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Revised Research Plan
Genetic structure, and assessment of the threat from hybridization in the Colorado endemic *Physaria bellii* (Bell's Twinpod)
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Physaria bellii Mulligan (Bell's Twinpod) is a Colorado endemic which has been classified as "imperiled" due to rarity (G2 S2) by the Natural Heritage Program ranking system (Colorado Natural Heritage Program (CNHP) 1999). *Physaria bellii* is a member of the Brassicaceae (mustard family) and is a self-incompatible perennial (Mulligan 1966a). It occurs on open shale and sandstone washes of the Niobrara and Pierre formations in Larimer, Boulder and Jefferson Counties. These formations occur in the foothills of the Front Range, and the plant is found between 5,200' and 5,800' elevation (CNHP 1999).

Physaria bellii has probably never been common due to its particular habitat preferences, although most populations are currently considered relatively stable. On the other hand, *P. bellii* faces threats which include habitat fragmentation, competition from exotic species, grazing and potential hybridization with a closely related congener. Although some ecological studies have been conducted (Supples 2001, Jennings 1989, Popp 1983, and Peterson and Harmon 1981), there are two important areas of research that have yet to be addressed. First, because *P. bellii* is a narrowly-distributed endemic, there is a need to study the genetic variation present in this species and how that variation is distributed within and among populations to determine their relative health. Secondly, because there have been reports of *P. bellii* hybridizing with the closely related *P. vitulifera*, it is necessary to examine this relationship to determine if hybridization threatens the future stability of *P. bellii* populations. I will explore these areas of research by answering the following questions:

1. How is genetic variation distributed in *P. bellii*? Genetic diversity is the variety of alleles and genotypes present in a group of organisms and is generated by mutation, as well as the effects of

migration, hybridization, selection and chance (Frankham et al. 2002). Variation is required for populations to evolve in the face of environmental change and is potentially partitioned among and within populations (Wright 1931). Populations with high amounts of genetic diversity possess adaptive evolutionary potential.

Harper (1977) proposed four major reasons why populations may be small: 1. There is a scarcity of available sites, which are separated by distances beyond normal dispersal, 2. The carrying capacity of the site is low, 3. The habitability of the site is of short duration because of successional displacement and 4. The site has not been fully exploited because colonization is in its early stages. Even though most *P. bellii* populations number in the hundreds or thousands, this species is potentially affected by the dynamics of 1. and 3. As mentioned above, the occurrence of *P. bellii* is intimately tied to the presence of the open, shale substrate that defines its habitat. It is an endemic with a relatively narrow range, about 40 km x 160 km (CNHP 1999, see map). Furthermore, suitable habitat within its range is patchy, not continuous. This patchiness is potentially exacerbated by humans building houses in the foothills of the Front Range, which is prime *P. bellii* habitat. In an argument put forth by Supples (2001), the distribution of *P. bellii* is shaped, on the one hand, by the forces of erosion exposing new substrate for *P. bellii* to colonize. On the other hand, the process of succession results in other plant species colonizing the site and competing with *P. bellii* for resources until it is no longer found at the site. The patchy nature of its habitat and the present distribution of *P. bellii* populations suggest the possibility that *P. bellii* populations are to some degree isolated from each other.

Whether geographical separation results in reproductive isolation is a question of whether gene flow takes place between populations. Gene flow is the movement of genes from one population to another (Wright 1969). The absence of gene flow is generally thought to have negative consequences, especially in small populations. A low rate of migration results in a higher probability of relatives mating, or inbreeding. Inbreeding depression results in reduced fitness due to the accumulation of

deleterious alleles (Franklin 1980). Populations that are small as well as isolated are prone to random changes in allele frequencies that are not counterbalanced by selection, a process known as genetic drift (Wright 1931). However, the total absence of gene flow is rare, and, as Wright (1931) demonstrated, only one migrant per generation can be enough to counteract the effects of drift. Ellstrand (1992) suggested that as methods of detecting gene flow have changed over the last several decades, evidence of the occurrence of gene flow has increased. Indeed, populations separated by hundreds or thousands of kilometers are often found not to be genetically isolated.

