# Assessing the threat from hybridization to the rare endemic *Physaria bellii* Mulligan (Brassicaceae)

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A B S T R A C T

Hybridization of a rare endemic with a more common congener can endanger the rarer species through gene swamping. This study used a combination of genetic, morphological and cytological analyses to assess the threat to Bell's twinpod (*Physaria bellii*), a rare endemic restricted to the Colorado Front Range, from hybridization with its more widespread congener *Physaria vitulifera*. Two populations of *P. bellii* at the southernmost end of its range were suspected of containing hybrid plants, based on field observations of intermediate leaf morphology. Inter-simple sequence repeat (ISSR) marker analysis confirmed that suspect individuals contained alleles from both *P. bellii* and *P. vitulifera*, with most hybrids genetically closer to *P. vitulifera*. 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*. The morphometric data supported the genetic data with Canonical Discriminant Analyses of both data sets showing similar patterns. The extent of the threat to *P. bellii* populations from hybridization with the more common *P. vitulifera* was assessed through cytological analysis of existing populations and through field and greenhouse crosses. While naturally occurring hybrids are tetraploid, diploid *P. vitulifera* populations do not occur close enough to the current range of diploid *P. bellii* to permit gene flow. Interspecific crosses yielded fewer normal seeds than intraspecific crosses, regardless of the ploidy levels of the parents. It does not appear that *P. bellii* is currently threatened by hybridization with *P. vitulifera*, and efforts to conserve this globally imperiled species should focus on the threat from potential habitat loss rather than from gene swamping.

## 1. Introduction

Naturally rare plant species are often specialists whose distributions reflect those 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, may bring formerly allopatric species into sympatry. If the species are closely related, hybridization can occur. Hybridization between a rare plant species and a more common congener may result in a decline in the number of genetically distinct individuals of the rare species. Because they have been genetically swamped by

the more common species, individuals of the rare species become rarer (Ellstrand and Elam, 1993; Rhymer and Simberloff, 1996; Lamont et al., 2003; Parsons and Hermanutz, 2006). 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; Keeler, 2004). Crosses between individuals of differing ploidy levels, itself a form of hybridization under Harrison's (1990) definition, often produce sterile offspring due to abnormal chromosomal pairing during meiosis (Avise, 2004).

Natural hybridization can be detected by comparing known parental types to putative hybrids using morphological and/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 (Rieseberg and Ellstrand, 1993; Burke and Hamrick, 2002). Recent studies demonstrate the value of using molecular and genetic data in addition to morphology to distinguish between hybridizing species (e.g. Swartz and Brunsfeld, 2002; Cattell and Karl, 2004). The presence of diagnostic DNA markers from both parent species in suspected hybrid individuals can be used to confirm that hybridization has occurred.

Bell's twinpod, *Physaria bellii* Mulligan is a diploid (2N = 8) herbaceous, self-incompatible, perennial member of the Brassicaceae (Mulligan, 1966, 1967; Kothera, 2006). It is pollinated by a group of generalist pollinators (Kothera, 2006), including honeybees (*Apis mellifera*), whose congeners are capable of flying up to 3 km on foraging flights (Dick, 2001), and is endemic to open shale and sandstone washes in two counties along the Front Range of northern Colorado, USA (Mulligan, 1966; Fig. 1). According to the standardized Natural

Heritage Methodology of Natureserve (Morse, 1986; Colorado Natural Heritage Program (CNHP)), which is used in the US and Canada, *P. bellii* has a ranking of G2/S2. This means that *P. bellii* is at risk of extinction because of its restricted range, which is itself potentially threatened by residential development and limestone mining, and due to the small number of populations (Spackman et al., 1997; Doyle et al., 2004). At the time this study was initiated, CNHP estimated there were approximately 20 *P. bellii* populations, and that several populations on private land had been lost in recent years. Ecological information for *P. bellii* populations in this study is given in Kothera et al. (2007).

The range of *Physaria vitulifera* Rydberg, with a known distribution that includes eight counties (Jennings personal communication), overlaps with but is more extensive than that of *P. bellii* (Fig. 1). *P. vitulifera* exists as morphologically identical tetraploid (2N = 4X = 16) and diploid (2N = 8) populations, while cytological studies of the rarer *P. bellii* have shown it to be consistently diploid (2N = 8; Mulligan, 1967). Ploidy levels of *P. vitulifera* populations located closest to *P. bellii* populations were not known.

