

DISSERTATION

POPULATION GENETICS AND INCIDENCE OF HYBRIDIZATION
IN THE RARE COLORADO ENDEMIC PLANT *PHYSARIA BELLII*

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fort Collins, Colorado

Summer 2006

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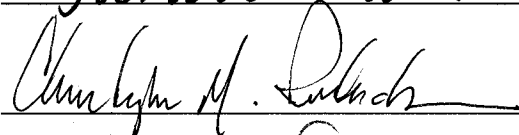
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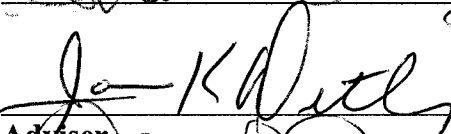
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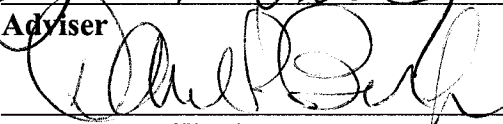
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ABSTRACT OF DISSERTATION
POPULATION GENETICS AND INCIDENCE OF HYBRIDIZATION IN THE
RARE COLORADO ENDEMIC PLANT *PHYSARIA BELLII*

Physaria bellii Mulligan (Brassicaceae) is a rare, herbaceous perennial endemic to shale and sandstone outcrops along the Front Range of northern Colorado, USA. Leaves were collected for genetic and morphological analyses from a sample of 10 *P. bellii*, 11 *P. vitulifera* Rydberg and two putative hybrid populations. The first part of the study estimated genetic diversity and structure of *P. bellii* populations using ISSR (Inter-Simple Sequence Repeat) markers. Genetic diversity of *P. bellii* populations was moderate (expected heterozygosity = 0.22), and a moderately high degree of population structure was found (F_{ST} ranged from 0.17 - 0.24). After dividing populations into a northern (populations 1-5) and a southern (populations 6-10) group, a hierarchical AMOVA partitioned most of the variation among individuals within populations (74%), with lesser amounts among populations within groups (20%) and between groups (6%). An analysis of genotypes suggests that gene flow is occurring among the southern *P. bellii* populations such that the ten sampled populations were grouped into eight genetic clusters. A Mantel test of the correlation between genetic and geographic distances was highly significant ($p < 0.001$), suggesting a clinal pattern of variation. In the second part of the study, genetic data from ISSR markers established that putative hybrids should be classified as such. The distribution of species-diagnostic markers in hybrids, including a small number of known F1 individuals, suggests that the natural hybrid populations are genetically more similar to *P. vitulifera* than *P. bellii*. Morphological data from leaf

measurements showed hybrids to be intermediate for three of five characters.

Morphological data supported the genetic data with Canonical Discriminant Analyses of both data sets showing similar patterns. Further assessments to examine whether *P. bellii* was threatened by hybridization with *P. vitulifera* included using root tip chromosome squashes to determine ploidy levels of *P. vitulifera* populations in proximity to *P. bellii* populations, and performing controlled interspecific and intraspecific crosses. The results indicate *P. bellii* is not currently threatened by hybridization with *P. vitulifera*, and efforts to conserve *P. bellii* should focus on the threat from potential habitat loss instead of from hybridization.

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Acknowledgements

Back in 2001, my advisor, Shanna Carney, as well as then department chair Joan Herbers and then Lab Coordinator Tracy Halward worked with me to rearrange my schedule of teaching duties and other obligations so that I did not end up having to take more than a few weeks off after Zach was born. Shanna accepted me as one of her first graduate students, even though I was a non-traditional student and my GRE scores left something to be desired. Thanks.

Fellow graduate teaching assistants (Chrissy Alba, Kelly Hardwicke, Dan Cariveau, Reesa Conrey), as well as the Lab Coordinators (now Donna Weedman and Brooke Byerly) accommodated my needs as far as what labs I taught and I appreciated that.

I received grant money from the City of Boulder Open Space and Mountain Parks Small Grant Program and the Colorado Native Plant Society Steinkamp Fund. Thanks to both for supporting this work.

Several faculty members and other professionals were a huge help when I needed assistance with permits, lab work, data analysis or other research related problems. Lynn Riedel from the City of Boulder Open Space and Mountain Parks was my contact person for working with several *P. bellii* populations and was always knowledgeable and helpful. Sean Warren from Ken Caryl Ranch helped me secure permits for working with one of the hybrid populations. Dave Anderson, Susan Spackaman-Panjabi, Jill Handwerk

and the staff from the Colorado Natural Heritage Program helped me find *P. bellii* as a study species and were generous in sharing their information. I benefited from conversations with Ruth Hufbauer, who was not a committee member, but often functioned as one. Phil Chapman, as well as being a great teacher, was also instrumental in helping me pick the right tests to analyze my data. Adam Henk helped me develop my gel-loading technique.

I gratefully acknowledge the assistance and contributions of the other members of my committee. Chris Richards went above and beyond to not only assist in the analysis of my data and preparation of the resulting manuscript, but also helped me to understand a great deal about population genetics. I also benefited from fruitful discussions and editing assistance from Sarah Ward, who generously offered me lab space to do the last bit of lab work on F1 hybrids. Scott Reid and Judy Harrington from Soil and Crop Sciences helped me get this work done in an unfamiliar lab. Mark Simmons helped me with questions related to systematics and was gracious when I flooded his office because I let the DI water run all night. Finally, Jim Detling stepped in to be my adviser after Shanna left the department. He has helped me do several rounds of paperwork for various things, and I appreciate his efforts to not let me slip through the cracks, and especially for encouraging me to request an assistantship in Fall 05.

Finally, I dedicate this work to my son, Zach. We are lucky monkeys indeed. I'm looking forward to this new chapter of our lives – on to the next adventure!! My family: Dad, Kris and Bart, John and Mary, and Ron and Lori, I couldn't have done it w/o you.

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INTRODUCTION

Conservation biology concerns itself with the perpetuation of rare species. The first theoretical work in this field determined that both genetic and evolutionary processes were needed to maintain populations of rare species (Soule 1980; Frankel and Soule 1981). However, Lande (1988) asserted that demographic processes associated with small population size (i.e., the Allee effect) were likely to extinguish rare populations before genetic processes had time to impact the population in a meaningful way. Subsequent work is supportive of the idea that rare plants often show genetic effects of finite population size (Barrett and Kohn 1991; Holsinger and Gottlieb 1991; Reed and Frankham 2003; Frankham 2005). Unfortunately, as Oostermeijer et al. (2003) noted, it is still infrequent that genetic data and other types of data are incorporated into the same study.

The term “rare plant” can imply a variety of life-history strategies. A species would be considered rare if it is infrequent per square hectare but occurs over a large range (Harper 1981). Conversely, a rare plant can exist in large populations (i.e. be locally abundant) in its preferred habitat, yet have a small range overall. Plant species of the second description are what Rabinowitz (1981) termed “classic endemics.” Adaptation to certain habitat conditions demonstrates how variation in landscape features drives natural selection to produce many endemic plant species (Kruckeberg and Rabinowitz 1985). These habitats often afford endemics the benefit of reduced competition from species less well-adapted to extreme conditions. Kunin and Gaston (1997) acknowledged that the very adaptations that permit a species to adjust to local conditions may function as constraints that prevent it from succeeding in a different

habitat, a process they termed the “rarity trap”. Because many locally endemic plant species are intimately tied to the existence of preferred habitat, processes that alter habitat quality and quantity may elevate their risk of extinction. This is not to say that all narrow endemics have a high risk of extinction; on the contrary, evidence exists that several plant species have persisted as patchily distributed populations for long periods of time (e.g. Mateu-Andres and Segarra-Moragues 2000; de Lange and Norton 2004, He et al. 2004; Prentis et al. 2004; Ge et al. 2005; Kim et al. 2005; Segarra-Moragues et al. 2005). Nevertheless, because their persistence is strongly linked to available suitable habitat, naturally rare species merit monitoring to ensure populations do not suffer from a loss of genetic diversity.

Rare plant species that occur near more common congeners face an additional threat besides habitat loss - they may hybridize with the congener. Hybridization is ubiquitous among angiosperms and it is thought that many, if not most extant angiosperm species have one or more hybridization events in their lineage (Soltis et al. 2003). Traditionally, especially in animal studies, hybrids have been viewed as evolutionary dead ends. This view was largely due to an acceptance of the Biological Species Concept (Mayr 1942), where one of the defining attributes of a species is reproductive isolation from other such entities. According to this view, “good” species do not hybridize. This line of thinking made sense in light of many hybridization events. Interspecific crosses often produced hybrids that were weak, inviable or otherwise less fit than their parents. However, hybridization has also been seen occasionally to have the opposite outcome, especially in the plant literature, where the offspring were more fit than their parents in certain habitats (Anderson 1948, 1949). Arnold (1997) elaborated on the evolutionary

importance of rare F1 hybrids and how subsequent backcrosses could produce offspring that were competitively superior to their parents, sometimes even in parental habitat. He termed this the Evolutionary Novelty Hypothesis.

A rare species can be affected by hybridization in a number of ways. First, the rare species loses reproductive potential because gametes are wasted when producing hybrid offspring that are often weak or infertile. Secondly, a rare species may suffer loss of habitat due to the encroachment of the congener or the hybrids or both, which reduces the amount of space available for the rare species. Thirdly, a rare species may become so highly introgressed through hybridization that few genetically pure individuals remain. This process is referred to as “swamping” because the degree of hybridization is so extensive that most remaining individuals are of hybrid origin (Ellstrand 1992, Ellstrand and Elam 1993, Levin et al. 1996). The outcome of hybridization events can be complicated when one or both parental species occur as populations with varying ploidy levels.

The initial detection of hybrids usually involves the observation of intermediate morphology, and there is often the assumption that early generation hybrids should show an intermediate phenotype when compared to the parental taxa. However, the expression of morphological characters can also be influenced by the pleiotropic action of other genes. Mallet (2005) reviewed current trends in hybridization and noted that advanced backcross hybrids may be indistinguishable from one of the parental taxa, so the frequency of hybridization in nature is almost certainly underestimated. Rieseberg and Ellstrand (1993) reviewed hybridization studies and examined patterns of inheritance for fitness-related traits. They found the percentage of first generation hybrids displaying

either an intermediate or a parental phenotype was about the same - 45%, and that the frequency of intermediate morphological characters decreased in subsequent generations. Thus, morphological measurements alone are insufficient to determine whether hybridization has occurred between a rare plant and a more common congener. A large number of molecular techniques are available (Nybom 2004, Mallet 2005), but several marker types are regularly used to answer questions involving rare plant species. Some examples of dominant markers that target and amplify nuclear DNA include RAPDs (Randomly Amplified Polymorphic DNA; Welsh and McClelland 1990, Williams et al. 1990), AFLPs (Amplified Fragment Length Polymorphism; Vos et al. 1995), and ISSRs (Inter-Simple Sequence Repeats; Gupta et al. 1994, Zietkiewicz et al. 1994).

This set of studies was initiated to examine the population genetics and incidence of hybridization in *Physaria bellii* (Brassicaceae). *Physaria bellii* is, by Rabinowitz's (1981) categorization, a classic endemic; it is restricted to shale and sandstone outcrops in two counties in Colorado, yet is locally abundant in these areas (Spackman et al. 1997, Doyle et al. 2004). The examination of genetic diversity and degree of differentiation in *P. bellii* was warranted because populations of this species are at risk of being lost to residential development and limestone mining along the Front Range. Furthermore, anecdotal evidence indicates the two southernmost populations of *P. bellii* contain hybrids of this species and the more common *P. vitulifera*. Because hybridization can threaten rare species (Ellstrand and Elam 1993, Rhymer and Simberloff 1996), this study also sought to establish whether hybridization has occurred and assess whether hybridization poses a threat to populations of *P. bellii*.

