Genetic and Morphological Analysis of Guanella Pass Botrychium Plants

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Introduction

This report presents genetic and morphological analysis of plants of *Botrychium* subgenus *Botrychium* occurring at a roadside habitat near Guanella Pass in Colorado and suspected of representing one or more new taxa. Plants of the putative new taxa were collected from the Guanella Pass site on July 15, August 12, and August 24, 2005, under the direction of Steve Popovich of the Arapaho National Forest. A single plant of the most common taxon was collected by Don Farrar near the 4th of July Trailhead near Eldora, Colorado on July 12. Horizontal starch-gel enzyme electrophoresis was conducted on these plants using standard procedures on July 26 and September 1, 2005.

Genetic data

The accompanying spreadsheet lists the allelic composition of Guanella Pass *Botrychium* plants and related species as determined by enzyme electrophoresis for 22 gene loci in 10 enzyme systems. Alleles are numbered in the order of their migration as detected within all species of *Botrychium* subgenus *Botrychium*, the low numbers migrating farthest from the origin. Fractional numbers are alleles detected after the originally detected alleles were numbered with integers. They do not carry any special meaning.

‘n’ represents null alleles, that is absence of any staining for loci never active in a particular species (e.g., AAT-3 in B. lineare) or any alleles not active at a locus where its heterodimer with another allele is active.

‘—‘ represents loci not staining for unknown reason in a given plant.

PGM-1 is a duplicated locus in most if not all diploid plants of B. campestre and B. lineare, ‘0’ being a fast duplicate of ‘1’. Thus 0-1 does not indicate recombinational heterozygosity.

At the top of the spreadsheet are listed all alleles detected for B. campestre and B. lineare in a survey of more than 100 plants from all areas of the species' ranges.

Next are listed the genotypes of 12 plants of a taxon found at Guanella Pass in Colorado (11 plants) and the 4th of July Trailhead near Eldora, CO (1 plant) that is closely related to B. campestre and B. lineare, herein designated as 'lincamp'. The first three plants display no heterozygous loci, the next nine plants display at least one heterozygous locus. All of these plants appear to have normal spores that are approximately the size of B. lineare and B. campestre.

Next are two plants found at Guanella Pass that morphologically and genetically appear to be hybrids between a 'lincamp' diploid and another unknown diploid. B. 'lincamp' x ??? is a pinnately lobed plant with linear ultimate segments. B. 'lincamp' x mont? is a plant with flabellate pinnae feasibly intermediate between B. 'lincamp' and B. montanum. Both plants appear to have normal spores.

Six of the Guanella Pass plants were electrophoresed a second time to confirm accuracy and repeatability of the tests.

Finally are listed all the alleles detected in B. montanum and B. simplex, diploid species capable of contributing most of the non-'lincamp' alleles found in the second of the putatively hybrid plants. These species, especially B. montanum, have morphologies that in combination with B. 'lincamp' could produce the required pinna outline.

Analysis of Results

<u>B. 'lincamp'</u> (in later reports this taxon will be referred to as B. 'bifurcatum')

These twelve plants have similar morphology that is intermediate between B. lineare and B. campestre. Upper pinnae are usually bifurcate with linear segments similar to those of B. lineare but about half as long as the pinnae of plants of B. lineare found elsewhere in Colorado. The basal pinnae are often broader with more than two lobes. The trophophore is sessile and the sporophore stalk is short as in both B. campestre and B. lineare.

Three of these plants are homozygous but contain some alleles unique to either B. campestre or B. lineare. These plants contain no alleles not present in one or the other of these species.

Nine of the plants are heterozygous at two to four loci. Five of the plants are heterozygous for alleles not known elsewhere in either B. campestre or B. lineare <u>or in any other diploid species</u>.

