# HOMOPLOID HYBRID SPECIATION IN A RARE ENDEMIC *CASTILLEJA* FROM IDAHO (*CASTILLEJA CHRISTII*, OROBANCHACEAE)

by

Danielle Leigh Clay

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Danielle Leigh Clay

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The following individuals read and discussed the thesis submitted by student Danielle Leigh Clay, and they evaluated her presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

| | |
|---|---|
| James F. Smith, Ph.D. | Chair, Supervisory Committee |
| Stephen J. Novak, Ph.D. | Member, Supervisory Committee |
| Marcelo D. Serpe, Ph.D. | Member, Supervisory Committee |
| David C. Tank, Ph.D. | Member, Supervisory Committee |

The final reading approval of the thesis was granted by James F. Smith, Ph.D., Chair of the Supervisory Committee. The thesis was approved for the Graduate College by John R. Pelton, Ph.D., Dean of the Graduate College.

# DEDICATION

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## ABSTRACT

Evidence to support the origins of a putative hybrid species with certainty must be determined using several lines of evidence: the presence of genetic additivity of parental marker alleles in a putative hybrid species, along with ecological or niche separation. Novel or transgressive morphological traits obtained through chromosomal rearrangements during hybridization may facilitate niche separation of the hybrid species from progenitor habitats. These evolutionary processes together enforce reproductive isolation and promote an independent evolutionary trajectory in hybrid species. By studying these evolutionary processes in putative hybrid species, researchers may identify hybrid species with confidence.

We employed multiple lines of evidence to examine a putative hybrid origin in the rare endemic *Castilleja christii*, which is known from only one population on 80 hectares at the summit of Mt. Harrison, Cassia Co., Idaho. We utilized granule-bound starch synthase II (*waxy*) to initially address hybridization between *Castilleja christii* and widespread species *C. miniata* and/or *C. linariifolia* in an area of sympatry. We aligned cloned sequences from all three *Castilleja* species and scored all direct sequenced individuals based on this alignment for the presence, absence, or a combination of species-specific indels and/or substitutions. Interestingly, all 230 direct-sequenced *Castilleja christii* individuals had no unique alleles, and contained both *C. miniata* and *C. linariifolia* sequences within their genomes, indicating that *C. christii* is likely of hybrid

origin. Morphologically, ANOVA and discriminant functions analyses tested among all three *Castilleja* species for 33 morphological characters revealed that *C. christii* shared traits with both parents while also displaying characters that were unique and transgressive. Ecological data were collected to address whether phenology, spatial, and/or ecological differences provide barriers to hybridization between the three sympatric *Castilleja* species at the summit of Mt. Harrison. Pollen mother cells were collected from all three *Castilleja* species at the summit to address cytological differences and the potential of polyploidy to act as a barrier to hybridization. All three taxa were found to be diploids (2N = 24). All three *Castilleja* species associated with different plant communities, were spatially distinct, and were found growing on different aspects of the summit. Based on these lines of evidence, we conclude that *Castilleja christii* is a stabilized homoploid hybrid derivative of *C. linariifolia* and *C. miniata* and is likely following an independent evolutionary trajectory from its progenitors.

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# CHAPTER 1: GENERAL INTRODUCTION

The evolution of populations has traditionally been examined in light of standing genetic variation and mutational input. Variation within and among populations can also arise via other evolutionary forces, such as hybridization or polyploidy. In this introduction, I briefly review the current and historical theories and experimental findings regarding hybridization, polyploidy, and speciation in plants. This is not an exhaustive review, but serves to contextualize my thesis research. I then review the main objectives and goals of the study, provide background information on the study species and study areas, and explain site-selection criteria.

## Theoretical Background

### Species Concepts

Studies of hybridization and speciation rely on the idea of what constitutes a species. Many theoretical species concepts exist, and each is generally specific to the types of questions researchers ask. The morphological species concept (Grant, 1981) is widely used in plant studies and denotes a species as "an assemblage of morphologically similar individuals that differs from other such assemblages." While this concept may be practical for field biologists, it is subjective, as different field biologists may emphasize different characters. Particularly in instances of cryptic hybridization, the morphological species concept would not recognize hybrids that closely resemble their progenitors, even if these were auto or allopolyploids that were reproductively isolated.

The biological species concept (Mayr, 1942) posits that a species is "a group of interbreeding (or potentially interbreeding) populations that are reproductively isolated from other such groups" and is a popular concept in studies of animal speciation. It is problematic in plants due to rampant hybridization and many instances of asexual reproduction (e.g., agamospermy). Many systematists oppose the biological species concept on the grounds that the ability to cross is not a sound feature to unite a biological species, as intercrossability is symplesiomorphic (reviewed in Soltis and Soltis, 2009).

The evolutionary species concept (Simpson, 1961; Wiley, 1978; Mayden, 1997) recognized species on the grounds of having a "unique evolutionary role, tendencies and historical fate." Hybridization is accommodated within this concept, as long as parental progenitors do not coalesce into one species (Soltis and Soltis, 2009). Similarly, in the absence of the parental progenitors' merging, instances of allopolyploidy or homoploid hybrid speciation yielding new species are in agreement with this species concept, as they would have their own evolutionary fates.

Perhaps the most widely accepted species concept in plants is the General Lineage Theory (De Queiroz, 1998, 2007). The General Lineage Theory incorporates other species concepts as evidence for speciation, and maintains that many different processes (e.g., natural selection, mutation, migration, and genetic drift) may occur at different times along the evolutionary path of a species. This theory provides a unified concept that states that "…species [are] separately evolving metapopulation lineages," and serves as a standard by which populations or species can be considered. Other processes involved in speciation can then be defined as subcategories or further lines of evidence to trace the trajectory of speciation (e.g., a monophyletic species, reproductively isolated species,

ecologically divergent species, morphologically distinct species). This concept fully incorporates speciation as a product of hybridization (homoploid hybrid speciation or allopolyploidy); as long as there is evidence to support lineages as evolving separately, they could be considered species unto themselves.

Interspecific Hybridization

Hybridization has historically been associated with studies seeking to understand how taxa are, or have become, reproductively isolated. In recent decades, hybridization has been alternatively viewed as an adaptive force within populations, which can lead to an increase in genetic variation and diversification among populations (Lewontin and Birch, 1966; Whitham, 1989; Mecham, 1960; Nagle and Mettler, 1969; Moore, 1977; Key, 1968; Barton, 1979). Hybrids can have varying levels of fitness depending on their fertility, and this dictates duration and adaptive significance of a hybrid within a community (Barton and Hewitt, 1985). Even when hybridization is rare, it can have important adaptive implications: increased gene flow across species boundaries may increase the chances of introgression (Anderson, 1949; Rieseberg and Wendel, 1993; Ellestrand et al., 1999; Martinsen et al., 2001), which in turn may produce an increase in genotypic or phenotypic diversity among and within populations due to increased allelic variation (Rieseberg et al., 2003). Over time, this may allow for the formation of new species or evolutionary lineages (Grant, 1981; Arnold, 1997; Rieseberg and Carney, 1998; Arnold et al., 1999). Though hybridization was initially thought to be rare in nature (Roberts, 1929), it has been hypothesized that between 40 and 80 percent of all angiosperm species have arisen via hybridization events (Grant, 1981; Whitham et al.,

1991; Arnold, 1994), further demonstrating the adaptive potential and evolutionary significance of this phenomenon.

Though hybridization is arguably important in the evolutionary history of plant species, it can also be genetically threatening to rare species. Gene flow via pollen or seed into a small population can affect small populations more than larger ones. Through hybridization, the alleles of the rare species could be effectively swamped by gene flow from another species. Over time, this could lead to loss of rare individuals, and eventually extinction of the rare species, as its alleles become diluted with those of the congener. If fertile offspring were not found to have a reduction in fitness, they could potentially compete for the same resources and thus outcompete and displace the rare species (Carney et al., 2000). The loss or breakdown of rare species can be rapid (Carney et al., 2000) or may take thousands of years (Goodfriend and Gould, 1996). Alternatively, if a rare species were more fertile than a common species, hybridization may threaten the common species with localized extinction (Anttila et al., 1998).

Hybrid individuals are often first observed in the field via morphological intermediacies of parental types (Anderson, 1949); however, because of potential backcrossing between generations of hybrids with parental species, hybrid individuals may not possess intermediate traits of both parental types (Rieseberg and Ellestrand, 1993; Burke and Hamrick, 2002). Whether hybridization is the cause of phenotypic diversity merits investigation into the causes of hybrid fitness and the mechanisms of hybrid zone formation and stability. Researchers have proposed many models to describe the dynamics of hybrid zones (Dobzhansky, 1940; Key, 1968; Remington, 1968; Endler, 1977; Moore, 1977; Barton, 1979; Barton and Hewitt, 1985; Harrison, 1986; Moore and

Price, 1993; Arnold, 1997; Campbell and Waser, 2007; Wang et al., 1997), each of which describe different fitness scenarios of hybrids and parental progenitors within the hybrid zone and how the environment and selection act on the hybrid population. A combination of environmental factors, natural selection, and hybrid fitness dictate how broad or narrow a hybrid zone can be, and are the tenets of the models noted above. For example, if hybrid fitness is determined via intrinsic or genetic circumstances alone, hybrids would likely be inviable after recombination (i.e., endogenous selection), thus the hybrid zone would be narrow (Campbell and Waser, 2007). Alternatively, if alleles generated during recombination were favored by selection in the environment (i.e., exogenous selection), hybrid individuals may exhibit varying degrees of fertility and may backcross with parental species, resulting in introgression and a potentially wide array of genotypes (Rieseberg et al., 1999; Jiggins and Mallet, 2000; Barton, 2001) and broader zones of hybridization. For these reasons, natural hybrid zones offer a unique opportunity to study the environmental effects on the genetic architecture of hybridizing taxa in the field, because a hybrid zone with fertile hybrids offers potentially hundreds of generations of recombination (Rieseberg et al., 1999).

## Polyploidy

Polyploidy, having two or more entire sets of genomes per cell, has been touted as an important method of speciation in plants, as it provides reproductive isolation due to differences in chromosome number (Müntzing, 1936; Clausen et al., 1945; Stebbins, 1947, 1950, 1971; Masterson, 1994). Polyploid individuals arise via genome duplication events that are achieved either by chromosome doubling or the fertilization of unreduced gametes (Soltis and Soltis, 2009). Approximately 50-70% of all angiosperm taxa are

hypothesized to have polyploid origins or have undergone genome duplication events (Grant, 1981; Levin, 1983; Masterson, 1994; Leicht and Bennett, 1997). There are two main forms of polyploidy: autopolyploids are formed via single genome duplication, while allopolyploids experience genome duplication post interspecific hybridization (Ramsey and Shemske, 1998). Polyploidy can occur within species or even within lineages of the same species on multiple occasions (Soltis and Soltis, 2009).

Polyploidy in plants has been historically attributed to allopolyploidy (Levin, 1983); however, recent evidence indicates that autopolyploidy may be underestimated as it is difficult to detect (Soltis et al., 2007). Interspecific hybridization resulting in allopolyploidy has been an important mechanism of diversification in angiosperms due to dramatic structural changes that take place in response to genome duplication and differences in gene expression due to new allelic variation produced during hybridization (Soltis and Soltis, 2009). This new allelic variation may allow the resultant allopolyploid lineage to outcompete or occupy niches novel to their progenitor species. Further, the importance of polyploidy in the diversification of angiosperms has been suggested to have been influential in the origins of eudicots and angiosperms (Buzgo et al., 2005; De Bodt et al., 2005; Soltis et al., 2009; Soltis and Soltis, 2009).

## Hybrid Speciation

Hybrids may become species through allopolyploidy or via homoploid hybrid speciation, in which hybrids share the same chromosome number as their progenitors. Allopolyploidy was traditionally thought to be more prevalent than homoploid hybrid speciation (Stebbins, 1950; Ramsey and Schemske, 1998; Rieseberg and Carney, 1998; Soltis and Soltis, 2000; Mallet, 2007), though the perceived rarity in homoploid hybrid

speciation events may be because natural homoploid hybrids are difficult to detect (Wolfe et al., 1998; Ferguson and Sang, 2001; Mallet, 2007). Whether derived via allopolyploidy or through homoploid hybrid speciation, hybrids are considered true species when they are ecologically distinct and reproductively isolated from their parental progenitors and maintain this over time (Hegarty and Hiscock, 2005).

