinnae above the basal pair ovate or elliptic, often pinnately lobed or divided, middle veins larger and more crowded. . . **21** *(the twice dissected, bipinnate and pinnate-pinnatifid species)*

3. Basal pinna pair often conspicuously enlarged and/or more strongly stalked than others, in large plants pinnately divided and replicating the central rachis and pinnae. . . **4**
3. Basal pinna pair not conspicuously larger or more strongly stalked and not pinnately divided. . . **6**

4. Trophophore sessile or nearly so; sporophore stalk shorter than trophophore. . .
   B. pumicola
4. Trophophore prominently stalked; sporophore stalk longer than trophophore. . . **5**

5. Middle pinnae narrowly attached, pinna bases acute to acuminate, light green. . .
   B. simplex var. **compositum**
5. Middle pinnae broadly attached, pinna bases cordate, bluish green. .
   B. simplex var. **fontanum**

6. Trophophore stalk longer than distance between first two pair of pinnae <u>and</u> sporophore stalk (at the time of spore release) much longer than the total length of the trophophore. . . **7**
6. Trophophore stalk shorter than distance between first two pair of pinnae <u>or</u> sporophore stalk (at the time of spore release) shorter than the total length of the trophophore. . . **8**

7. Pinnae round to fan-shaped, outer margin rounded, entire; trophophore lax. .
   B. simplex
   (see above for differentiation of western varieties of B. simplex)
7. Pinnae rhombic, outer margin straight, coarsely toothed; trophophore stiffly upright. . .
   B. montanum

8. Basal pinna span less than 60°; pinnae narrowly spatulate to linear or rhombic. . . **9**

8. Basal pinna span greater than 60°; pinnae broadly spatulate to fan-shaped. . . **11**

9. Pinnae as broad as long, adnate to the rachis, strongly decurrent. . . ***B. adnatum***

9. Pinnae elongate, more or less linear to narrowly wedge shaped. . .**10**

10. Pinnae narrowly spatulate to wedge shaped, often shallowly cleft into non-spreading lobes, largest pinnae usually not basal. . . ***B. campestre***

10. Pinnae linear, often deeply cleft into widely spreading lobes, basal pinnae usually the largest. . . ***B. lineare***

11. Span of basal pinnae 60° to 120°. . . **12**

11. Span of basal pinnae greater than 120°. . . **17**

12. Trophophore sessile or short-stalked. . . **13**

12. Trophophore distinctly stalked. . . **14**

13. Trophophore sessile; pinna outer margins entire to coarsely dull-toothed to shallowly cleft into non-spreading lobes; basal sporophore branches long, often branched and twisted; basal pinnae seldom bearing sporangia*. . . ***B. spathulatum***

13. Trophophore sessile to short-stalked; pinna outer margins sharply toothed, sometimes deeply cleft into spreading lobes with toothed outer margins; basal sporophore branches short, unbranched and straight; basal pinnae often bearing sporangia*. . . ***B*. *ascendens***

14. Sporophore tall, its stalk (at time of spore release) equal to or exceeding the total length of the trophophore; sporophore branches spreading, not overlapping; pinnae fan-shaped and entire to shallowly lobed. . . ***B. minganense***

14. Sporophore short, its stalk (at time of spore release) 3/4 or less the entire length of the trophophore; sporophore branches ascending and overlapping; pinnae entire to deeply cleft into 2 (4) lobes. . . **15**

15. Pinnae entire or symmetrically cleft, outer margins regularly dentate; basal pinnae often bearing supernumerary sporangia*. . . ***B. ascendens***

15. Pinnae entire or asymmetrically cleft into a larger upper and smaller lower lobe; basal pinnae seldom bearing supernumerary sporangia*. . . **16**

16. Plants in the field with a whitish-green appearance (changing to green following collection and storage); outer pinna margins entire to crenulate; pinnae evenly spaced. . . **B. pallidum**

16. Plants dull green; outer pinna margins entire to irregularly toothed; space between 1st and 2nd pinna pair disproportionately large. . . **B. gallicomontanum**

17. Plants pallid (whitish) when fresh; pinnae short and broadly attached to the rachis, mushroom shaped, entire to 2-4 lobed with upper lobe(s) larger. . **B. pallidum**