Among the nine heterozygous plants, seven different multilocus genotypes are present. This result is in sharp contrast to population genetic analysis from all other *Botrychium* populations examined by Farrar or reported by other researchers in this extent of plant to plant variability. Populations of diploid *Botrychium* species generally show no within population variability with all plants being homozygous. In occasional populations that do possess some degree of allelic variability, more than 90% of individual plants are homozygous. These results, obtained from various known species of *Botrychium*, have generally been attributed to a high level of self fertilization of bisexual gametophytes, possibly due to the difficulty of sperm transfer in the underground habitat of the gametophytes.

The Guanella plants display the degree of heterozygosity and plant to plant variability expected of predominantly out crossing diploid plants. The plant to plant variability and the number of different multilocus genotypes is inconsistent with the Guanella plants being allotetraploids with fixed heterozygosity. More likely, all twelve of these plants are members of a single diploid taxon that differs from other *Botrychium* species in being predominantly outcrossing. This could occur if the gametophytes of this taxon are functionally unisexual, e.g., by undergoing an extended period of unisexuality before changing sex or becoming bisexual.

An array of genetically different heterozygous individuals could also be produced by extensive hybridization between two different species, but this would require that at least one of the parent species have plant to plant allelic variability at eight different loci. Such plant to plant variability has not been found in any of the known diploid species. This hypothesis would also require that the two species be sufficiently closely related to produce hybrids with non-abortive spores. This could be the case for B. lineare and B. campestre which are sister species with a genetic identity closer than that between most species. However, hybridization between known genotypes of B. campestre and B. lineare could not produce the genotypes containing alleles not present in those two species. It also seems unlikely that a B. campestre x B. lineare cross would possess a phenology distinctly later than that of either parent--a requirement necessitated by the absence of either typical B. campestre or typical B. lineare at the site at the time the Guanella plants were located. [Because of their early phenology B. lineare and B. campestre could have been present earlier in the season but senesced by the time the Guanella ‘lincamp’ plants were located.]

A third possibility is that the Guanella plants are a product of hybridization between B. lineare and a new taxon of later phenology that has not yet been detected. That taxon would have to be genetically variable as described above and possess the non-lineare alleles present in the Guanella plants. If the Guanella plants are first generation hybrids this would require presence of two undetected species at the site.

In consideration of the requirements of the above scenarios, the simplest hypothesis is that these 12 Guanella plants are a new diploid taxon closely related to B. lineare and B. campestre but containing alleles not present in either of those species. [Absence of these alleles in homozygous individuals is not unexpected given the low number of plants sampled.] The new taxon is further characterized by an out-crossing breeding behavior previously unknown in *Botrychium*. The occurrence of a plant of the same morphological and genetic composition at a second site (near Eldora, CO), also in the absence of B. campestre and B. lineare, further supports these plants being an independent taxon.

Also of significance is the presence of the PGI ‘1’ allele in the Guanella taxon. This allele is rare in European genotypes of B. lunaria but otherwise unknown in diploid species. Yet it <u>is</u> present in several North American tetraploid species, including B. minganense, B. matricariifolium, B. pinnatum and B. paradoxum. These species contain fixed heterozygosity at many loci indicating their production by allopolyploid speciation and necessitating the occurrence of the PGI ‘1’ allele in one of their parental diploids. For B. minganense, B. pinnatum and B. matricariifolium, this allele may have been provided by a B. lunaria genotype now limited to Europe. For B. paradoxum a B. lunaria parentage seems unlikely. Because B. paradoxum lacks an expanded trophophore it seems probable that one or both of its parent diploid species would have highly reduced pinnae, like those of the Guanella plants.

