
**Fitness effects of inbreeding and outbreeding on
golden paintbrush (*Castilleja levisecta*):
Implications for recovery and reintroduction**

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PREFACE

This report is the result research conducted by the Institute for Applied Ecology (IAE) under contract with a public agency, the Washington Department of Natural Resources, Natural heritage Program under Contract No. 03-218. IAE is a non-profit organization dedicated to natural resource conservation, research, and education. Our aim is to provide a service to public and private agencies and individuals by developing and communicating information on ecosystems, species, and effective management strategies and by conducting research, monitoring, and experiments. IAE offers educational opportunities through 3-4 month internships. Our current activities are concentrated on rare and endangered plants, habitat management and invasive species.

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INTRODUCTION

Background

Castilleja levisecta (golden paintbrush) once occupied prairies and grasslands throughout the Puget Trough and Willamette Valley. Habitat destruction and alteration over the past century have resulted in substantial declines in native vegetation in this ecoregion, and several species, including golden paintbrush, are now listed by state and federal agencies as threatened or endangered. All remaining populations of golden paintbrush occur in Washington and British Columbia; the species is considered to be extirpated in Oregon.



Figure 1. *Castilleja levisecta* (golden paintbrush).

Castilleja levisecta is an herbaceous perennial that appears to reproduce only by seed. Many populations are declining, and past research indicates that disturbances such as fire and mowing may be useful for maintaining or expanding existing populations (USFWS 2000). Like most paintbrushes (Heckard 1962), this species is a hemiparasite – its roots penetrate the roots of neighboring plant species and derive nutrients, carbohydrates, and possibly other secondary compounds from these hosts.

The Recovery Plan for golden paintbrush (USFWS 2000) identifies population reintroduction, development of propagation methods, and studies of the pollination biology of the species as high priority actions to meet recovery objectives. Because no populations of this species are known to remain in Oregon, population reintroduction will be crucial to recovery in this region as well as portions of the range to the north.

The importance of mating system: inbreeding and outbreeding

Creating new populations from propagated material should consider the source of these materials as well as their genetic relationship to one another. When plants self or mate with close relatives their offspring may show negative effects related to inbreeding depression. On the other hand, individuals that are distantly related may produce offspring that show higher fitness than either parent, or their offspring can have reduced vigor due to outbreeding depression (see reviews by Kaye 2001a and Hufford and Mazer 2003). The reintroduction plan for *C. levisecta* (Caplow 2002) recommends that the risks of inbreeding and outbreeding depression be examined experimentally.

Inbreeding depression – Inbreeding depression occurs when close relatives mate (or plants self-fertilize) and their offspring display reduced vigor or fitness. Inbreeding depression is a well-known and studied phenomenon, and often occurs in small, fragmented, or isolated populations, or when mating is frequent between close neighbors. It may result when deleterious recessive alleles are paired (creating homozygotes) so that their negative effects are expressed in the progeny. When these genes are not paired (as after outcrossing), they may be masked by a more favorable allele (as a heterozygote), so the progeny function normally. Inbreeding depression may also result from loss of heterozygote advantage. In plants, inbreeding depression can be expressed at any stage in the life cycle, including seed germination, seedling establishment, plant growth rate and survival, flowering, and seed production. Populations suffering from inbreeding depression can often benefit from out-crossing with individuals in other populations, which may result in higher heterozygosity, improved health of individuals, and greater population viability. This is one factor used to support the use of multiple sources of plant materials in restoration (one side of the Single or Multiple Source debate [Kaye 2001a]).

Outbreeding depression – Outbreeding depression, which is a reduction in fitness of progeny from distant parents, has a much shorter history of study and is less documented and understood than inbreeding depression. A recent literature search (Kaye 2001a) found 468 papers on inbreeding depression but only 25 references to outbreeding depression. Even so, this hot topic in genetic and conservation research has been demonstrated in various organisms, including salmon (Gharrett 1999), fruit flies (Aspi 2000), and chimpanzees (Morin et al.