The two species are readily distinguishable in the field by leaf (Fig. 2) and fruit characteristics. However, plants with intermediate leaf morphology have been observed in the two southernmost populations classified as *P. bellii* by CNHP (H1 and H2 in Fig. 1). This finding necessitated characterizing the putative hybrids and determining if hybrids existed further north, where several populations of the two species occur close enough for gene flow to occur. If hybridization was widespread, the already imperiled *P. bellii* could be further endangered by hybridization with *P. vitulifera* and subsequent gene swamping.



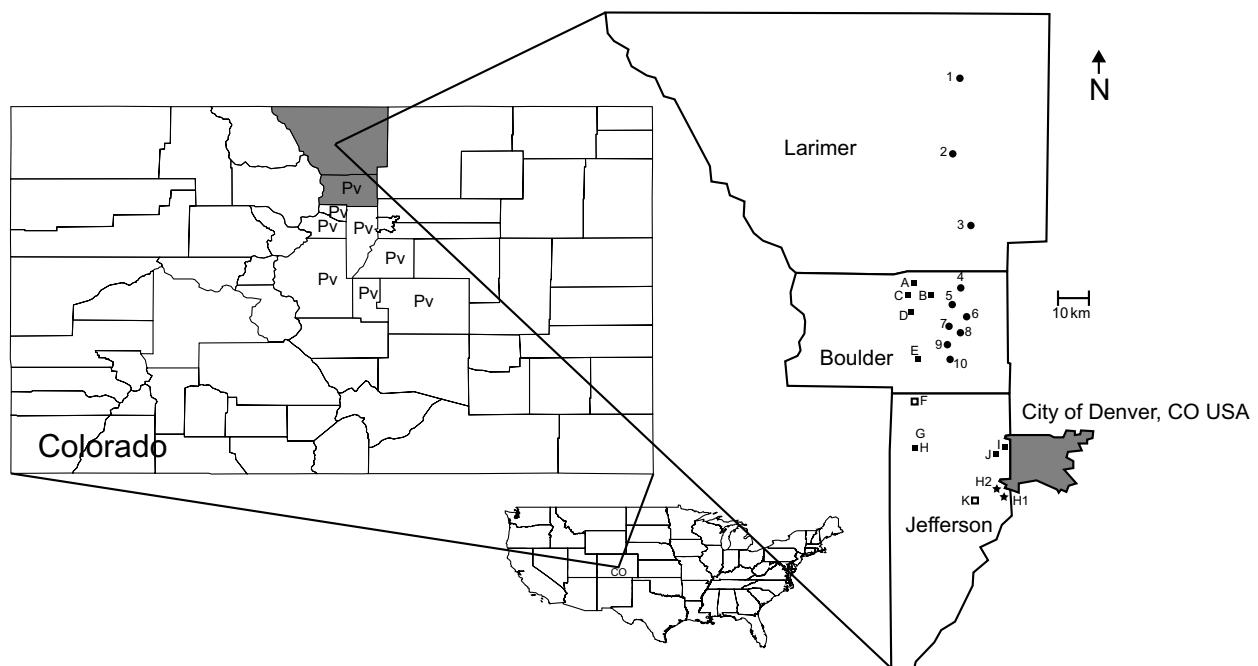Fig. 1 – Map showing locations of *Physaria* populations sampled for this study. Circles (1–10) = *P. bellii*; filled squares (A–E, H–J) = tetraploid *P. vitulifera*; open squares (F, K) = diploid *P. vitulifera*; stars (H1, H2) = putative hybrid populations. Shading = range of *P. bellii*. See Results for information on population G. Counties within the range of *P. vitulifera* shown with the designation "Pv".
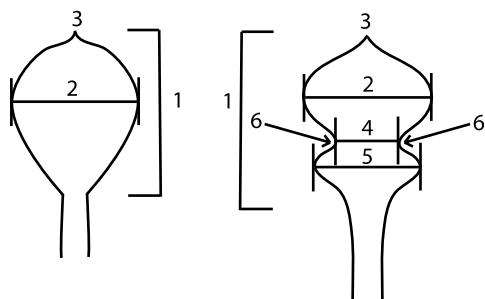
**Fig. 2 – Illustrations of *P. bellii* (left) and *P. vitulifera* leaves showing leaf morphology characteristics. 1 = *length*; 2 = *width*; 3 = *toptooth* (here, present); 4 = *W1*; 5 = *W2*. The *P. vitulifera* leaf shown has one pair of sinuses (6), which were counted for the character *pairsinuses*. Leaves often had one or more teeth around the margin (*numberteeth*), which were left off this illustration for clarity.**