17. Plants deep green to pea green; pinnae narrowly attached to the rachis, usually symmetrical, fan-shaped, entire or variously cleft into lobes. . . **18**

18. Pinnae well spaced; pinna texture delicate; margins finely toothed or crenulate. . . **B. crenulatum**

18. Pinnae nearly touching to overlapping; pinna texture firm; margins entire to undulate, occasionally toothed or cleft into several segments. . . **19**

19. Pinnae asymmetrical with the lower half larger; sporophore stalk at spore release shorter than or equal to the length of the trophophore. . . **B. tunux**

19. Pinnae symmetrical, sporophore stalk at spore release longer than the trophophore. . . **20**

20. Basal pinna span 120° to 180°, basal side margin slightly concave; basal pinnae sessile or nearly so; pinnae not overlapping the rachis. . . **B. lunaria**

20. Basal pinna span greater than 180°, basal side margin strongly recurved; basal pinnae stalked; pinnae overlapping the rachis. . .**B. yaaxudakeit**

21. Trophophore lustrous; sporangia bright yellow-green before spore release. . . **22**

21. Trophophore dull or glaucous; sporangia dull green before spore release. . . **24**

22. Upper pinna bases obtuse (angle > 90°) to cordate (>180°); pinna apices rounded; sporophore stalk equal to trophophore length; sporophore pinnately branched. . **B. pinnatum**

22. Upper pinna bases acute (angle < 90°); pinna apices angular; sporophore stalk shorter than trophophore length; sporophore ternately branched (divided into three main branches). . . **23**

23. Trophophore outline triangular (equilateral) with basal pinnae nearly as large as the central rachis and pinnae; pinna pairs 3-4; pinnae narrowly ovate to oblong. . . ***B. lanceolatum*** subsp. ***lanceolatum***
23. Trophophore outline narrowly triangular to broadly ovate; basal pinnae not disproportionately enlarged; pinna pairs 5-6; pinnae ovate to elliptic. . .
B. alaskense

24. Trophophore stalk longer than the average distance between pinnae; basal pinnae usually bearing supernumerary sporangia*. . . ***B. pedunculosum***
24. Trophophore stalk equal to or shorter than the average distance between pinnae; basal pinnae seldom bearing supernumerary sporangia*. . . **25**

25. Trophophore sessile or inconspicuously stalked. . . **26**
25. Trophophore clearly stalked. . . **27**

26. Basal pinnae often conspicuously elongated; pinna lobes parallel to converging; sporophore ternately branched (divided into three main branches). . . ***B. michiganense***
26. Basal pinnae not conspicuously elongated; pinna lobes spreading; sporophore pinnately branched. . . ***B. echo***

27. Pinnae shallowly dissected, not overlapping. . .
B. hesperium var. hesperium
27. Pinnae deeply dissected and strongly overlapping. . .
B. hesperium var. fenestratum

All species occasionally produce sporangia on the lowermost pinnae.

C. 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, 1999) and Farrar (1998, 2001) 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 probably 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*. 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).

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 to produce a set of relative similarity values called genetic identities (GI) between populations or species. Farrar (1998, 2001) conducted enzyme analysis on more than 3000 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 5. 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 5. **Nei's Unbiased Measures of Genetic Identity** (Farrar 2001)

Species	lineare	pallidum	lunaria	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
lunaria				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

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. lunaria 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 or subspecies 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 early 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. lunaria* 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.

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*. We have recently determined that the basic genotype of European *B. lunaria* is very different from that of common North American *B. lunaria*, and that the European genotype of *B. lunaria* occurs in the mountains of Alaska and the Yukon, and that European *B. lunaria* is the probable parent of a number of North American allotetraploid taxa (see Table 2), including *B. yaaxudakeit* which is the allotetraploid derivative of European *B. lunaria* X American *B. lunaria*. Mary Stensvold is currently studying genetic differentiation in European *B. lunaria* and its phylogenetic relationships to other moonwort species.

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.

D. 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, pair and segregate perfectly in meiosis (see discussion below). Sterility of hybrids maintains distinction of species.