<u>B. ‘lincamp’ x ??? (Farrar # 13015)</u>

This plant is twice pinnate in its lower pinnae with linear ultimate segments. Although its spores appear to be normal, this plant is heterozygous at 5 gene loci suggesting that it may be of hybrid origin. It shares many alleles with the ‘lincamp’ taxon indicating that that taxon could be one of its parents, but no other known diploid *Botrychium* is a good candidate for the other parent. The only other twice pinnate diploid moonwort *Botrychium* is B. lanceolatum with angled, tapering and pointed segments unlike those of the Guanella plant. B. lanceolatum could contribute the PGM-1=3 and AAT-1=1 alleles, but it could not contribute the PGI=3 allele or the 6PGD=3 allele present in the Guanella plant. Furthermore B. lanceolatum is monomorphic at half its loci for alleles that are not expressed in this Guanella plant, making it unlikely that B. lanceolatum or any of its allotetraploid derivatives has participated in the formation of the Guanella plant.

Although this Guanella plant shares most of its alleles (including the unique 6PGD=3 allele) with the ‘lincamp’ taxon discussed above, it also contains three alleles not yet detected in the ‘lincamp’ taxon and two alleles not present in either B. lineare or B. campestre. Of the three alleles present in this Guanella plant that are not present in the ‘lincamp’ taxon, all three are found only in B. lunaria and are not common in that species. PGI=3 is also present in B. simplex and B.

montanum, PGM-1=3 is also present in B. simplex, B. lanceolatum, the allotetraploids of B. lanceolatum, and in the allotetraploid B. paradoxum. AAT-1=1 is present in most diploid species, but not in B. lineare or B. campestre. None of these plants has a morphology that in combination with the ‘lincamp’ taxon would likely produce the twice pinnate Guanella plant.

The above data leave only two plausible hypotheses for explaining the twice-pinnate Guanella plant:

1) It may be the result of hybridization between the ‘lincamp’ taxon and another, as yet undiscovered, species. Supporting this hypothesis are the unique morphology and three alleles not detected in the ‘lincamp’ taxon.

2) It may be an alternative expression of the ‘lincamp’ taxon that also happens to possess three rare alleles not detected in the small sample of plants with the ‘lincamp’ morphology. Supporting this hypothesis is the rare presence of the 6PGD=3 allele in the ‘lincamp’ taxon and the failure to find a second new species in the area despite intensive search.

B. ‘lincamp’ x montanum ? (Farrar13018)

This plant differs from the ‘lincamp’ taxon in possessing broad flabellate pinnae. It is similar to the ‘lincamp’ taxon in the bifurcation of its lower pinnae and in its short sporophore stalk. The plant is superficially similar to the allotetraploid species B. ascendens but differs from that taxon in its broadly attached pinnae and in its shallowly lobed rather than sharply tooth outer pinnae margins. It also differs from B. ascendens at 10 of 22 gene loci, making it highly improbable that the Guanella plant is the same as or closely related to B. ascendens.

This Guanella plant possesses six heterozygous loci and alleles at five of these loci that are not present in the ‘lincamp’ taxon whereas the other allele at four of these loci is present in the ‘lincamp’ taxon. These data strongly suggest that this plant has been generated through hybridization between the ‘lincamp’ taxon and another diploid species. In this case, unlike the twice pinnate plant discussed above, the non ‘lincamp’ alleles are present in other diploid species that could also conceivably combine with ‘lincamp’ to produce the morphology of this Guanella plant. The 6 allele at MDH-2 is present in B. montanum and B. simplex, the 3 allele at PGM-1 is present in B. simplex, B. montanum, B. lanceolatum and B. lunaria, the 2 allele at AAT-2 is present in B. simplex, B. lunaria and B. crenulatum, the 3 allele of DIA-3 is present in all diploid species except B. lineare and B. pallidum, and the 4 allele of IDH is present in B. lunaria, B. montanum, B. mormo and B. lanceolatum. In comparison of these species as possible parents of the Guanella plant, B. montanum, B. simplex and B. lunaria each possess four of the five needed alleles. The most uncommon of these alleles is MDH-2=6, AAT-2=2 and IDH=4. The first two of these are present in B. simplex and the first and third are present in B. montanum. The second and third are present in B. lunaria, but only in European genotypes of this species. Thus B. montanum and B. simplex emerge as the most likely non-‘lincamp’ parent of this Guanella plant.