The objectives of the research we report here were to use morphological, molecular and cytological analyses to establish whether hybrids exist between *P. bellii* and its more common congener *P. vitulifera*, examine the extent of hybridization between the two species, and to assess the threat from such hybridization to the continued survival of *P. bellii*.

## 2. Materials and methods

*Sampling*: During the summer of 2002, leaf tissue was sampled from 30 individuals from each of ten *P. bellii* populations and the two suspected hybrid populations (1–10 and H1, H2 in Fig. 1). Half of the *P. bellii* populations contained less than 1000 individuals and half contained more than 1000 individuals (Kothera et al., 2007). For each sampled population, a 300 m transect, which covered most, if not all of the population, was placed starting at the edge of a population. One individual was sampled at the beginning of the transect, and every 10m thereafter moving along the transect towards the interior of the patch. 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. In addition, the eleven *P. vitulifera* populations nearest to known *P. bellii* and suspected hybrid populations were also sampled, but in a different manner because the populations were smaller, and the systematic method used for *P. bellii* would have under sampled the *P. vitulifera* populations Therefore, between 8 and 12 randomly selected *P. vitulifera* plants were sampled from each population, representing about one-third to one-half of the population. One representative leaf was collected from each plant for morphology measurements, and another was removed for DNA extraction. All leaves were placed on ice immediately after collection and then stored at −80°.

Leaf tissue was also collected from one population of *P. acutifolia* near Woods Landing, WY. In Wyoming, this species has previously been classified as *P. vitulifera* (Jennings, 2004), and the location of the Woods Landing *P. acutifolia* population close to the northern end of *P. bellii*'s range provided another possible opportunity for hybridization. Twelve individuals were randomly sampled, representing most of the population.

*Seed germination*: Seed was collected during the summer of 2002 from *P. bellii* populations 1–10 and during the summer of 2003 from *P. vitulifera* populations A–K and one of the two putative hybrid populations (H2). 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 min, which can increase germination relative to no treatment by surface sterilizing the seed or softening the pericarp to allow water into the seed (Ervin and Wetzel, 2002). The seeds were then placed on wet paper towels in a petri dish, placed in a dark drawer for five days at room temperature, and watered when needed.

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, Colorado, USA). To simulate the rocky, well-drained soil that characterizes *Physaria* habitats, the potting soil was overlain with a 2 cm layer of sand. Seedlings were transplanted into 4 in. pots when they were 6–8 cm high and placed in a Colorado State University greenhouse in Fall 2003. 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 in June 2004.

*Greenhouse and field crosses*: No steps were taken to prevent selfing while performing greenhouse crosses, since both *Physaria* species are self-incompatible (Mulligan, 1966, 1967). Mature anthers were removed from one pollen donor with clean forceps and dragged lightly across the stigmas of open flowers on one inflorescence of the pollen recipient. The forceps were cleaned with 95% ethanol between pollinations to minimize cross-contamination. Fruits were collected approximately two months after pollination occurred. Details of greenhouse and field crosses are presented in Table 1. Thirty-two of the total of 45 *P. bellii* and *P. vitulifera* plants grown for greenhouse crosses failed to produce flowers, or

**Table 1 – Results from controlled greenhouse and field pollinations between *Physaria bellii* and *Physaria vitulifera***

| Pollen recipient | Pollen donor | | |
|---|---|---|---|
| | Pb | 2NPv | 4NPv |
| *A. Greenhouse* | | | |
| Pb | – | 0(1) | 0(20) |
| 2NPv | 0(1) | – | 5(9) |
| 4NPv | 0(12) | 0(10) | 52(48) |
| *B. Field* | | | |
| Pb | – | 0(22) | 0(13) |
| 2NPv | 3(56) | – | – |
| 4NPv | 21(58) | – | – |

The number of normal seeds is shown, with the number of crosses performed in parentheses. Abbreviations: 2N = diploid, 4N = tetraploid, Pb = *P. bellii*, Pv = *P. vitulifera*. Crosses which were not of interest and therefore not attempted are designated by "–".

produced flowers when there were no other plants flowering and thus could not be crossed with another plant.