<u>Allopolyploid speciation</u>—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.

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 <u>auto</u>tetraploid. In most cases, 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 <u>allo</u>tetraploid is thus fully capable of reproducing, dispersing, and evolving as an independent species with its own distinctive morphology and behavior.

About 60% 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.

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, because, in *Botrychium*, self-fertilization maintains homozygosity within each parental contribution, *Botrychium* allotetraploids usually display only two different alleles per locus in an individual plant. Presence of this fixed (always present) heterozygosity at many gene loci in every member of a population or species is a clear indication of polyploidy, especially in strongly selfing plants such as Botrychium where selfing eliminates non-fixed (recombinational) heterozygosity.

AA — Two diploid species with 2 sets of homologous chromosomes

A — Haploid spores produced by meiosis grow into gametophyte plants that produce gametes by mitosis

BB

B

AB — Intraspecific hybrid. This plant is sterile. It cannot produce viable spores because the chromosome sets A and B (referred to as homeologous chromosomes) are too different to pair perfectly in meiosis.

2X — Chromosome doubling

AABB — Allopolyploid (tetraploid) resulting from replication of chromosomes without subsequent nuclear division. This plant is fertile because each initial set of chromosomes can now pair in meiosis with its duplicated copy, ie. A pairs with A, B with B.

AB AB AB AB AB — Spores now carry fixed heterozygosity having one allele from each parent. Gametophytes derived from them produce male and female gametangia in close proximity.

