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Creating Microsatellite Primers for *Botrychium*

Brittney K. Ellis
Lake Forest College, ellisbk@lakeforest.edu

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Abstract

Botrychium sensu stricto (Ophioglossaceae) is a genus with cryptic species that are difficult to delineate on the basis of their limited morphology and high incidence of hybridization events. Various means have been used to determine the phylogeny of Botrychium based on both genetic and morphological factors. We designed species-specific microsatellite primers to better determine species relationships between the allotetraploid Botrychium matricariifolium and the diploid species Botrychium angustisegmentum. We examined seventeen individuals, from both B. matricariifolium and B. angustisegmentum. We found that both B. matricariifolium and B. angustisegmentum amplified on the majority of DNA on our gels for eight of twelve runs. Amplified loci on the Beckman Coulter CEQ 8000 runs were associated with bands visualized on the gel. Preliminary results with a high percentage of missing data showed a genetic difference between the two species. There was little variation within the populations, however we may see more by using our primers to test other populations of B. matricariifolium and B. angustisegmentum may also prove beneficial to look at other species entirely, particularly Botrychium pallidum.

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LAKE FOREST COLLEGE

Senior Thesis

Creating Microsatellite Primers for *Botrychium*

by

Brittney K. Ellis

April 15, 2014

The report of the investigation undertaken as a Senior Thesis, to carry one course of credit in the Department of Environmental Studies.
Botrychium sensu stricto (Ophioglossaceae) is a genus with cryptic species that are difficult to delineate on the basis of their limited morphology and high incidence of hybridization events. Various means have been used to determine the phylogeny of Botrychium based on both genetic and morphological factors. We designed species-specific microsatellite primers to better determine species relationships between the allotetraploid Botrychium matricariifolium and the diploid species Botrychium angustisegmentum. We examined seventeen individuals, from both B. matricariifolium and B. angustisegmentum. We found that both B. matricariifolium and B. angustisegmentum amplified on the majority of DNA on our gels for eight of twelve runs. Amplified loci on the Beckman Coulter CEQ 8000 runs were associated with bands visualized on the gel. Preliminary results with a high percentage of missing data showed a genetic difference between the two species. There was little variation within the populations, however we may see more by using our primers to test other populations of B. matricariifolium and B. angustisegmentum may also prove beneficial to look at other species entirely, particularly Botrychium pallidum.
To my parents, Robert Ellis and Rebecca Whitmore, for their unwavering support of my academic pursuits and personal endeavors.
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Literature Review

The phenomena of polyploidy

Polyploidy is defined as the addition of a complete set of chromosomes to the genome that is caused by the inheritance of an additional set of chromosomes (Otto, 2007). Changes in ploidy levels occur in both animals and plant lineages, and the phenomenon is often ongoing (Otto & Whitton, 2000). There are two different mechanisms of polyploidy (Otto, 2007). An allopolyploid is created when a hybridization event between two distinct individuals occurs, while an autopolyploid is formed when chromosomes from an individual plant are duplicated. Allopolyploidy refers to a species of hybrid origin (Otto & Whitton, 2000). Studies indicate that polyploidization has been and continues to be an evolutionary success (Otto, 2007). Several occurrences of polyploidy are ancient events and have been the predecessors of species rich groups (Otto, 2007). Ploidy can effect gene regulation and developmental processes, which can cause changes in a species morphological features, breeding system, and ecological tolerances (Otto & Whitton, 2000).

Changes in ploidy account for 2 to 4% of speciation events in flowering plants (Otto & Whitton, 2000). In ferns ploidy accounts for 7-31% of speciation events (Otto & Whitton, 2000). At the population level, beneficial mutations arise more often in large populations, without regard to ploidy. Thus polyploids are not as beneficial as haploids in this case. However, in small populations, mutations are rarer, thus polyploidy is more beneficial (Otto, 2007). It is important to understand that polyploids are not simply doubled diploids. When gene expression is altered, genes face differing levels of selection, causing improved function. The polyploid will always differ from its diploid progenitor(s), as it has often has higher fitness and more specialized functions.
Duplicated genes equal higher fitness levels in most species as polyploidization causes genes to have a plethora of functions they wouldn’t attain as a haploid or diploid, such as larger cells and overall plant size. Polyploidy also guarantees that heterozygosity will be enhanced in future generations of the plant (Otto, 2007). Ploidy may be beneficial to evolution because increased diversification and qualities of adaptation may result from restructured and extra genetic material that occurs as a result of polyploidy (Perrie, 2010). Unfortunately, the significance of polyploidy to biodiversity remains unclear, although the phenomenon occurs often (Otto, 2007).

It is considered the norm in Pteridophytes to regularly undergo increases in ploidy level (Perrie, Shepherd, De Lange, & Brownsey, 2010). Estimates range from approximately 7-31% of fern speciation events are coupled with an increase in ploidy (Perrie et al., 2010), making this one of the most remarkable mutations that can occur in fern species (Otto, 2007). This is relatively high in relation to the angiosperms, in which only 2-4% of speciation events are a result of ploidy. Almost half of recent changes in haploid chromosome numbers have been a result of polyploidy in ferns (Otto & Whitton, 2000). This can make it particularly challenging to construct phylogenies within pteridophytes as tetraploid species remain a challenge to delineate. Even detecting polyploids is a challenge; as a result, numerous phylogenies remain unresolved (Otto & Whitton, 2000). An example of this is the family Ophioglossaceae which contains the genus *Botrychium s.s.* Although some studies have done genetic testing in order to resolve relationships within the genus, tetraploid species still present a challenge to constructing accurate phylogenies (Williams & Waller, 2012; Dauphin, Vieu, & Grant, 2014).
Introduction to Botrychium