Field crosses were conducted to generate $F_1$ hybrids in 2003, and again in 2005 because of the low success rate of greenhouse crosses. 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 $F_1$ hybrid seeds were produced in this fashion, of which six germinated. All six artificial $F_1$ hybrid seeds were consumed, either to obtain chromosome counts or in the process of genetic analysis, whereby they were not used for chromosome counts.

*Cytological analysis of P. vitulifera and suspected hybrid populations*: Two to three day old seedlings of wild collected plants were soaked in a 2 mM solution of 8-hydroxyquinoline for 4–6 h in a dark drawer and fixed overnight at 4° in a 3:1 solution of 100% ethanol: glacial acetic acid. Fixed root tips were hydrolyzed in 1N hydrochloric acid for five minutes, 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 population were recorded.

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, and squashed for viewing under a light microscope.

*Leaf morphology*: The following morphometric measurements were made on leaves from 244 *P. bellii*, 100 *P. vitulifera* and 58 putative hybrid individuals sampled as described above. 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*) (Fig. 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.

*ISSR analysis*: ISSR (Inter-simple sequence repeat; Gupta et al., 1994; Zietkiewicz et al., 1994) 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 $F_1$ hybrids grown from seed produced by controlled pollinations. Following a screen of candidate ISSR primers, three anchored dinucleotide repeat primers supplied by the University of British Columbia were selected for use in this study based on their repeatability and the number of polymorphisms generated

**Table 2 – Primer information for ISSR markers used in the analysis of *Physaria bellii*, *Physaria vitulifera* and their putative hybrids**

| Primer name | Sequence | Annealing temperature | Total number of 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%) |
| Fragment sizes ranged from 280 to 1350 bp. | | | | |

(see Table 2). Genomic DNA was extracted from 100 mg of frozen leaf tissue using DNeasy Plant Mini Kits (Qiagen, Valencia, California, USA). ISSR reactions were carried out in 25 µl volumes containing 16.2 µl HPLC water, 2.6 µl 10× *Hot-Taq* PCR buffer (GeneSys, Buffalo, NY, USA), 1.5 µl MgCl (25 mM), 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). Amplification was performed 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, followed by 2 min at 94° for the initial denaturation step. This was followed by 35 cycles of 94° for 30 s, primer-specific annealing temperature (see Table 2) for 45 s and 72° for 90 s, with a final extension step at 72° for 20 min. Amplification products were resolved on 4% denatured polyacrylamide gels, silver stained using the method of Bassam and Caetano-Anollés (1993), allowed to air dry and scanned to create a permanent record. Bands were visually scored as present or absent in each individual. PCR reactions with Primer 809 were performed twice and run on separate gels, and amplification products were checks for inconsistencies. In every case, banding patterns on the first and second gels were identical.

*Physaria acutifolia*: DNA was extracted from twelve individuals from the Woods Landing, WY *P. acutifolia* population and screened with two of the three ISSR primers used with the other *Physaria* taxa, here defined as *P. bellii*, *P. vitulifera* and their suspected hybrids. The PCR, electrophoresis and silver staining protocols were the same as those used for the other two *Physaria* taxa.

*Statistical analysis*: 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, 2003). 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 (SAS, 2003). 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 the SAS Procedure Glimmix, which is designed for such distributions.

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 the 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. Finally, the same set of five characters was analyzed with the SAS Procedure Candisc, which performs a canonical discriminant analysis (CDA) 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).

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. The 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. Therefore *locus6* was excluded from the discriminant analysis and the CDA, leaving 26 informative markers.

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.

Finally, the program HINDEX (Buerkle, 2005) was used to estimate the relative genetic contribution of the parent species to each of the 58 natural hybrids, as well as three known $F_1$s. Maximum-likelihood estimates were based on data from 50 ISSR marker loci for which parental allele frequencies differed by 0.05 or more.

## 3. Results

*Controlled pollinations and ploidy levels*: One hundred and eighty three inter-species crosses were performed, resulting in 24 seeds, for a success rate of 3%. By comparison, intra-species crosses were more successful: 67 crosses yielded 57 seeds (85% seed set; Table 1). This trend was especially evident in crosses between tetraploid *P. vitulifera* individuals. In contrast, no seeds were produced when *P. bellii* was the pollen recipient.