Reasons why homoploid hybrid species are difficult to detect can be attributed to the complexity of reproductive isolating mechanisms, which require intensive study and multiple lines of evidence. Several models exist to explain how hybrids become reproductively isolated in sympatry despite a shared ploidy level with their progenitors. The most widely accepted model is Grant's (1958) 'recombinational speciation,' where the two parental species differ by two or more chromosomal rearrangements. Early work by Müntzing (1930) influenced this model. Unequal crossing over during meiosis in examples of hybridization generally yields sterile offspring. Müntzing supposed that chromosomal rearrangements in later generation hybrids could lead to novel combinations of chromosomal sterility factors via chance, yielding a fertile, stabilized hybrid species, reproductively isolated from its parents in sympatry due to a chromosomal incompatibility despite a common ploidy level. Alternatively, or concomitantly with chromosomal rearrangements, genic sterility factors could also lead to reproductive isolation in hybrid species. Therefore when testing the model of recombinational speciation (Grant, 1958) both chromosomal arrangements and genic factors are generally explored in a hypothesized homoploid hybrid speciation event (Rieseberg, 1997).

Another method of reproductive isolation between hybrids and parental progenitors is 'transgressive segregation' (Grant, 1975), in which novel combinations of parental alleles in hybrids may allow them to become ecologically distinct in niches unoccupied by their parents (Tanksley, 1993; Rieseberg et al., 1999). This process is driven by the adaptive potential of the combination of alleles from both parents producing extreme or 'transgressive' phenotypes in hybrids (Rieseberg, et al., 1999; Hegarty and Hiscock, 2005), and has been empirically demonstrated in natural and artificial *Helianthus* hybrids through a comparison of adaptive quantitative trait loci (QTL) (Rieseberg et al., 2003). In this example, the hybrid origins of three diploid *Helianthus* species (*H. anomalus, H. deserticola*, and *H. paradoxus*) were examined by crossing the supposed parent species, *H. annuus* and *H. petiolaris* in controlled crosses. The three diploid species of hybrid origin occupy niches novel to their parents: *Helianthus anomalus* is adapted to sand dunes, *H. deserticola* occupies desert basins, and *H. paradoxus* is found in salt marshes. The phenotypes of these species were successfully resynthesized in the laboratory and were discovered to tolerate the same extreme ecological niches as the hybrid species they morphologically resembled. When analyzed using QTL mapping, each synthetic hybrid matched the adaptive chromosomal segments in the natural hybrids (Rieseberg et al., 1993). This research elegantly demonstrates the correlation between adaptive QTLs and extreme phenotypes generated by hybridization and chromosomal rearrangements, and how this combination allows hybrids to become reproductively or spatially isolated from their parental progenitors and each other via survival in novel or extreme ecological niches (Rieseberg et al., 1993).

## Thesis Research

The objective for my thesis research was to characterize a putative hybridization event in a rare endemic species of *Castilleja* (*C. christii* N. Holmgren, Orobanchaceae) with sympatric diploid species *C. miniata* Gray and *C. linariifolia* Benth. I investigated this event using morphological, molecular, ecological, and cytological analyses. Specifically, I was interested in the degree of hybridization, if hybrids could be characterized based on phenotypic traits, and whether hybrids were ecologically distinct from their progenitors. When molecular research indicated that *C. christii* and all field identified putative hybrids were genetically identical and shared the genomes of *C. miniata* and *C. linariifolia*, the focus of my research became to characterize a homoploid hybrid speciation event using data collected to initially address hybridization.

### Background to Study System

The genus *Castilleja* Muntis, commonly referred to as paintbrush, is a member of the Orobanchaceae, a family of hemi- and holoparastites included in the Lamiales (Olmstead et al., 1993; Olmstead et al., 2001). This genus includes approximately 180 annual and perennial herbaceous species, and is found throughout North, Central, and South America, with the highest concentration of species occurring in the western United States (Tank and Olmstead, 2008).

Speciation and diversification within this genus is generally attributed to the ease with which species hybridize and experience subsequent genome duplication (allopolyploidy; Ownbey, 1959; Heckard, 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Chuang and Heckard, 1993). Consequently, the base haploid chromosome number for the genus is $n = 12$, with wide ranging ploidal variation both

within and among species (Heckard and Chuang, 1977). Some *Castilleja* species are polyploid, though it is unclear if these species were generated via allo- or autopolyploidy (Heckard and Chuang, 1977; Heckard et al., 1980). Further, ploidal variation among sympatric *Castilleja* species has been suggested as a barrier to reproduction (Heckard and Chuang, 1977; Hersch and Cronn, 2009).

Hybridization and polyploidy have been attributed to the complexities in morphology within the genus; determining *Castilleja* species in the field can be difficult, as species overlap in almost every morphological character and the genus as a whole is presumed to be recently diverged (Holmgren, 1984). Egger (1994) notes that because hybridization in *Castilleja* is well-known and may occur frequently in areas of sympatry, the validity of parental species descriptions should not be called into question and instead should be strictly interpreted, with hybrids being recognized based on intermediate morphological variation. Hybridization in *Castilleja* can be widespread, where introgression between hybrids and parents produces a complete breakdown of morphological species distinctions in areas of parental overlap; or hybridization may be more localized, with F1 hybrids occurring only rarely among their progenitors (Heckard, 1968; Heckard and Chuang, 1977; Egger, 1994).

Species in the subtribe Castillejinae are hemiparasitic (in contrast to other members of the family Orobanchaceae, which are holoparasites). They have the ability to photosynthesize and can also acquire solutes, water, and defense compounds from their hosts (Hansen, 1979; Stermitz and Harris, 1987; Adler and Wink, 2001). Luna (2005) suggested that *Castilleja* is not host specific. The hemiparasitic nature of *Castilleja* and the relationships of species with their hosts may influence certain morphological traits

(e.g., stem branching, stem height; Holmgren, 1971), which can contribute to complications in the evaluation of the origins of a hybrid species when exploring this hypothesis using morphology or chemistry.

Study Species and Field Sites

Species observed during this master's thesis include the rare endemic Christ's paintbrush (*Castilleja christii*) and two sympatric *Castilleja* species, *Castilleja miniata* and *C. linariifolia*. *Castilleja christii* is critically imperiled, and at high risk of extinction due to extreme rarity, therefore it has a G1 global rank (CPC, 2005). Additionally, *Castilleja christii* is considered a candidate species for Federal Endangered Species Status by the U.S. Fish and Wildlife Service (CPC, 2005). *Castilleja miniata* and *C. linariifolia* have widespread distributions and multiple ploidy levels are reported (*C. miniata*: $n$ = 12, 24, 48, 60; *C. linariifolia* $n$ = 12, 24; Heckard and Chuang, 1977). The ploidy level for *Castilleja christii* was formerly unknown, however using chromosome counts, we discovered *C. christii* is a diploid (Chapter 4).

*Castilleja christii* was first described by Noel Holmgren in 1973, who noted the bracts of this species were yellow to yellow-orange, and are different than bracts of *C. miniata* and *C. linariifolia,* which are generally red or reddish-orange (Holmgren, 1984). Field based observations of intermediate forms between *Castilleja christii* and other congeneric species led us to explore putative hybridization events occurring on Mt. Harrison.

Data were collected for this thesis at several field locations across Idaho (Figure 1.1). Field sites were chosen based on the presence of one or more species of interest. Because we were first interested in hybridization between *Castilleja christii* and *C.*

*miniata* and/or *C. linariifolia*, sites isolated from the population of *C. christii* were chosen as controls to maximize detection of species-specific molecular markers and morphological traits. The criteria associated with the selection of control sites were based on presence of a particular parental species and the presence of at least 25 plants in a 25 meter radius. Only one species per plot were sampled at control sites (N). Control sites were established: on lower portions of Mt. Harrison, Cassia Co, ID (N = 1); Mt. Independence, Cassia Co, ID (N = 2); Wildhorse Campground near Mt. Borah, Custer Co., ID (N = 1); the Cotterell mountains, Cassia Co., ID (N = 1); and the Boise National Forest, Boise Co., ID (N = 2).

The single field site for *Castilleja christii* was on 80 hectares at the summit of Mt. Harrison, ID (Cassia Co.) and is inclusive of the entire range of *C. christii*. Here, putative parental populations of *C. miniata* and *C. linariifolia* sympatric with *C. christii* were collected during the summer of 2009.

Plot Establishment

Plots at both control sites and at the summit of Mt. Harrison were circular, 25 meters in diameter, and 25 plants were sampled within each plot. Occasionally, more than 25 individuals were collected per plot to increase the number of total samples. A center point was established at each plot and its coordinates were determined using GPS. A wooden stake was driven into the ground at each center point and was flagged. Elevation, slope, aspect, soil type, weather, and a brief description of plant cover within the plot were recorded. Five, one $m^2$ micro-plots were established per plot, one at center point and the others 12 meters from the center point in each cardinal direction to estimate percent cover of woody plants, forbs, and grasses. This procedure also allowed us to assess

differences in structural characteristics between plots, both within and outside the range of *Castilleja christii*. (See Chapter four for specific methodologies pertaining to ecological sampling.)

Seven plots (n) at control sites were established (*C. miniata,* n = 4; *C. linariifolia*, n = 3). Three types of plots were established at the Mt. Harrison site, with number of plots following each type: (A) plots containing only *C. christii* (n = 4); (B) plots containing *C. miniata* and *C. christii* (n = 7); (C) plots containing *C. linariifolia* (n = 1); and (D) plots containing *C. christii* and *C. linariifolia* (n = 1). In total, 13 plots on Mt. Harrison were established. To sample as widely as possible throughout the range of *Castilleja christii*, plots were established at least 100 m apart. We hoped to have equal sample sizes at the Mt. Harrison site for both *C. linariifolia* and *C. miniata*; however, *C. linariifolia* was only in a few small patches growing sympatrically with *C. christii*. One plot at the summit was approximately 150 meters from any *C. christii* plants, but was not sampled as a control plot as the plot was close enough for potential cross-pollination.

Once plots were established, transects were run from the center point in random directions, extending out 25 meters. Random directions were determined by one person spinning a compass dial, and the other person telling them when to stop. Occasionally, transects were not established randomly, and individuals were collected opportunistically to increase sample size. Only one species per transect was sampled. Plants were measured in a one meter belt transect, and all plants were flowering when measured. If at the end of a transect, 25 individuals were not sampled, another randomized transect would be run.

## Thesis Organization

This thesis is organized based on each line of evidence we used to support our hypothesis of homoploid hybrid speciation in the rare endemic *Castilleja christii.* Chapter Two is titled: "Using Single-Copy Nuclear Genes to Evidence Homoploid Hybrid Speciation in a Rare Species of *Castilleja*". We utilized granule-bound starch synthase II (*waxy)* to initially address hybridization between *Castilleja christii* and *C. miniata* and/or *C. linariifolia* in an area of sympatry. We aligned cloned sequences from all three *Castilleja* species and scored all direct sequenced individuals based on this alignment for the presence, absence, or a combination of species-specific indels and/or substitutions. Interestingly, we found that *Castilleja christii* contains both *C. miniata* and *C. linariifolia* sequences within its genome, supporting a hybrid origin in *C. christii*. Cytological analyses indicated that all three *Castilleja* species are diploid at the summit of Mt. Harrison and morphological analyses separate the three species based on a combination of floral characteristics and transgressive traits in *C. christii*. Further, our evidence suggests that *Castilleja christii* is a homoploid hybrid species between *C. miniata* and *C. linariifolia*, which is a novel discovery in *Castilleja.*

Chapter Three is titled: "Morphological Examination of Homoploid Hybrid Speciation in the Rare Endemic *Castilleja christii* (Orobanchaceae)." In this chapter, we determine whether morphological data corroborate molecular results (i.e., homoploid hybrid speciation in *Castilleja christii*), whether *C. christii* can be considered a distinct species based on morphology alone, and what morphological characters are important for the identification of all three sympatric species. We found that three taxa are evident from our analyses, and *Castilleja christii* has several traits that are transgressive from other

sympatric *Castilleja*; however, morphology alone does not negate the possibility of ongoing hybridization. Along with data from other chapters, we have evidence to support *Castilleja christii* as being a homoploid hybrid species between *C. miniata* and *C. linariifolia.* A key to species is also included for ease in identification of *Castilleja* at the summit of Mt. Harrison.