1992). Some animal studies have found a positive effect of outbreeding, however, such as in bats (Rossiter et al. 2001). Among plants it may occur in larkspur (Waser and Price 1991, 1994), skyrocket (Waser et al. 2000), a carnivorous pitcher plant (Sheridan and Karowe 2000), Hawaiian silversword (Friar et al. 2001), a Mediterranean borage (Quilichini et al. 2001), a subshrub (Montalvo and Ellstrand 2001), and an exotic roadside weed (Keller et al. 2000). In many cases, crossing between unrelated individuals results in F1 progeny with increased fitness, followed by the expression of outbreeding depression in later generations (Hufford and Mazer 2003). Most researchers (e.g., Lynch 1991, Waser 1993) believe that there is hybrid vigor in the first generation followed by reduced fitness in later generations from loss of ecological adaptation (at least one of the original parents was poorly adapted to the site) and/or disruption of coadapted gene complexes.

In this paper we examine the mating system of *Castilleja levisecta* and explore the effects of inbreeding and outbreeding on F1 progeny fitness at multiple life history stages. The results have direct bearing on reintroduction and recovery actions for the species because the risks associated with inbreeding and outbreeding need to be identified in order to assist with seed selection and genetic management of outplanting activities.

METHODS

Pollination experiment

We performed controlled cross-pollination between individuals of differing known heritage. These crosses were of four major types: self-pollination, crosses between siblings, crosses between non-sibling plants from the same source population, and crosses between individuals from different populations. The plants used in these crosses were grown in pots from seed collected in wild populations on Whidbey Island and San Juan Island, Washington in September, 2000. Sources of plants and the number of each type of cross are listed in Table 1. Genetic identities among the three source sites (False Bay [San Juan Island], West Beach, and Fort Casey [Whidbey Island]) were all very high (0.93-0.94, Godt and Hamrick [2002]) although their geographic distances ranged from 13.3 to 45.4 km (Table 1). Seeds from individual plants were kept separate at the time of collection, so that maternal lines could be documented. Crosses between siblings involved pollination between two plants grown from seed taken from the same maternal parent.

Flowers of *Castilleja levisecta* are borne on a raceme, and each flower has one pistil and stigma as well as a single ovary with numerous ovules. The mean number of ovules per flower in *C. levisecta* is 183 ± 5.8 (Kaye, unpubl. data). Each fruit is a capsule and the seeds are typically very small (~ 0.5 mm). The flowers are protogynous; their pistils extend beyond the opening of the flower and the stigma becomes receptive prior to anther dehiscence (Figure 2). We grew all plants in a screen house to prevent insects from visiting the experimental flowers and moving pollen between plants.

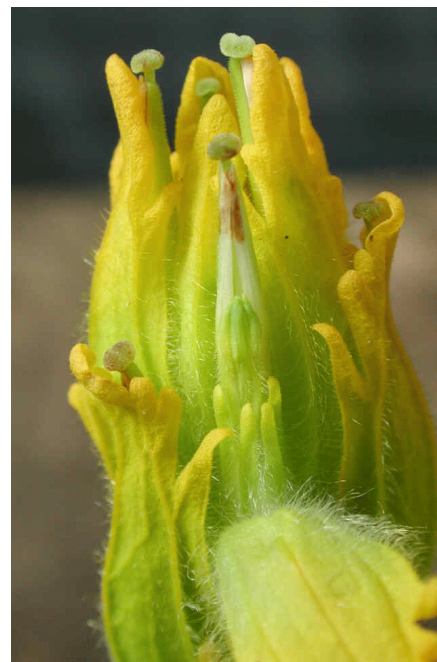


Figure 2. The inflorescence of *Castilleja levisecta* is a raceme, and the individual flowers are partly covered by a showy yellow bract (shown here pulled back to expose a flower). The receptive stigmas can be seen as two-lobed, rounded structures emerging above the flowers.