Gene flow can lead to several phenomena. Gene flow between populations should have the effect of increasing the effective size (Ne) of the populations (Ellstrand 1992). A higher Ne should counter processes that reduce variation, such as drift and inbreeding depression. However, another possible result of gene flow is hybridization, which could result in outbreeding depression (Templeton 1986). Populations are presumed to have some degree of local adaptation, and the introduction of novel genetic material can disrupt this adaptation. Another potential consequence of gene flow is "genetic assimilation" where genes of a more abundant species are introgressed into a rarer species and the number of pure individuals decreases (Reisberg et al. 1989). This phenomenon will be addressed in more detail in the next section where I discuss how *P. bellii* may be threatened by hybridization with a common congener, *P. vitulifera.*

Hamrick and Godt (1990) reviewed allozyme data for 449 plant species to evaluate the genetic diversity found in rare or endemic plants. They found that endemics had less diversity than widespread species in terms of percentage of polymorphic alleles, but at a population level, had the same degree of diversity as widespread species. Determining the population genetic structure of *P. bellii* is relevant because knowing how the genetic variation is distributed within and among populations will lend support to one of two scenarios. First, *P. bellii* variation could be partitioned as though some populations are reproductively isolated from each other. In this case we would expect to see most of the

variation among populations. This could cause the species to enter what Gilpin and Soule (1986) refer to as an "extinction vortex", where individuals in an isolated population interbreed, which leads to reduced fitness, reduced numbers, and eventually extinction of that population. More likely, present levels of gene flow may appear sufficient to prevent this fate. I hypothesize the latter will be the case for *P. bellii.*

2. What is the nature of the relationship between *Physaria bellii* and its closely related, spatially proximate congener *P. vitulifera*?

It is implied in the above discussion that hybridization is always a negative consequence of gene flow. Furthermore, the prevailing view until recently was that hybridization was unlikely to be evolutionarily important because hybrids were always less fit than their parents in every habitat. However, Arnold et al. (1999) reviewed several studies that showed that, while not common, hybridization occasionally results in genotypes that are as fit or more fit than their parents in certain habitats, which themselves may be viewed as hybrid. If hybrids can be as fit or more fit than their parents in certain situations, new evolutionary lineages could become established. The study of hybridization, then, can give insights into the process of speciation.

It has been anecdotally noted (on the basis of leaf morphology) that *P. bellii* and *P. vitulifera* may be hybridizing in Jefferson County. (S. Spackman, Botanist, CNHP, pers. comm.). *Physaria vitulifera* is more common and has a larger range than *P. bellii* (USDA Plants Database 2003, CNHP 1999). As mentioned previously, a potential consequence of gene flow between populations is "genetic assimilation" (Reisberg et al. 1989), where one species is overwhelmed by another species and the number of genetically pure individuals is diminished. In the case of *Cercocarpus traskiae* (Catalina mahogany) Reisberg et al. found that the number of genetically pure individuals was reduced to five because of extensive hybridization with the more common C. *betuloides*. Recommendations were made to remove sympatric *C. betuloides* and propagate *C. traskiae* from cuttings to increase population

numbers. *Physaria bellii* clearly is not in danger of being immediately assimilated. However, an assessment of the degree and extent of hybridization would help clarify whether *P. bellii* is likely to face a widening of the hybrid zone between it and the more common *P. vitulifera*. *Physaria vitulifera* occurs in Boulder County, although I have not found evidence that it occurs sympatrically with *P. bellii*. *Physaria vitulifera* does not appear to occur in Larimer County, although a small number of populations have been reported across the state line in Wyoming (Wyoming Natural Diversity Database 2002).

An important aspect of characterizing the relationship between *P. bellii* and *P. vitulifera* is examining life history and morphological differences between the two species and their purported hybrids. Leaf shape differs between the species, and the purported hybrids show an intermediate leaf shape. These differences can be quantified by taking measurements of leaves of each species and the hybrids. Another morphological characteristic that can be examined and potentially quantified is the pattern of trichomes (hair-like bristles) present on the leaf surfaces of all Brassicaceae. Because hybrids are formed due to overlapping phenology of the two species, I will also characterize flowering times for each species and their hybrids to determine the degree of overlap.