Ferns (Division Pteridophyta) are the second most diverse group of land plants after the angiosperms with 45 families (Smith, et al., 2006; Christenhusz et al., 2011; Dauphin et al., 2014). The phylogeny of Botrychium s.s. within the family Ophioglossaceae has been revised numerous times (Clausen, 1938; Kato, 1987; Dauphin et al., 2014). The family Ophioglossaceae is thought to be the most closely related to the family Psilotaceae. These two families are sisters to the rest of the ferns (Pryer et al., 2004). The genus Botrychium is most related to the genera Sceptridium which used to be part of the old genus of Botrychium sensu lato before the two genera were recognized as separate (Wagner & Wagner, 1993). Separate sporophore and trophophore structures are the characteristic most often used in identification of Botrychium and they are characteristics that all species within the family Ophioglossaceae share (Dauphin et al., 2014). Many unusual features make the family unique from other families including: limited secondary growth in the rhizome, sheathing leaf bases, circular bordered pits, subterranean and non-chlorophyllous gametophytes, and the absence of circinate venation, root hairs, and sclerenchyma (Wagner, 1990). Botrychium s.s. species are often overlooked despite their large diversity and range as a result of their small stature of one to 15 cm and limited morphological characteristics (Williams & Waller, 2012; Dauphin et al., 2014). As morphological differences among Botrychium are often extremely subtle, they are considered to be cryptic species (Dauphin et al., 2014). Paris and Wagner (1989) described a cryptic species as having poor morphological differentiation, exhibiting reproductive isolation, and incidences of misinterpretation of taxa as members of a single broader species. Classifying this genus has been difficult, as constructing
phylogenies of *Botrychium* presents a major challenge as a result of the limited morphology and high incidence of polyploidy which occurs within this genus (Hauk & Haufler, 1999).

Figure 1: One of our specimens of *B. matricariifolium* from the Upper Peninsula of Michigan. A. depicts the fertile sporophore and B. shows the sterile trophophore, the main morphological features that allow for species delineation within the cryptic genus *Botrychium* (Wagner, 1981).

The genus now contains 30 species (Table 1; Hauk, 2003; Dauphin, 2014). Species within *Botrychium* occur mainly in grasslands, meadows, and forests, and are often associated with light to moderate disturbance (Farrar & Johnson-Groh, 1990). Their distribution ranges in size from long, broad ranges to locally restricted ranges (Wagner and Wagner, 1990; Farrar & Johnson-Groh, 1991; Dauphin et al., 2014). *Botrychium* has ranges throughout North America, Africa (Atlas Mountains), Asia, Australia, Pacific Islands, New Zealand, and Patagonia (South America). The most studied *Botrychium* ferns are in North America and Europe, where it is suspected the greatest amount of species diversity exists. Ranges outside of North America and Europe still remain poorly investigated (Dauphin et al., 2014). *Botrychium* only occurs in a natural setting and is problematic to grow in captivity. Propagation of the species in captivity is nearly impossible because the gametophytes have a symbiotic relationship with mycorrhizal fungi (Johnson-Groh & Lee, 2002; Winther & Friedman, 2007). In general, genetic variability depends on the parent being heterozygous for at least some traits so that different alleles can be present in the gametes (Dauphin et al, 2014). However, the main mode of reproduction in moonworts is intragametophytic selfing, which is when two gametes from the same gametophyte combine. This leads to homozygosity.
Table 1: Thirty Identified Species of *Botrychium* s.s.

<table>
<thead>
<tr>
<th><em>Botrychium</em> s.s. species</th>
<th>Ploidy Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>acuminatum</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>alaskense</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>ascendens</td>
<td>Diploid</td>
</tr>
<tr>
<td>boreale</td>
<td>Diploid</td>
</tr>
<tr>
<td>campestre</td>
<td>Diploid</td>
</tr>
<tr>
<td>crenulatum</td>
<td>Diploid</td>
</tr>
<tr>
<td>echo</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>gallicomontanum</td>
<td>Diploid</td>
</tr>
<tr>
<td>hesperium</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>angustisegmentum</td>
<td>Diploid</td>
</tr>
<tr>
<td>lanceolatum</td>
<td>Diploid</td>
</tr>
<tr>
<td>linear</td>
<td>Diploid</td>
</tr>
<tr>
<td>lunaria</td>
<td>Diploid</td>
</tr>
<tr>
<td>matricariifolium</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>michiganense</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>minganense</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>montanum</td>
<td>Diploid</td>
</tr>
<tr>
<td>neolunaria</td>
<td>Diploid</td>
</tr>
<tr>
<td>pallidum</td>
<td>Diploid</td>
</tr>
<tr>
<td>paradoxum</td>
<td>Diploid</td>
</tr>
<tr>
<td>pedunculosum</td>
<td>Diploid</td>
</tr>
<tr>
<td>pinnatum</td>
<td>Diploid</td>
</tr>
<tr>
<td>pseudopinnatum</td>
<td>Diploid</td>
</tr>
<tr>
<td>pumicola</td>
<td>Diploid</td>
</tr>
<tr>
<td>simplex</td>
<td>Diploid</td>
</tr>
<tr>
<td>socorrense</td>
<td>Diploid</td>
</tr>
<tr>
<td>spathulatum</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>virginianus</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>X watertonense</td>
<td>Tetraploid</td>
</tr>
<tr>
<td>yaaxudakeit</td>
<td>Tetraploid</td>
</tr>
</tbody>
</table>

Understanding the phylogeny of *Botrychium* remains of concern, as 18 out of 30 species are polyploids (Dauphin et al., 2014). Furthermore, ferns within the genus *Botrychium* are considered of interest to conservation as nine are listed as rare in North America (Ahlenslager & Lesica, 1996). The classification of and differentiation between
moonworts is important because many species are afforded different levels of protection (Ahlenslager & Lesica, 1996). Changes in ploidy as well as hybridization events also cause many relationships within the species to remain hard to delineate (Ahlenslager & Lesica, 1996). This is particularly prevalent within the section Lanceolatum, as relationships with tetraploid species still remain unresolved within this division of the phylogeny (Williams & Waller, 2012; Dauphin et al., 2014).