Chapter Four is titled: "Phenological, Ecological, and Spatial Differences Contributing to the Evolutionary Success of the Homoploid Hybrid *Castilleja christii* (Orobanchaceae)." Field data were collected to address the potential for phenology and/or environmental differences to provide barriers to hybridization between three sympatric *Castilleja* species at the summit of Mt. Harrison. Additionally, pollen mother cells were collected from all three *Castilleja* species at the summit to address cytological differences and the potential of polyploidy to act as a barrier to hybridization. All three taxa were found to be diploids (2N = 24). All three *Castilleja* species were found to be ecologically distinct in the field in that they associate with different plant communities, and are spatially distinct and were found growing on different aspects of the summit; this indicates that *C. christii* has established itself ecologically from its progenitors. Anecdotal evidence suggests that the flowers of *C. christii* open earliest at the summit, followed by *C. miniata*, and lastly *C. linariifolia*, lowering the chances of the genome of *C. christii* becoming swamped by its progenitors via gene flow. Additionally, *C. christii* had a much higher germination rate in the lab than either of the other *Castilleja* species, indicating that it is highly fertile and may compete successfully with (or even outcompete) *C. miniata* and *C. linariifolia* in an area of sympatry.

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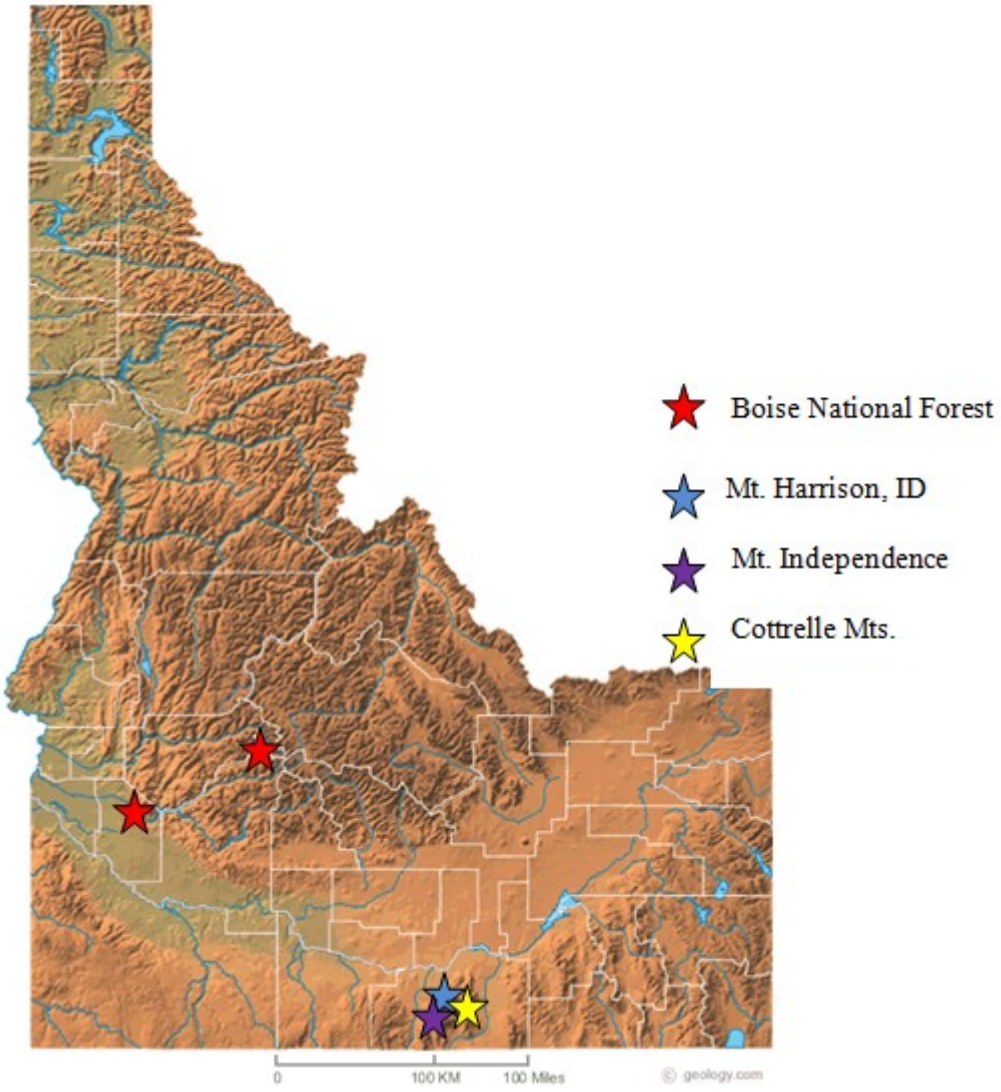


**Figure 1.1 Map of study area and locations sampled while exploring a potential hybrid origin of endemic *Castilleja christii* between *C. miniata* and *C. linariifolia*. The single *Castilleja christii* site sampled was on Mt. Harrison, Cassia Co., Idaho (purple star). Sites outside of the range of *Castilleja christii* (other stars) were sampled to obtain control specimens, which were utilized for species-specific bands in molecular analyses.**

# CHAPTER 2: USING SINGLE-COPY NUCLEAR GENES TO EVIDENCE HOMOPLOID HYBRID SPECIATION IN A RARE SPECIES OF *CASTILLEJA*

## Introduction

Interspecific hybridization followed by reproductive isolation of hybrid offspring from their progenitors has been considered of central importance in plant speciation (Grant, 1981; Abbott, 1992; Arnold, 1997; Rieseberg, 1997; Soltis and Soltis, 2009). Hybrids can become reproductively isolated from their parents via either a change in ploidy (i.e., allopolyploidy) or may become reproductively isolated while sharing a common ploidy level (homoploid hybrid speciation). While polyploidy is relatively common in plants with estimates ranging from 30 to 80% of species containing polyploid genomes (Leicht and Bennett, 1997), homoploid hybrid speciation is thought to be rare, with only a few plant species rigorously tested to verify they originated in this way (Rieseberg, 1997; Gross and Rieseberg, 2005; James and Abbott, 2005).

Polyploidy is relatively easily maintained. Hybrids share parental genotypes, yet are reproductively isolated from their parents by having double the amount of genetic material. Allopolyploids are also easily detected through combinations of chromosome counts and molecular markers (Soltis and Soltis, 2009). In contrast, homoploid hybrid speciation is considered rare because of the implications associated with the formation of hybrid species: hybrids must become genetically isolated from parental species while sharing the same chromosome number (Rieseberg, 1997). Hybrids can achieve this

through transgressive segregation (Grant, 1975) from parental progenitors, and through genetic recombination (Grant 1980; Rieseberg, 1997). Early work by Müntzing (1930, 1934, 1938) and later work by Stebbins (1942, 1945, 1950), Grant (1958, 1963, 1981), Templeton (1981) and McCarthy et al. (1995) influenced the 'recombinational speciation' model (Grant, 1958). Genomes of two hybridizing species may be dissimilar, leading to unequal crossing over during meiosis in their hybrids. The recombinational model (Grant, 1958) proposes that if the parental taxa differ by two or more chromosomal rearrangements, their hybrids would be heterozygous and partially sterile, as 75% of their gametes would be unbalanced and therefore inviable. This reduction of fertility in the F1 hybrids will constitute a barrier to introgression with parental species, however backcrossing and introgression between F1 hybrids may yield novel, chromosomally balanced genotypes with restored fertility and recombinant karyotypes in later hybrid generations (Rieseberg, 1997). These fertile offspring would be partially resistant to introgression with parental taxa and, because of genetic recombination, may move toward speciation if other factors aiding their survival are in place.

The recombinational model of speciation has several supplementary implications for hybrid species' successful establishment. One challenge is escape from and establishment outside of the hybrid zone. The consequences of remaining within the hybrid zone involve: 1) genetic swamping by parental progenitors via backcrossing and recombination (Barton, 2001), 2) a lack of viable offspring from intraspecific crosses in the hybrid species, or 3) F2 offspring that become outcompeted by parental genotypes within the community (Abbott et al., 2003). Therefore, hybrids that are ecologically isolated from parental species have a better chance at successful establishment and

persistence (Templeton, 1981). Similarly, if a hybrid species has a fitness advantage over parental species (McCarthy et al., 1995), or is self-compatible, which would unify unbalanced gametes and become paired and balanced through crossing over in meiosis, a hybrid derivative may become stabilized and may compete effectively with parental types (Grant, 1981; McCarthy et al., 1995). Lastly, if there is a selective advantage for the hybrid phenotype, homoploid hybrid speciation can occur in obligate outcrossing species (McCarthy et al. 1995).

Newly formed hybrid species may achieve higher levels of fitness or isolation from parental taxa through 'transgressive segregation' (Grant, 1975) or 'external isolation' (Grant, 1981), where novel combinations of parental alleles in hybrids may allow them to become ethologically or geographically distinct in niches unavailable to their parents (Grant, 1981; Rieseberg, Whitton, and Gardner, 1999; Abbott et al., 2010). This process is driven from the adaptive potential of the combination of alleles from both parents to produce extreme or 'transgressive' phenotypes in hybrids (Rieseberg, Archer and Wayne, 1999; Hegarty and Hiscock, 2005), and has been empirically evidenced in natural and artificial *Helianthus* hybrids (Rieseberg et al., 2003), *Gossypium* (Wendel et al., 1991; reviewed by Wendel and Cronn, 2003) and other genera (e.g., Wolfe et al., 1998; Ferguson and Sang, 2001; Wang et al., 1997; Howarth and Baum, 2005; James and Abbott, 2005; Mir et al., 2006; Poke et al., 2006). Several authors have made clear that both recombinational speciation and external isolation are equally important in the successful formation and establishment of a homoploid hybrid species and that homoploid hybrid speciation is unlikely to occur without partial ecological and/or spatial

separation from progenitor species (Rieseberg, 1997; Buerkle et al., 2000; Abbott et al., 2010).

Difficulty in detecting homoploid hybrid speciation may be due to the complexities in the formation of diploid hybrid species because many hybrids are subject to sterility or hybrid breakdown, but also because homoploid hybrids are difficult to detect, especially if the species is not recently derived (Rieseberg, 1997; Ferguson and Sang, 2001). Hybrid species are often first identified in the field using morphology, however this can be misleading, as morphological resemblance does not always denote a close evolutionary relationship (Maki and Murata, 2001) and morphological characters are more likely than molecular markers to diverge rapidly after speciation (Rieseberg and Ellstrand, 1993; Rieseberg and Morefield, 1994). For these reasons, molecular markers used along with morphological characters may obtain a clearer picture of homoploid hybrid speciation events. Several studies of homoploid hybrid plant species have been confirmed using molecular markers (Rieseberg, 1997; Abbott et al., 2000; Harris, 2002; James and Abbott, 2005). Molecular markers have disproved homoploid hybrid hypotheses in other studies (Rieseberg et al., 1990; Spooner et al., 1991; Wolfe and Elisens, 1993; Dubouzet and Shinoda, 1999; Maki and Murata, 2001).

Initial molecular work into the exploration of homoploid hybrid speciation began by utilizing isozyme marker technologies, which rely on the detection of a combination of parent-specific alleles in a hybrid individual (Soltis and Kuzoff, 1995; Abbott et al., 2000; Maki and Murata, 2001). More recently, bi-parentally inherited nuclear DNA gene regions such as the internal transcribed spacer (ITS) and intergenic spacer (IGS) regions of ribosomal DNA have been used to support homoploid hybrid hypotheses (Abbott and

Lowe, 1996; Rieseberg et al., 1996; Baumel et al., 2002; Kelly et al., 2009). These regions are rapidly evolving and display high rates of mutation, as they are transcribed and not translated, and have been used to determine the origins of hybrid species by comparing phylogenies of related taxa. For example, a hybrid between two parental species inherits one copy of each parental nuclear genome, therefore in PCR two copies are detected per individual. Hybrids are detected phylogenetically because each copy of the parental genome in the hybrid would cluster with each respective parent in a tree. However, if a hybrid were older, it may have already undergone mutations, recombination, concerted evolution, or drift that would potentially confound the detection of its hybrid origin (Ferguson and Sang, 2001; Àlvarez and Wendel, 2003; Tank and Olmstead, 2009).