Figure 3. Pollinated flowers of *C. levisecta* were marked with color-coded thread.

To prevent unwanted self-pollination, all crosses first involved emasculation, i.e., removing the anthers from the flowers identified as pollen recipients (dames) prior to anther dehiscence. We then used forceps to remove a mature, dehiscing anther from a pollen donor plant (sire) and applied pollen directly to the stigma of the dame. Each dame flower on a raceme was marked with color coded thread tied around its base (Figure 3).

When fruits matured, they were removed from the dames and returned to the lab for examination under a dissecting microscope. Each fruit was measured for length and width, then opened to count normally developed seeds as well as undeveloped ovules. We used this information to calculate the proportion of total ovules that set seed.

Table 1. Crossing types, source populations involved, and sample sizes (number of crosses conducted). Geographic distances between populations are given in parentheses.

Cross type	source populations used	sample size
self	Fort Casey	2
	West Beach	17
		Total= 19
sibling	False Bay	3
	West Beach	9
		Total= 12
within-population	False Bay	5
	Fort Casey	7
	West Beach	23
		Total= 35
between-population	False Bay × Fort Casey (45.4 km)	6
	False Bay × West Beach (33.1 km)	9
	Fort Casey × West Beach (13.3 km)	12
		Total= 27

Propagation of crossed progeny

To germinate seeds from each cross, the seeds were placed on germination pads moistened with distilled water in individual plastic boxes with tight fitting lids. We used 50 seeds from each cross except where fewer were available. The seeds were then exposed to cool temperatures (4 °C) for six weeks, followed by alternating warm temperatures (15 °C and 25 °C) for two weeks. This method has resulted in high germination rates (generally > 75%), depending on source population) in previous studies with *C. levisecta* (Wentworth 1998, Kaye 2001b). After these treatments we counted the total number of seedlings emerging in each box.

Up to ten seedlings from each cross were then planted in fine potting soil in 5 x 5 cm by 6 cm deep pots in a greenhouse. The plants were watered from beneath by flooding the greenhouse bench for 30 minutes, twice per week. After 4 weeks of growth, the small plants were then repotted into 10 x 10 cm by 9 cm deep pots with a seedling of *Eriophyllum lanatum*, an herbaceous plant in the Asteraceae that proved to be a superior host in earlier propagation experiments with *C. levisecta* (Kaye 2001b). The potted plants were then moved out of doors to a flat garden bed and allowed to grow an additional 8 weeks. All plants were fertilized once per week throughout this period. The total growth period from first potting to the end of the experiment was 89 days (ending 30 June 2003). We measured plant size on the 89th day as number of stems, length of each stem, and number of flowering racemes.

Analysis of fitness measures (seed set, seed germination, plant growth, flowering)

To determine the effect of crossing distance on fitness in *Castilleja levisecta*, we performed separate statistical analyses on each measure of plant performance we observed (Table 2). We tested the hypothesis that pollination-type had no effect on seed set with a Kruskal-Wallis one-way ANOVA on ranks combined with Kruskal-Wallis multiple-comparison Z-value tests to compare individual treatments. These non-parametric tests were used because the seed-set data, especially residuals for sibling crosses, did not conform to the assumptions of normal distribution and equal variances required for standard ANOVA. We examined seed germination and flowering with logistic regression to determine if the odds of germination or

flowering differed among the treatments. Our examinations of these data suggested that their distributions were not strictly binary, so we used quasi-likelihood methods. And finally, we used one-way ANOVA to test for a treatment effect on plant growth expressed as the sum of all stem lengths on each plant, followed by Fisher's protected LSD multiple-comparison test to compare individual treatments.

Table 2. Fitness measures used to evaluate the effects of inbreeding and outbreeding on *Castilleja levisecta*, the statistical analysis implemented in this study, and sample sizes for each treatment.