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. Both populations containing

suspected hybrids were tetraploid (2N = 16), as were eight of the sampled populations of *P. vitulifera* (A, B, C, D, E, H, I and J). The seeds collected from population G were depleted before a satisfactory squash protocol was developed, and thus no reliable chromosome counts were made for this population. Another two *P. vitulifera* populations were diploid (F and K). Population K was geographically the closest *P. vitulifera* population to the putative hybrids, approximately 2 km from population H1. Triploid cells with 12 chromosomes were observed in three germinated seeds from field crosses between *P. bellii* and tetraploid *P. vitulifera* individuals.

*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). In contrast, 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%. The plot of the two canonical variables is shown in Fig. 3.

*Genetic analysis of hybrids*: The ISSR analysis yielded six species-diagnostic bands: two in *P. bellii* and four in *P. vitulifera*. The number of species-diagnostic markers present in hybrids varied widely: 10 individuals (16%) had the first *bellii* marker and six (10%) had the second. Of the four *vitulifera* markers, 50 hybrids (82%) had the first, 43 (70%) had the second, 57 (97%) had the third and 35 (57%) had the fourth. All suspected hybrids 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%) putative hybrids had markers from both parental species. The three known $F_1$ hybrids generated from controlled crosses displayed more of an equal distribution of species-specific markers compared to the putative

**Table 3 – Differences of least-squares means for four leaf morphology characters measured in *Physaria bellii* (Pb), *Physaria vitulifera* (Pv) and their putative hybrids (H)**

| Comparison | Toptooth | Length | Numberteeth | Width |
|---|---|---|---|---|
| Pb vs. Pv | −3.43** | 6.24*** | −3.23** | 0.11 |
| Pb vs. H | −1.61 | 1.43 | −2.07* | 2.06 |
| H vs. Pv | 0.13 | 2.31* | 0.17 | −1.98 |

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 ($N = 397$).
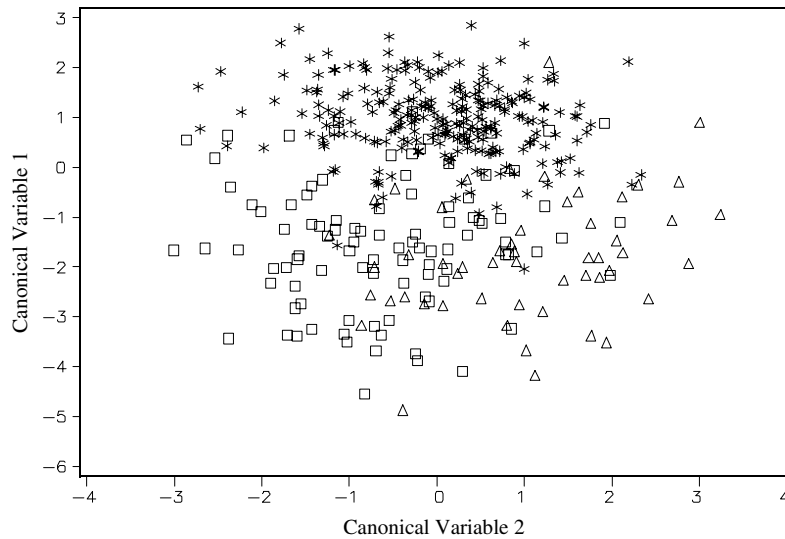
**Fig. 3 – Canonical discriminant analysis of *Physaria* leaf morphology data. Star = *P. bellii*; square = *P. vitulifera*; triangle = putative hybrid.**
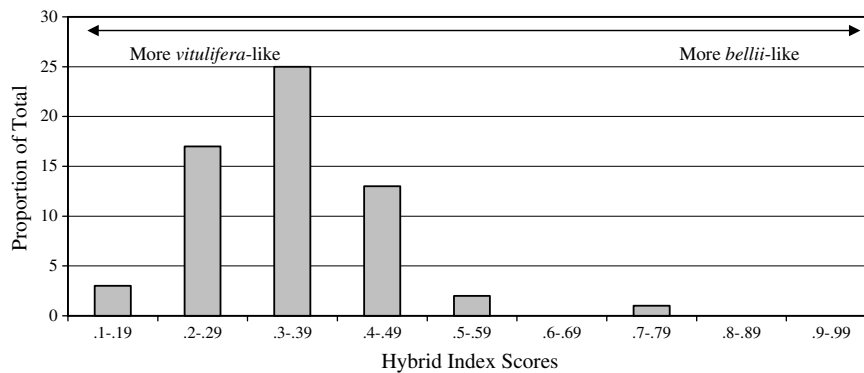