**Polyploidy Within *Botrychium***

Classification of *Botrychium* has been complicated by the high incidence of polyploidy, which has resulted from hybridization between members of the twelve diploids (Dauphin et al., 2014). *Botrychium* may be more susceptible to polyploidy because of the evolved-robustness hypothesis. According to Otto (2007), organisms with an alternation of generations reproduction cycle with haploid and diploid phases undergo more beneficial mutations than diploids as a result of a higher ploidy level, and they suffer less inbreeding depression (Otto, 2007). Ahlenslager and Lesica (1996) studied the sterile hybrid, *B. X watertonense*, along with its putative parent progenitors, *B. hesperium* and *B. paradoxum*. They found that these three species are stable entities, with varying genotypes (Ahlenslager, 1996). The mean height of *B. X watertonense* was greater than its parents, signaling hybrid vigor (Ahlenslager & Lesica, 1996). Hybrid vigor is defined as a hybrid that demonstrates enhanced performance over its parent species (Ahlenslager & Lesica, 1996). Wagner et al. (1983) stated that high incidences of hybridization in *Botrychium* may be evidence that intragametophytic out crossing is common within the species. Highly inbred populations display less hybrid vigor (Ahlenslager & Lesica, 1996). This could be especially important in *Botrychium*, where intragametophytic selfing leads to significant inbreeding depression.
Hauk & Haufler (1999) looked at 16 species of *Botrychium* for isozyme variation. They determined that *Botrychium* are primarily interbreeding, by determining interrelatedness between species via the amount of shared banding patterns. Hauk & Haufler (1999) concluded that *Botrychium* are indeed evolving at diploid and polyploidy levels. Polyploidy is significant to *Botrychium* as it is a common phenomenon, however it causes delineation of the species to remain conflicted as it is nearly impossible to decipher tetraploid relationships with their parent progenitors through the use of only morphological characters. Thus looking at genetic properties of these species is instrumental in discovering tetraploid relationships within *Botrychium*.

**Identifying Tetraploid Species**

It is difficult to identify tetraploids, as their morphological characteristics are similar to that of both their parent species. Numerous studies have looked at the origin of the tetraploid species. Wagner and Wagner (1993) postulated that *B. angustisegmentum* and *B. pallidum* were the putative parent species of *Botrychium*. This hypothesis was based strictly on morphological factors. Genetic markers alleviate this problem by clearly defining the species of moonworts and identifying the putative parent species. Genetic data determined that the *B. ascendens* found in Newfoundland were consistent with those found throughout North America (Zika & Farrar, 2009). *Botrychium ascendens* was found to be a tetraploid species that was distinct species from *B. lineare* and *B. campestre* because it had many alleles not present in either of those species (Zika & Farrar, 2009). Zika and Farrar (2009) suggest that *B. ascendens* is derived from ancient hybridization between *B. crenulatum* and the *B. lineare/campestre* complex. This is one example of how genetic data can be used to better infer relationships within a phylogeny.

Allozymes can be used to infer ploidy levels. Stensvold, Farrar, and Johnson-
Groh (2002) used allozyme data along with morphological characteristics in order to confirm that *B. tunux* and *B. yaaxudakeit* were separate species from *B. lunaria*, which they have been confused with. Allozyme data confirmed that *B. tunux* is a diploid and *B. yaaxudakeit* is a tetraploid. *B. matricariifolium* is difficult to identify as it is an allotetraploid with a large range of morphological features (Williams & Waller, 2012). The species may be a product of multiple lineages (Paris & Wagner, 1989), evidenced by the fact that it grows together with morphologically similar species (Wagner, 1993). There is also a wide range of morphological variation within genetically similar individuals (Williams & Waller, 2012; Fig. 2). As it is not possible to delineate *B. matricariifolium* based on morphological variables, further genetic testing is needed in order to place the species correctly into the phylogeny and determine if it is a hybrid swarm consisting of multiple hybridization events.

**Species Delineation**

Paris and Wagner (1989) argue that there is a disparity between theory and practice of how pteridophytes are delineated. In theory they could be delineated based on the biological species concept, which is defined as a group of populations that are inter-fertile and reproductively isolated from other groups and occupy a specific niche in nature. Delineation could also include the evolutionary species concept, which includes a single lineage of populations that maintain their own identity from other lineages, fit into their own ecological niche, and display a unique evolutionary history (Paris & Wagner 1989). What distinguishes the evolutionary species concept from the biological species concept is that it includes both sexually and asexually reproducing organisms. Paris and Wagner (1989) argued that using only morphology to delineate ferns was leading to underestimates of diversity. Wagner (1983) outlined the issues between practice and
theory of delineating *Botrychium* ferns. The Wagners proposed a method of delineating ferns based upon species they co-exist with, on the basis of whether or not they can interbreed successfully. Interbreeding successfully means that they are not discreet species, and that genetic characters are most likely not fixed. Up to seven species can grow together in one habitat, and their reproductive habits may help to show if the taxa are biologically discreet or not (Wagner, 1981). A species that does not interbreed with the other species in its habitat is most likely a biologically distinct species. The method of mutual associations is the ability of two taxa to remain distinct species within a habitat (Wagner, 1981). Mixed populations are thus of value in species delineation. In Wagner (1981), the method of mutual associations was used to delineate the validity of four new moonworts as distinct species: *Botrychium crenulatum*, *Botrychium paradoxum*, *Botrychium mormo*, and *Botrychium montanum*. However, as delineating species based on morphological variables and behavior in the field is often impractical, genetic testing in the lab can be advantageous for determining species relationships.