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Chapter 1: Population genetics of the rare Colorado endemic plant *Physaria bellii*

Mulligan (Brassicaceae)

ABSTRACT

Physaria bellii (Brassicaceae) is a rare, outcrossing perennial endemic to shale and sandstone outcrops along the Front Range of northern Colorado, USA. This species is locally abundant and considered presently stable. However, the Colorado Natural Heritage Program ranks *P. bellii* as G2/S2 because it exists as a small number of populations, and because it occupies a narrowly defined habitat deemed desirable for human uses. Leaf tissue from ten populations was collected and analyzed with ISSR (Inter-Simple Sequence Repeat) markers to discern the amount of genetic diversity and degree of population subdivision in *P. bellii*. Genetic diversity was moderate (0.22) and consistent across several methods of measurement. A moderately high degree of population structure was found (F_{ST} calculated using two algorithms ranged from 0.17 - 0.24), as was evidence of low levels of inbreeding. A hierarchical AMOVA, where populations were divided into a northern (populations 1-5) and a southern (populations 6-10) group, partitioned most of the variation among individuals within populations (74%), with lesser amounts among populations within groups (20%) and between groups (6%). A Principal Coordinates analysis of genetic distances was consistent with the north – south distribution of populations, as was a minimum spanning network based on pairwise F_{ST} values between adjacent populations. An analysis of genotypes revealed a high likelihood that gene flow is occurring among the southern *P. bellii* populations such that the ten sampled populations were grouped into eight genetic clusters. A Mantel test of

the correlation between genetic and geographic distances was highly significant ($P < 0.001$). The pattern of variation thus appears to be clinal, and efforts to conserve this species should involve preserving enough populations so that gene flow between populations is not interrupted.

INTRODUCTION

Early work in the field of conservation biology asserted the importance of maintaining both genetic and evolutionary processes in populations of rare species (Soulé 1980; Frankel and Soulé 1981). Nevertheless, genetic data are lacking for many plant species of concern. Population genetic analyses should be an integral component of rare plant management for several reasons (Schemske 1994). First, genetic information can provide a baseline to which monitoring data can be compared over time. Also, by providing diversity estimates, genetic studies can provide some measure of population viability (Reed and Frankham 2003) and guide sampling efforts should reestablishment of extirpated populations become necessary. Finally, evaluating the scale of dispersal and gene flow can aid efforts to mitigate the effects of inbreeding and drift in rare plant populations (Ellstrand 1992; Ellstrand and Elam 1993).

Plant rarity is a function of both a species' abundance and the range it occupies (Gaston 1994). Rabinowitz (1981) outlined seven forms of rarity, each of which has a different combination of three factors: geographic range (wide or narrow), habitat specificity (high or low) and local population size (large or small). Plant species with small geographic ranges and high habitat specificity are classic endemics, and many are threatened or endangered. Often, rarity is equated with vulnerability, but this is not

always the case (Mace and Kershaw 1997; Rosenzweig and Lomolino 1997). Naturally rare species are often locally adapted to stressful habitats many other plants find unsuitable (e.g. Pepper and Norwood 2001; Mattner et al. 2002; Mateu-Andres and Segarra-Moragues 2000; de Lange and Norton 2004; Segarra-Moragues et al. 2005). Kunin and Gaston (1997) refer to this level of specialization as the “rarity trap” where plants are so well adapted to a narrowly defined habitat that they are precluded from expanding into other habitats. *Physaria bellii* Mulligan (Bell’s Twinpod) is an example of this phenomenon.

Physaria bellii is a specialist in terms of its preferred habitat, and is restricted to sloping shale and sandstone washes of the Niobrara, Pierre, Lykins and Fountain/Ingleside formations between elevations of 1580 and 1760 meters along the Front Range in Colorado, USA (Figure 1.1; Spackman et al. 1997; Doyle et al. 2004). These geological formations have a patchy distribution, and as a result, *P. bellii* does as well. According to the system used by the Colorado Natural Heritage Program, *P. bellii* is ranked G2/S2, since there is a limited number of populations, and because this species depends on a habitat which is itself potentially threatened (Spackman et al. 1997, Doyle et al. 2004).

This endemic species is an herbaceous, diploid ($2N = 8$), perennial member of the Brassicaceae. Previous work suggests *P. bellii* is an early successional species whose presence is tied to small erosional disturbances that keep its preferred habitat relatively free of competitors (Supples 2001). *Physaria bellii* forms rosettes of leaves and bears several to many inflorescences, which flower from April through May, and set seed around the middle of July. Mulligan’s experiments (Mulligan 1966), as well as

greenhouse crosses conducted as part of a related study (Kothera et al. 2006), showed this species to be self-incompatible. The fruit is a small (4-6 mm) inflated silique consisting of two valves, each of which can hold a maximum of two seeds.

In contrast to species which have always been rare, recently rare species often occur as small, isolated populations in a formerly continuous habitat that has been fragmented (e.g. Llorens et al. 2004; Jacquemyn et al. 2004; Xiao et al. 2004; Hensen and Oberprieler 2005). Moreover, unlike many naturally rare species whose populations are already discontinuous, populations of newly-rare species can have their viability compromised by the process of fragmentation (Frankham et al. 2002; Charlesworth 2003). Thus, while some rare species require intervention to remain viable, others need only periodic monitoring to ensure their continued persistence. For these reasons, naturally rare species with discontinuous distributions can serve as models to predict the genetic consequences of population subdivision in recently rare species (Fleishman et al. 2001).

Protocols that involve dominant genetic markers, such as RAPDs (Randomly Amplified Polymorphic DNA; Welsh and McClelland 1990, Williams et al. 1990), AFLPs (Amplified Fragment Length Polymorphism; Vos et al. 1995), and ISSRs (Inter-Simple Sequence Repeats; Gupta et al. 1994, Zietkiewicz et al. 1994) are widely used in the conservation of rare plants. ISSR markers were chosen for this study because their use is methodologically straightforward, and they have a higher stringency than RAPDs (Wolfe et al. 1998). As with all dominant markers, ISSRs are biallelic, meaning that a band is scored as either present or absent. Consequently, heterozygotes are not

distinguishable from homozygous dominant individuals, which precludes a direct estimate of inbreeding.

This paper describes a population genetic study of *P. bellii*. We used a panel of ISSR markers to generate a series of complimentary measures to estimate the genetic diversity and the scale of genetic differentiation in this naturally rare endemic. The results are the first to describe population genetic parameters for this species, and will provide a valuable baseline for future management actions.

MATERIALS AND METHODS

Sampling

Ten populations of *P. bellii* were sampled across its geographic range (Larimer and Boulder counties; Figure 1.1). At the time this study was initiated, this number represented approximately half of the known populations. Two additional populations in Jefferson County were excluded because they were found to contain interspecific hybrids (Kothera et al. 2006). Additional populations have since been found, bringing the known number of populations of *P. bellii* to approximately 30 (Doyle et al. 2004).

During the summer of 2002, plant tissue was collected for this and a companion study (Kothera et al. 2006) that examined the incidence of hybridization between *P. bellii* and the closely related *P. vitulifera*. The sampling method was designed to systematically collect representative, unbiased genetic diversity across this species' range (Huenneke 1991; Owuor et al. 2003). For each sampled population, a 300m transect was started at the edge of a population and sampling commenced towards its interior. One individual was sampled at the beginning of the transect, and every 10m thereafter. The

sampled individual was chosen by flipping a coin to determine whether to walk left or right off of the transect, followed by rolling a six-sided die, which determined the number of steps taken before stopping to sample the closest individual. Sampling continued this way until 30 individuals from each population were collected. Leaf tissue was placed into individually numbered plastic bags and placed on ice until it could be stored at -80°C pending subsequent DNA extraction.

Molecular genotyping

Three “anchored” ISSR primers were used for this study. Each primer had one, two or three additional nucleotides on either the 3’ or 5’ end of the primer (Table 1.1). Using anchored ISSR primers is thought to increase their specificity by reducing the number of fragments of DNA that will bind with the primer, while still generating acceptable levels of polymorphism (Gupta et al. 1994, Zietkiewicz et al. 1994, Wolfe et al. 1998).

Genomic DNA was extracted from 100 mg of frozen leaf tissue using DNeasy Plant Mini Kits (Qiagen, Valencia, California, USA) according to the manufacturer’s protocol. The concentration of DNA was quantified using a fluorometer and diluted with AE buffer from the Qiagen kits to a concentration of 10 ng/ μl . ISSR reactions were carried out in 25 μl volumes containing 16.2 μl HPLC water, 2.6 μl 10x *Hot-Taq* PCR buffer (GeneSys, Buffalo, NY, USA), 1.5 μl MgCl (25mM), 0.5 μl dNTP mix (100 mM), 2 μl primer (10 mM), 0.2 μl *Hot-Taq* Polymerase (GeneSys, Buffalo, NY, USA) and 2 μl template DNA (10 ng/ μl). Reactions were carried out on a 96-well plate in a Hybaid thermal cycler with an initial cycle of 95° for ten minutes to activate the *Hot-Taq*

polymerase and an additional 2 minutes at 94°. This was followed by 35 cycles of 94° for 30 seconds, annealing temperature (Table 1.1) for 45 seconds and 72° for 90 seconds. There was a final extension step at 72° for 20 minutes.

Four percent denatured polyacrylamide gels were used to resolve fragments. PCR product and formamide-based loading dye were heated in the thermal cycler at 95° for five minutes to separate the DNA strands before loading the gel. A 100bp DNA ladder was used with reference bands ranging from 100 to 1500 bp (GeneSys, Buffalo, NY, USA). To minimize the effects of electrophoresis and staining on band variability, individuals from each population were divided into two groups and run on separate gels, thus any anomalies seen in one run and not the other could be investigated. In addition, some individuals' DNA underwent a second round of PCR and subsequent electrophoresis, which was then compared to previous runs to ensure that bands were being produced consistently. In every case, banding patterns on the first and second gels were identical. Gels were silver stained according to the method of Bassam and Caetano-Anollés (1993), allowed to air dry and scanned to create a permanent record.

Data Analysis

The ISSR banding pattern of each individual was scored as the presence or absence of every polymorphic band, resulting in a data matrix of 1's and 0's for each of the 300 individuals. Only bands that could be scored consistently among populations were used.

Several measures of genetic diversity were calculated for this study. Nei's (1978) unbiased estimate of heterozygosity, h was estimated using the approach of Lynch and

Milligan (1994) as implemented by the software Tools for Population Genetic Analysis (TFPGA; Miller 2000). Also, a Bayesian estimate of heterozygosity, h_s , was determined in Hickory, which requires comparatively fewer assumptions regarding the data (Holsinger et al. 2002, Holsinger and Lewis 2003) Estimates of heterozygosity calculate the probability that two alleles for a given locus are not identical by descent, and when averaged over all loci represent a measure of the genetic diversity at the level of the population. The Shannon index of phenotypic diversity, I , (Shannon and Weaver 1949) was calculated using PopGene version 1.32 (Yeh and Boyle 1997). Finally, the percentage of polymorphic loci, P , among all ISSR fragments was also calculated.

Given *P. bellii*'s nearly linear distribution along the Front Range, some detectable regional differences might exist among sampled populations. A hierarchical Analysis of Molecular Variance (AMOVA) was estimated using Arlequin (Version 3.0; Excoffier et al. 2005). The ten study populations were divided into a northern group (populations 1-5) and a southern group (populations 6-10). Statistical significance of the proportion of variance associated with fixation indices was determined with permutation tests against a null distribution generated by the data. The AMOVA procedure in Arlequin also provided estimates and significance of pairwise F_{ST} values.

A measure of linkage disequilibrium (LD) across the sampled loci using exact tests was obtained using Arlequin. LD is a nonrandom association between pairs of loci (Hartl and Clark 1997). The overall level of LD in *P. bellii* populations was assessed using a method similar to that used by Miyashita et al. (1999) and Tero et al. (2003), which compared the frequency of instances of LD to the number of possible combinations of loci to determine if significant LD was present.

A Bayesian analysis of genetic structure was performed in Hickory (version 1.0; Holsinger and Lewis 2003), which uses Markov's chain Monte Carlo (MCMC) simulations to produce posterior distributions based on the data. The default parameters of the program were used, which included using uniform priors, a burn-in period of 5,000 iterations, a run of 25,000 iterations and a thinning factor of 5. Each model was run five times to ensure the Markov chain was converging, and to make certain the results were consistent. The results were then averaged for the purpose of model comparison. Further comparisons were made between models with similar results by comparing posterior distributions of the parameter θ_B , which is analogous to F_{ST} .

To visualize differences among populations, a Principal Coordinates Analysis (PCOA) of Nei's (1978) unbiased genetic distances between populations was performed using the program GenAlEx (Peakall and Smouse 2001). A graph of the first two principal coordinates was generated, which plotted each *P. bellii* population as a point. Also, pairwise F_{ST} values were used to construct a minimum spanning network (MSN), with each population as a node, to assess whether genetic relationships were consistent with geographic ones. Finally, a Mantel test (Mantel 1967) was performed, which correlated population-level genetic distance (Nei 1978) and geographic distance matrices between populations (TFPGA; Miller 2000).