Fitness measure (response variable)	Statistical analysis
Seed set (proportion of ovules developing into normal seeds)	Kruskal-Wallis one-way ANOVA on ranks with Kruskal-Wallis multiple-comparison Z-value test. Sample sizes: self= 19, sibling= 12, same population= 35, different population= 27.
Seed germination (odds of seeds germinating)	Logistic regression, quasi-likelihood. Sample sizes: self= 8, sibling= 10, same population= 35, different population= 26.
Plant growth (total stem length)	One-way ANOVA, Fisher's protected LSD multiple-comparison test. Sample sizes: self= 6, sibling= 9, same population= 35, different population= 25.
Flowering (odds of producing a flowering stem)	Logistic regression, quasi-likelihood. Sample sizes: self= 6, sibling= 9, same population= 35, different population= 25.

RESULTS

Effects of inbreeding and outbreeding on fitness

Seed set – *Castilleja levisecta* appears to be almost completely self-incompatible, and seed-set appears to increase as the genetic relationship among mating individuals becomes more distant. The proportion of ovules that set normal seeds was significantly affected by pollination cross-type in Kruskal-Wallis ANOVA ($df=3$, χ^2 corrected for ties= 44.3, $P < 0.0001$). Self-pollinations had the lowest seed-set, averaging 0.7%, which was significantly less than the average $33\% \pm 7\%$ (SE) for sibling crosses (Figure 4). Crosses between individuals from different populations had much higher seed-set ($\bar{x} = 80\% \pm 5\%$) than those between siblings, while matings between non-sibling plants from the same population had intermediate seed set ($\bar{x} = 71\% \pm 4\%$).

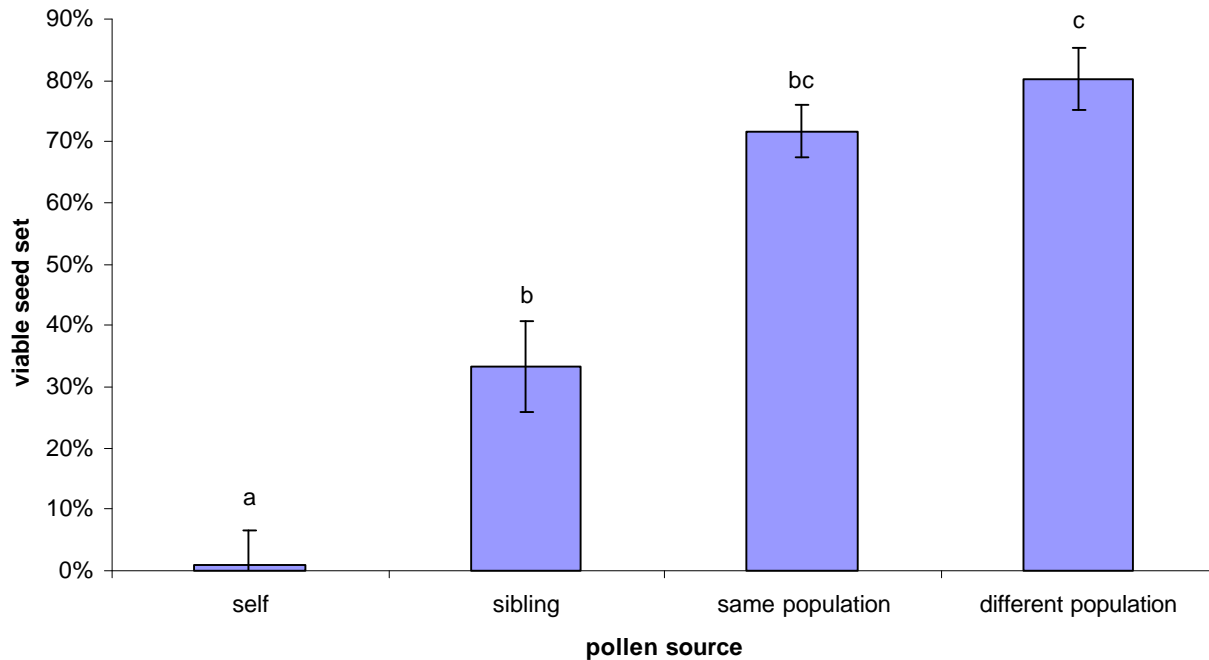


Figure 4. Mean (± 1 SE) percentage seed-set in *Castilleja levisecta* from different pollination cross-types. Bars with the same letter do not differ at the $\alpha = 0.05$ level of probability.