**Delineation of *Botrychium* based on Morphology**

The simplest way that *Botrychium* has been delineated is through its morphological attributes. The Wagners delineated several species of *Botrychium* on the basis of morphological characteristics including: *Botrychium crenulatum*, *B. paradoxum*, *B. mormo*, *B. montanum*, *B. pinnatum*, *B. pedunculosum*, *B. pallidum*, *B. spathulatum*, *B. linear*, *B. hesperium*, *B. ascendens*, and *B. campestre* (Wagner, 1981; Wagner, 1986; Wagner, 1990; Wagner, 1994). It was important Wagner to stick to a set pattern when making taxonomic judgments (Wagner, 1981). The Wagners also used characteristics such as lamina indumenta, stipe alation, involucre shape, frond size, and pinnate shape to
classify species, as *Botrychium* have such a reduced morphology (Wagner, 1994).

Wagner and Wagner (1983) delineated two species on *Botrychium, B. echo* and *B. hesperium,* on the basis of the sterile trophophore. Wagner (1983) recognized that much taxonomic research was still needed into *Botrychium.*

**Delineation of *Botrychium* Using Genetics**

More recent studies use a combination of genetics and morphology. Brunsfeld and Swartz (2002) resolved the confusion between two species, *Botrychium minganense* and *Botrychium crenulatum.* These two ferns are often mistaken for one another in the field, even by experts (Wagner, 1993). These two species have different levels of environmental protection, making the distinction between the two species of interest to conservation efforts within Washington, Oregon, Idaho, and Montana (Brunsfeld & Swartz, 2002). Using DNA markers and morphological characteristics to assess the two species’ distinctness, the results showed well-defined and distinctly separate *B. minganense* and *B. crenulatum* clusters. Brunsfeld and Swartz (2002) demonstrated the importance of using genetic and morphological variables to delineate cryptic species.

Genetic testing in the lab has become instrumental in the delineation of species within *Botrychium,* where work in the field based only on morphology is limited. Different methods of genetic testing can help to create a more accurate phylogeny. Data on ploidy levels can be useful for separating species, estimating hybridization levels, and separating individuals. Wagner and Wagner (1981, 1990b, 1993) used chromosome squashes to count the chromosomes within *Botrychium,* thus chromosome counts have been obtained for most species within the genus. Tetraploid species present a particularly difficult challenge in constructing accurate phylogenies. This is particularly prevalent in
the *lanceolatum* clade in which many species relationships remain unresolved (Hauk & Haufler, 1995; Williams & Waller, 2012; Dauphin *et al.*, 2014) updated and revised the *lanceolatum* section of *Botrychium* by adding the allotetraploid *B. matricariifolium*. The *lanceolatum* clade was defined morphologically as being composed of a diploid species with a deltate trophophore (Hauk *et al.*, 1995). Hauk and Haufler (1999) postulated that *B. angustisegmentum* (previously *B. lanceolatum angustisegmentum*) was one of the putative parent species of *B. matricariifolium*. However the complex relationships among the genotypes made it impossible to truly resolve the origins of *B. matricariifolium*.

Farrar and Johnson-Groh (1991) discovered a new prairie moonwort based on its morphological differences from other species within the same habitat. Starch-gel enzyme electrophoresis was run on the new species *B. gallicomontanum*, leading to the finding that *B. campestrum* and *B. simplex* were the parent species of *B. gallicomontanum*. This finding led to the hypothesis that *B. gallicomontanum* was a tetraploid species. Similarly, *B. ascendens* is hypothesized to have been derived from an ancient hybridization between *B. crenulatum* and *B. lineare*. Based on morphological and genetic factors Zika and Farrar (2009) hypothesized that it had originated in North America and at some point migrated through Canada to Newfoundland. Wagner and Grant (2002) used enzyme electrophoresis data as well as morphological features to determine that *B. alaskense* is intermediate between *B. lunaria* and *B. angustisegmentum*. Morphology plays a pertinent role today in the delineation of *Botrychium* despite new advances made in genetics.

**Hybridization**

Meyer (1981) was the first to ascertain that a species of moonwort was in fact, a hybrid. He hypothesized that *B. angustisegmentum* and *B. lunaria* were, in fact, the putative parent species of *B. matricariifolium*. Although untrue, subsequently high
incidences of hybridization events were found to be occurring in *Botrychium*. In the field, researchers recognize fertile hybrids based on their morphology and sterile hybrids based on inviability (Wagner, 1986; Williams & Waller, 2012). This is confirmed in the lab through chromosome counts or flow cytometry (Wagner, 1993; Table 1). Numerous outcomes can occur as a result from hybridization, including the creation of infertile hybrids or the production of ecologically viable hybrids that give rise to new species (Wagner, 1993). Hybridization and ploidy have resulted in a large diversity within *Botrychium* (Hauk & Haufler, 1999), and these phenomena continue to occur within the species.