Structure (Prichard et al. 2000) was used to explore whether the ten sampled populations represent distinct genetic entities, or if there was evidence of gene flow. The model of no population admixture was selected and it was assumed that allele frequencies were correlated within populations. The most likely number of clusters was associated with the value of K that had the lowest $\ln \Pr(X|K)$ (estimated log probability of the data)

value, although when the probabilities associated with two values of K differ by only a few units, the smaller number of K is preferred (Pritchard et al. 2000). When the program was initially run with K varying from 2-15 clusters, the results indicated the most likely number of clusters was eight. Subsequent testing increased the length (50K burn-in, 100K data collection) and number of runs (ten), with values for K ranging from 6-10 clusters. To estimate the stability of the clustering, we ran 50 separate runs at the optimal K value and calculated the Similarity Coefficient as described by Rosenberg et al. (2002).

RESULTS

Genetic variability within populations

Thirty-nine polymorphic markers were scored for this study. Table 1.1 details the sequence, annealing temperature, total number of bands and degree of polymorphism for each of the three ISSR primers. ISSR fragments ranged in size from 280 to 1350 base pairs. It is notable that each of the 300 individuals in the study had a unique multilocus genotype. The majority of fragments were found in more than one population, but populations 2 and 8 had one unique allele each. Values for genetic diversity measures are given in Table 1.2. Patterns of diversity are similar across diversity measures. Levels are moderate, with the two northernmost populations (1 and 2) showing the greatest diversity, followed by the southernmost populations. The populations in the middle of *P. bellii*'s range exhibited lower levels of diversity.

Linkage disequilibrium was examined at the population level by Arlequin. There were 15,210 possible combinations of pairs of loci when each population was considered

separately (all pairwise combinations of 39 markers x ten populations). Of this total, 732 pairs, or 4.8%, showed significant linkage at the 0.05 level. This is approximately what would be expected by chance.

Genetic structure at different hierarchical levels

The AMOVA (Table 1.3) indicated that most (74%) of the molecular variation in *P. bellii* exists among individuals within populations, with lesser amounts among populations within the northern and southern groups (20%) and between the northern and southern groups (6%). Permutation tests suggest that each fixation index was significantly different from the null distribution (over all $F_{ST} = 0.24, p = 0.00$; $F_{SC} = 0.21, p = 0.00$; $F_{CT} = 0.06, p = 0.01$).

Pairwise F_{ST} values between populations were also highly significant from the null distribution ($p < 0.0001$) and ranged from 0.108 to 0.361. Population 6 was associated with consistently high pairwise F_{ST} values. The distribution of populations in the network constructed from pairwise F_{ST} values generally follows their geographic distribution, with the southern group clustering together and the northern group arranged in linear fashion (Figure 1.2).

The Deviance Information Criterion (DIC; Spiegelhalter et al. 2002) generated by each run of Hickory was used to compare models. Lower values indicate a better fit of the model to the data. Similar DIC values, 1494 vs. 1502, were obtained for the full (where both θ_B and f , analogous to the inbreeding coefficient, F_{IS} , are calculated) and the $f = 0$ (where only θ_B is calculated) models, respectively. F_{ST} calculated using the full model was 0.230 and its 95% credible interval did not include zero (range = 0.200 -

0.267). The within-population inbreeding coefficient, f , was 0.773 (range = 0.456 - 0.988). F_{ST} calculated using the ' $f=0$ ' model was 0.169 (range = 0.142 - 0.198). The similarity in DIC values for the full and the $f=0$ models indicates there are not appreciable levels of inbreeding and the populations are in H-W equilibrium. When comparing the difference in posterior distributions of Θ_B from the full vs. the $f=0$ models, the credible interval was found not to contain zero, suggesting the calculation of f affected the value of Θ_B . The other available models produced higher DIC values, which precluded their use to best explain these data.

Multivariate analysis of between-population relationships

The first two principal coordinates explained 42 and 27% of the total variance respectively and separated the populations into two groups that correspond fairly well with the geographic groups used for the hierarchical AMOVA (Figure 1.3). The southern group of populations as defined for the AMOVA (populations 6-10) clusters together, but population 5 is also present in this grouping. Population 6 is outside the cluster of southern populations. The remaining populations in the northern group occupy a distinct portion of the graph. Mantel test results show a significant correlation between the genetic and geographic distances among populations ($r^2 = 0.590$, $p < 0.001$).

The reticulation observed in the MSN of populations was reinforced in the analysis performed by Structure. Similar estimated log probability values were associated with $K = 8$ (-4388.4) and $K = 9$ (-4381.7) clusters. We chose to use the smaller (parsimonious) value in subsequent analyses. The similarity coefficient (Rosenberg et al.2002) calculated among 50 runs at $K = 8$, was 0.98. Table 1.4 lists the

proportion of the ten sampled populations that was assigned to each cluster when $K = 8$. Six of the eight clusters (Clusters A-C, E, G and H) contain over 78% of one *P. bellii* population each (populations 1-6 from Table 1.1). Clusters D and F each contain between 20% and 58% of each of the four southernmost populations (populations 7-10). Cluster A contains 78% of population 4 and 34% of population 10. Cluster B contains 79% of population 5 and 46% of population 7.

DISCUSSION

This study utilized several complimentary measures to describe the genetic diversity and degree of genetic differentiation in the endemic plant *P. bellii*. One set of measures (Nei's unbiased heterozygosity, Shannon Index, PCOA, Mantel Test) was based on genetic distances. A second set of measures (AMOVA, MSN, calculations using Hickory) focused on population divergence based on approaches that partitioned heterozygosity. The third set of measures (Structure analysis) examined the degree of LD at the population level and estimated the number of genetic groups into which the ten *P. bellii* populations would cluster. These measures were chosen because their use allowed the maximum amount of information to be obtained from a dominant marker data set. As a result, the ISSR analysis revealed that populations are not redundant in terms of the genetic information contained in each. In other words, each *P. bellii* population contributes to the overall levels of diversity in this species. This is a noteworthy finding, considering that the levels of genetic diversity and degree of genetic divergence are almost certainly underestimated because ISSRs are dominant markers. As the results

could be used in future management decisions, it was important that the data be fully analyzed in this manner.

The AMOVA partitioned variation in *P. bellii* in a manner consistent with an outcrossing species with a patchy distribution. Thus, while most of the genetic variation (74%) existed within populations, some variation was also present among populations within the northern and southern groups (20%), and between these groups (6%; Table 1.3). Several other studies of outcrossing endemic species studied at three hierarchical levels (within populations, among populations within groups and between groups) show similar patterns (Sales et al. 2001; Jacquemyn, et al. 2004; Juan et al. 2004).

Furthermore, permutation tests with the fixation indices indicate significant genetic structuring at all hierarchical levels tested. *Physaria bellii* populations show a strong north to south orientation (Figure 1.1), which reflects the patchy distribution of the rocky substrates preferred by this species. Carpenter (1997) concluded that *P. bellii* is the dominant species in its preferred habitat, which is characterized by a high percentage of bare ground (Supples 2001). Noting a negative correlation between the presence of *P. bellii* and percent vegetation cover, Supples (2001) proposed a mechanism for the persistence of *P. bellii* in its preferred habitat. Because *P. bellii* is found significantly more often on sloped than on flat habitats, it may benefit from periodic erosional disturbances which remove or reduce competition (Donahue et al. 1983, cited by Supples 2001). The process by which favorable habitat is maintained is likely limited to the exposed rocky substrates of the above-mentioned geologic formations, which themselves display a patchy distribution. Thus, *P. bellii* would not be expected to expand its range under normal conditions.

Model comparison in Hickory was informative in determining whether populations of *P. bellii* exhibit appreciable levels of inbreeding, and whether significant genetic structure is present in this species. Estimating the inbreeding coefficient, F_{IS} (“ f ” in Hickory), from dominant markers can be difficult or even unreliable (Holsinger and Lewis 2003). For this reason, Hickory was used to obtain a qualitative measure of inbreeding only, in addition to an estimate of Θ_B . The small difference between the DIC values of the full versus the $f=0$ model (1494 vs. 1502) is consistent with low levels of inbreeding in *P. bellii*, as these results indicate no reason to choose the full over the $f=0$ model. Thus, the value of f from the full model (0.77) is likely spurious (Holsinger and Lewis 2003). The other possible models, f -free and $\Theta_B = 0$, were inadequate to explain these data because of their comparatively larger DIC values (data not shown). Further comparison between the full vs. the $f=0$ models was warranted because the value of Θ_B associated with each model differed (0.23 vs. 0.17 for the full vs. $f=0$ models, respectively). If the value of Θ_B is influenced by the estimation of f , then a model where f is not estimated is the preferred model (Holsinger and Lewis 2003). The 95% credible interval for the difference in posterior distributions of Θ_B did not contain zero, thus the value of Θ_B was affected when Hickory was required to calculate a value for f . This finding, along with a comparable DIC value, suggests the $f=0$ model, where $\Theta_B = 0.17$, is preferable to the full model.

The results from the analysis performed by Structure (Table 1.4) indicate that gene flow occurs among the southern group of *P. bellii* populations around Boulder, CO. When similar estimated log probabilities are associated with two values of K , the smaller number of clusters is a better fit to the model (Pritchard et al. 2000). Thus, there were

likely eight genetic *P. bellii* populations instead of ten. Clusters D and F in Table 1.4 contain a mixture of each of the four southernmost populations, which is strongly suggestive of gene flow among them (Prichard et al. 2000). This is somewhat unexpected because the sampled populations were at least 1 km apart, although the southern populations were closer together than the populations in the northern group. Alternatively, a similar pattern of results from Structure could also be observed if the southern populations were descended from a common source. However, generalist pollinators such as honey bees (*Apis mellifera*) were observed at *P. bellii* flowers during this study and may be able to occasionally fly the distances required to transfer pollen from one population to another (Dick 2001). Furthermore, a small number of pollen or seed migrants can result in measurable gene flow (Ellstrand 1992). This finding, along with evidence of a high degree of population differentiation, indicates that occasional gene flow occurs, but at a rate that maintains genetic differentiation among populations. These results are in contrast to other recent studies, which found the number of sampled populations consistent with the number of populations as determined by Structure (Tero et al. 2003; Segarra-Moragues et al. 2005).

The graphical representations of inter-population relationships using PCOA and a MSN (Figures 1.2 and 1.3) are consistent with the presence of gene flow among the southern populations of *P. bellii*. In both cases, populations in the southern group cluster together and populations in the northern group are arranged in a linear fashion, which follows their geographic distribution fairly well (Figure 1.1). Population 5 appears with the southern group in the PCOA, although its position in the MSN corresponds with its geographic position. This population was originally placed in the northern group because

it was so highly differentiated from population 6. Upon closer inspection, population 6 appears to be an outlier in both analyses, a view supported by high pairwise F_{ST} values between this and all other *P. bellii* populations (range 0.30 – 0.36). Therefore, population 5 may belong to either group; although as part of the northern group it was distinct from the southern group in the AMOVA.

The Mantel test revealed a significant correlation between the geographic and genetic distances among *P. bellii* populations ($r^2 = 0.590, p < 0.001$). Along with other evidence from this study, this finding suggests that the variation observed in this species is clinal. Populations of *P. bellii* are currently considered stable but may be vulnerable for two reasons. First, the linear distribution of populations suggests that the current scale of gene flow would be altered with the removal of even a few *P. bellii* populations. For this reason, the critical populations are probably those north of the city of Boulder, as they provide a conduit for gene flow between the northern and southern groups. In addition, a recent survey by Doyle et al. (2004) noted that a significant number of occurrences of *P. bellii* are on private land, which is considered to be of high value for residential development and limestone mining. Populations could be extirpated if *P. bellii* habitat is used for other purposes, because the species lacks formal protection. Thus, periodic monitoring of *P. bellii* populations, designed to evaluate current levels of genetic diversity and divergence, would most likely benefit this species. Also, continuance of on-going educational efforts by local government open lands programs will help inform the public of threats to *P. bellii*. In this way, informed management decisions can be made that minimize the loss of diversity in this vulnerable species.