AABB — Pairing of identical gametes through self fertilization faithfully reproduces the tetraploid genotype.
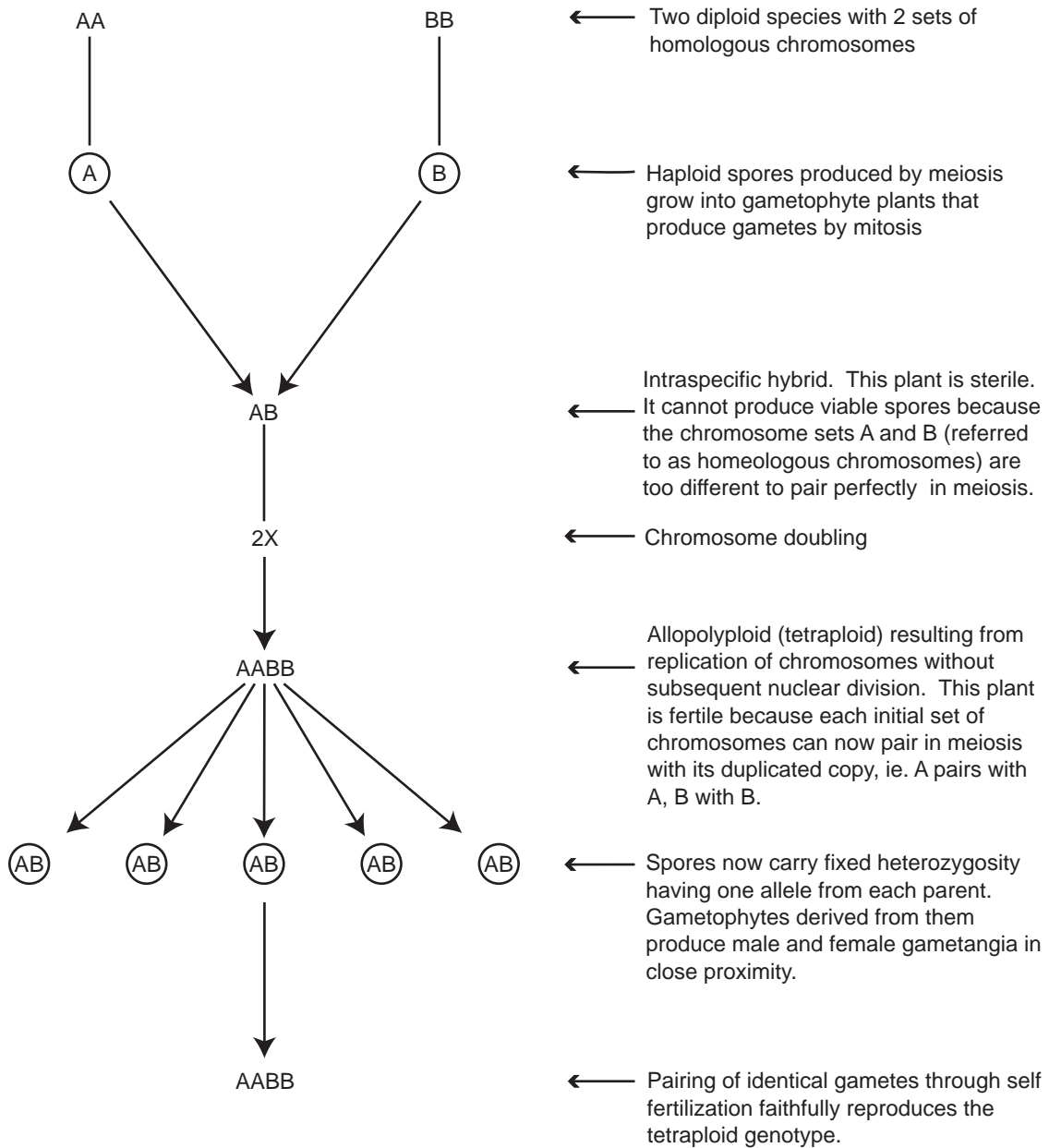
Figure 2. Origin of tetraploid species of *Botrychium*. Each letter represents one set of chromosomes. AA and BB represent two different diploid species.
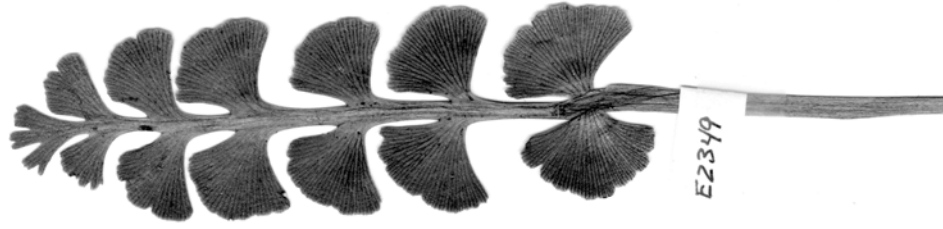
Detecting ancestral parents of allotetraploid species.—When the typical isozyme patterns of all the diploid species have been determined, it is possible to determine which of these patterns, when combined, produce the pattern observed in a given tetraploid species. The answer is sometimes clear, as in 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 time of 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. In some cases, 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 2 lists the probable parents of polyploid Botrychium. Figures 1, 3, 4, and 5 illustrate the morphological intermediacy of allotetraploid species between their probable ancestral diploid parents.
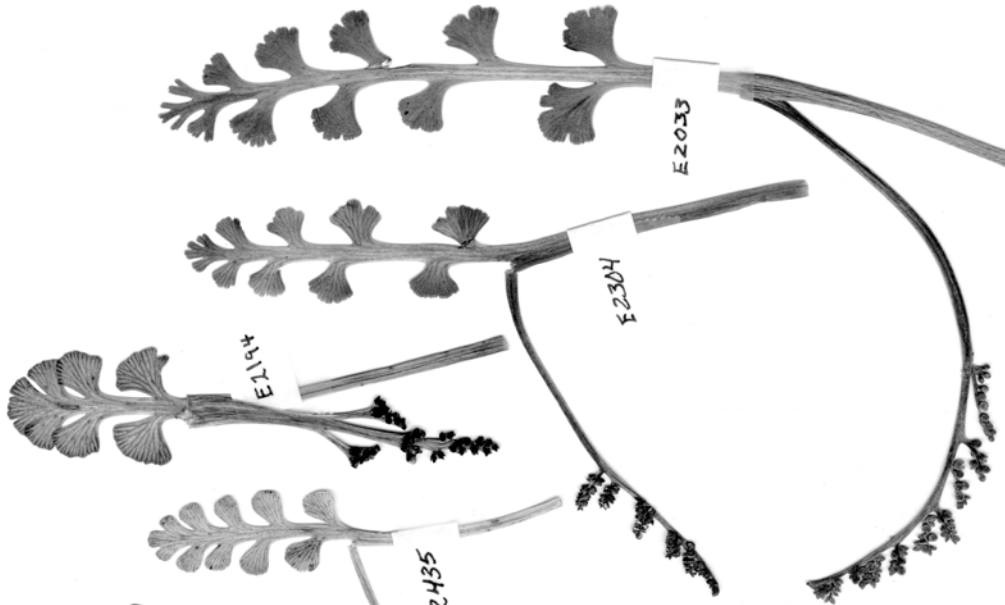
Multiple origins of allotetraploids.—Allotetraploid species result from a past hybridization event between two diploid species followed by chromosome doubling. It is reasonable to expect that such an event, involving the same two species happened more than once. That is, the same allotetraploid will 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 3.