3. Do varying ploidy levels in *Physaria vitulifera* act as an effective barrier to hybridization, thus keeping the two species distinct entities? An early study by Mulligan (1966b) found that some populations of *P. vitulifera* are tetraploid (4N, or twice the normal number of chromosomes, 4N=16), while others are diploid (2N=8). *Physaria bellii* is a diploid species with 2N=8 (Mulligan 1966a). Normally, the number of chromosomes in the gametes of both parents has to be equal for fertile offspring to be produced. Therefore, if some populations of *P. vitulifera* are found to be tetraploid, this would potentially act as an effective barrier to hybridization between these species in those areas because matings would form sterile triploid offspring. Additional populations of both species have been documented since Mulligan's work (1966b), and I will investigate whether ploidy level serves as a reproductive barrier, hypothesizing that a tetraploid condition in *P. vitulifera* would prevent effective

hybridization with *P. bellii* where their ranges overlap.

Methods

To answer Research Question 1 and part of Question 2, I am using ISSR (Inter-Simple Sequence Repeat) markers.

Papers using this technique were first published by Zietkiewicz, et al. (1994) and Gupta et al. (1994). The method takes advantage of the idea that Simple Sequence Repeats (SSRs), also known as microsatellites, are ubiquitous throughout an organism's genome. SSRs are short sequences (two to five base pairs) of repeated nucleotides. For example, AGAGAGAGAG, or $(AG)_5$ is a dinucleotide repeat. The ISSR technique uses one primer that is complementary to a microsatellite. The Polymerase Chain Reaction (PCR) is used to amplify target sequences. The PCR product is run on an electrophoretic gel, and the resulting bands represent the DNA sequence between two inverted microsatellites. One way to limit the number of fragments amplified is to "anchor" the primer by adding one or more nucleotides at the beginning or end of the primer that are not part of the repeat. A primer with the sequence $(AG)_5CT$ is an example of an anchored primer. I am using several anchored and several non-anchored primers in my research.

ISSRs are dominant markers, which means that data are collected by noting the presence or absence of polymorphic bands on a gel and that heterozygotes are indistinguishable from homozygous dominant ("band present") individuals. Certain assumptions are made during data analysis to compensate for this, namely that the populations are in Hardy-Weinberg equilibrium. ISSRs have two

advantages over the other widely-used dominant marker systems reported in the current literature: they use a higher annealing temperature than RAPDs (Randomly Amplified Polymorphic DNA) and are more straightforward to use than AFLPs (Amplified Fragment Length Polymorphisms). The higher annealing temperature used in ISSRs increases the chances of correct amplification of fragments.

The other aspect of Research Question 2 is finding morphological and life history differences between *Physaria bellii* and *P. vitulifera*. To that end, I have collected leaves from populations of both species and the purported hybrids. I will take four measurements per leaf and run one-way ANOVAs to see if there are statistically significant differences in leaf shape between the species and the hybrids. I also plan to examine and compare the pattern of trichomes on the surface of the leaves of the two species and the hybrids to determine if there are quantifiable differences between the three groups.

To quantify the events of flowering and seed set, I will take weekly observations of two populations of each species and the two hybrid populations, for a total of six populations visited each week. At each observation, I will record the number of inflorescences, number of flowers open per inflorescence and the ratio of inflorescence size to rosette size. To accomplish this, I will set up a semi-permanent transect that runs from the edge of the population into the middle, and make an observation at each of five one meter squared plots that are spaced every 10m. I will also obtain measurements of the following: abundance of flowering versus non-flowering *P. bellii* plants per one meter plot, the size classes of those plants, duration of flowering, seed set and associated plant species found at each site. Finally I will examine pollinator activity, by selecting one of the five one meter squared plots and expand it to nine meters squared (three meters on a side) for the purposes of pollinator observations. I will make two morning and two afternoon observations of one hour each at each site (for a total of 8 observations per taxa), and record the number of flowers visited per hour, as well as the types of pollinators and the number of different plants each pollinator visits. Samples of each type of pollinator will also be collected for future identification.