Nuclear markers have also been used in conjunction with maternally-inherited chloroplast DNA to detect species of hybrid origin by comparing incongruence between gene trees (e.g., Soltis and Kuzoff, 1995; McKinnon et al., 2001; Okuyama et al., 2005; Kim et al., 2008). However, the susceptibility of nuclear ribosomal DNA to concerted evolution and the often relatively low variation within the chloroplast genome at the intraspecific level have limited the precision with which homoploid hybridization can be identified (Kim et al., 2008). Further, even with well-supported tree topologies where incongruence may infer a hybridization event, these phylogenetic patterns can result from many alternative processes, such as recombination between alleles or genes, lateral gene transfer, incomplete lineage sorting, or orthology/paralogy conflation (Frajman et al., 2009).

To avoid the potential of concerted evolution and to distinguish between hybridization and other events influencing discordance among gene trees with confidence, low-copy or single-copy nuclear genes have been used successfully in phylogenetic studies of allopolyploids (e.g., Cronn et al., 1999; Ferguson and Sang, 2001; Popp and Oxelman 2001; Doyle et al., 2003; Mason-Gamer et al., 2004; Popp et al., 2005, Huber et al., 2006) and some diploids (e.g., Wendel and Cronn, 2003; Howarth and Baum, 2005; Joly and Bruneau, 2006; Poke et al., 2006). Like ITS and IGS, low-copy and single-copy nuclear genes are biparentally inherited; however, they are less likely to become homogenized over time (concerted evolution), aiding in the detection of hybridization or hybrid speciation events (Tank and Olmstead, 2009).

It is desirable to use a combination of low-copy nuclear genes and chloroplast DNA or other unlinked DNA sequence regions when approaching evolutionary questions using molecular markers, as an increase in marker sample size may increase the accuracy of the study, aiding in resolution and support. Moreover, the combination of maternally and bi-parentally inherited markers is valuable in studies of hybridization, as these may be used to distinguish between other stochastic events such as gene duplication, recombination, and lineage sorting within the genome (Pamilo and Nei, 1988; Linder and Rieseberg, 2004; Frajman et al., 2009), can be used to infer rates of introgression and hybridization (i.e., Arnold, 1993; Brubaker et al., 1993; Welch and Rieseberg, 2002) and have been used to confirm species of hybrid origin (Rieseberg and Soltis, 1991; Wendel et al., 1991; Wolfe et al., 1998; Mallet, 2007).

Speciation and diversification within the genus *Castilleja* Muntis is generally attributed to the ease with which species hybridize and experience subsequent genome

duplication (allopolyploidy; Ownbey, 1959; Heckard 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Chuang and Heckard, 1993). The base haploid chromosome number for the genus is $n = 12$, with wide ranging ploidal variation both within and among species (Heckard and Chuang, 1977). Some species are polyploid themselves, though it is unclear if these species were generated via allo- or autopolyploidy (Heckard and Chuang, 1977; Heckard et al., 1980). Ploidal variation among sympatric *Castilleja* species has been suggested as a barrier to reproduction (Heckard and Chuang, 1977), which may be the only barrier to hybridization in zones of sympatry (Holmgren, 1984) other than contextual pollinator-mediated selection (Hersch and Roy, 2007). Hybridization events within the genus have largely been inferred from observations of morphological intermediacy in the field and verified via cytological studies in the lab or with molecular markers (Ownbey, 1959; Holmgren, 1984; Heckard, 1968; Heckard and Chuang, 1977; Egger, 1994; Hersch and Roy, 2007; Hersch-Green and Cronn, 2009).

*Castilleja christii* N. Holmgren is a rare endemic restricted to 80 hectares at the summit of Mt. Harrison, in southeastern Idaho, and is at high risk of extinction due to extreme rarity, earning it a G1 global rank (CPC, 2005). Additionally, *Castilleja christii* is considered a candidate species for Federal Endangered Species Status by the U.S. Fish and Wildlife Service (CPC, 2005). This species occurs in sympatry with *Castilleja miniata* Gray and *C. linariifolia* Benth, both of which have widespread distributions and multiple ploidy levels (Heckard and Chuang, 1977). At the summit of Mt. Harrison, all three sympatric *Castilleja* species were found to be diploid (Chapter 4).

*Castilleja christii* was first described by Holmgren (1973), who noted the bracts of this species were yellow to yellow-orange, and differed from those of *C. miniata* and

*C. linariifolia,* which are generally red or reddish-orange (Holmgren, 1984). Field-based observations of intermediate forms between *Castilleja christii* and other congeneric species led us to explore putative hybridization events occurring on Mt. Harrison. Morphological data indicate that *Castilleja christii* is distinct from *C. miniata* and *C. linariifolia*, and has traits that are transgressive and novel from these two species (Chapter 3). *Castilleja christii* is ecologically distinct and common at the summit of Mt. Harrison, while other sympatric *Castilleja* species tend to be less common at the summit (Chapter 4).

In this chapter, we use a combination of universal chloroplast microsatellites and the granule-bound starch synthase II single-copy nuclear gene (*waxy)* to address potential homoploid hybrid speciation in the rare endemic paintbrush, *Castilleja christii*. Specifically, we aim to (1) use molecular data to assess putative hybridization events between sympatric *Castilleja* species at the summit of Mt Harrison and (2) verify if individuals in the field identified as *Castilleja christii* are genetically a combination of *C. miniata* and *C. linariifolia.* These results will be used in our broad-scale study to address our hypothesis of homoploid hybrid speciation in this rare endemic.

## Methods

Plants

Leaf material from *Castilleja christii*, *C. miniata, C. linariifolia*, and field identified putative hybrids was collected in the field and placed in silica gel (for a list of criteria for species identification, see Chapter 3 and/or Appendix C.1). In total, 724

collections were made from within control plots outside of Mt. Harrison (*Castilleja miniata* or *Castilleja linariifolia*), within plots on Mt. Harrison (*C. miniata, C. linariifolia,* and *C. christii*), or opportunistically to increase sample size of *C. christii* (Figure 1.1).

DNA Analyses

Genomic DNA was obtained from silica-dried leaves from a subsample of 283 individuals using a commercial DNA extraction kit (DNeasy, QIAGEN,Valencia, CA, USA) according to the manufacturer's protocol. In most cases, DNA was used from the same plants as were used in morphological and cytological analyses (see Appendix A.1).

Single-Copy Nuclear Genes

Primers used for amplification of the nuclear gene encoding granule-bound starch synthase II (*waxy*), optimized for use within the Lamiales, were obtained from Tank and Olmstead (2009) (Figure 3.1). Amplifications of exons 7-13 were attempted for the entire region (7F-13R), and for smaller regions separately (7F-9R; 9F-11R; 11F-12R; 12F-13R). The PCR conditions for these amplifications were as follows: 94° for two minutes, 80° for 5 minutes, 94° for 1 minute and 30 seconds, 50° for two minutes, 72° for 2 minutes; repeat 29 times; followed by a final extension of 72° for 15 minutes. The reactions were carried out on a PTC-200 or PTC-100 thermocycler (Bio-Rad; Hercules, CA). Product quality and quantity were verified using 2% agarose/Tris-borate-EDTA (1 X TBE) gels stained with ethidium bromide (mini-gels).

Because some *Castilleja* species are known to have multiple ploidy levels, initial reactions with good products were cloned to address intraspecific allelic variation using

PGEM-T easy vector systems cloning kits (Promega; Madison, WI). Three of the regions were first attempted (7F-9R; 9F-11R; 11F-12R; Figure 3.1), as these were the most informative (D. Tank, pers. comm.). For each species of *Castilleja*, 5-10 positive clones from three individuals per plot and per gene region were selected for sequencing. Reactions were cleaned using Exo-sap (Affymetrix, Cleveland, OH) and sequenced using a Li-Cor simultaneous bidirectional sequencing kit (Lincoln, NE), according to Li-Cor standard manufacturer's protocols. Clones were separated on 6.5% polyacrylamide gels and visualized on a Li-Cor LongreadIR automated sequencer (Li-Cor Biotechnology Division, Lincoln, Nebraska). Sequences were visualized and bands verified using E-seq, version 3.0 (Li-Cor Biotechnology Division, Lincoln, NE). Each sequence was then converted to a FASTA file using Align IR (Li-Cor Biotechnology Division, Lincoln, NE) and checked for accuracy with a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/). Sequences were aligned manually in PhyDE (http://www.phyde.de/) and indels and substitutions were scored for each species. To ensure that our data were in agreement with other published *Castilleja* sequences of the same gene region, GenBank accessions were also referenced (Appendix B.2).

Based on alignments from cloned individuals, only 11F-12R regions proved to be informative and contained species-specific indel regions and substitutions, therefore we continued with direct sequencing procedures using only this region. Direct sequencing for purified PCR products was sent to Genewiz, Inc. (Genewiz, Inc. South Plainfield, NJ, www.genewiz.com ). Sequence files and corresponding pherograms received from Genewiz were verified using Chromas Lite software (LC Sciences, Houston TX) to visualize pherograms.

The neighbor – joining method (Saitou and Nei, 1987) was applied to cloned sequences from all three *Castilleja* species from this study, along with *Castilleja* species within the clades containing *C. miniata* and *C. linariifolia* (see Tank and Olmstead, 2009). Only the 11F – 12R intron region was analyzed because most cloned sequences in this study were from that region. The aforementioned sequences were analyzed using PAUP* (Swofford, 2002) and dendrograms obtained with the neighbor-joining algorithm. Ambiguous alignments (e.g., - 1 bp indels, or repeats) were eliminated.

Chloroplast Microsatellites

Chloroplast universal microsatellite loci (ccmp 6, ccmp7, ccmp8, ccmp9, and ccmp10 (Demesure, 1995) were employed in PCR reactions for a subsample of individuals from *Castilleja christii, C. linariifolia*, and *C. miniata*, both from control and Mt. Harrison sites using the same thermocycler specifications used for the *waxy* gene regions. Bands were run on 5.5% polyacrylamide genotyping gels with both a 700 and 800 internal lane spacer region. Gels were visualized on a Li-Cor LongreadIR automated sequencer (Li-Cor Biotechnology Division, Lincoln, Nebraska). Bands were read and base pair lengths compared in E-seq, version 3.0 (Li-Cor Biotechnology Division, Lincoln, NE).

## Results

### Single Copy Genes (*waxy*)

#### Cloning

Within the 11F-12R region (Figure 2.1) five species-specific substitutions and three indels were detected between *C. miniata* and *C. linariifolia* (Table 2.1). Based on cloning, two *Castilleja miniata* and *C. linariifolia* haplotypes were detected, however only one version of each was detected in our subsample of *Castilleja miniata* and *C. linariifolia* from plots within the range of *C. christii* (see Appendix B.1; "Clone A" in both *C. miniata* and *C. linariifolia*; Figures 2.2, 2.3; Table 2.1). *Castilleja christii* and field-identified putative hybrids were genetically identical to each other; therefore, for the rest of this chapter, *C. christii* will include all field-identified putative hybrid individuals. *Castilleja christii* clones matched only haplotype A of either *C. linariifolia* or *C. miniata* sequences, with no alleles unique to *C. christii* detected (Table 2.1).

#### Direct Sequencing

We evaluated all collections of *Castilleja christii* and a subsample from each plot for *C. miniata* and *C. linariifolia.* Because *Castilleja christii* and *C. miniata* were initially proposed as potentially hybridizing, we did not sequence as many *C. linariifolia* individuals. Nevertheless, the variation we detected within the genomes of *C. miniata* and *C. linariifolia* was equivalent to GenBank accessions of the same *waxy* regions, therefore inequality in sample size was not an issue. In total, 283 individuals were directly sequenced and compared with alignments from cloned samples and GenBank accessions

for species-specific indels and/or substitutions (*C. christii* N = 230; *C. miniata* N = 38; *C. linariifolia* N = 11).