**Hybridization in Botrychium**

Parental lineages have been delimited through various mechanisms including morphology, karyology, molecular phylogenetics, and isozyme analysis. However, a few studies have looked at relationships between diploid and polyploidy species of *Botrychium* s.s. using plastid DNA regions and Amplified Fragment-Length Polymorphisms (AFLPs). These studies include Hauk et al., (2003), Hauk and Hawke (2012), Williams and Waller (2012), and Dauphin et al., 2014. The phylogeny of the genus is constantly changing as a result of advances in genetic analyses. These examples convey the importance of both morphological and molecular studies on identification and delineation of *Botrychium*.

Hauk, et al. (2003), built on former Wagner principles of morphology in which the main distinguishing factor in Ophioglossaceae was the division of the frond into the trophophore and sporophore segments, along with other distinguishing features such as leaf venation, degree of leaf dissection, sporangia structure, and gametophyte construction. In their study, DNA sequencing and morphology were used in order to
address phylogenetic relationships within Ophioglossaceae along with character evolution. They found that molecular and morphological data showed similar relationships of major groups of Ophioglossaceae, which confirmed other modern interpretations of phylogeny and evolutionary characters in Botrychium. Hauk and Hawke (2012) examined relationships amongst 23 species of Botrychium s.s. using data from three plastid DNA regions and by analyzing cpDNA sequence variation. The study confirmed the current phylogeny of Botrychium within the three confirmed clades. Relationships among the five subclades remain unresolved, however, with the discovery of species of that are diploid and tetraploid.

Williams and Waller (2012) used flow cytometry, chloroplast sequences, and AFLPs in order to better analyze phylogenetic relationships within Botrychium (Fig. 2). Flow cytometry was used to confirm the relative ploidy level of samples and to confirm putative hybrid individuals. AFLPs help to study intricate relationships between population and species and are particularly useful in population genetics studies. These markers are helpful in analyzing population variation and reconstructing phylogenies (Koopman, 2005; Meudt & Clarke, 2007; Koopman et al., 2008; McKinnon et al., 2008; Williams & Waller, 2012). They may be able to offer more than chloroplast DNA in terms of placing cryptic species within a phylogeny (Bardy et al., 2010; Williams & Waller, 2012). However, they are limited in the scope because they are unable to reveal much about relationships between hybrids and putative parental species. The study both confirmed B. matricariifolium as an allotetraploid along with two other species within the clade, and reconciled relationships in Botrychium by constructing a new phylogeny. The study suggested that many sections within the phylogeny be amended based on the new data including placing B. echo and B. hesperium within the Lanceolatum section and
adding a Minganense section to accommodate tetraploid species. Williams and Waller (2012) suggested that more work needs to be done within _lanceolatum_ in order to resolve relationships within _Botrychium_. A more accurate phylogeny could be constructed by looking at additional plastid regions, more neutral markers (such as microsatellites), and nuclear regions.

Dauphin _et al._ (2014) used the same methods as Hauk (2012) as well as Williams and Waller (2012). Dauphin _et al._ (2014) looked at taxa outside of North America using three noncoding plastid DNA regions. They created a new molecular phylogeny of _Botrychium s.s._ by using non-coding plastid DNA regions. The samples used were from moonworts outside of North America, and the research confirmed the identity of several morphologically cryptic species within _Botrychium s.s._ Few studies have used nuclear DNA to analyze the putative parent species in moonworts and to reconstruct phylogenetic trees of inheritance, although both specific and universal primers are available (Dauphin _et al._, 2014). Nuclear DNA markers have proven difficult to interpret in polyploidy ferns (Dauphin _et al._, 2014). The study both confirmed recently identified species within _Botrychium_ and recently described phylogenies. By looking at the chloroplast DNA, morphologically cryptic species were separated, particularly within the _lunaria_ clade. Dauphin _et al._ (2014), agreed with Williams and Waller (2012) in that section _lanceolatum_ needed more research in order to understand relationships within the clade.
Figure 2: A new phylogeny suggested by Williams & Waller (2012). Relationships within *Lanceolatum* remain unresolved despite this updated phylogeny of *Botrychium*. Bold lettering represents allotetraploid species. The thickest lines represent >90% support in all three analyses, and the medium lines represent <90% support in one or two analyses.
What are Microsatellites?

Microsatellites are currently one of the most effective ways to solve ecological questions. They are inexpensive and allow ecologists to use molecular markers without purchasing specialized equipment (Selkoe & Toonen, 2006). Microsatellites are essentially a repeat (5-40 times) of one to six nucleotides. In regards to species-species marker isolation, primers must be created to bind to and amplify the target regions (Selkoe & Toonen, 2006). Microsatellite primers usually do not work across a wide ranging taxonomic groups, thus new primers must be developed for new species (Selkoe & Toonen 2006). The process of creating primers and isolating microsatellite markers is fast and inexpensive. Therefore, laboratories can expect to develop and receive microsatellite loci for their target species within three to six months (Selkoe & Toonen 2006). Microsatellites are extremely sensitive markers and can thus reveal genetic diversity, breeding systems, and genetic structures of species in natural populations (Jiminez, Hoderegger, Csencics, & Quintanilla, 2010).