To answer Research Question 3, I am growing *Physaria bellii* and *P. vitulifera* plants to use in controlled crosses in the greenhouse.

I have had propagation problems with both species, but have worked out a method that results in about 50% germination. I have about 12 plants of each species in the greenhouse and have recently started more seed. I am planning to test whether seed can be produced from crosses between *P. bellii* and *P. vitulifera* from areas where it was found to be diploid and from areas where it was found to be tetraploid. Because each of the 5-10 inflorescences on both *Physaria* species is distinct, it will be relatively simple to treat each inflorescence independently of the others for the purposes of cross pollinations, as long as there is only one pollen type per inflorescence. Inflorescences will be bagged as described below so that seed paternity can be assured. In the meantime, I will perform root tip chromosome squashes with *P. vitulifera* seed collected from populations in Boulder and Jefferson Counties to determine chromosome numbers. A colleague in Germany has germinated seed I sent him and confirmed that *P. bellii* has 2N=8 chromosomes (M. Lysak, pers comm.).

If the plants grown in the greenhouse fail to flower, I will perform crosses in the field, using *P. bellii* plants as pollen donors and *P. vitulifera* plants from both diploid and tetraploid populations as the pollen recipients. To accomplish this, I will carefully bag unopened inflorescences of *P. vitulifera* plants with fine meshed fabric such as bridal tulle to prevent pollination by insects. Several days later, I will take pollen collected from *P. bellii* and apply it to the stigmas of opened *P. vitulifera* flowers that were covered with fabric. As both *Physaria* species are self-incompatible, pollen from at least two plants will be mixed to increase the chances that at least some of the pollen will be compatible. I will carefully re-bag the fabric around the inflorescences again and return in several weeks to collect any seeds that have formed. If permission can be secured, I will perform crosses with *P. vitulifera* as the pollen donor and *P. bellii* as the pollen recipient.

If seed is not produced from a cross between a tetraploid *P. vitulifera* and a diploid *P. bellii*, then

it is unlikely *P. bellii* is in danger of genetic swamping by the more common species where *P. vitulifera* is tetraploid. Another potential consequence of this type of cross is reduced fitness for both species by "wasting" their gametes in a process that will not result in any viable offspring, a phenomenon called "gamete wastage" (Elstrand and Elam 1993). If crosses between diploid *P. vitulifera* and *P. bellii* produce viable seed, then hybridization would potentially be confined to that area where those diploid populations are found sympatrically.

Progress to date

I spent about a year trying to optimize two other markers systems and learning the ins and outs of doing this kind of lab work. First I tried microsatellite primers that were meant for other species in the Brassicaceae. I did eventually get enough of a band for three of the primers that I was able to send it out to get sequenced, but the results were ambiguous. I then tried RAPDs but dealt with contamination problems and was not getting consistent results. I heard about ISSRs and borrowed a few primers from Sarah Ward in Soil and Crop Sciences. These primers produced bands relatively easily, although I did end up using polyacrylamide gels instead of agarose to get the best resolution.

At this point, I have eight primers that look promising for ISSR work. I spent a lot of time this summer modifying reaction conditions and proportions of reagents trying to get consistent bands with 1.5% agarose gels. I found much better resolution with 4% denaturing polyacrylamide gels. I am using silver staining to visualize the bands.

During the summer of 2002, I used a systematic random sampling method to collect leaf samples and seed from 30 individuals from each of 10 *P. bellii* populations and the two purported hybrid populations. My choice of populations represents the range of this species along the Front Range (see maps). I also haphazardly collected leaves and seeds from 10-15 individuals from each of 12 *P. vitulifera* populations from Boulder and Jefferson counties. I have extracted DNA from all of the 10 *Physaria bellii* populations, leaving two purported hybrid and about 10 *P. vitulifera* yet to go. I am in

the process of running gels and generating data, but have not finished this work. I plan to run gels to complete the population structure work first, then look for species-specific markers among the same primers I used for the population work to complete the hybridization work (Research Question 2).