All direct sequenced *Castilleja miniata* and *C. linariifolia* individuals were distinct at specific locations of the *waxy* gene region of interest (11F-12R; Figure 2.1; Table 2.1); however, *C. christii* was found to share the genomes of both *C. miniata* and *C. linariifolia* (Figure 2.2). At the first indel (Table 2.1, Indel 1) in direct-sequenced *Castilleja christii* individuals, *C. christii* has a double peak in chromatograms, displaying a combination of both parental *waxy* sequences, while *C. linariifolia* and *C. miniata* have single peaks (Figure 2.2, bp 127 in *C. christii*; Table 2.1, Indel 1). It was impossible to read the chromatograms of *Castilleja christii* after Indel 1, as each peak was double after Indel 1. Often it was impossible to tell which haplotype from each parent was present in direct-sequenced individuals due to the presence of double peaks within *Castilleja christii.*

*Castilleja christii* cloned 11F-12R sequences clustered within both *C. miniata* and *C. linariifolia* clades, except for one *C. christii* cloned sequence that clustered with *C. pilosa*, due to this clone sharing an indel unique to *C. pilosa* (Figure 2.6).

Chloroplast Microsatellites

Of the four regions we utilized, chloroplast universal microsatellite (ccmp) 6, 7, 8, and 10 produced clear bands. These microsatellites were not informative at determining the maternal parentage of *Castilleja christii*, as all three species were of the same band lengths at all four loci, approximately 120 bp (Figure 2.4).

## Discussion

Genetic Evidence Supporting a Hybrid Origin in *Castilleja christii*

Evidence to support the recent origins of a putative hybrid species with certainty must be determined by the presence of genetic additivity of parental marker alleles and few, if any, unique alleles in a putative hybrid species (Gallez and Gottlieb, 1982; Rieseberg et al., 1990; Wolfe and Elisens, 1995; Morrell and Rieseberg, 1998). In all sequenced samples, *Castilleja christii* shared the genomes of *C. miniata* and *C. linariifolia*, with no unique alleles detected (Table 2.1; Figure 2.2). Similarly, neighbor-joining analyses indicate that *Castilleja christii* shares the genome of both *C. linariifolia* and *C. miniata*, because clones from *C. christii* cluster within independent clades containing either parental species (Figure 2.6). We expected to find this pattern in *Castilleja christii*, given that a hybrid will inherit one copy from each nuclear parental genome. Other studies using nuclear genes to examine hybridization have also looked for this pattern, and hybrids between species have been verified in this way (Small et al., 2004; Gross et al., 2003; Sang and Zhang, 1999).

Our results are surprising, because since the discovery of *Castilleja christii* on Mt. Harrison in the 1950s, many botanists have studied this plant and while some have speculated that *C. christii* may potentially hybridize with other local *Castilleja* species, no one suspected that it may be of hybrid origin. Holmgren (1973) first described the species as endemic, restricted to the summit of Mt. Harrison, ID, with morphological features that separate out easily from other *Castilleja* species. It is not surprising that botanists have never suspected a hybrid origin for *Castilleja christii*, because for some characters, this species is morphologically distinct from its progenitors (Chapter 3);

however, in this study, we discovered *C. christii* shares the genomes of *C. miniata* and *C. linariifolia.* From this example, it is easy to see why homoploid hybrid speciation is difficult to detect (Rieseberg, 1997).

The placement of one clone with *Castilleja pilosa* in neighbor-joining analyses is not readily explained. *Castilleja pilosa* is not found on Mt. Harrison, or even in the region surrounding this area (Holmgren, 1984). The placement of this one clone of *Castilleja christii* with *C. pilosa* is entirely due to the presence of a sequence in an indel that is identical to the sequences in *C. pilosa*, but different from other clones of *C. christii.* This may indicate convergence at the molecular level, may reflect an older relictual sequence either due to common ancestry and incomplete lineage sorting, or reflect an earlier presence of *C. pilosa* in the vicinity of Mt. Harrison.

Using molecular markers to detect homoploid hybrid speciation has been successful in many studies (e.g., Rieseberg and Soltis, 1991; Wendel et al., 1991; Wolfe et al., 1998; Mallet, 2007); however, genetic patterns detected in hybrid taxa may be falsely interpreted due to other stochastic events. Like phenotypic characters, molecular markers of related taxa may be shared and alleles retained among closely related species if those species were derived from a polymorphic ancestor (Rieseberg, 1997). *Castilleja miniata* and *C. linariifolia* belong to different clades within *Castilleja* (Tank and Olmstead, 2009), therefore the probability of common ancestral (symplesiomorphic) or convergent evolution to explain the pattern of genetic variation in *C. christii* is unlikely. Putative diploid hybrid species share a combination of alleles with their progenitors. This genetic pattern may be due to hybridization or the hybrid taxon may be ancestral to its supposed progenitors, which is a concern in the detection of hybrid species using only bi-

parentally inherited characters (Rieseberg et al., 1990). It is unlikely that *Castilleja christii* is ancestral to either *Castilleja linariifolia* or *C. miniata*, as both *C. linariifolia* and *C. miniata* are widespread throughout the western U.S.A. (Holmgren, 1984). Because *Castilleja christii* is endemic to Mt. Harrison, which was likely glaciated approximately 10,000 years ago (Anderson, 1931), *C. christii* is unlikely to be more than 10,000 years old. Further, due to a lack of unique alleles in *Castilleja christii*, it is likely that this speciation event is relatively recent, as *C. christii* may not have had time for the generation of novel alleles through point mutations (i.e., Golding and Strobeck, 1983). While *Castilleja christii* has transgressive phenotypic traits novel to its progenitors, it also exhibits a combination of parental traits (Chapter 3). Therefore, *Castilleja christii* likely has not had sufficient time for its morphological traits to coalesce, which is typical of early generation hybrid species (Morrell and Rieseberg, 1998).

Alternatively, perhaps *Castilleja christii* had a broader distribution in the past than suspected, or occurred in other areas. Due to the presence of cirques and moraines on Mt. Harrison, it is likely that glaciers were present during the Pleistocene era (Anderson, 1931). At that time, the distribution of *Castilleja christii* may have been much larger and lower in elevation, when the glaciers covered the majority of Mt. Harrison and/or the surrounding mountains and lower lying areas. Due to potential adaptations that restricted *Castilleja christii* to an alpine environment, the distribution of the species may have followed the glaciers as they receded to higher elevations of Mt. Harrison, and why we see *C. christii* restricted to the summit of Mt. Harrison today. However, this scenario is unlikely because *Castilleja christii* does not have any unique base pair substitutions or insertion deletion events and the *C. miniata* and *C. linariifolia* clades are less than five

million years old (D. Tank, pers. comm.), indicating that *C. christii* likely originated on Mt. Harrison.

The potential for homoploid hybrid speciation to occur repeatedly and in different locations and times has been reported (Abbott, 1992; Rieseberg et al., 1996) and evidenced in a putative hybrid species between *Castilleja miniata* and *Castilleja linariifolia* in a previous study exploring the putative allopolyploid origin of *Castilleja crista-galli* Rydb. (Mathews and Lavin, 1998). Ownbey (1959) originally described *Castilleja crista-galli* as potentially of hybrid origin between *C. miniata* and *C. linariifolia*, as this species occurs in areas of sympatry and *C. crista-galli* appears to be morphologically intermediate between the two. More recent research by Mathews and Lavin (1998) has indicated that *Castilleja crista-galli* is not intermediate in morphology between its two putative progenitors, but clusters more closely with *C. miniata* and other closely related *Castilleja* species than with *C. linariifolia*. Further, while the authors retained specific status of *C. crista-galli* due to ecological separation, they are cautious, as *C. crista-galli* did not show any unique combinations of fixed genetic characters. Mathews and Lavin (1998) concluded that they did not have enough genetic evidence to support *C. crista-galli* as an allopolyploid, and instead suggested that this species may be a homoploid hybrid species.

In contrast to *Castilleja crista-galli, C. christii* was not originally inferred to be of hybrid origin by Holmgren (1984) due to the presence of species-specific morphological traits (Chapter 3), which are different than those traits found in *C. crista-galli* (Holmgren, 1984). Moreover, while *C. crista-galli* and *C. christii* share the genomes of *C. linariifolia* and *C. miniata,* they are ecologically, spatially, and morphologically distinct from each

other, and for these reasons are considered separate species. Other examples of recurrent homoploid hybrid speciation are found in the literature, most notably evidenced in *Helianthus* homoploid hybrids (Rieseberg et al., 1990; Rieseberg et al., 2003), where two diploid *Helianthus* progenitor species *H. annuus* and *H. petiolaris* gave rise to three different diploid *Helianthus* species, which all are ecologically distinct in niches unavailable to their parents: *Helianthus anomalus* is adapted to sand dunes, *H. deserticola* occupies desert basins, and *H. paradoxus* is found in salt marshes. Similarly, *Castilleja christii* is restricted to the summit of Mt. Harrison, has glandular hairs on all above-ground parts of the foliage and stems, and yellow to yellow-orange bracts, while *C. crista-galli* is found in extreme northwestern Wyoming and adjacent Montana, lacks glandular hairs below the inflorescence, and has red to purplish bracts (Ownbey, 1959; Heckard and Chuang, 1977).

Botanists have searched the mountains adjacent to Mt. Harrison for other populations of *Castilleja christii*; however, no additional populations have been found. In our study, however, some individuals that were identified as *Castilleja miniata* from adjacent Mt. Independence were found to have a copy of the *Castilleja linariifolia waxy* sequence (Appendix B.3; Figure 2.5). These are likely F1 hybrids between *Castilleja miniata* and *C. linariifolia.* If these plants were possibly hybrid species, the environmental conditions on Mt. Independence are different than those at the summit of Mt. Harrison and therefore "*Castilleja christii*" may not develop there. Therefore, genotype by environment interactions on Mt. Harrison may be selecting for the morphological and genetic features that have arisen in *Castilleja christii*.

*Castilleja christii* All Contain One Copy of the Waxy Gene from Each Parent

It is surprising that we only encountered *Castilleja christii* individuals with both parental *waxy* sequences, because in classic examples of Mendelian inheritance, we would expect to see half of all progeny from a selfed F1 hybrid between *C. miniata* and *C. linariifolia* with both parental sequences and a quarter with either *C. miniata* or *C. linariifolia* sequences. In cases of homoploid hybrid speciation, it is generally unlikely to see strict additivity at all loci in the absence of asexual reproduction, particularly in outcrossing species where introgression, F1s and F2s may all have contributed to the stabilized hybrid species (Soltis and Soltis, 2009). *Castilleja* is typically an outcrossing genus (though see Chuang and Heckard, 1992) and known for the ease with which species hybridize, so the question remains as to why both parental copies of the *waxy* gene are found in *Castilleja christii.*

One potential explanation may be selection acting on the *waxy* gene in favor of individuals with both parental copies of *Castilleja christii.* The *waxy* or granule-bound starch synthase II enzyme synthesizes linear glucan (amylose) (Nelson and Pan, 1995). This enzyme plays a role in seed, endosperm, and tuber formation (Smith et al., 1997, and refs. therein), but also has shown to be expressed in later developmental stages, such as floral and meristematic tissue and reproductive events such as seed filling (Merida et al., 1999). Craig et al. (1998) discovered that any changes to the starch synthase II molecule resulted in highly contorted starch granules, potentially because the organization of amylopectin had been altered. In *Castilleja christii,* its unique environment at the summit of Mt. Harrison may necessitate copies from both parents to effectively synthesize starch, and individuals inheriting the gene from only one parent do not properly organize

amylopectin in this particular environment. This may be an explanation for why *Castilleja christii* is common at the summit of Mt. Harrison, while *C. miniata* and *C. linariifolia* are restricted and isolated to only a few sites at the summit: potentially the combination of parental sequences of the *waxy* gene code for more well-adapted individuals in this alpine environment. Seed germination studies from collections of all three *Castilleja* species at the summit of Mt. Harrison indicate that *C. christii* has a high germination rate and seedling survival (see Ch. 4; ~80%; seedlings were robust and one seedling was raised to flowering), while the germination and seedling survival rates of *C. miniata* and *C. linariifolia* were lower (~25%; seedlings were delicate and none survived). Though these tests were informal and were limited by the lack of a reciprocal study of seed germination from the summit of Mt. Harrison, they may indicate that the combination of *waxy* sequences from both parents in *Castilleja christii* may have given this species a fitness advantage over parental species at this specific site in the *waxy* genome. Additionally, the combination of alleles from both parents in *Castilleja christii* may be what restricts this species to the summit of Mt. Harrison and why it is not found elsewhere.