Seed germination – We found no evidence that seed germination was affected by cross type. Logistic regression did not detect any significant difference in germination rate among the mating types tested here ($df= 3,75$; $\chi^2= 0.32$; $P= 0.957$). The overall seed germination rate averaged $84.5\% \pm 3\%$ (SE). Note that few seeds were produced from self and sibling crosses, so that little statistical power was available to detect differences between these matings and other types. This rate of germination is typical of wild-collected seeds, which tend to have greater than 75% germination (Kaye 2001b) under the same environmental conditions as applied in the current study.

Plant Growth – Plant size was affected substantially by mating type ($df= 3,71$; $F= 0.12.72$; $P< 0.00001$). F1 plants from self-pollinations grew an average of $55 \text{ cm} \pm 19 \text{ cm}$ ($n= 6$) of stem in 89 days, and were not significantly different from plants resulting from crosses between siblings ($\bar{x}= 79 \text{ cm} \pm 16 \text{ cm SE}$, $n= 9$) (Figure 5). However, both of these cross types produced plants significantly smaller than non-sibling crosses in the same population ($\bar{x}= 130 \text{ cm} \pm 8 \text{ cm SE}$, $n= 35$), which were in turn significantly smaller than plants from between-population crosses ($\bar{x}= 162 \text{ cm} \pm 9 \text{ cm SE}$, $n= 25$).

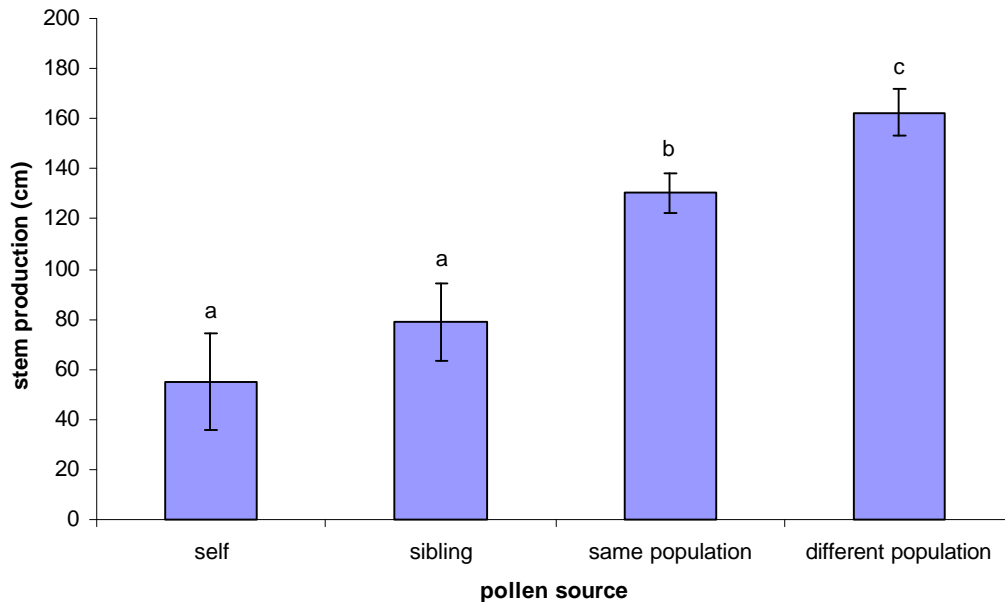


Figure 5. Mean (± 1 SE) plant size (total stem production) of *Castilleja levisecta* plants grown from seed produced by different pollination types. Bars with the same letter do not differ at the $\alpha= 0.05$ level of probability.