Of these two, B. montanum is a more likely candidate based on its thick texture, stiff posture, broadly adnate pinnae with dentate margins, and sporophore-trophophore separation well above ground level. In opposition to either B. montanum or B. simplex as a parent are the very short stalks of both trophophore and sporophore in the Guanella plant. Also in opposition are several alleles at monomorphic loci of the non ‘lincamp’ parent (five in B. montanum, three in B. simplex) that are not expressed in the Guanella plant and the absence of either of these species in the Guanella Pass vicinity. Thus the match with B. montanum is not as good as might be expected and again raises the possible presence of an as yet undiscovered species that would better fit as the non-‘lincamp’ parent of this Guanella plant.

This Guanella plant also appears to have normal spores rather than the variable sizes and empty and collapsed spores typical of interspecies hybrids. This could be explained by the F1 hybrid having undergone restoration of fertility through chromosome doubling. However, no additional plants of this morphology have yet been detected in the vicinity to indicate that this is an independently reproducing taxon. (A somewhat similar plant was collected at Winter Park but was prepared as a herbarium voucher before a fresh sample could be taken for allozyme analysis.) It should also be noted that the out-crossing behavior of the ‘lincamp’ taxon at the Guanella site would also promote hybridization with other species.

Conclusions

The eleven plants from Guanella Pass plus the single plant from the 4th of July Trailhead near Eldora that are labeled herein as ‘lincamp’ most likely represent a single diploid taxon. Because of the number of alleles and aspects of morphology it shares with B. campestre and B. lineare, ‘lincamp’ is certainly a close relative of those species, but because it contains alleles derived from both species as well as alleles not present in either of those species it must be considered distinct from those species. It is further unique in its plant to plant variability (9 of 12 plants display different multilocus genotypes) that is probably derived from a predominantly out-crossing breeding system. The level of taxonomic distinction, i.e., variety, subspecies or species, will be determined following further study.

Two plants from Guanella Pass are unique in both their morphologies and in their genotypes. Both share many alleles with the ‘lincamp’ taxon and may have been derived by hybridization of that taxon with another *Botrychium* species. Presence of such hybrids might in fact be expected due to the out-breeding nature of ‘lincamp’. However, there are several problems with this hypothesis.

First, no known species fits the projected morphology and genotype necessary to produce the twice pinnate plant, and the species most likely to have served as the non-‘lincamp’ parent of the flabellate plant (B. montanum) is not known to be present in Colorado. Second, both of these Guanella plants appear to have normal spores rather than abortive spores expected in first generation hybrids. Consequently alternative hypotheses must also be considered.

It is possible that these two Guanella plants have undergone chromosome doubling to become fertile independently reproducing species. This origin does not solve the problem of imperfect fits for the non-‘lincamp’ parents, but does allow the original hybridization to have taken place at another time and place. It seems improbable though that these independent allotetraploid taxa would happen to occur as single individuals (no other similar plants have been confirmed anywhere) in association with the ‘lincamp’ taxon.

Another possibility is that these two unique plants are simply morphological and genetic extensions of the ‘lincamp’ taxon, i.e., they are members of the ‘lincamp’ taxon in which the improbable coincidence of different morphology and different alleles has occurred.

In consideration of all the evidence, the most likely hypothesis is that these two plants are first generation hybrids in which spores maintain an appearance of normalcy. If this is the case, then it is also likely that one or two additional new taxa exist in the area with genotypes and morphologies capable of producing the two Guanella plants in genetic combination with the ‘lincamp’ taxon.

The Guanella Pass site is of great biological significance because of the presence of at least one and possibly three new taxa of *Botrychium*. This significance is amplified by the out-breeding behavior displayed by the new ‘lincamp’ taxon, which contrasts with previous observations of predominant self-fertilization in *Botrychium* species. The mechanism underlying its high level of out-breeding may provide important insight into how the speciation process in *Botrychium* has generated the morphological and genetic variation displayed in the current array of diploid species.