26

Diploid **Allotetraploid** **Diploid**

E2203

E2197

E2358

B. pinnatum

B. lunaria

B. lanceolatum

3921

E1818

E2240

E2031

B. lineare

B. ascendens

B. crenulatum

Figure 4.

Diploid **Allotetraploid** **Diploid**

E2760

E1864

E1863

E998

B. lanceolatum *B. "michiganense"* *B. pallidum*

E2102

3873

3871

E2235

3882

B. lanceolatum *B. echo* *B. lineare*
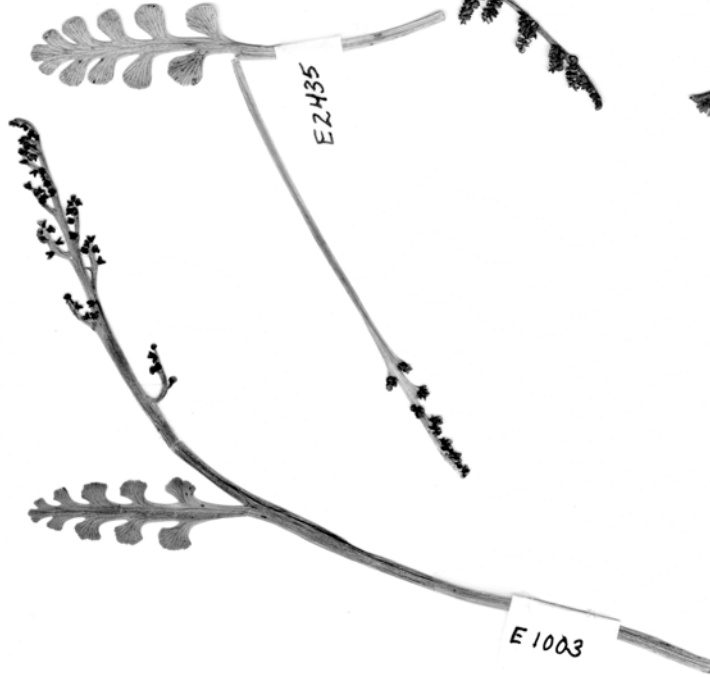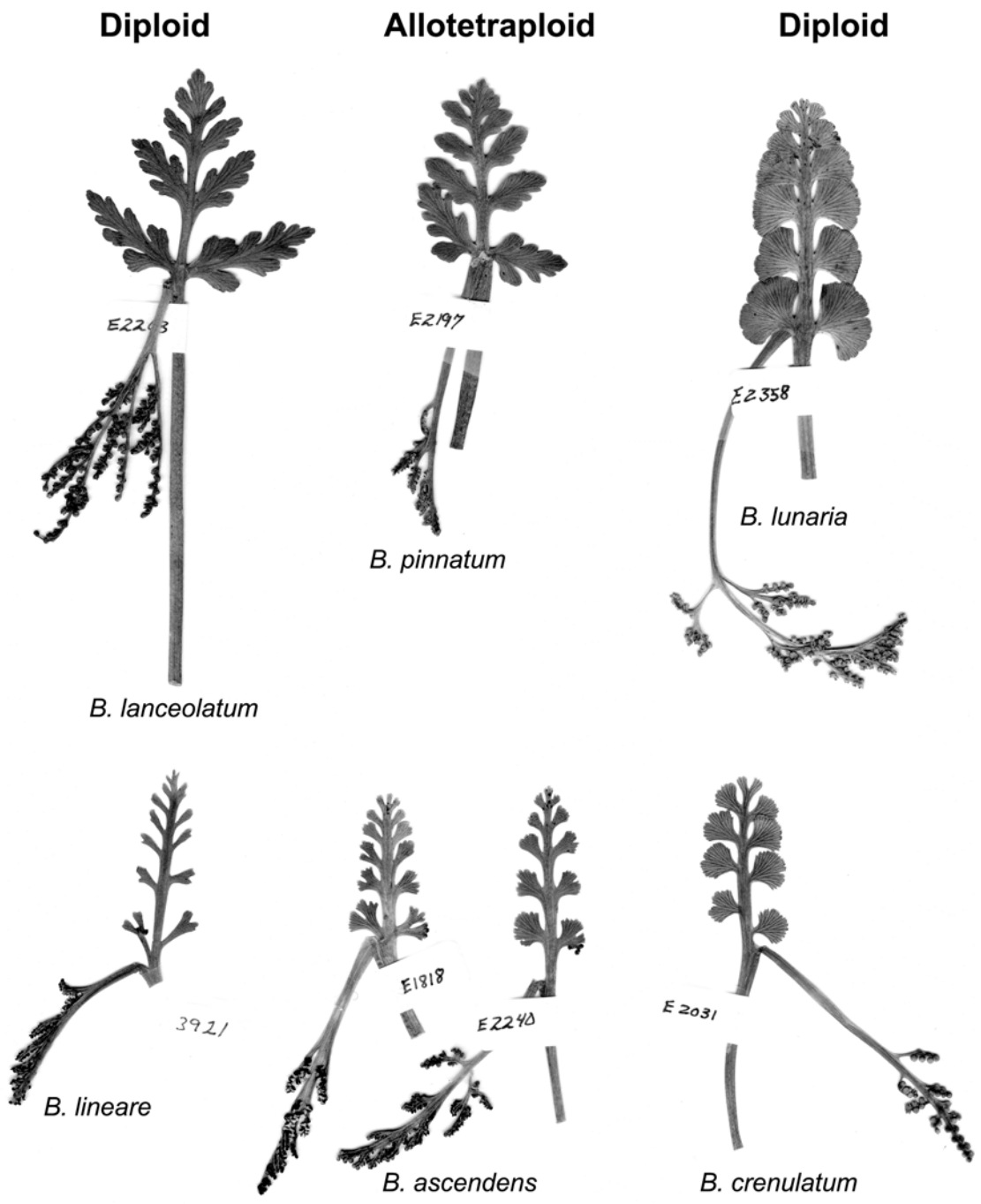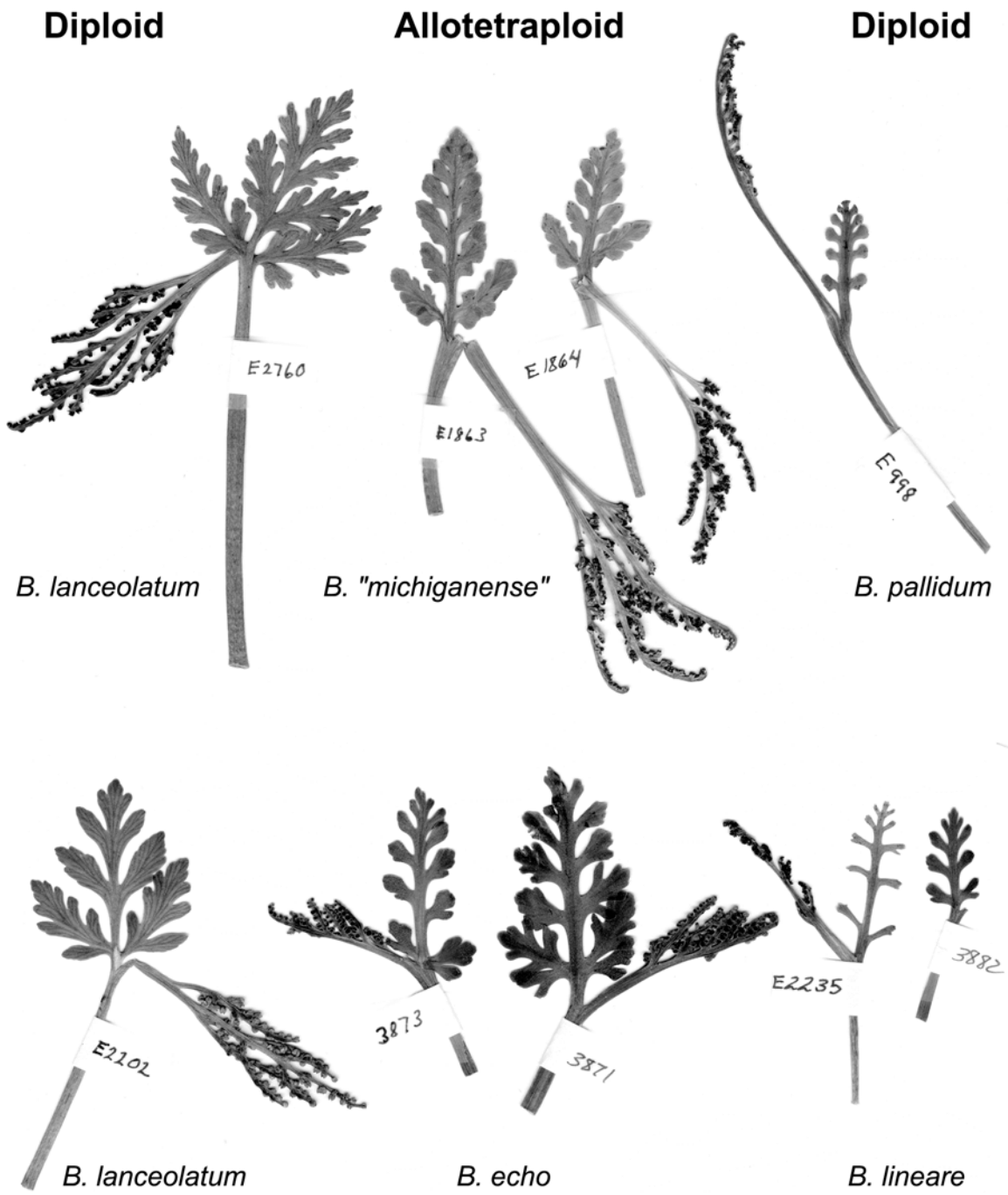
Figure 5.