Alternatively, the *waxy* gene could be linked to other genes that are selected against when only one copy is present in the *Castilleja christii* genome, therefore successful *C. christii* individuals have copies from both parents. Although in some studies of homoploid hybrid speciation, hybrid derivatives shared large portions of parental genomes (Rieseberg et al., 2003), a study involving *Heliconus* butterflies has shown that ecological divergence and reproductive isolation from parental species may be dependent on only a few introgressed genes shared in a homoploid hybrid species

(Jiggins et al., 2008). In these cases, Jiggins et al. (2008) warns that it may be difficult to identify species of hybrid origin unless the introgressed 'speciation genes' are examined directly because at alternative loci the hybrid species may resemble parental sequences of either parent.

Perhaps the most reasonable explanation as to why each *Castilleja christii* individual sequenced had both parental copies of the *waxy* gene is chromosomal recombination: the presence of both parental alleles in *Castilleja christii* may have become fixed within the genome as a homozygotic condition, with all *Castilleja christii* individuals from intraspecific crosses inheriting both parental "alleles" (now paralogs) regularly. For example, two species of the same ploidy level may differ by two reciprocal translocations. Hybrids would be heterozygous for both parental chromosomal arrangements (Figure 2.5). Progeny between F1 crosses or backcrosses with parents would result in 75% of hybrid gametes that are unbalanced and inviable; however, 25% would be balanced and viable. Of the viable gametes, half would retain parental chromosomal patterning while half would be recombinant. If recombinant F2 offspring were selfing or if the recombinant karyotype was of higher fitness than parental types, these recombinant karyotypes would be balanced, viable, and fertile, and because recombinant karyotypes would be fixed as a homozygotic condition within the hybrid genome, these would be passed on to offspring, thus facilitating hybrid speciation. If recombinational speciation was the reason for the pattern of genetic variation seen in *Castilleja christii,* this species would now continually pass both copies of the *waxy* gene to offspring because it is a fixed homozygous condition. Studies investigating granule

bound starch synthase in *Castilleja* may provide evidence for the reasons *Castilleja christii* is always found with both parental *waxy* copies.

Other Evidence to Support a Hybrid Origin in *Castilleja christii*

Results from our other studies of the homoploid hybrid origin of *Castilleja christii* corroborate our genetic findings in this chapter. Briefly, *Castilleja christii* displays phenotypic, life history, and morphological traits that are transgressive and novel from its putative progenitors and morphological data between the three species are significantly different (Chapter 3). Often, species determined to be of hybrid origin in other studies have exhibited morphological traits novel to those of their progenitors (Heiser, 1947, 1949; Grant, 1950, 1954c, 1966b; Schwarzbach et al., 2001) and the provisional delimitation of species can be determined by the presence of at least one fixed or non-overlapping morphological character (Wiens, 2007). Transgressive characters in *Castilleja christii* were floral in nature (Chapter 3). Floral traits have been suggested to be under strong pollinator-mediated selection in several other studies (Campbell et al., 1997; Rieseberg, Whitton, and Gardner, 1999; Rieseberg et al., 2003; Rosenthal et al., 2002; Gross et al., 2004; Hersch and Roy, 2007). Further, most examples of homoploid hybrid species are ecologically and spatially distinct from parental progenitors, which may aid in the successful establishment of the hybrid species (Rieseberg, 1997; Gross and Rieseberg, 2005). *Castilleja christii* is spatially and ecologically isolated at the summit of Mt. Harrison, and each species appears to display a preference for specific plant associations and certain directional aspects at the summit (Chapter 4). The combination of strict genetic additivity in *Castilleja christii* and the absence of unique alleles coupled with pollinator-mediated selection acting on phenotypic expression with that of physical

ecological divergence of *Castilleja christii* from other sympatric *Castilleja* species on Mt. Harrison is evidence for hybrid speciation.

Suggestions for Further Research

Our data provide sound evidence to support *Castilleja christii* as a homoploid hybrid species, which is a novel discovery within the genus. Speciation and diversification in *Castilleja* is generally attributed to the ease with which species hybridize and experience subsequent genome duplication (allopolyploidy; Ownbey, 1959; Heckard, 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Chuang and Heckard, 1993). To further shed light on the mechanisms of hybrid speciation in *Castilleja christii* and to gain insights into the evolution of the genus *Castilleja,* our initial findings must be tested with other lines of evidence. First, we suggest that more research be conducted with other nuclear gene regions to check for additivity at other loci. Other maternally inherited markers should also be used to determine the maternal parentage of *Castilleja christii*.

Additionally, the *waxy* gene specifically should be explored to provide an explanation as to why we see only strict genetic additivity at the locus utilized in this study. Progeny arrays could be used in this instance to study how the *waxy* gene is inherited and if it is linked with other genes that could promote the prolific nature and phenotype of *Castilleja christii* at the summit of Mt. Harrison. Recombinational speciation can be tested using quantitative trait loci analyses in *Castilleja christii* and its progenitors in an attempt to artificially recreate the *Castilleja christii* phenotype, which would in theory be at least partially reproductively isolated from its parents.

The discovery of *Castilleja christii* as a homoploid hybrid species of potentially recent origin is exciting, as it provides the opportunity for chronicling the continued differentiation of *C. christii* from *C. linariifolia* and *C. miniata.* Further, by studying the speciation process of *Castilleja christii,* we gain insight into the continued speciation and diversification within the genus *Castilleja*, while furthering our knowledge of the mechanisms and processes involved in homoploid hybrid speciation, of which only a handful of empirically tested examples exist in the literature.

## Conclusions

Evidence to support the origins of a putative hybrid species with certainty must be determined by the presence of genetic additivity of parental marker alleles and few, if any, unique alleles in a putative hybrid species (Morrell and Rieseberg, 1998). In this study, *Castilleja christii* was found to share the genomes of both *C. linariifolia and C. miniata* at a specific *waxy* gene region (Figure 3.2), and no unique alleles were discovered within the genome of *C. christii*. We conclude that *Castilleja christii* is a stabilized hybrid derivative of *C. linariifolia* and *C. miniata*. *Castilleja christii* is ecologically and spatially distinct at the summit of Mt. Harrison (Chapter 4) and expresses morphological traits that are transgressive from its progenitors (Chapter 3), providing evidence for transgressive segregation (Grant, 1975) and support *C. christii* as a hybrid species, despite a shared ploidy level among congenerics. Taken together, these data suggest that *Castilleja christii* is likely following an independent evolutionary trajectory from its progenitors. Further research using different molecular markers will strengthen support of a homoploid hybrid origin in *Castilleja christii* and will provide answers to the complexities of speciation within this dynamic genus.

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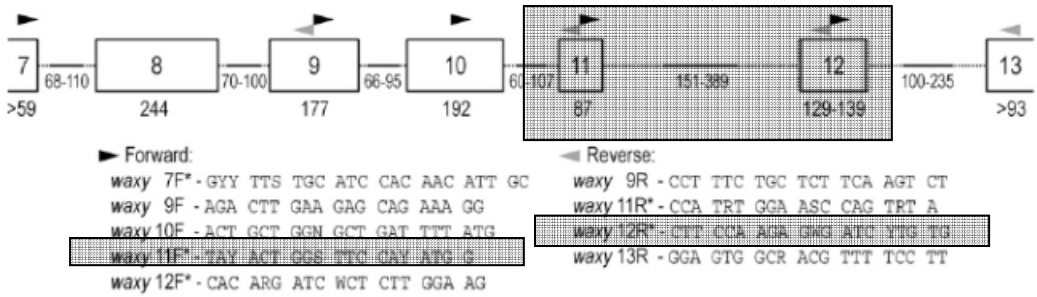


**Figure 2.1. Diagram of the 3' waxy gene used in this study. Though this study attempted the primers from entire length of this gene region, only the 11F and 12R primers were used for direct sequencing in this study (highlighted here) (adapted from Tank and Olmstead, 2009).**

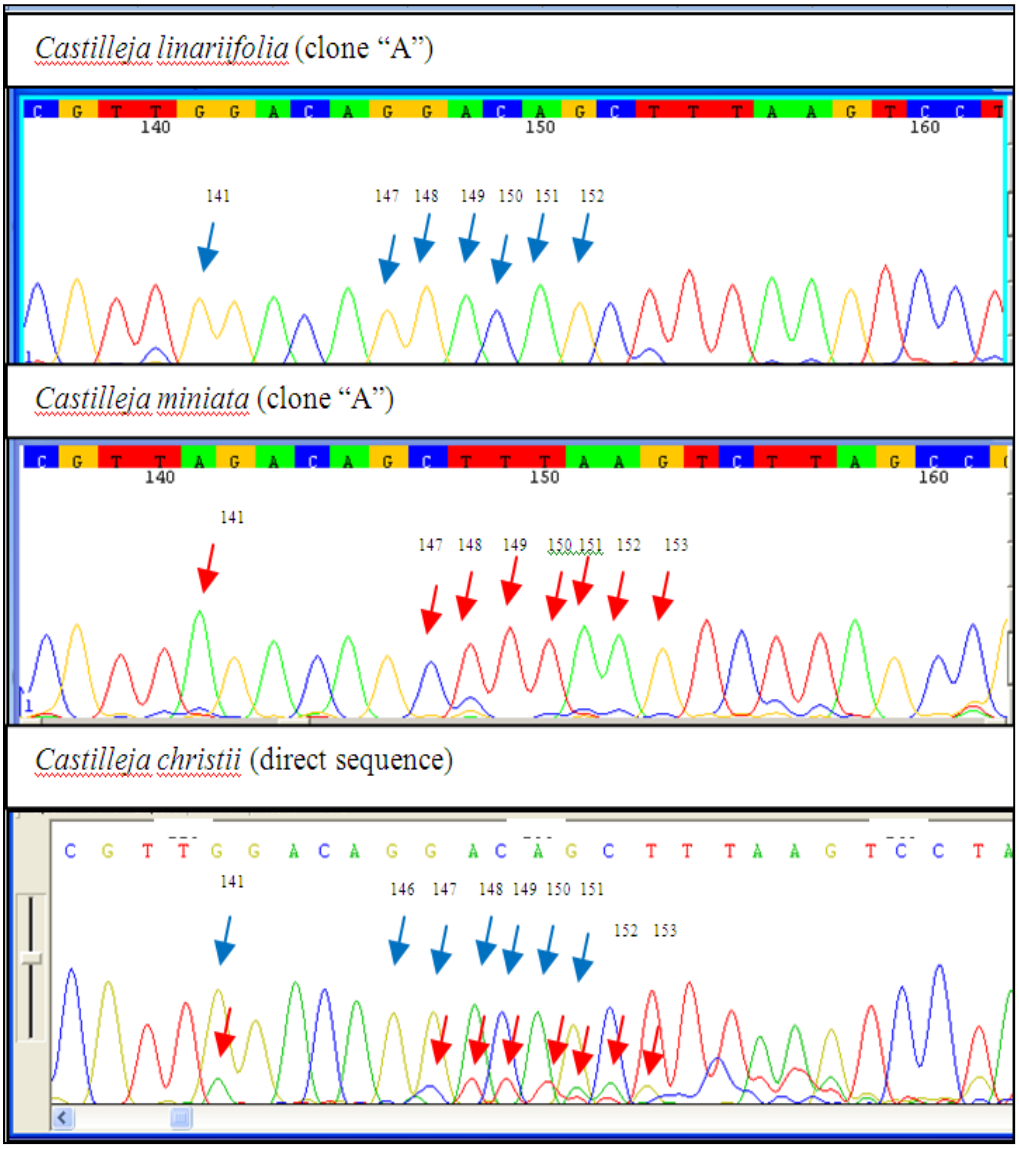


**Figure 2.2 Pherograms generated from cloned individuals of *Castilleja linariifolia* and *C. miniata,* depicting species-specific regions containing several defining substitutions (black arrows) and indels (red arrows = *C. miniata;* blue arrows = *C. linariifolia*). The lowest panel depicts *Castilleja christii*, which contains the additive genetics of both *C. linariifolia* and *C. miniata* genomes at a *C. linariifolia* indel region, as shown by the presence of both blue and red arrows (or peaks) from both parents. Every *Castilleja christii* individual sequenced had the same combination of parental alleles, as shown above.**