**Fig. 4 – Histogram showing hybrid index scores for suspected hybrids (*N* = 61). An individual's score represents *P. bellii*'s genetic contribution. Thus individuals with scores towards 0.1 are more *vitulifera*-like and individuals with scores towards 0.99 are more *bellii*-like. Average score = 0.34.**

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*.

Hybrid index values represented the proportion of *P. bellii*'s genetic contribution to the 58 natural and three known F$_1$ hybrids. For the natural hybrids, the average hybrid index was 0.34, indicating that *P. vitulifera* has made a greater contribution to these hybrids than *P. bellii* (Fig. 4). In contrast, hybrid index values for the known F$_1$ individuals were 0.37, 0.51 and 0.75.

The SAS Procedure Discrim was successful at grouping individuals into the correct taxa. All individuals of the parental species and most of the putative hybrids (95%) were classified correctly. The CDA clearly separated *P. bellii* and *P. vitulifera* individuals while generating a distribution of putative hybrids wider than and located between that of the parental species (Fig. 5).

The three taxa formed discrete clusters in the UPGMA. 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*: Twelve *P. acutifolia* individuals were analyzed with UBC primers 890 and 841 and scored for the 28 polymorphic bands associated with those primers (Table 2). Over half of the *P. bellii* and *P. vitulifera* markers (*n* = 17; 61%) were not present in any *P. acutifolia* individual. There were no species-specific or monomorphic markers found in all *P. acutifolia* individuals. However, three markers in several *P. acutifolia* individuals were not found in the other two species.

## 4. Discussion

*Feasibility and extent of hybridization between P. bellii and P. vitulifera*: Our results confirm that natural hybridization between *P. bellii* and *P. vitulifera* does occur and that the two southernmost *P. bellii* populations contain hybrid individuals. First, the analysis of leaf morphology data shows the hybrids to be dissimilar to either parental species (Fig. 3; Table 3). Overall,

hybrids were intermediate in their leaf morphology, although there was little difference in leaf sinus size between hybrids and *P. vitulifera*. Second, hybrid indices constructed from ISSR markers indicate natural and known $F_1$ hybrids are genetically unlike either parent species, with natural hybrids being more *vitulifera*-like and $F_1$s being more genetically intermediate (Fig. 4). Furthermore, the relatively small proportion of *P. bellii* genes in natural hybrids suggests that this species has not recently contributed genes to the hybrid populations. Third, visual portrayals of informative markers from the ISSR data set via the CDA underscore the genetic distinctiveness of the three taxa, as well as how the hybrids appear genetically similar to *P. vitulifera* (Fig. 5). Finally, groupings in the UPGMA supported the existence of three discrete entities.

The differences in CDAs for the molecular versus the morphological data call attention to the difficulty of relying on phenotypes to establish hybridization (Figs. 3 and 5). 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, underscoring the need to incorporate genetic data in studies of hybridization.

*Assessment of threat to P. bellii from hybridization*: The presence of hybrids implies that the two species were at one time in closer proximity than they are at present. In addition, the location of H1 and H2 make it unlikely that present day populations of *P. bellii* are contributing genes to those populations. However, some *P. vitulifera* populations are currently located close enough to *P. bellii* populations for gene flow to occur. Despite this, our results indicate the threat to *P. bellii* from hybridization with *P. vitulifera* and subsequent gene swamping is relatively small for several reasons. First, controlled pollinations, although admittedly few in number, indicated that hybrid seed formation is more difficult than intraspecific seed formation (Table 1), suggesting that at least partial reproductive isolating mechanisms exist between these two species. Second, and perhaps more relevant to the assessment of the degree of threat to *P. bellii*, is the distribution of diploid and tetraploid *P. vitulifera* populations, which

was unknown before this study. The finding that *P. vitulifera* populations are tetraploid close to *P. bellii* populations, along with the fact that searches in these areas have not resulted in individuals with intermediate leaf morphology (Riedel, personal communication) makes it probable that any hybrids formed are sterile triploids. This result is positive news for the conservation of *P. bellii*, as it does not appear to be in danger of genetic swamping. Third, the 50 km gap between the southernmost present-day *P. bellii* population (#10) and hybrid populations H1 and H2 may have served to slow or halt the pace of hybridization, as diploid *P. vitulifera* populations are now located too far away from *P. bellii* populations for pollination to occur.