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 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* SUBGENUS *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 initiate 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 1% 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, 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.**

<u>Distribution of variability</u>.—The sexual life cycle, although it fails to generate genetic variability through recombination in *Botrychium*, 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 further 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).

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 morphological traits as well as the same genotype. 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 almost exclusively 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 intergametophytic selfing as described above. In the second case, the two types remain distinct not only because of selfing, but also because they are genetically incompatible—occasional 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. 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 is often assumed to be much 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.

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) is a powerful deterrent to successful reproduction by isolated gametophytes (Peck et al. 1990).

As discussed above, in *Botrychium* species individual gametophytes regularly become bisexual and all genetic studies examining these plants 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 great 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. This evidence indicates restriction of migration such that 1) populations more than a few miles apart may be effectively isolated and 2) that suitable uncolonized habitat at these distances have a low probability of receiving a sufficient number of spores to assure colonization.

Genetic variability.—It is important to note 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 unpublished).

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, and thus do not accumulate in the genome 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.

It is probably safe to assume that 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 (63%) 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).

Genetic vulnerability to environmental change.—There is no reason to believe that historically plants of *Botrychium* have reproduced differently in the past than now. Underground bisexual gametophytes are characteristic of all Ophioglossaceae and of their closest relatives, the Psilotaceae. If low genetic variability is due to intragametophytic selfing which, in turn, is imposed by the underground environment, then we can reasonably assume that *Botrychium* species have always maintained 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, *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, fitness is not a function of population size.

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 do 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 (see Life History) strongly suggest that moonwort *Botrychium*s rely heavily on their mycorrhizal partner for photosynthates as well as 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. **This lack of genetic variability in *Botrychium* should not be a concern in assessing species or population viability**.