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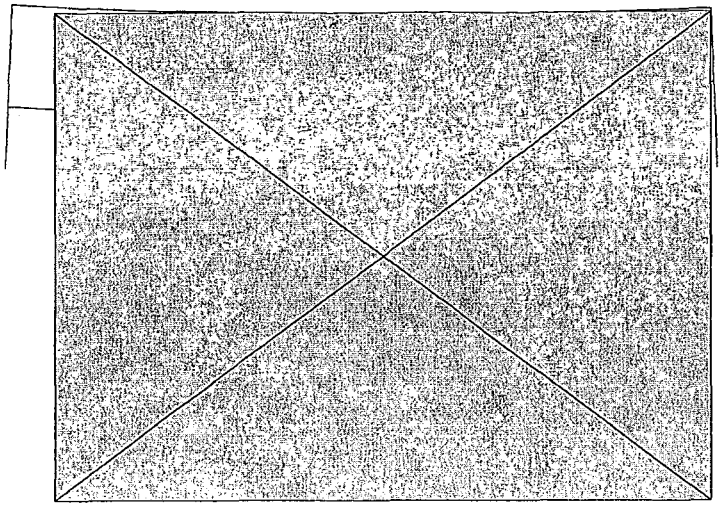
Distribution of Known Occurrences of Bell's Twinpod (Physaria belli) in Colorado



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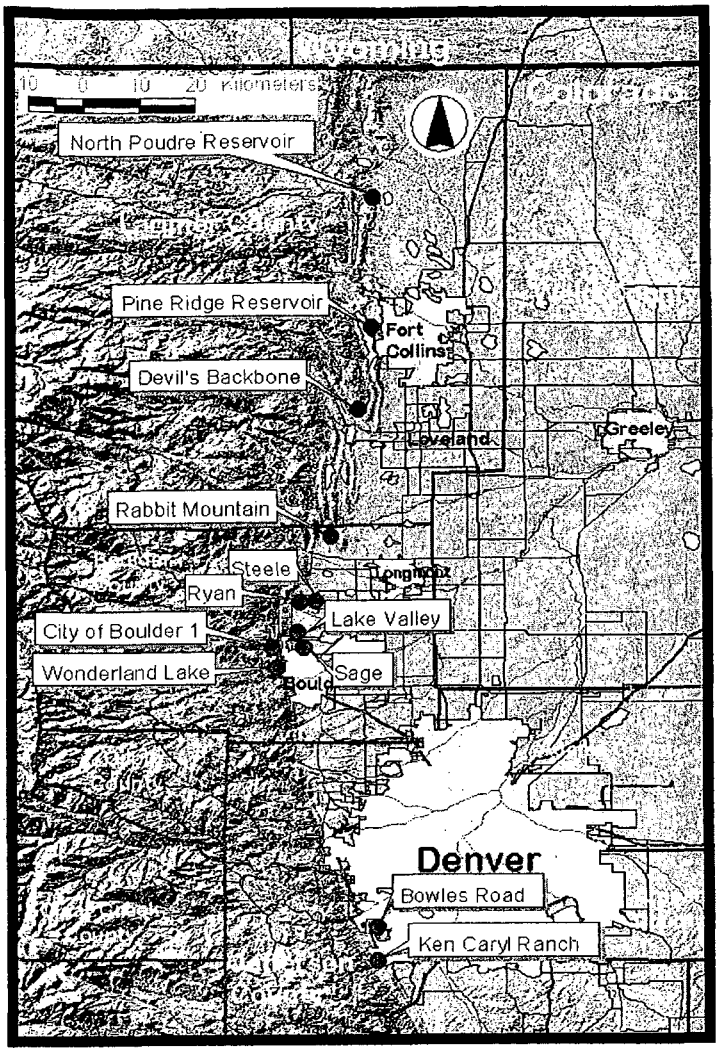
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Fort Collins
Devil's Backbone
Loveland
Greeley
Rabbit Mountain
Steele
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Longmont
Lake Valley
City of Boulder 1
Wonderland Lake
Boulder
Sage
Denver
Bowles Road
Ken Caryl Ranch