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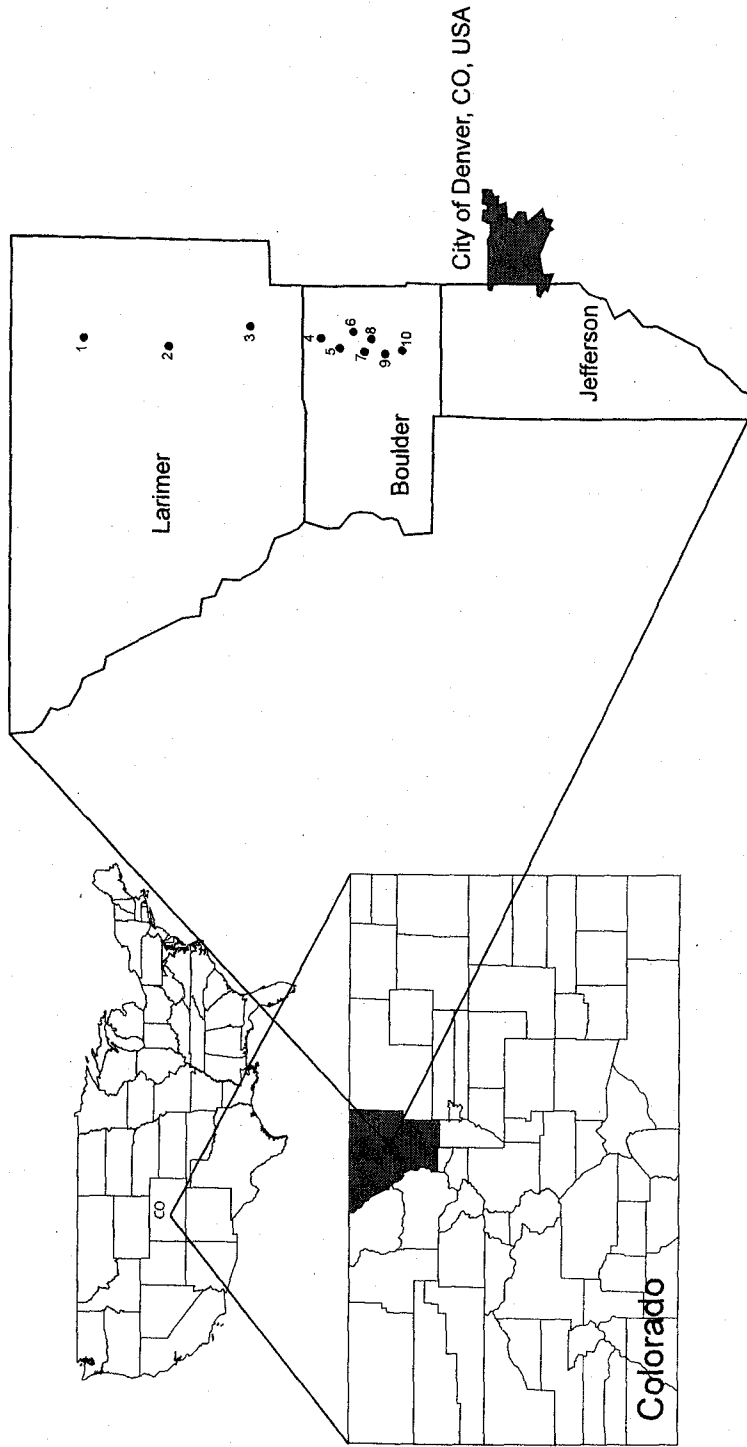


Figure 1.1. Map of *Physaria bellii* populations in this study.

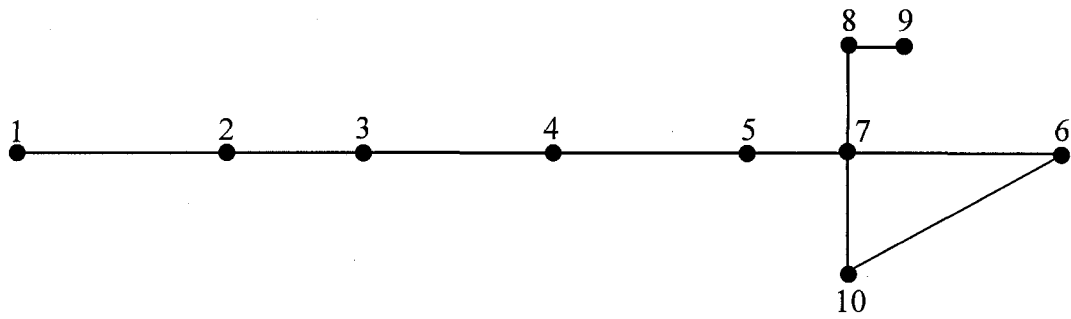


Figure 1.2. Network of ten *Physaria bellii* populations based on pairwise F_{ST} values generated in Arlequin. Population 1 is the northernmost population and 10 is the southernmost. Populations 6-10 comprise the southern group.

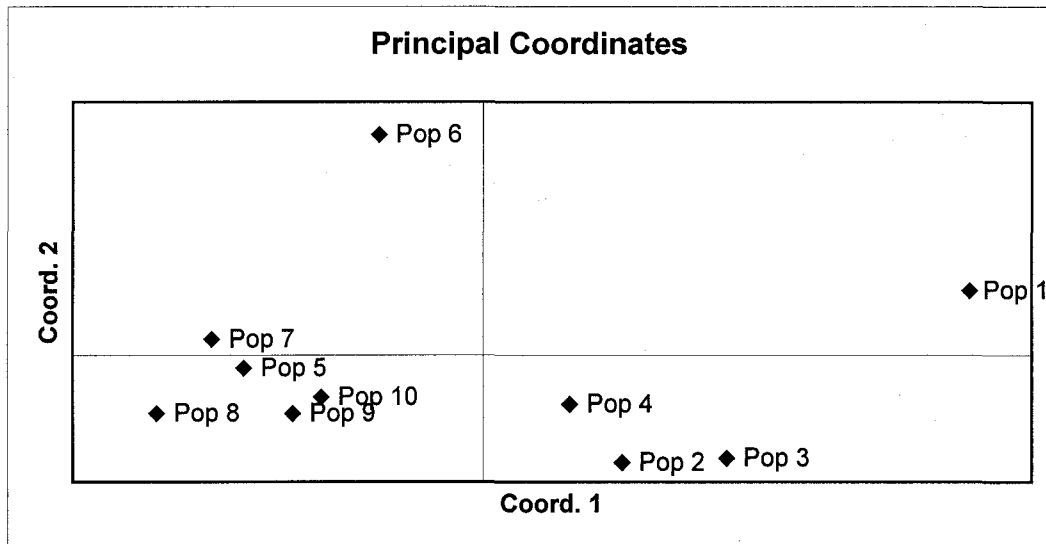


Figure 1.3. Plot of the first two Principal Coordinates using Nei's (1978) genetic distance calculated among populations of *P. bellii*. The first and second Principal Coordinates explained 42 % and 27% of the variance, respectively.

Table 1.1. Primer information, number of bands, and degree of polymorphism for the three primers used in this study.

Primer Name	Sequence	Annealing Temperature	Total Bands	Number (Percent) Polymorphic Fragments
UBC890	VHV(GT) ₇	51°	22	11 (50%)
UBC809	(AG) ₈ T	47°	16	11 (69%)
UBC841	(GA) ₈ YC	48°	24	17 (71%)
Total = 39 Polymorphic Markers				

Where V=G,A, or C; H=A,T or C and Y=T or C

Table 1.2. Genetic diversity measures for populations of *Physaria bellii*. Nei's (1978) unbiased heterozygosity (h), a Bayesian equivalent of heterozygosity (h_s), Shannon Index (I), number of bands, percent polymorphic loci and number of individuals sampled per population (N). Populations are listed from the northernmost to the southernmost.

Population	h	h_s	I	# Bands	P	N
1. North Poudre	0.2464	0.2526	0.3673	30	48	30
2. Pine Ridge	0.2415	0.2643	0.3749	34	55	30
3. Devil's Backbone	0.2135	0.2288	0.3234	28	45	30
4. Rabbit Mountain	0.2355	0.2530	0.3586	31	50	30
5. Ryan	0.1767	0.2005	0.2731	28	45	30
6. Steele	0.1989	0.1958	0.2973	26	42	30
7. Lake Valley	0.1956	0.2180	0.2973	26	42	30
8. Sage	0.2093	0.2298	0.3139	27	44	30
9. City Boulder	0.2193	0.2317	0.3319	28	45	30
10. Wonderland Lake	0.2297	0.2398	0.3477	31	50	30
Averages	0.2166	0.2310	0.3258			

Table 1.3. AMOVA analysis of pairwise differences between 39 polymorphic ISSR loci from populations of *Physaria bellii*. Each of ten populations was placed into either the northern group (populations 1-5 in Table 1.2) or the southern group (populations 6-10 in Table 1.2) for this analysis, which was performed using Arlequin (Excoffier et al., 2005).

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation
Between groups	1	93.820	0.350	5.67
Among populations within groups	8	330.840	1.226	19.88
Within populations	290	1330.667	4.589	74.44
Total	299	1755.327	6.134	

Table 1.4. Proportions of the ten *P. bellii* populations that grouped into each of eight clusters in the Structure analysis. For example, .856 of population 1 was placed into cluster E. Proportions greater than .20 of a particular population are in bold.

Population Number	Cluster							
	A	B	C	D	E	F	G	H
1	0.032	0.010	0.033	0.006	0.856	0.014	0.002	0.047
2	0.004	0.005	0.807	0.012	0.018	0.038	0.000	0.116
3	0.012	0.001	0.170	0.002	0.018	0.006	0.000	0.792
4	0.783	0.009	0.101	0.005	0.001	0.027	0.017	0.057
5	0.061	0.787	0.051	0.011	0.000	0.078	0.010	0.002
6	0.003	0.046	0.002	0.003	0.001	0.081	0.863	0.001
7	0.004	0.458	0.026	0.206	0.000	0.272	0.033	0.001
8	0.002	0.044	0.008	0.578	0.000	0.361	0.006	0.001
9	0.033	0.018	0.035	0.415	0.002	0.480	0.002	0.016
10	0.341	0.075	0.043	0.296	0.004	0.210	0.028	0.004

Chapter 2: The Real Deal? Seeking evidence of hybridization in the rare Colorado endemic *Physaria bellii* Mulligan (Brassicaceae)

ABSTRACT

Rare plant species are often confined to a small patches of suitable habitat. If conspecifics are brought into sympatry by changes to their preferred habitat, hybridization may occur. Furthermore, the outcome of a hybridization event can be confounded when one parental species occurs as populations of varying ploidy levels. This study used genetic and morphological analyses to examine hybridization between two endemic *Physaria* species, *P. bellii*, and the more common *P. vitulifera*. Genetic data from ISSR (Inter-Simple Sequence Repeat) markers showed putative hybrids to be genetically more like *P. vitulifera* than like *P. bellii*, but *P. bellii*-diagnostic markers were present in a small number of hybrid individuals. This view was supported by genotyping three known F1 individuals, which had a higher frequency of *P. bellii* diagnostic markers. Morphological data from leaf measurements showed hybrids to be intermediate for several characters, with the exception of the leaf sinus trait that was statistically closer to *P. vitulifera*. Morphological data positively supported the genetic data with Canonical Discriminant Analyses of both data sets showing similar patterns. Whether the rarer *P. bellii* was threatened by hybridization with the more common *P. vitulifera* was assessed in two ways. First, ploidy levels were estimated with root tip chromosome squashes from seed collected from the sampled *P. vitulifera* populations, which were located in proximity to *P. bellii*, and from the putative hybrid populations. Existing hybrids are tetraploid, and *P. bellii* and diploid *P. vitulifera* do not occur closely enough to easily

allow modern day gene flow. Second, greenhouse and field crosses were performed to estimate the ease with which hybridization occurs. Interspecific crosses yielded fewer normal-looking seeds than intraspecific crosses, regardless of the ploidy levels of the parents. Thus, it does not appear that *P. bellii* is currently threatened by hybridization with *P. vitulifera*, and efforts to conserve *P. bellii* should focus on the threat from potential habitat loss instead of from hybridization.