Flowering – The rate at which plants flowered was also significantly different among plants from different cross-types (logistic regression; $df= 3,71$; $\chi^2= 8.77$; $P= 0.033$). On average, 11% ($\pm 3\%$ SE) of crosses between non-sibling individuals from the same population produced offspring that flowered (Figure 6), and the odds of these plants flowering was not significantly different from self or sibling crosses. However, the odds of flowering for plants from crosses between different populations was 1.1 to 4.6 times higher than within population crosses.

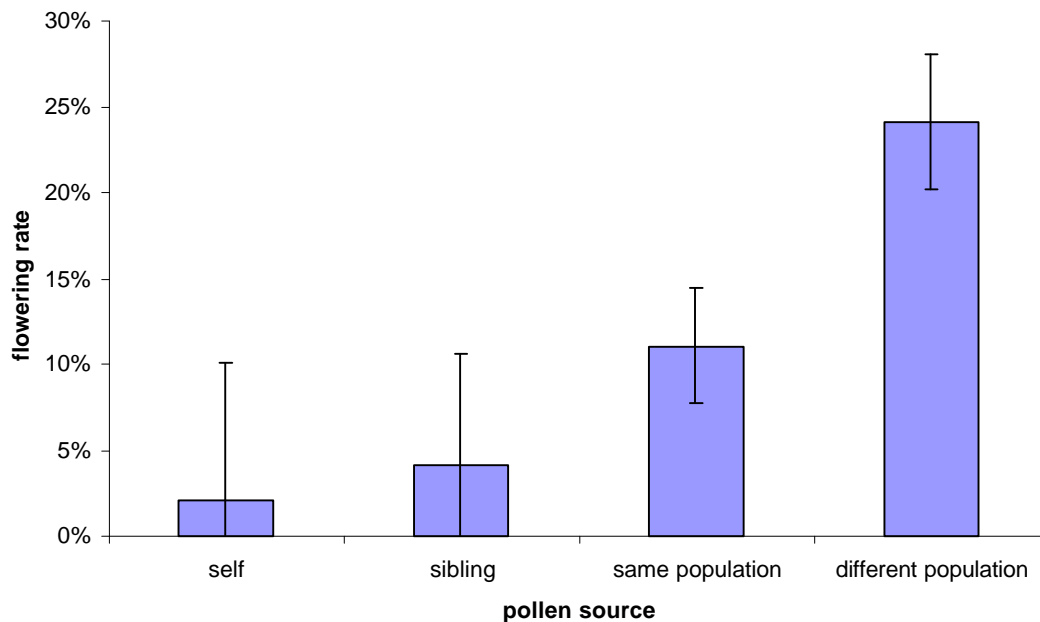


Figure 6. Mean (± 1 SE) percentage flowering of F1 plants from different cross types.

DISCUSSION

Mating system of *Castilleja levisecta*

Castilleja levisecta appears to require out-crossing for reliable seed set because the species possesses barriers to self-fertilization. These barriers include protogyny in the flowers which may prohibit self-pollination in the field (but possibly not geitonogamy by insects), as well as genetic or physiological self-incompatibility. Although the mechanism of self-incompatibility is unknown, it appears to block seed set almost completely, with the exception that a small number of seeds may be produced in fruits from self-pollinations. We found that among 19 selfings, only 8 produced fruits with any filled seeds and these seeds generally numbered only 1 or 2, while in outcrossed plants fruits containing seeds were produced from all pollinations.

Self-incompatibility may be typical in the genus *Castilleja*. Although we found no published studies that specifically address compatibility within species of this genus, Heckard (1968), in his extensive study of polyploidy in *Castilleja*, stated that most species appear to be self-sterile and no seed is produced unless pollinators are present.