The natural hybrids we sampled from populations H1 and H2 were all tetraploid. The nearest *P. vitulifera* population to the hybrids (K), is diploid and could have been involved in an initial hybridization event with diploid *P. bellii*. However, this is unlikely to have occurred recently as the nearest pure *P. bellii* population is presently approximately 50 km north of the hybrid populations. Because apparently suitable habitat exists within this gap (Anderson, personal communication), it is possible that some *P. bellii* populations have been extirpated, perhaps due to residential development along the Front Range.

Given that the *P. vitulifera* population nearest the two hybrid populations is diploid, the following scenario may describe the establishment of the hybrids. After the initial hybrids were formed, chromosome doubling occurred, resulting in allotetraploidy. The preponderance of *vitulifera*-diagnostic markers in present day hybrids suggests continued introgression of *P. vitulifera* genes after the initial hybridization event. We found no triploid individuals among plants sampled from populations H1 and H2, but they may exist if diploid *P. vitulifera* genes are currently introgressing into these hybrid populations. Viable triploid seed was produced from field crosses, but those seeds were used for chromosome squashes and genetic analysis and not grown to maturity, so the fertility of this class of hybrids is unknown. An alternative scenario where *P. bellii* produced unreduced ovules, thereby allowing the production of tetraploid offspring from
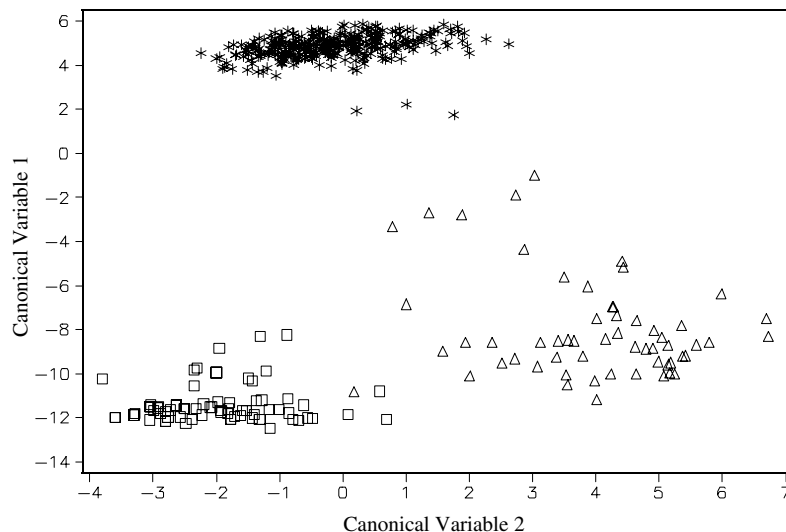


Fig. 5 – Canonical discriminant analysis of *Physaria* ISSR data. Star = *P. bellii*; square = *P. vitulifera*; triangle = putative hybrid.

crosses with tetraploid *P. vitulifera* seems less likely because of the closer proximity of a diploid *P. vitulifera* population to the present-day hybrids.

*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 because over half of the ISSR markers found in *P. bellii* and *P. vitulifera* were not present in any *P. acutifolia* samples.

## 5. Conclusions

Several recent papers have documented the decline of a rare plant species through hybridization with a more common congener (Brock, 2004; Klips and Culley, 2004; Hardig et al., 2005; Burgess and Husband, 2006; Zika, 2006). Our results suggest that hybridization between *P. bellii* and *P. vitulifera* is possible and has happened in the past; however, gene swamping by its more widespread congener does not appear to pose a significant current threat to *P. bellii*. On the other hand, remaining *P. bellii* populations do risk extinction through habitat loss from residential development and limestone mining. Efforts to preserve this imperiled endemic should therefore focus on habitat conservation and protecting against further loss of populations through human activity.

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