INTRODUCTION

Naturally rare plant species are often specialists whose distributions reflect that of their preferred habitats (Kruckeberg and Rabinowitz 1985, de Lange and Norton 2004, Gustafson and Latham 2005). Alterations to this habitat, as a result of human or natural disturbance, can affect rare plants in several ways. Besides the obvious condition of less available space, disturbance can bring formerly allopatric species into sympatry. If the species are closely related, hybridization can be a potential outcome. Harrison (1990) defined hybridization as “successful matings in nature between individuals from two populations or groups of populations, which are distinguishable on the basis of one or more heritable characters”. One potential outcome of hybridization is genetic swamping of the rare species, whereby its numbers decline because matings produce hybrid individuals (Ellstrand and Elam 1993). Furthermore, if the more common species exists as diploid and tetraploid populations, hybrids between this and a diploid species will likely be variable in their fitness because some will be diploid and others triploid.

Hybridization is ubiquitous among angiosperms and it is thought that many, if not most, extant angiosperm species have one or more hybridization events in their lineage

(Soltis et al. 2003). This is somewhat counter-intuitive because both prezygotic and postzygotic reproductive isolating mechanisms exist that often prevent or minimize the frequency of hybridization (Rieseberg and Carney 1998). Consequently, while many hybridization events produce weak or inviable offspring, hybridization occasionally produces offspring that are more fit than their parents in certain habitats (Anderson 1948, 1949). The Evolutionary Novelty Hypothesis (Arnold 1997) elaborated on the evolutionary importance of rare F1 hybrids and how subsequent backcrosses could produce offspring that were competitively superior to their parents, sometimes even in parental habitat.

If hybridization occurs between a rare plant species and a more common congener, the number of genetically distinct individuals of the rare species may decline. If reproductive isolating mechanisms are weak, introgression can occur whereby the rare species will cross with hybrids, as well as with the other parental species (Levin et al. 1996, Wolf et al. 2001, Brock 2004). Over time, individuals of the rare species become rarer because they have been genetically swamped by the more common species (Ellstrand and Elam 1993, Rhymer and Simberloff 1996, Burke and Hamrick 2002, McKinnon et al. 2004). Suitable habitat becomes occupied by hybrids, which can be problematic for rare species that have particular habitat requirements. In extreme cases, hybridization can lead to the extinction of rare plant populations (Ellstrand 1992, Soltis and Gitzendanner 1999).

The effects of hybridization can be complicated by the existence of polyploid forms of one or both parental species. Some plant species exist as populations with differing ploidy levels (e.g. Johnson et al. 2003, Husband 2004, Keeler 2004, Tate and

Simpson 2004). Crosses between individuals of differing ploidy levels, itself a form of hybridization under Harrison's (1990) definition, often produce offspring which are less fit than their parents because chromosomes do not pair properly during meiosis (Young and Murray 2000, Husband and Schemske 2000, Avise 2004). Mulligan (1967b) found that the more common species in the present study, *Physaria vitulifera* Rydberg exists as tetraploid and diploid populations (Figure 2.1). However, Mulligan's work was incomplete, and ploidy levels for *P. vitulifera* populations located close to populations of the rarer *P. bellii* were unknown when this study was initiated. If those *P. vitulifera* populations were diploid, *P. bellii* could be threatened by genetic swamping. Conversely, if those *P. vitulifera* populations were tetraploid, crosses would result in inviable triploid offspring and *P. bellii* would probably not be threatened by swamping. It was thought that hybridization was possible because two putative hybrid populations exist in Jefferson County (populations H1 and H2 on Figure 2.1; Spackman, et al. 1999, Doyle et al. 2004).

Natural hybridization can be detected by comparing known parental types to putative hybrids using morphological or molecular analyses. Although it is intuitive to assume that hybrids will have morphology intermediate to the parental species, this is not necessarily the case (Reiseberg and Ellstrand 1993, Mendelez-Ackerman 1997, Majumder et al. 1997, Burke and Hamrick 2003, Tsukaya et al. 2003). Several recent studies demonstrate the value of using molecular and genetic data in addition to morphology to distinguish between hybridizing taxa (Swartz and Brunsfeld 2002, Cattell and Karl 2004, Rossetto 2005). The presence of diagnostic DNA markers from both parents in suspected hybrid individuals can be used to confirm that hybridization has

occurred. Methodologically straightforward markers, such as ISSRs, RAPDs and AFLPs are appropriate for this purpose.

This study assessed the extent of hybridization between *Physaria vitulifera* and *P. bellii*. The objectives were to: 1) Use molecular and morphological techniques to characterize putative hybrids, 2) Determine ploidy levels of potential parent and putative hybrid *Physaria* populations, and 3) Conduct controlled crosses between the two species to assess the ease of hybrid formation.

MATERIALS AND METHODS

Study Species and Study Area

Physaria bellii is endemic to open shale and sandstone washes in two counties along the front range of Colorado, USA (Mulligan 1966). It is ranked by the Colorado Natural Heritage Program (CNHP) as a G2/S2 species, meaning it is globally imperiled due to its restricted range and significant threats to its habitat, which include residential development and limestone mining (Spackman, et al. 1999, Doyle et al. 2004). At this time, however, *P. bellii* populations are considered stable and, while vulnerable, this species receives no formal protection. *Physaria bellii* is distinguishable in the field from *P. vitulifera* by leaf (Figure 2.2) and fruit characteristics. *Physaria vitulifera* has a larger range than *P. bellii* (extending through at least six counties in Colorado). Populations of *P. bellii* are consistently diploid ($2N = 8$; Mulligan 1966). Tetraploid and diploid populations of *P. vitulifera* are morphologically indistinguishable (Mulligan 1967b).

During the summer of 2002, leaf tissue was sampled from ten *P. bellii* populations, and the two putative hybrid populations (H1, H2). Eleven *P. vitulifera*

populations in proximity to *P. bellii* and putative hybrid populations were also sampled. Plant tissue was collected from 8-12 arbitrarily selected plants per population. One representative leaf was collected from each plant for leaf morphology measurements, and another was removed for DNA extraction. Leaves were placed on ice immediately after collection and then stored at -80 degrees.

In addition to collections made from the above-mentioned *Physaria* species, leaf tissue was collected from one population of *P. acutifolia* near Woods Landing, WY. This species was of interest because it had previously been classified as *P. vitulifera* (Jennings 2004). Furthermore, the location of the *P. acutifolia* population was close to the northern end of *P. bellii*'s range and therefore represented a possible opportunity for hybridization. Twelve arbitrarily chosen individuals were sampled, representing most of the population.

Seed Germination

Seed was collected during the summers of 2002 and 2003 from all *P. bellii*, *P. vitulifera* populations from which leaf tissue had been sampled, as well as one of the two putative hybrid populations. Seed was germinated to produce plants for controlled pollinations in the greenhouse and also for use in determining ploidy levels of *P. vitulifera* populations located near *P. bellii* and the putative hybrid populations. Seeds were soaked in undiluted household bleach for 10 minutes, then placed on wet paper towels in a Petri dish and placed in a dark drawer for five days at room temperature, and watered when needed.

Greenhouse and Field Crosses

Germinated seeds to be used for greenhouse pollinations were transferred from petri dishes to four-cell packs filled with moist potting soil (Metromix 200, Denver Clay, Denver, CO). Seedlings were transplanted into 4 inch pots when they were 6-8 cm high. To simulate the rocky, well-drained soil that characterizes *Physaria* habitats, the potting soil was overlain with a 2 cm layer of sand. Plants were grown in a large greenhouse, and light and temperature were not adjusted. After five months, the plants were transplanted into one gallon pots containing potting soil and a 2 cm layer of sand. They began to flower eight months after germination.

No steps were taken to prevent selfing while performing greenhouse crosses, since both *Physaria* species are self incompatible (Mulligan 1966, 1967a). One or two mature anthers were removed from the pollen donor with clean forceps and dragged lightly across the stigmas of all open flowers (usually one or two) on one inflorescence of the pollen recipient. Details of greenhouse and field crosses are presented in Table 1. Fruits were collected approximately two months after pollination occurred. Thirty-two of the total of 45 *P. bellii* and *P. vitulifera* plants failed to produce flowers, or produced flowers when there were no other plants flowering and thus could not be crossed with another plant.

Field crosses were conducted in 2003 and 2005 to generate F1 hybrids. Unopened inflorescences were wrapped in fine mesh fabric to exclude pollinators. Ten days later, when it was likely that some flowers would be open, those inflorescences were unwrapped, pollen was applied to the stigmas of any open flowers using the method described above, and the inflorescence was rewrapped. Seeds were collected two months

later. A total of 24 F₁ hybrid seeds were produced from field crosses, of which six germinated and were used for chromosome counts and genetic analysis.

Chromosome squashes

Physaria vitulifera chromosome numbers were determined by treating actively growing root tips from germinated seeds of wild collected plants. Seedlings were soaked in a 2 mM solution of 8 hydroxyquinoline for 4-6 hours in a dark drawer, after which they were fixed overnight at 4° in a 3:1 solution of 100% ethanol: glacial acetic acid. The following day, root tips were soaked in a 1N hydrochloric acid solution for five minutes to hydrolyze the cells, macerated on a slide under a drop of 2% aceto-orcein stain, flamed briefly, and squashed for viewing with a light microscope. A representative mitotic chromosome spread was photographed for each individual using a digital camera. Chromosomes from at least five cells per root were counted, and chromosome numbers for a minimum of five individuals from each *P. vitulifera* population were recorded.

Seed for root tip chromosome counts was collected from putative hybrid population H2. Additionally, unopened flowers were collected in 2005 from suspected hybrid population H1 to determine the ploidy level from pollen mother cells. Immature anthers were stained with a drop of 2% aceto-orcein stain on a slide, squashed for viewing and viewed with a light microscope.

Leaf Morphology

Leaves from 244 *P. bellii*, 100 *P. vitulifera* and 58 putative hybrid individuals were examined to determine whether measurable differences existed between the putative

hybrids and each of the parental species. Two discrete characters were recorded: *toptooth*, the presence (1) or absence (0) of a tooth at the apex of the leaf, and *numberteeth*, the number of teeth per leaf. In addition, two continuous characters were measured using digital calipers: the length of the leaf measured from the apex to the beginning of the petiole (*length*) and the width of the leaf at the widest point (*width*) (Figure 2.2).

A second analysis was conducted to examine whether the sinuses observed on putative hybrid leaves were quantitatively different than those found in *P. vitulifera*. Three characters were measured: *pairsinus*, the number of pairs of sinuses per leaf, *W1*, the width of the leaf at the narrowest part of the first sinus, and *W2*, the width of the leaf at the next widest point distal from the apex of the leaf.

The variables *length*, *width*, *numberteeth*, *W1* and *W2* were transformed to achieve a normal distribution after generating the appropriate exponential transformation with a SAS macro that performed a Box-Cox transformation (*boxglm.sas*; Friendly 2002, SAS Institute, Inc. 2005). The variable *numberteeth* was transformed with a square root transformation and was approximately normal after transformation.

The characters *length*, *width*, *numberteeth*, *W1* and *W2* were analyzed using ANOVAs with the SAS Procedure Mixed with the Least Squares Means option. Four possible models were tested, each with different combinations of random and repeated statements. Model choice was determined by comparing the AIC (Akaike Information Criterion) (Akaike 1973) for each of the models run for each character, and choosing the simplest model with the smallest AIC. The variables *pairsinuses* and *toptooth* were not normally distributed and were thus examined with SAS Procedure Glimmix, which is

designed for such distributions. Only the first two models described above were tested, as the Procedure does not accept repeated statements.

Five leaf morphology characters (*length*, *width*, *toptooth*, *numberteeth* and *pairsinus*) were selected for a multivariate analysis to determine whether as a group they discriminated among taxa. Even though *P. bellii* leaves lack sinuses, the *pairsinus* character was included here because it is an important means of telling the parental taxa apart in the field. First, the SAS Procedure Stepdisc was used to determine whether all five characters were informative in discerning among taxa. All five characters were retained and analyzed with SAS Procedure Discrim, using the nonparametric and nearest neighbor options, which used the data to place individuals into one of three taxa and provides a measure of how well the leaf data represented three discernable groups. The number of nearest neighbors, *k*, was set at 20 because similar results were obtained when *k* was any number between 5-20. Finally, the same set of five characters was analyzed with SAS Procedure Candisc, which performs a canonical discriminant analysis (CDA) of the data. This Procedure searches for linear combinations of variables that show the greatest differences among groups and provides a two-dimensional representation of the data. The SAS Procedures Stepdisc and Candisc assume normally distributed data, but can be used descriptively with other kinds of distributions (SAS 1999).