Many species of plants maintain a mixed-mating system in which both self- and cross-pollination serve to fertilize embryos. However, because *Castilleja levisecta* is nearly self-incompatible, the vast majority of seeds produced in its populations are likely from insect-mediated crosses between different individuals. This prediction is consistent with findings from a recent genetic study (Godt and Hamrick 2001) of allozyme diversity in *C. levisecta*, which found no evidence for inbreeding in its populations. The authors also noted that there could be strong selection against inbred individuals and our measurements of fitness traits of inbred plants support this assumption. In addition, heterozygosity is generally higher than expected in populations of *C. levisecta* (Godt and Hamrick 2001), and self-incompatibility and failure of flowering in inbred individuals could have contributed to this.

Effects of inbreeding and outbreeding on fitness

We found evidence for effects of crossing distance on fitness of *Castilleja levisecta* at multiple stages in the life cycle of the species. Both inbreeding depression and outbreeding advantage

(hybrid vigor) were detected. For example, inbreeding, including selfing and inbreeding among siblings (which, on average, share 50% of their alleles), reduced seed production and plant growth of the F1 generation significantly when compared to crosses between non-siblings from the same or different populations (Figures 4, 5 and 7). Inbreeding depression was not detected in seed germination or flowering rate, but this may be because of insufficient statistical power due to the small sample sizes available for the inbred treatments.

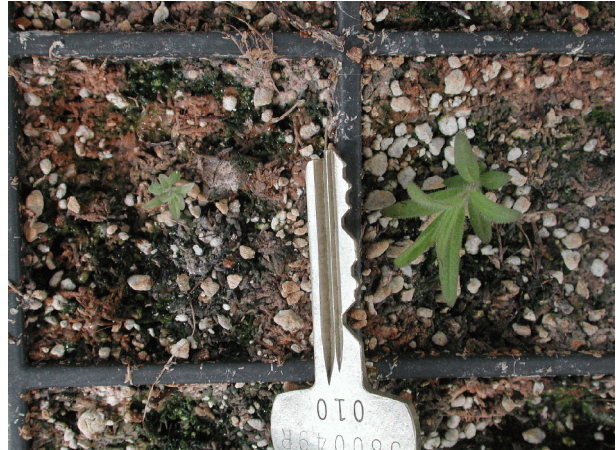


Figure 7. *Castilleja levisecta* individuals (30 days old) from self-pollination (left) and outbreeding (right).

One recent example of inbreeding depression (Richards 2000) in a perennial plant, white campion (*Silene alba*), showed that isolated populations had high inbreeding depression (in the form of low seed germination success), crosses between related individuals resulted in reduced germination success, and gene-flow was higher between unrelated individuals. This study is important because it demonstrates the potential for a “rescue-effect” for populations experiencing inbreeding depression by intentionally mixing unrelated individuals into such a population. Although inbreeding has not been detected in *Castilleja levisecta* populations to date (Godt and Hamrick 2002), small populations could experience a decline in seed production due to insufficient numbers of genetically compatible individuals. In addition, inbreeding depression could accumulate in very small populations in which the surviving members are genetically related. In either case, intentionally introducing pollen or individual plants from other populations (preferably nearby sites) could improve seed production and play a role in population recovery.

Outbreeding depression was not detected at any level in our study of seed set and F1 plant traits. Instead, we found significant increases in fitness of plants produced by crosses of individuals from different populations. In particular, plant growth and flowering rate were substantially higher in outbred plants (Figures 5 and 6). Crosses between plants from different populations produced F1 individuals that produced 25% more stem length, on average, than

plants from within-population crosses. Further, flowering was 1.1 to 4.6 times more likely in outbred plants than those from within population matings.