Microsatellites can help with population genetic studies. For example, they can provide reliable estimates of long distance dispersal (DeGroot, Verduyn, Wubs, Erkens, & During, 2012). Microsatellites can help to better understand genetic impacts of long distance dispersal in pteridophytes (Selkoe & Toonen, 2006; DeGroot et al., 2012). Microsatellites are able to provide estimates of migration patterns and quantify relationships of species and individuals; therefore they are very effective for use in studies of population genetics (Selkoe & Toonen, 2006). Some issues can arise when using microsatellites, affecting the reliability of data. However, if loci are selected correctly during the isolation process, most of these can be avoided (Selkoe & Toonen, 2006).
Microsatellites in Use for Species Delineation

Microsatellites can be used for population studies or to determine interspecies relationships (Selkoe & Toonen, 2006). DeGroot et al. (2012) used microsatellite markers to analyze the genetic diversity of four separate populations of calciole ferns. The study was done in the Netherlands in an area known as the Kuinderbos where these plants are rare. Samples were collected from throughout Europe in order to provide a reference for how much existing variation was present in the Kuinderbos (Degroot et al., 2012). The microsatellites showed that genetic diversity was separated among populations. Most of the populations showed high levels of inbreeding and thus low genetic variation. Degroot et al., (2012) found that diploids and polyploids were capable of and frequently did colonize long distance via single spore establishment. It was found that isolated habitats receive spore rains including self-fertilizing genotypes, thus limited gene flow via long-distance dispersal could conserve certain genotypes.

Jimenez et al. (2010) used microsatellites to detect allozyme diversity in Iberian populations of the fern Dryopteris aemula. Jimenez et al., (2010) developed eight microsatellite loci and cross-amplified other ferns as well. Five loci were used to study the genetic diversity of D. aemula and it was found that the microsatellite markers designed could be used on closely related species (Jiminez et al., 2010). Microsatellites were used to study the genetics of D. aemula and it was found that there was low genetic variation within the species. Thus they hypothesized that there was a high incidence of inbreeding occurring in D. aemula. Jiminez et al., (2010) also found that long distance dispersal was more effective in D. aemula than other species.

Species within Botrychium are cryptic and hybridize often (Dauphin et al., 2014). As a result, the numerous allotetraploids within the phylogeny are problematic to
delineate. Genetic methods such as chloroplast testing and AFLP’s have been unable to determine relationships in section lanceolatum (Williams & Waller, 2012; Dauphin et al., 2014). Specifically it has been difficult to ascertain relationships between the tetraploid, _B. matricariifolium_ and other species within _Botrychium_. Currently no microsatellites have been developed specifically for _Botrychium_. Doing so would help to resolve species relationships within the genus, particularly for _B. matricariifolium_ and other alloployploids. The second chapter will introduce _Botrychium_ and the two species we studied in particular, _B. angustisegmentum_ and _B. matricariifolium_. It will also present our findings in regards to the designing and implementing of microsatellites for these two specific species.
Creating Microsatellite Primers for *Botrychium*

Brittney Ellis, Evelyn Williams

**Introduction**

*Botrychium* s.s. (Family Ophioglossaceae), also known as the moonworts, is a genus of ferns that are exceptionally prone to polyploidy and hybridization events (Dauphin *et al.*, 2014). Thus delineating species within *Botrychium* remains a challenge. *Botrychium* are also considered cryptic species, defined as a species with poor morphological differentiation, reproductive isolation, and misinterpretation of taxa as members of a single broader species (Paris, 1983). This is important to note because ferns within the genus *Botrychium* are considered rare, with nine endangered species list candidates and nine listed as rare in Canada. Thus it is of importance to be able to distinguish between *Botrychium*, as different species fall under different levels of protection under the Federal Endangered Species Act (Ahlenslager & Lesica, 1996).

Morphological as well as genetic factors have been used to delineate species within *Botrychium*. The allotetraploid, *B. matricariifolium*, has been hard to place within the phylogeny as it shares morphological features with other species within the genus. For example, the species *Botrychium michiganense* is very morphologically similar to *B. matricariifolium*. *Botrychium michiganense* was described after *B. matricariifolium*, and has an overlapping range. Based on morphological factors, Wagner and Wagner (1993) postulated that *B. angustisegmentum* (Fig. 1) was one of the two putative parent species of *B. matricariifolium*. Based on isozyme variation, Hauk and Haufler (1999) hypothesized that *B. angustisegmentum* was indeed one of the putative parent species of *B. matricariifolium*; however, this could not be confirmed because of complex
relationships amongst the genotypes. Williams and Waller (2012) used AFLPs in order to better infer relationships between species in *Botrychium* and thus construct a more accurate phylogeny. The study found evidence that *B. angustisegmentum* is the maternal parent and recommended that further research needed to be done within the *lanceolatum* section that encompasses *B. matricariifolium* in order to confirm species relationships. *Botrychium matricariifolium* and similar species may also be the product of repeated hybridization events between *B. angustisegmentum* and another species, but genetic studies up to now have not used the right tools to confirm this.

In order to further explore relationships within *Botrychium*, we decided to use microsatellite markers to explore the relationship between *B. matricariifolium* and possible parent species *B. angustisegmentum* (Fig. 1). We chose to use microsatellites as they allow us to see hybridization relationships between species, which are beyond the scope of AFLPs. Microsatellite primers usually do not work across wide ranging taxonomic groups, thus new primers must be developed for new species (Selkoe & Toonen, 2006). The goals of this study are to design working microsatellite primers for *B. matricariifolium* and *B. angustisegmentum* to further explore relationships between the species.
Materials & Methods

All specimens were collected from the Upper Peninsula of Michigan in 2009 by Evelyn Williams. She extracted DNA using Qiagen DNeasy Plant Mini-prep kits from *B. matricariifolium* and *B. angustisegmentum* specimens. Individuals for each species were collected from single populations. *B. matricariifolium* samples were from the Van Meer site in the U.P, while the *B. angustisegmentum* samples were from the Trap Hills site (Fig. 2 and 3). Microsatellite libraries were created at The Field Museum specifically for *Botrychium* using *B. matricariifolium* and *B. angustisegmentum* DNA. Williams followed the “Microsatellite Isolation with Dynabeads 2003” protocol by Glenn and Schable. After sequencing the potential primers on an ABI 3730, we found microsatellite motifs using CodonCode Aligner. We designed primers using the flanking regions of the motifs, usually 20-30 base pairs in front of and behind the motif. We started out with 25 primer pairs. Primers derived from *B. matricariifolium* have the prefix “BM”, and those from *B.
angustisegmentum have the prefix “BL”. Primers were constructed by Sigma-Aldrich.