ISSR Analysis

ISSR (Inter-Simple Sequence Repeat) markers were generated for *P. bellii*, and *P. vitulifera* individuals, as well as for putative and known hybrids. ISSRs are dominant markers which generate sufficient levels of polymorphism to conduct genetic analyses at

the population level (Gupta et al. 1994, Zietkiewicz et al. 1994, Wolfe et al. 1998). One primer is used for each PCR reaction, targets a particular microsatellite repeat and amplifies the region between inverted repeats. Sixty-two ISSR markers were used to discern among the three taxa and included fragments ranging in size from 280 bp to 1350 bp (Table 2). This set of markers included 39 polymorphic markers used for the *P. bellii* population genetic analyses, plus monomorphic and species-diagnostic markers. Data were generated by visually scoring each marker as present or absent in each individual.

ISSR markers were generated for a total of 445 individuals, 300 *Physaria bellii* (from 10 populations), 87 *P. vitulifera* (from 11 populations), 58 putative hybrids (from 2 populations) and three known F1 hybrids grown from seed produced by controlled pollinations. The same statistical tests used for the leaf morphology data were used for the ISSR data. Initially, a stepwise discriminant analysis using the SAS Procedure Stepdisc was used to select markers which best discriminated among the three taxa. This resulted in paring the data set down from 62 to 27 markers. These 27 markers were analyzed by the SAS Procedure Discrim as described above. A CDA was then performed on the set of retained loci. One marker, *locus6*, was problematic because the data for this locus were perfectly correlated with the first canonical variable (see Results). Therefore *locus6* was excluded from the CDA and the discriminant analysis.

A UPGMA (Unweighted Pair Group Mean Average) phenogram based on Nei's (1978) unbiased minimum distance was generated from the original set of 62 loci scored for 445 individuals by the program TFPGA (Tools for Population Genetic Analysis; Miller 2000). One thousand bootstrap (Felsenstein, 1985) replicates were performed to assess branch support.

Six species-diagnostic markers (defined as being present in at least 90% of one of the parental taxa) were used to construct a hybrid index (Anderson 1949) for each taxon, as well as for the three known F1 hybrids produced by controlled pollinations (Figure 2.3). Each index was constructed by assigning an individual one point for each *P. vitulifera*-diagnostic marker it had, and one point for each *P. bellii*-diagnostic marker it did not have. Thus, *P. vitulifera* individuals would be expected to have the highest index scores and *P. bellii* individuals the lowest index scores. Hybrids would be expected to have intermediate hybrid index scores when compared to the parental taxa.

Physaria acutifolia

DNA was extracted from twelve individuals from one *P. acutifolia* population in Albany County, WY, and screened with two of the three ISSR primers used with the other *Physaria* taxa. The PCR, electrophoresis and silver staining protocols were the same as those used for the other two *Physaria* taxa.

RESULTS

Controlled Pollinations and Ploidy levels

Table 1 summarizes the results from all controlled pollinations performed in this study. One hundred and eighty three inter-species crosses were performed, 127 with *P. bellii* as the pollen donor, 23 with diploid *P. vitulifera* as the pollen donor and 33 with tetraploid *P. vitulifera* as the pollen donor. Seed was considered normal-looking and therefore viable if it was smooth in appearance and similar in size to wild-collected seed. A total of 24 normal-looking seeds were produced from inter-species crosses, for a success rate of 3%. By comparison, intra-species crosses were more successful: 67

crosses yielded 57 seeds. In particular, crosses between tetraploid *P. vitulifera* individuals yielded slightly more than one seed per cross (48 crosses and 52 seeds).

Cells from germinated field-collected seed had either eight chromosomes, in which case they were diploid, or 16 chromosomes, in which case they were tetraploid. Triploid cells (12 chromosomes) were observed in three germinated seeds from field crosses between *P. bellii* and tetraploid *P. vitulifera* individuals. Eight of the sampled populations of *P. vitulifera* were tetraploid ($2N = 16$): A, B, C, D, E, H, I and J, and two *P. vitulifera* populations were diploid ($2N = 8$), F and K. One putative hybrid population, H2, was assessed through root tip squashes and found to be tetraploid. The other putative hybrid population, H1, was assessed through examination of pollen mother cells only. This population also appeared to be tetraploid, based on two squashes of fair quality. The seeds collected from one Jefferson county *P. vitulifera* population, G, were depleted before a satisfactory squash protocol was developed, and thus no reliable chromosome counts were made for this population. One diploid *P. vitulifera* population, K, was geographically the closest *P. vitulifera* population to the putative hybrids, approximately 2 km from population H1. The other diploid *P. vitulifera* population, F, was approximately 20 km of the putative hybrid populations.

Leaf Morphology

In the first analysis of leaf characters, the Least Squares Means comparisons revealed significant differences between *P. bellii* and *P. vitulifera* for three of the four characters (Table 3). However, the hybrids were significantly different from each parental species on only one of the four characters. In the second analysis of leaf

characters, 69 of 100 *P. vitulifera* leaves (69%) and 27 of 53 putative hybrid leaves (51%) had measurable sinuses. *W1*, and *pairsinus* were not significantly different between taxa ($t = 0.68, p = 0.512$; $t = 0.23, p = 0.824$ respectively). The character *W2* was marginally significantly different between *P. vitulifera* and hybrids ($t = -2.26, p = 0.047$).

The SAS Procedure Stepdisc retained all five characters (*length*, *width*, *toptooth*, *numberteeth* and *pairsinus*) as informative for use in the SAS Procedure Discrim, where all but one *P. bellii* individual and 79% of *P. vitulifera* individuals were correctly classified. The Discrim Procedure was less successful at classifying hybrids based on their leaf characteristics, with 26 (68%) of 53 individuals classified as hybrids. Eleven putative hybrids (21%) were classified as *P. bellii* and 16 (30%) were classified as *P. vitulifera*, for an error rate of 51%. This is not surprising, however, since hybrids frequently resemble their parental species for a number of morphological characters.

The plot of the two canonical variables is shown in Figure 2.4. Individuals of the parental species are fairly well separated, although the points are not tightly clustered together. Putative hybrid individuals cluster in a distinct part of the graph, but also are spread over the space occupied by the parental taxa.

Genetic Analysis of Hybrids

The ISSR analysis yielded six species-diagnostic bands: two in *P. bellii* and four in *P. vitulifera*. Interestingly, all six occurrences of a *P. bellii* marker in *P. vitulifera* were from the *P. vitulifera* population (K) located closest to the hybrid populations. The hybrid indices (Figure 2.3) show ISSR genotypes of the putative hybrids to be a mixture

of markers from the parental species, although they are generally more *vitulifera*-like. The percentage of suspected hybrids which had at least one of the four *P. vitulifera* markers ranged from 60% to 95%, and the percentage of suspected hybrids which had *P. bellii* markers was 9% and 14%. All hybrid individuals had at least one species-diagnostic marker: one had one marker, ten had two markers, 24 had three markers, 16 had four markers, six had five markers and one had all six markers. Eleven (19%) purported hybrids had markers from both parental species.

The three known F1 hybrids generated from controlled crosses displayed more of an equal distribution of species-specific markers compared to the putative hybrids. One individual had two species diagnostic markers from each parental species, one had one marker from each parental species and the third had two markers from *P. vitulifera* (Figure 2.3).

SAS Procedure Discrim performed on the set of 26 ISSR markers deemed informative by the SAS Procedure Stepdisc did a good job of grouping individuals into the correct taxa. All individuals of the parental species and most of the putative hybrids (95%) were classified correctly and the error rate was low. The first canonical variable of the CDA did a very good job of separating the *P. bellii* and *P. vitulifera* individuals (Figure 2.5). The putative hybrids showed comparatively more spread to their distribution, and the data points for several individuals were located approximately midway between the centers of the cluster of hybrids and one of the parental taxa.

Figure 2.6 shows a UPGMA phenogram generated in TFPGA using Nei's (1978) unbiased genetic distances between the 10 *P. bellii*, 11 *P. vitulifera* and 2 putative hybrid populations. The three taxa formed discrete clusters. The group with the two putative

hybrid populations had the highest bootstrap support at 0.82. The cluster containing all *P. vitulifera* populations had 0.53 bootstrap support and the cluster with all *P. bellii* populations had 0.43 bootstrap support.

Physaria acutifolia

Representatives from all four taxa (*P. bellii*, *P. vitulifera*, *P. acutifolia* and putative *P. bellii* x *P. vitulifera* hybrids) were electrophoresed on one gel to determine whether the marker patterns generated for *P. acutifolia* individuals sampled from Albany County WY were similar to *P. vitulifera* or if they appeared to be a distinct entity. Twelve *P. acutifolia* individuals were scored for 27 ISSR markers originally scored for the other three taxa. Over half of the *P. bellii* and *P. vitulifera* markers (n = 17; 63%) were not present in any *P. acutifolia* individual. There were no markers found in all *P. acutifolia* individuals but not in any of the other three taxa. However, three markers were found in some *P. acutifolia* individuals and not found in the other species.

DISCUSSION

The results from this study suggest that the two *Physaria* populations located in Jefferson County, CO, previously classified as *P. bellii*, instead contain hybrids, as seen from both molecular and morphological analyses. Hybrid indices constructed from species-diagnostic ISSR markers show the putative hybrids to be unlike either parental species, although they appear more *vitulifera*-like (Figure 2.3). The putative hybrids possessed an admittedly low frequency of *P. bellii*-diagnostic markers, but this frequency lends support to the idea that the hybrids are not simply misclassified *P. vitulifera*

populations. Also, the small number of known F1 hybrids displayed more intermediate genotypes compared to the putative hybrids, which demonstrates the adequacy of the chosen markers and reinforces the idea that the putative hybrids are highly introgressed with *P. vitulifera* genes. A second line of evidence that the putative hybrids are correctly identified as such was from the analysis of the entire ISSR data set. The discriminant analysis correctly classified most individuals with their respective taxa, including putative hybrids. The visual portrayal of informative markers via the CDA underscores the genetic distinctiveness of the three taxa, as well as how the hybrids appear genetically similar to *P. vitulifera* (Figure 2.5). Finally, the UPGMA phenogram using the entire ISSR data set grouped the two putative hybrid populations together, and placed them with the group of *P. vitulifera* populations (Figure 2.6). Although there is some criticism of using bootstrap values alone to make phylogenetic determinations (e.g. Brown 1994) it is reasonable to view the UPGMA results as supportive of the idea that the hybrids are separate taxonomic entities compared to the parental taxa (McDade 1990). The low bootstrap values are likely a result of the many ways the populations could be ordered within the parental species groups.

The analyses of leaf morphology data also support the assertion that the putative hybrids are not *P. bellii* populations. The linear regression analysis of leaf morphology data with the SAS Procedures Mixed and Glimmix indicate that the hybrids are different from either parental species (Table 3). In the first analysis, leaves from putative hybrids differed significantly from those of each parental taxon on just one of the four characters, suggesting that the hybrids, overall, were intermediate in their leaf morphology. In contrast, the parental species differed for three of the four characters. However, in the

second analysis, the non-significant and marginally significant results from the sinus measurement comparisons can be interpreted as meaning the depth of the sinuses of a hybrid leaf are similar to those of a *P. vitulifera* leaf. Thus, if a hybrid has sinuses (which only 51% did in this study), it appeared *vitulifera*-like rather than intermediate in leaf morphology. The leaf morphology data, when analyzed with the SAS Procedures Stepdisc, Discrim and Candisc, did a fairly good job of grouping individuals with their respective taxa, but it was apparent that this data set was more variable than the molecular data set (see below).

The differences in CDAs for the molecular versus the morphological data underscore the difficulty of relying on phenotypes to establish hybridization. Comparing Figures 3 and 4, one can see a higher degree of overlap among taxa in the CDA of leaf morphology data versus the molecular data. One possible explanation of why CDAs performed on two data sets from the same individuals would yield slightly different results is due to the types of data evaluated for each analysis. Each locus scored for the ISSR analysis had only two possible values, 1 or 0. On the other hand, two of four leaf characters (*length* and *width*) used in that CDA were continuous, one (*numberteeth*) had multiple values and one (*toptooth*) consisted of presence-absence data like the ISSR markers. The higher degree of variation associated with continuous and meristic trait measurements, compared to binary data, could explain the wider spread of data points associated with the leaf morphology data. In extreme cases, this variability might obscure a biological signal, which is a good reason to incorporate molecular data in studies of hybridization (e.g. Swartz and Brunsfeld 2002, Cattell and Karl, 2004).