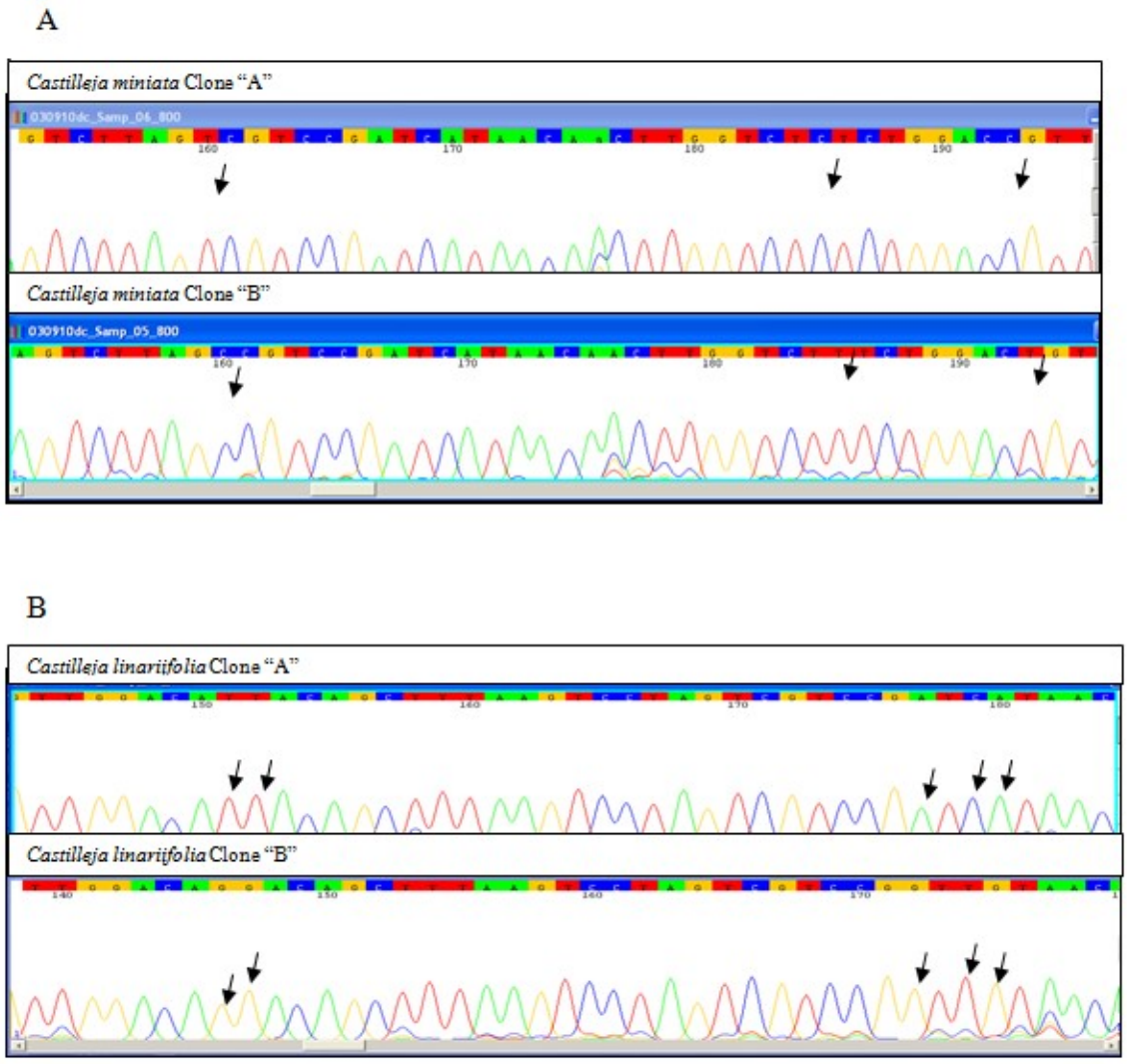


**Figure 2.3 Pherograms depicting intraspecific variation in *Castilleja miniata* and *C. linariifolia*. A) Images of the *waxy* gene region 11F-12R for two cloned *Castilleja miniata* samples. B) Images of the *waxy* gene region 11F- 12R for two cloned *C. linariifolia* samples. Arrows indicate intraspecific variation found within *C. miniata* or *C. linariifolia* at control sites. Across our range of samples (*C. miniata* N = 38; *C. linariifolia* N = 11), only "Clone A" individuals were detected in direct-sequenced individuals for both species.**

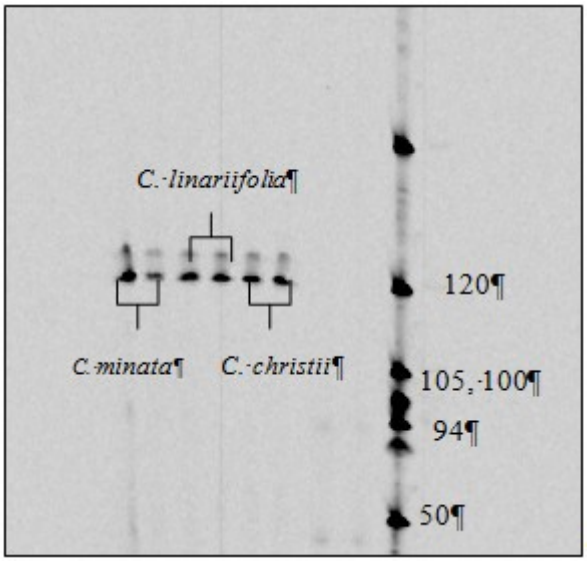


**Figure 2.4 Universal chloroplast microsatellites. Universal chloroplast microsatellite (ccmp 7) images of *Castilleja miniata, C. linariifolia* and *C. christii* used in this study. *Castilleja miniata* and *C. linariifolia*, are both from sites outside of the range of *Castilleja christii* to eliminate any potential introgression with each other or *C. christii.* Band lengths were approximately 120 bp long for each species for all four universal chloroplast regions.**

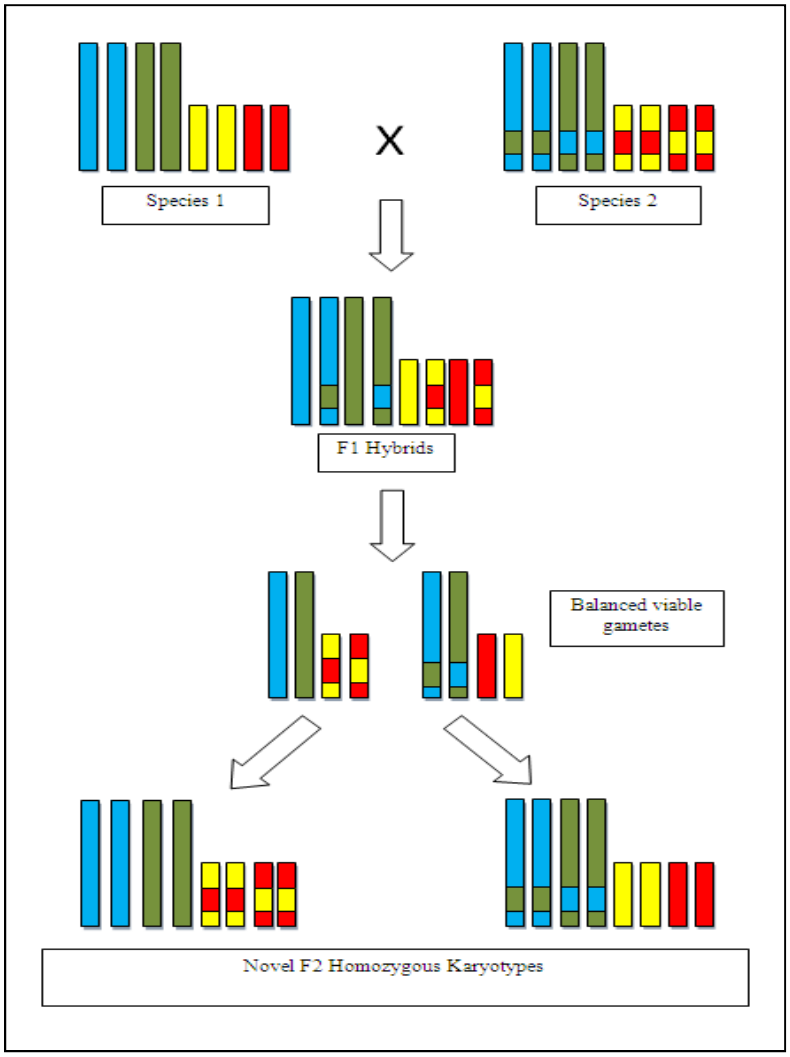


**Figure 2.5 A simple model of recombinational speciation. Two species are of the same ploidy level but differ by two reciprocal translocations. Hybrids would be heterozygous for both parental chromosomal arrangements. Progeny between F1 crosses or backcrosses with parents would result in 75% of hybrid gametes that are unbalanced and inviable (not shown); however, 25% would be balanced and viable (shown here). Of the viable gametes, half would retain parental chromosomal patterning while half would be recombinant. If recombinant F2 offspring were selfing or if the recombinant karyotype was of higher fitness than parental types, these recombinant karyotypes would be balanced, viable, and fertile but partially sterile with parental types. These fixed homozygotic genotypes would be inherited by subsequent generations, thus facilitating hybrid speciation. (Adapted from Rieseberg, 1997.)**

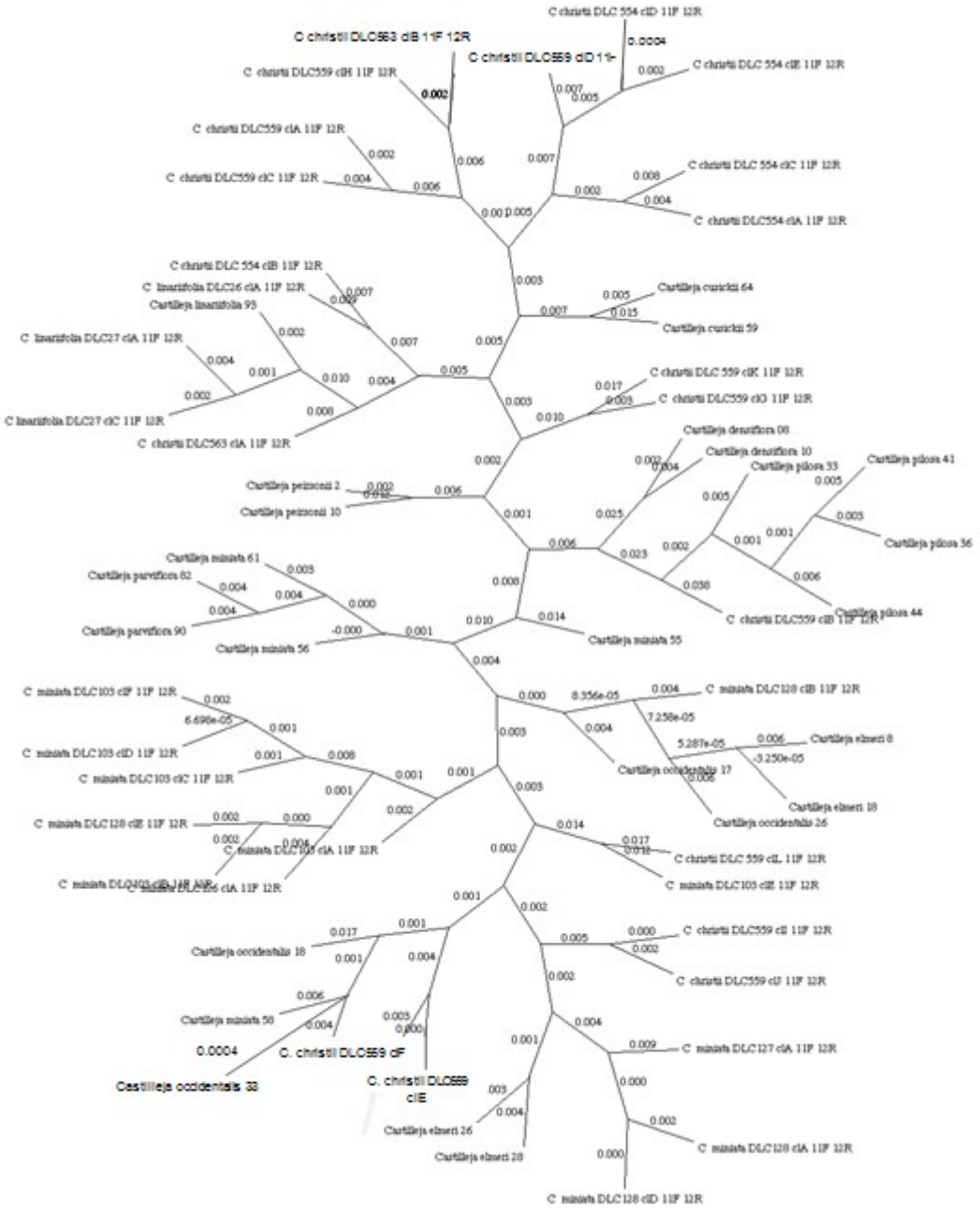


**Figure 2.6 Unrooted neighbor-joining tree for different clades of *Castilleja* species. Constructed using PAUP* software for sequences from the 11F – 12R *waxy* intron region of *Castilleja linariifolia* and *C. miniata* clades (sequences obtained from D. Tank; see Tank and Olmstead, 2009). The aforementioned sequences were run along with cloned 11F-12R *Castilleja christii* sequences to address a putative hybrid origin in *C. christii. Castilleja christii* clustered within both *C. miniata* and *C. linariifolia* clades, except for one cloned *C. christii* cloned sequence that clustered with *C. pilosa*, due to this clone sharing an indel unique to *C. pilosa.* Numbers at the nodes indicate neighbor-joining distances.**