After receiving the primers, we ran Polymerase Chain Reactions (PCR) in order to make sure that DNA was present and make sure that the primers worked in Botrychium for our 17 specimens of B. matricariifolium and B. angustisegmentum. We amplified loci using a one-step M13 PCR protocol, with 10 µl PCR reactions (5 µl 2x MyTaq Mix, 0.1 µl BSA, 0.25 µl M13-tagged forward primer, 0.5 µl reverse primer, 3.15 µl water, and 1.00 µl of DNA). We used the following PCR program: 94°C for 3 min, (94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, 72°C for 10 min repeat 13 times). The first four times we ran the PCR, our primers failed and we were unable to see the amplified PCR product (Fig. 4a). We changed the PCR program from 13 repeats to 40 repeats, which corrected this problem for the next eight PCR runs (Fig. 4b).
Figure 2: Distributional range of *B. matricariifolium*. Our samples were collected by Evelyn Williams at the Van Meer site in the Upper Peninsula of Michigan.

Figure 3: Distributional range of *B. angustisegmentum*. Our samples were collected by Evelyn Williams at the Trap Hills site in the Upper Peninsula of Michigan.

After running PCR, we ran the product on a 1% agarose gel for approximately 30 minutes. We used SYBR green dye to stain the DNA and photographed each gel. We recorded bands for 25 primers (Table 1). We used these to determine what primers most
likely would be successful and should be run on the Beckman Coulter Ceq 8000 to assess variability. The Beckman Coulter Ceq 8000 uses M13 dye to tag the DNA, which travels through a gel, and passes in front of a laser. The laser excites the tagged DNA, and compares the fluorescence with a ladder standard. Analysis after the run infers the DNA fragment sizes in comparison to the ladder. We used a two-step M13 PCR protocol to label the loci. In step one of the two-step M13 PCR protocol, we used the PCR recipe from above with the same PCR program. Step two was added directly to the finished step one (5 µl subtotal reaction): 2.5 µl 2x MyTaq Mix, 0.25 µl BSA, 0.125 µl MgCl2, 0.25 µl M13 dye, 187.5 with the following program: 94°C for 3 min, (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 10 min, hold, repeat 2 x 27). We ran the labeled PCR product on a Beckman Coulter Ceq 8000. We compared the successful primers to their original gels to see if the two agreed.

Results

We found nine primers that amplified and had successful Beckman Coulter Ceq 8000 runs (Table 1). Looking back on the successful runs, we noticed that the primers that amplified the most successfully on the gels showed up on the Beckman Coulter Ceq 8000. Primers that were unclear on the gels generally did not appear on the Beckman Coulter Ceq 8000. Furthermore, gels with multiple bands, often showed up on the Beckman Coulter Ceq 8000 with multiple loci. There is more than one locus on some of the primers because multiple loci could have amplified on a chromosome and thus be close together, or could have amplified on two separate chromosomes and be distanced further apart. Two loci could also be located on different chromosomes that happen to be the same size. Gels that showed double banding often amplified at two separate loci on the Beckman Coulter Ceq 8000. Primer BM8 is an example of a primer that showed
double banding on the gel and also peaked at multiple loci on the Beckman Coulter Ceq 8000 (see figure 4B.).

A.

![Image](image1.png)

B.

![Image](image2.png)

**Figure 4:** A. An unsuccessful gel run using the 1 x 13 protocol from 1/20/14. B. A successful gel run using the 1 x 40X protocol from 2/4/2014. We ran BM5, BM6, BM7, BM8, and BM9. BM5, BM6, and BM8 amplified successfully on the gel, with BM7 displaying double bands. BM8 was the only primer to successfully amplify on the Beckman Coulter Ceq 8000.

Both *B. angustisegmentum* and *B. matricariifolium* primers amplified on the Beckman Coulter Ceq 8000. Primers BL1, BL2, BL4, BL5, BM1, BM3, BM8, BM13, BM16, and BM17, showed multiple bands on the gel and amplified successfully on the Beckman Coulter Ceq 8000. Primer BM2 exhibited multiple bands on the gel, and the
primer amplified on the Beckman Coulter Ceq 8000, however the primer proved to be uninteresting in terms of variation. Primer BM5 appeared on the gel, however without the double banding prevalent in the previously mentioned primers, and successfully amplified on the Beckman Coulter Ceq 8000. Unsuccessful Beckman Coulter Ceq 8000 runs also showed up poorly on the gels, these included: BL3, BM4, and BM6.