It is possible that tetraploid *P. bellii* x *P. vitulifera* hybrids resulted from an initial hybridization event between diploid individuals of both species, an assertion supported by the presence of a diploid *P. vitulifera* population located in close proximity to the present-day hybrids. This is unlikely to have occurred recently because the nearest pure *P. bellii* population is presently approximately 50 km north of the hybrid populations. There may have been additional introgression by *P. vitulifera* after the formation of the initial hybrids, which could explain why, genetically, the hybrids are genetically *vitulifera*-like, rather than having more intermediate genotypes. At some point after hybridization, chromosome doubling occurred, where the gametes of the hybrids failed to reduce during meiosis. This process allows chromosomes which are unlike in the parental species to pair properly during the formation of gametes (Avice 2004, Hegarty and Hiscock 2005). No triploid individuals were observed while performing chromosome squashes in either hybrid population, but they may exist if diploid *P. vitulifera* genes are currently introgressing into the hybrid populations. Viable triploid seed was produced from field crosses, but germinated seeds were used for chromosome squashes and genetic analysis and not grown to maturity, so the fertility of this class of hybrids is unknown.

Assessment of threat to *P. bellii* from hybridization

Physaria bellii is found in only two counties in Colorado (Larimer and Boulder), and although populations appear to be currently stable, this species' narrow range and specific habitat preferences make it vulnerable to losses from human activities such as residential development and limestone mining (Spackman et al. 1999, Doyle et al. 2004)

Several recent studies on other rare plant species have documented the phenomenon of hybridization between rare and common congeners (Brock 2004, Klips and Culley 2004, Hardig et al. 2005). Since evidence from this study suggests that hybridization has likely occurred in the past, hybridization and introgression with *P. vitulifera* could pose a threat to *P. bellii* populations. However, several lines of evidence support the assertion that *P. bellii* populations are not currently threatened by hybridization with *P. vitulifera*. First, controlled pollinations to produce hybrid seeds demonstrated that hybrid seed formation is more difficult than intraspecific seed formation (Table 1), suggesting that some reproductive isolating mechanisms are in place for these two species. Also, the *P. vitulifera* populations closest to extant *P. bellii* populations were found to be tetraploid, and the triploid offspring formed from these crosses would most likely be sterile. Furthermore, searches of these locations have not found individuals with intermediate leaf morphology (L Reidel, personal communication). Diploid *P. vitulifera* populations, which would have a better chance of producing fertile offspring because they have the same number of chromosomes as *P. bellii*, are located too far away from *P. bellii* populations for pollination to occur. *Physaria vitulifera* is found at higher elevations than *P. bellii*, and flowers later in the season. In some years, the two species overlap in flowering times, but in some years they do not, limiting opportunities for interspecific cross-pollination. The location of the hybrid populations is difficult to explain as the two hybrid populations are located approximately 1 km apart, and the nearest known *P. bellii* population is located approximately 50 km away to the north. Some time in the past, the two *Physaria* species may have had ranges that overlapped to a greater extent than they

do today. The specific reason that the current range of *P. bellii* does not extend further south remains unknown.

Physaria acutifolia

At the time this study was initiated, it was thought that *P. vitulifera* existed in Wyoming within approximately 70 km of the northernmost *P. bellii* population in Larimer county which would imply that hybridization was possible at both ends of *P. bellii*'s range. However, a subsequent examination of herbarium specimens conducted by Jennings (2004) indicated that the species in question was *P. acutifolia*, not *P. vitulifera*. Moreover, Jennings (2004) asserted that *P. vitulifera* probably was never found in WY. The results from the current study support Jennings' determination with 63% of ISSR markers found in *P. bellii* and *P. vitulifera* not present in the putative Wyoming *P. acutifolia* samples. Plants in this population also had ISSR bands not found in any *P. bellii* or *P. vitulifera* individuals in this study.

Rare plants face a variety of threats to their continued survival. Many challenges faced by rare plant populations are directly connected to human activities. Other factors, such as hybridization, can potentially have an impact, but their influence on the fate of rare plant species is unknown. It is important, then, to assess what actually constitutes a threat to a rare species. Although several recent papers have documented the decline of a rare plant species because of hybridization with a more common congener (Brock 2004, Klips and Culley 2004, Hardig et al. 2005), this does not appear to be the case for *P. bellii*. Rather, any hybridization event appears to have happened far enough in the past that *P. bellii* populations do not appear to be threatened at present. On the other hand, *P. bellii* populations face the real threat of habitat loss through residential development and

limestone mining (Spackman et al. 1999, Doyle et al. 2004). Therefore, management efforts to conserve this species should focus on protecting against the loss of populations through human activity.

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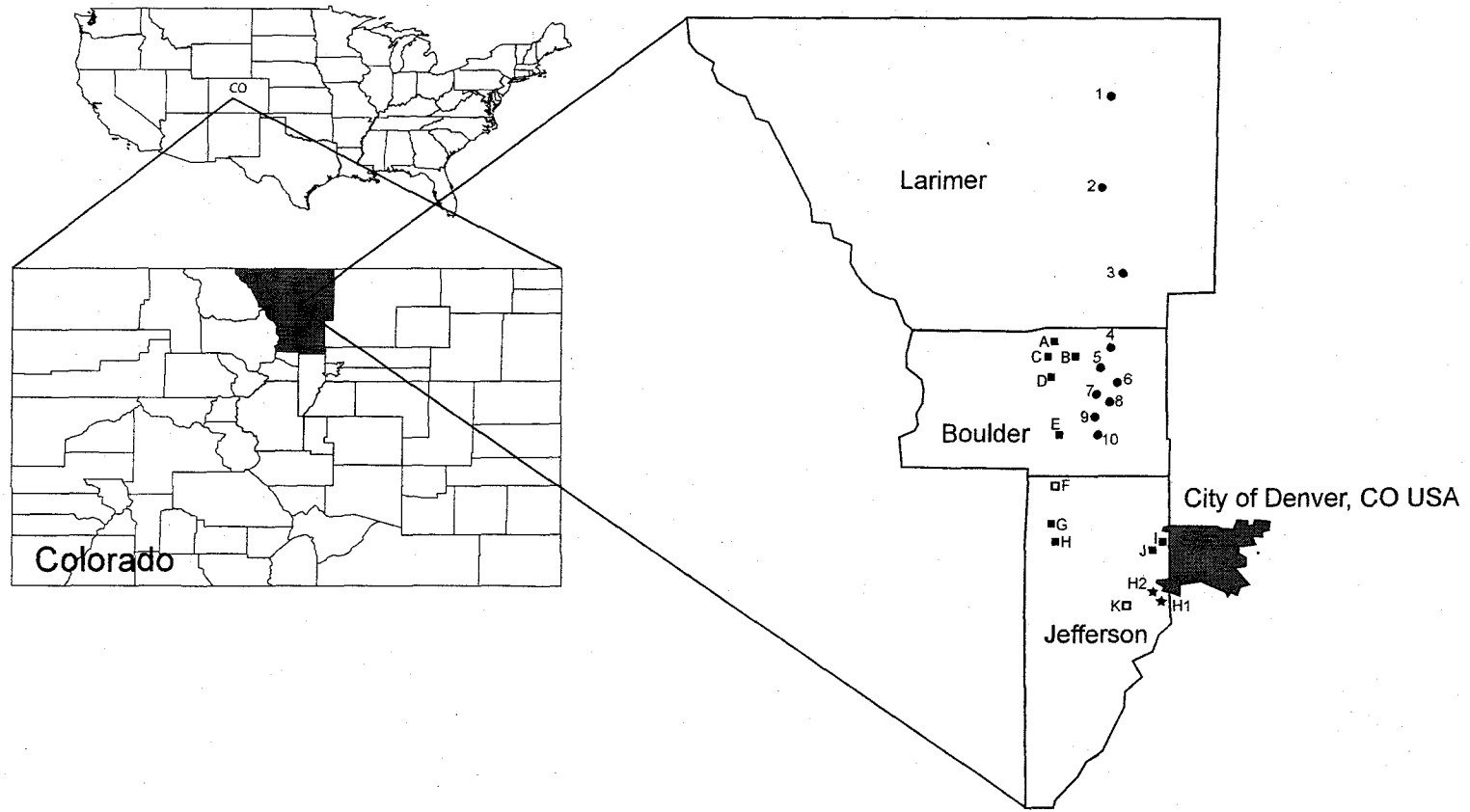


Figure 2.1. Map showing locations of *Physaria* populations sampled for this study. Circles (1-10) = *P. bellii*; filled squares (A-E, G-J) = tetraploid *P. vitulifera*; open squares (F, K) = diploid *P. vitulifera*; stars (H1, H2) = putative hybrid populations. Gray shading = range of *P. bellii*.

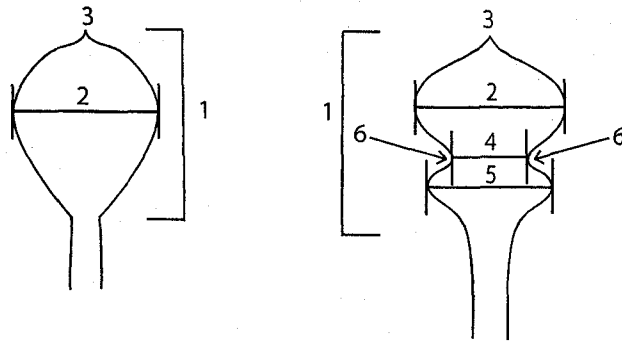


Figure 2.2. Illustrations of *P. bellii* (left) and *P. vitulifera* leaves showing leaf morphology characteristics. 1 = length; 2 = width; 3 = *toptooth* (here, present); 4 = *W1*; 5 = *W2*. The *P. vitulifera* leaf shown has one pair of sinuses (6), which were counted for the character *pairsinuses*. Leaves often had one or more teeth around the margin (*numberteeth*), which were left off this illustration for clarity.

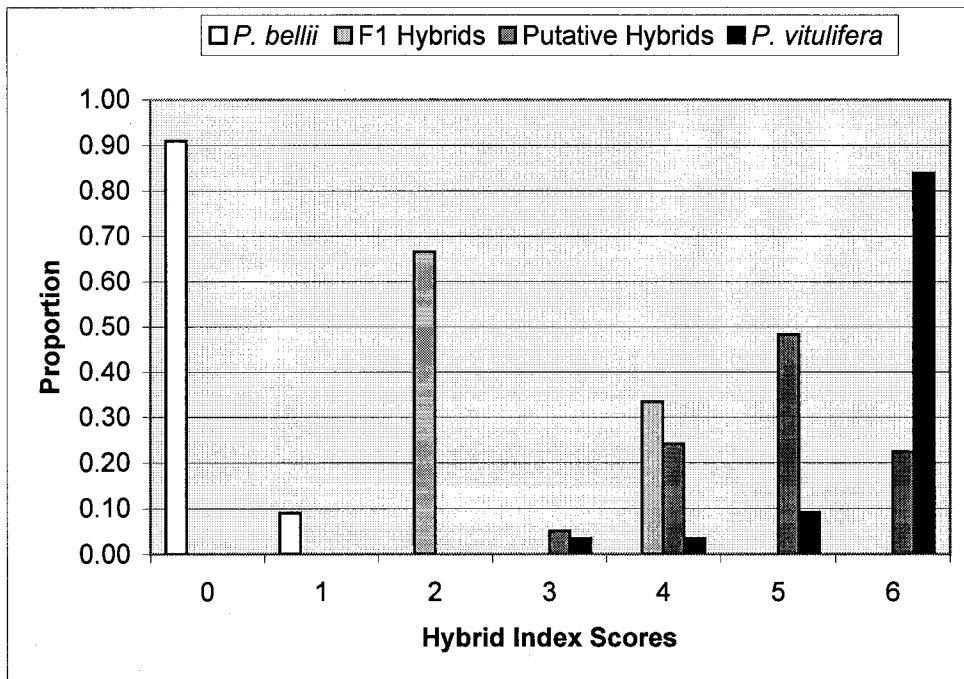


Figure 2.3. Histogram of hybrid index scores for *Physaria bellii* (N = 300), known F1 hybrids (N = 3), putative hybrids (N = 58) and *P. vitulifera* (N = 87), showing proportion of each group with a given index score. Scores toward zero are more *P. bellii*-like and scores toward six are more *P. vitulifera*-like.