One concern about this study is that multiple populations were used for each treatment and pooled for analysis. For example, sibling crosses involved two different source populations, and their relative decline in fitness might best be compared only to their parents or other plants from the same population. One reason for this concern is that differences in heterozygosity among populations could affect the severity of inbreeding depression detected in sibling crosses. However, we believe that our analysis with pooled data is justified because the levels of heterozygosity in these populations were similar (and high, 0.174 at West Beach and 0.231 at False Bay; Godt and Hamrick 2002). In addition, measurements of plant fitness of parental plants of all crosses detected no significant differences in stem production or flowering (Kaye, unpubl. data). Another concern is that the crosses between populations to detect outbreeding effects could differ if the various pairs of populations crossed differed in their genetic similarity. For example, a between-population cross between two populations that were similar genetically might result in a different effect than a cross between two genetically distant populations. The pairs of populations used for crosses in our study all had high and similar genetic identities (0.93 - 0.94, Hamrick and Godt 2002), despite variability in their geographic distances (Table 2).

Although we did not detect outbreeding depression in the F1 generation, this phenomenon may yet act in *Castilleja levisecta*. Several studies (reviewed in Kaye 2001a and Hufford and Mazer 2003) have found that expression of outbreeding depression may be delayed to the F2 or F3 generations. Even so, it may not occur at all, and some studies have found no risk of outbreeding depression even for very long-distance crosses. For example, in a study of partridge pea (*Chamaecrista fasciculata*, an annual legume) Fenster and Galloway (2000) collected plants from various populations ranging from 100 m to 1000 km apart, performed controlled crosses, and grew the parents and progeny in common gardens. They found that first-generation hybrids between plants from different populations outperformed their parents, regardless of the geographic distance between sources. By the third generation, however, this increase in fitness declined. The level of decline varied with distance between parent populations, with crosses between plants from < 1000 km apart yielding third-generation

plants at least as vigorous as their original parents. Thus, crosses of up to 1000 km had a short-term beneficial effect, and little long-term risk (at least through the third generation).

There have been too few studies of out-breeding depression to make generalizations about the level of risk, however. Some studies have documented negative effects of outbreeding across short distances (tens of meters to 100 m) (Price and Waser 1979, Waser and Price 1989, 1991, 1994) or between different habitats (Montalvo and Ellstrand 2001), while others have found great variability in the effects of outbreeding, even in the same species (e.g., Waser et al. 2000). The threat of outbreeding depression is one argument against mixing seed sources during plant restoration (Kaye 2001a). It is also one of the dangers of moving plants a great distance to a restoration area where they could interbreed with a local population.

Outbreeding advantage may play a role in the formation of new species through hybridization in *Castilleja*, which is a process that has been documented in the genus (Heckard 1962), especially in the Intermountain West (Heckard and Chuang 1977). In addition, the presence of hybrid swarms in mixed species populations of *Castilleja* may be facilitated by the lack of inter-specific mating barriers (Anderson and Taylor 1983) and hybrid vigor of mixed species crosses.

Recommendations for recovery and reintroduction

1. If seed production is found to be low in any small, wild populations of *Castilleja levisecta*, the intentional importation of pollen or transplanted individuals should be considered to restore compatible mating types (or genetically unrelated individuals) to the population. This potential recovery action should be evaluated along with other explanations for low seed production, including insufficient pollination and resource limitation (due to climatic effects, interspecific competition, or other factors).
2. Seed collection for reintroduction projects should be managed to spread collections among many individuals and emphasize non-neighbors, if possible. Neighboring plants may be more genetically related than distant plants in the same population and more likely to cross-pollinate with each other, thus increasing the potential for inbreeding

depression. In addition, seeds from different individual plants should be stored separately.

3. Reintroduction projects should consider the genetic relationship of individuals in founder populations and take steps to minimize the chances that members of the new population are siblings or close relatives. Specifically, non-relatives should be planted close to one another and relatives should be kept distant.
4. Mixing plants from different source populations at reintroduction sites may improve the fitness of their progeny, at least in the short term. At this time, this recommendation should be considered only for adjacent populations in regions where the species is still extant (e.g., Whidbey Island or San Juan Island) and when there is some suggestion that the population is declining or is already very small, or in areas where the species has been extirpated (such as the Willamette Valley) so that any risks of outbreeding depression and local genetic contamination are minimized.

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