We noticed that in the tetraploid species *B. matricariifolium*, there were often multiple peaks that amplified (up to four) and in the diploid species *B. angustisegmentum*, the loci showed at most two peaks on the Beckman Coulter Ceq 8000. The reason for this is that *B. angustisegmentum* is a diploid species, it has two copies of each chromosome, and therefore it is feasible for two peaks to appear on the Beckman Coulter Ceq 8000 for this species. There can be two alleles at different sizes and peaks at most in *B. angustisegmentum*. *Botrychium matricariifolium* can have up to four peaks because it is a tetraploid species. This is why we possibly see differing numbers of alleles between *B. matricariifolium* and *B. angustisegmentum*. Our Beckman Coulter CEQ 8000 results showed that *B. angustisegmentum* and *B. matricariifolium* were in fact distinct species. We calculated the genetic distance between samples, and then ran a Principal Coordinates Analysis which showed that even with 37% missing data, the two species seem genetically different from each other and there was some variation within a species (See Fig. 5)
Figure 5. A Principle Coordinates Analysis of *B. angustisegmentum* and *B. matricariifolium*. An ordination that calculated the genetic distance between samples. The two species are shown as genetically distinct and display some variation based on our data from the Beckman Coulter CEQ 8000 runs.
Table 1.

Locus numbers and data on various factors such as whether the product amplified on the gels and Beckman Coulter CEQ 8,000. If the product amplified on the Beckman Coulter CEQ 8,000, data on the size of the microsatellite/loci was recorded.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Present</th>
<th>Absent</th>
<th>Amplified Product on Beckman Coulter Ceq 8000</th>
<th>Approximate size of Microsite Locus/Loci</th>
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<tr>
<td>BL1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>86.67, 158.13</td>
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<tr>
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<td></td>
<td>X</td>
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<td></td>
<td></td>
<td>X</td>
<td></td>
</tr>
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<td>BL4</td>
<td>X</td>
<td></td>
<td>X</td>
<td>162.74, 181.69, 257.98</td>
</tr>
<tr>
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<td>X</td>
<td></td>
<td>X</td>
<td>124.21, 181.71</td>
</tr>
<tr>
<td>BL6</td>
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<td></td>
<td>X</td>
<td></td>
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<tr>
<td>BM1</td>
<td>X</td>
<td></td>
<td>X</td>
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</tr>
<tr>
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<td>X</td>
<td></td>
<td>X</td>
<td>110</td>
</tr>
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<td></td>
<td></td>
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<td>X</td>
<td>277.86</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>X</td>
<td></td>
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</tr>
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<td>X</td>
<td></td>
<td>X</td>
<td>90.57, 94.97, 108.53</td>
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<tr>
<td>BM9</td>
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<tr>
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<tr>
<td>BM11</td>
<td>X</td>
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<tr>
<td>BM20</td>
<td>X</td>
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**Discussion**

DNA visualized on the gel had a high incidence of amplifying on the Beckman Coulter CEQ 8000 as well. In our first four rounds of PCR, we were unable to visualize bands on the gel until we changed the program from 13 cycles to 27. More cycles allowed us to visualize the bands on the gel because in the one step process, more cycles were needed in order for the primers to attach to the DNA so that it could be amplified. There could be more bands on a gel because each primer could be amplifying more than one locus. At each loci there could be two (diploid) or four (tetraploid) alleles. *Botrychium matricariifolium* always has four sets of chromosomes, but it could be homozygous and therefore have the same alleles at the locus. *B. angustisegmentum* has two sets of chromosomes and *B. matricariifolium* has up to four, however, the gel is not
of a fine enough scale to determine this information. Running the product on the Beckman Coulter CEQ 8000, allowed us to visualize the alleles located at each loci. When there are multiple bands on the gel, it represents different loci that have amplified. These loci could be close together or on two completely different chromosomes.

The loci that amplified in both *B. matricariifolium* and *B. angustisegmentum* were somewhat variable. This may be because both samples were collected from within the same population. This may mean that the populations that we looked at may be undergoing intragametophytic selfing which may account for the increased homozygosity and decreased variation (Otto, 2007; Perrie, 2010; Dauphin *et al.*, 2014). The Principal Coordinates analysis of Genetic Distance showed a small amount of variation within the two species and confirmed that they were distinct from one another. However, there was a high rate of missing data (37%), which can be attributed to a run on the Beckman Coulter CEQ 8000 in which the ladder failed. It may also be attributed to a few primers that failed to amplify on the Beckman Coulter CEQ 8000. The microsatellites would be more effective if they had shown more variation. The shorter peaks in some of the runs make it harder to use the microsatellites; however this issue could be solved by adding more PCR product. By testing more populations we may see more variation within the two species.

In future studies using these microsatellite primers, it may be beneficial to look at populations of *B. matricariifolium* and *B. angustisegmentum* from other areas then where our samples were collected. We may also see increased variation if we looked at different species of *Botrychium*, in particular *B. pallidum*. Working on the PCR program could also be beneficial in order to get rid of some of the bands on the gel and sequencing the DNA found in the gel bands to make sure what we are sequencing is a microsatellite.
Conclusion

The microsatellite primers we designed worked on to amplify the DNA on the gels as well as for both *B. matricariifolium* and *B. angustisegmentum*. When multiple bands showed up on the gel for a particular primer, it also tended to multiply at multiple loci when run on the Beckman Coulter Ceq 8000. However, the Beckman Coulter Ceq 8000 runs showed minimal variation between individuals. The Principal Coordinates Analysis of genetic distance showed that *B. angustisegmentum* and *B. matricariifolium* were distinct species, and had a small amount of variation within their respective populations. This may be attributed to the fact that individuals from both *B. matricariifolium* and *B. angustisegmentum* were collected from one population each. For future studies, more variation may be seen by testing individuals from different populations or different species of *Botrychium* completely, in particular *B. pallidum* to further resolve discrepancies within the phylogeny.
References