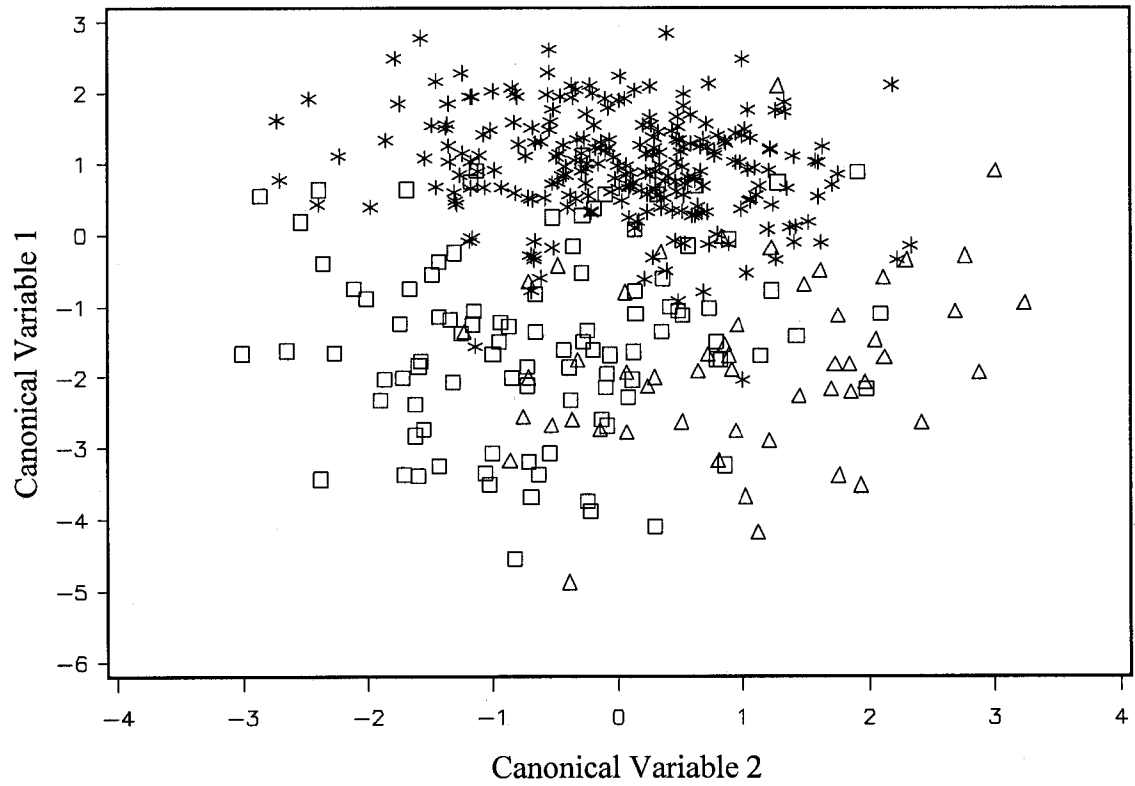


Figure 2.4. Graph of first and second canonical variables from the SAS Procedure Candisc performed on *Physaria* leaf morphology data. Star = *P. bellii*; square = *P. vitulifera*; triangle = purported hybrid.

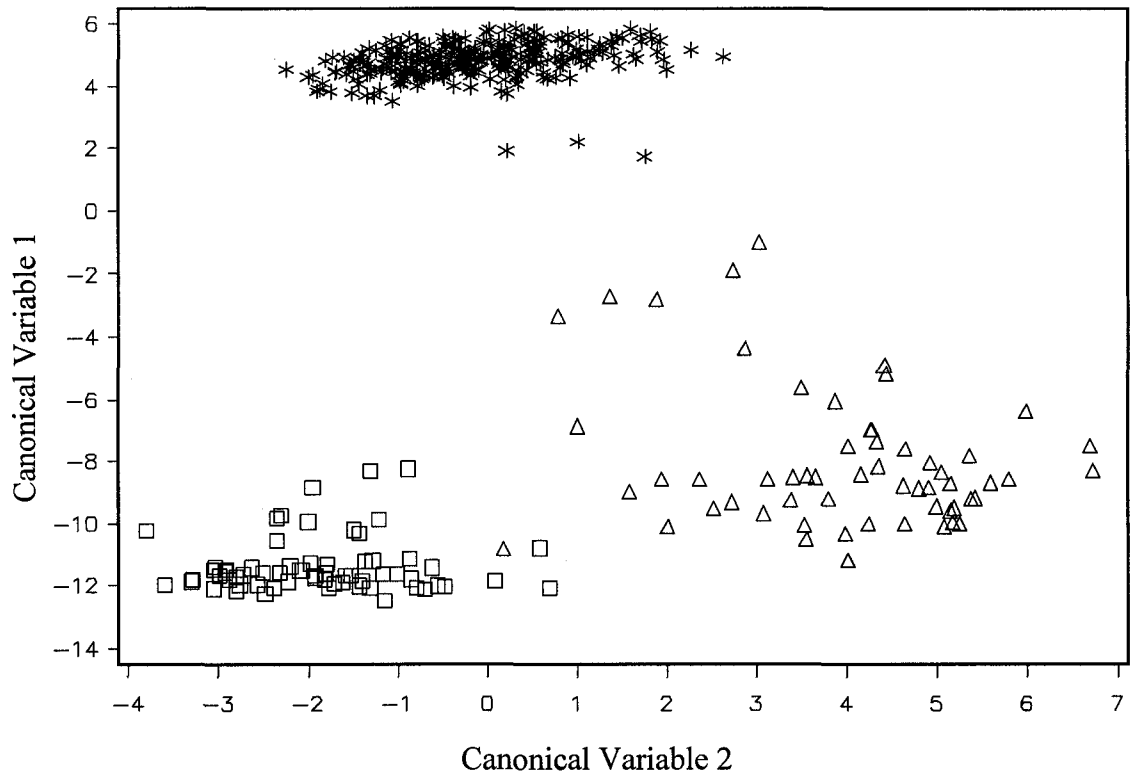


Figure 2.5. Graph of first and second canonical variables from the SAS Procedure Candisc performed on *Physaria* ISSR marker data. Star = *P. bellii*; square = *P. vitulifera*; triangle = purported hybrid.

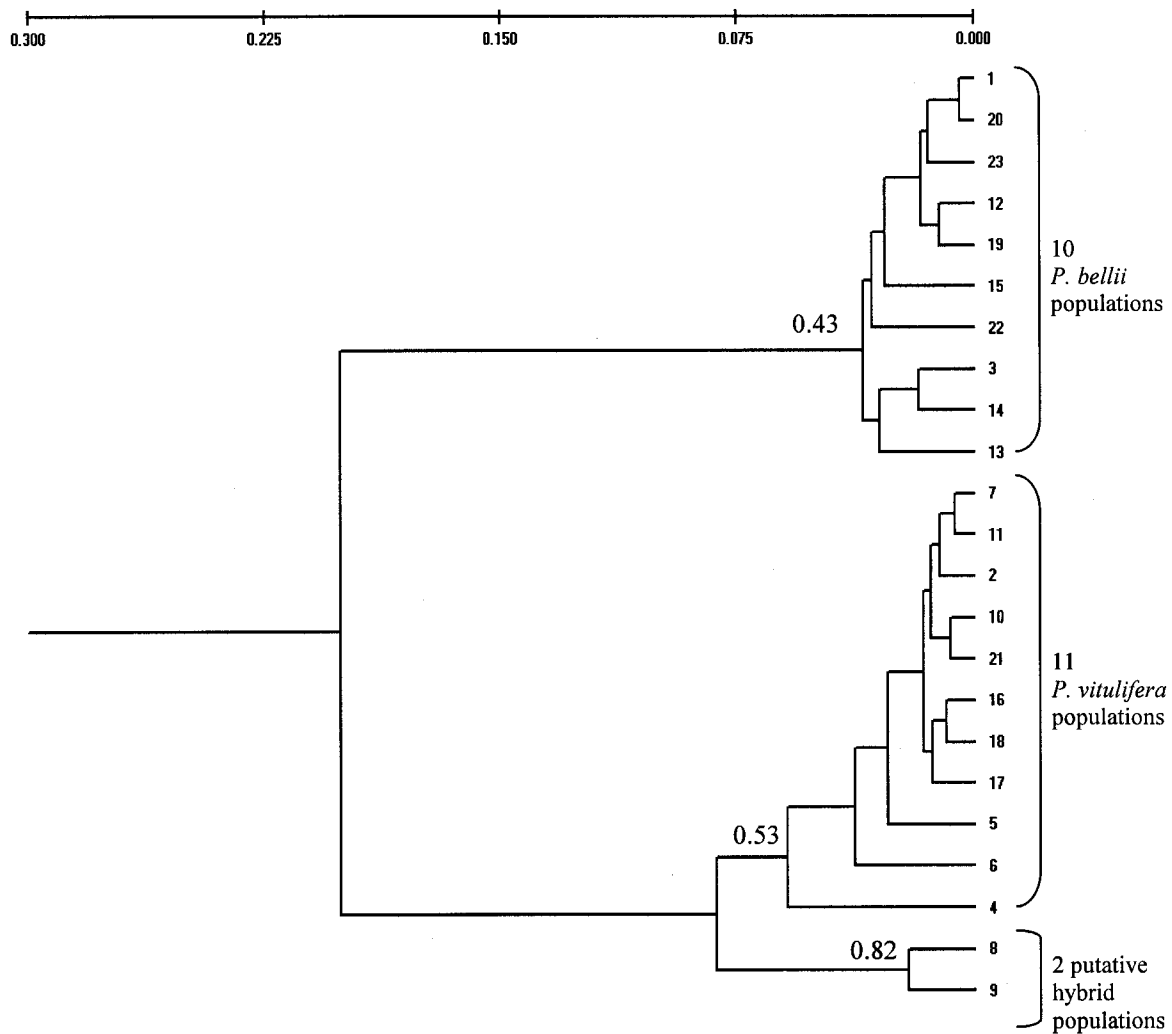


Figure 2.6. UPGMA dendrogram of *Physaria* populations sampled in this study. Numbers above selected nodes indicate bootstrap support over 1000 replicates in TFPGA (Miller 2000).

Table 2.1. Results from controlled greenhouse and field pollinations between *Physaria bellii* and *P. vitulifera*. The number of normal-looking seeds is shown, with the number of crosses performed in parentheses. Abbreviations: 2N = diploid, 4N = tetraploid, Pb = *P. bellii*, Pv = *P. vitulifera*.

		Pollen Donor					
		Greenhouse			Field		
		Pb	2N Pv	4N Pv	Pb	2N Pv	4N Pv
Pollen	Pb	0(0)	0(1)	0(20)	0(0)	0(22)	0(13)
	2N Pv	0(1)	0(0)	5(9)	3(56)	0(0)	0(0)
	4N Pv	0(12)	0(10)	52(48)	21(58)	0(0)	0(0)

Table 2.2. Primer information for ISSR markers used in the analysis of *Physaria bellii*, *P. vitulifera* and their putative hybrids.

Primer Name	Sequence	Annealing Temperature	Total Bands	Number (Percent) Polymorphic Fragments
UBC890	VHV(GT) ₇	51°	22	11 (50%)
UBC809	(AG) ₈ T	47°	16	11 (69%)
UBC841	(GA) ₈ YC	48°	24	17 (71%)

Table 3.3. Differences of Least-Squares Means from the SAS Procedure Mixed for four leaf morphology characters measured in *Physaria bellii* (B), *P. vitulifera* (V) and their putative hybrids (H). Values are *t* values for comparisons between pairs of taxa.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. See text for descriptions of each character. (n=397)

Comparison	<i>toptooth</i>	<i>length</i>	<i>numberteeth</i>	<i>width</i>
B vs. V	-3.43**	6.24***	-3.23**	0.11
B vs. H	-1.61	1.43	-2.07*	2.06
H vs. V	0.13	2.31*	0.17	-1.98