**Table 2.1 List of cloned taxa and positions of insertions and deletions (Indels) or substations. These are noted below by their location from the beginning of the waxy intron 11F.**

| Cloned taxon | Position from beginning of 11F Intron | Type | Sequence |
|---|---|---|---|
| *C. christii* Cl. A | 115-124 | substitution 1 | CCTTTCCGAC |
| *C. christii* Cl. B | | | CCTTTCCGAC |
| *C. linariifolia* Cl. A | | | CCTTTCCGAC |
| *C. linariifolia* Cl.B | | | CCTTTCCGAC |
| *C. miniata* Cl. A | | | CCATTCCGGC |
| *C. miniata* Cl. B | | | CCATTCCGGC |
| *C. christii* Cl. A | 143-152 | indel 1 | GGACAGGACAG |
| *C. christii* Cl. B | | | AGACAG----- |
| *C. linariifolia* Cl. A | | | GGACAGGACAG |
| *C. linariifolia* Cl.B | | | GGACAGGACAG |
| *C. miniata* Cl. A | | | AGACAG----- |
| *C. miniata* Cl. B | | | AGACAG----- |
| *C. christii* Cl. A | 175-176 | substitution 2 | CG |
| *C. christii* Cl. B | | | TG |
| *C. linariifolia* Cl. A | | | CG |
| *C. linariifolia* Cl.B | | | TG |
| *C. miniata* Cl. A | | | CA |
| *C. miniata* Cl. B | | | CA |
| *C. christii* Cl. A | 184-188 | substitution 3 | TTATT |
| *C. christii* Cl. B | | | TTATT |
| *C. linariifolia* Cl. A | | | TTATT |
| *C. linariifolia* Cl. B | | | TTATT |
| *C. miniata* Cl. A | | | TTGGT |
| *C. miniata* Cl. B | | | TTGGT |
| *C. christii* Cl. A | 227-230 | indel 2 | CTA |
| *C. christii* Cl. B | | | C-A |

| | | | |
|---|---|---|---|
| *C. linariifolia* Cl. A | | | CTA |
| *C. linariifolia* Cl. B | | | CTA |
| *C. miniata* Cl. A | | | C-A |
| *C. miniata* Cl. B | | | C-A |
| *C. christii* Cl. A | 234-239 | substitution 4 | GGGATC |
| *C. christii* Cl. B | | | GGGACC |
| *C. linariifolia* Cl. A | | | GGGATC |
| *C. linariifolia* Cl. B | | | GAGAAC |
| *C. miniata* Cl. A | | | GAGAAC |
| *C. miniata* Cl. B | | | GAGAAC |
| *C. christii* Cl. A | 251-268 | indel 3 | TAACAATCGTTATTGAAACTAT |
| *C. christii* Cl. B | | | TAACAATCGTTATTGAAACTAT |
| *C. linariifolia* Cl. A | | | TAACAATCGTTATTGAAACTAT |
| *C. linariifolia* Cl. B | | | TAACAATCGTTATTGAAACTAT |
| *C. miniata* Cl. A | | | ----------CTAT |
| *C. miniata* Cl. B | | | ----------CTAT |
| *C. christii* Cl. A | 370-378 | substitution 5 | TTTATGTTT |
| *C. christii* Cl. B | | | TTTATATTT |
| *C. linariifolia* Cl. A | | | TTTATGTTT |
| *C. linariifolia* Cl. B | | | TTTATATTT |
| *C. miniata* Cl. A | | | TTTATATTT |
| *C. miniata* Cl. B | | | TTTATATTT |

# CHAPTER 3: MORPHOLOGICAL EXAMINATION OF HOMOPLOID HYBRID SPECIATION IN THE RARE ENDEMIC *CASTILLEJA CHRISTII* (OROBANCHACEAE)

## Introduction

Interspecific hybridization followed by reproductive isolation of hybrid offspring from their progenitors has been considered of central importance in plant speciation (Grant, 1981; Abbott, 1992; Arnold, 1997; Rieseberg, 1997; Soltis and Soltis, 2009). Hybrids can become reproductively isolated from their parents via either a change in ploidy (i.e. allopolyploidy) or may become reproductively isolated while sharing a common ploidy level (homoploid hybrid speciation). While polyploidy is relatively common in plants with estimates ranging from 30 to 80% of species containing polyploid genomes (Leicht and Bennett, 1997), homoploid hybrid speciation is thought to be rare, with only a few plant species rigorously tested to verify they originated in this way (Rieseberg, 1997; Gross and Rieseberg, 2005; James and Abbott, 2005).

Polyploidy is relatively easily maintained. Hybrids share parental genotypes, yet are reproductively isolated from their parents by having double the amount of genetic material. Allopolyploids are also easily detected through combinations of chromosome counts and molecular markers (Soltis and Soltis, 2009). In contrast, homoploid hybrid speciation is considered rare because of the implications associated with the formation of hybrid species: hybrids must become genetically isolated from parental species while

sharing the same chromosome number (Rieseberg, 1997). Thus, new diploid hybrid species may be outcompeted while in sympatry or parapatry with parental types.

Niche separation between hybrid and parent species is crucial to avoid potential genetic swamping or competition from parental species (Lewontin and Birch, 1966; Grant, 1981; Templeton, 1981; Schulter, 1998; Burkle et al., 2000). In empirically tested cases of homoploid hybrid speciation, most stabilized hybrid species were found to be ecologically or spatially distinct from their progenitors, thus minimizing the potential of genetic swamping and reinforcing reproductive isolation (Abbott, 1992; Arnold, 1997; Rieseberg, 1997; Brennan et al., 2009). Discovering how homoploid hybrid species achieve ecological divergence and which adaptations allow recruitment outside of parental niches involves a complex series of questions and examinations that make studying homoploid hybrid speciation a difficult task (Rieseberg, 1997).

Ecological or spatial divergence can occur in several different ways. An intermediate habitat between parental types may allow a hybrid to easily colonize such a location. This scenario has been documented in the homoploid hybrid *Iris nelsonii*, which occupies a divergent habitat that combines features from all three parental progenitors: *Iris fulva* occupies shady, shallow riparian areas surrounding bayous; *I. hexagona* inhabits sunnier, deeper swamp waters; and *I. brevicaulis* inhabits drier upland pastures and forests. *Iris nelsonii* combines these features and is found in areas of shady, deep water cypress swamps (Arnold, 1993).

Secondly, through hybridization, hybrid species can generate novel or transgressive phenotypes, which may allow hybrid species to become better suited to environments outside of those of their progenitors. A classic example of this is the hybrid

speciation of sunflowers, *Helianthus*: the parental species *H. annuus* and *H. petiolaris* occupy mesic clay and sandy soils, respectively. Their hybrid species derivatives all display transgressive traits that enable them to occupy niches novel to their parents: *H. anomalus* is restricted to sand dune habitats and has more succulent leaves, which may allow for resistance to desiccation from blowing sand in their sand dune habitats (Schwartzbach et al., 2001); *H. deserticola* occupies arid desert areas and has adaptations for the desert environment including rapid flowering, small narrow leaves, and reduced boron uptake (Rieseberg et al., 2003); and *H. paradoxus* occupies desert salt marsh habitats and is adaptive in that it has the ability to reduce the toxic effects of sodium and other mineral ions through active exclusion (Lexer et al., 2003), internal sequestration, and increased leaf succulence (Welsh and Rieseberg, 2002). Schwartzbach et al. (2001) explored transgressive character expression in *Helianthus anomalus* by looking at a combination of morphological and ecophysiological characters to explain the success of this species in habitats that are novel from its parental species. Many of the characters that differed between *Helianthus anomalus* and its parental taxa were morphological in nature, which were speculated to assist with conditions experienced in the hybrid species' habitat. The adaptation of transgressive traits in hybrid species have been explained by studies using quantitative trait loci (QTL; reviewed in Rieseberg et al., 1999), which have indicated that genetically divergent lineages have adapted transgressive phenotypes through hybridization, and these phenotypes assist hybrid species in their evolutionary independence (Lai et al., 2005). Though *Helianthus* hybrids are an extreme example of phenotypic adaptation to novel environments, any type of trait value in a hybrid species, whether intermediate, a combination of parental types or traits that are novel or

transgressive from that of parents may allow for higher fitness for hybrids in novel environments, which is facilitated by the adaptive potential of hybridization (Barton, 2001; Ellstrand and Schierenbeck, 2000; Schwarzbach et al., 2001).

Within the genus *Castilleja,* the combination of allopolyploidy and interspecific hybridization has significantly contributed to the evolution of the genus (Ownbey, 1959; Heckard, 1968; Heckard and Chuang, 1977; Hersch-Green and Cronn, 2009; Tank and Olmstead, 2009). Hybrids may become genetically isolated from parental species via polyploidy, which seems to be the only barrier to hybridization in zones of sympatry (Holmgren, 1984) other than contextual pollinator-mediated selection (Hersch and Roy, 2007). Hybridization events within the genus have largely been inferred from morphological observations in the field and verified via cytological studies in the lab or with molecular markers (Ownbey, 1959; Holmgren, 1984; Heckard, 1968; Heckard and Chuang, 1977; Egger, 1994; Hersch and Roy, 2007; Hersch-Green and Cronn, 2009).

Determining *Castilleja* species in the field can be difficult, as species overlap in almost every character and the genus as a whole is presumed to be recently diverged (Holmgren, 1984). Egger (1994) notes that because hybridization in *Castilleja* is well-known and may occur frequently in areas of sympatry, the validity of parental species descriptions should not be called into question and instead should be strictly interpreted, with hybrids being recognized based on intermediate morphological variation.

On 200 acres at the summit of Mt. Harrison (Cassia county, Idaho), the rare endemic Christ's paintbrush (*Castilleja christii* N. Holmgren) occurs in sympatry with *Castilleja miniata* Gray and *C. linariifolia* Benth. *Castilleja christii* is critically imperiled, and at high risk of extinction due to extreme rarity, therefore it has a G1 global

rank (CPC, 2005). Additionally, *Castilleja christii* is considered a candidate species for Federal Endangered Species Status by the U.S. Fish and Wildlife Service (CPC, 2005). *Castilleja miniata* and *C. linariifolia* have widespread distributions throughout North America and multiple ploidy levels (Heckard and Chuang, 1977).

*Castilleja christii* was first described by Holmgren (1973), who noted the bracts of this species were yellow to yellow-orange, and are different than bracts of *C. miniata* and *C. linariifolia,* which are generally red or reddish-orange (Holmgren, 1984). Field based observations of intermediate forms between *Castilleja christii* and other congeneric species led us to explore putative hybridization events occurring on Mt. Harrison. Analyses of molecular data have indicated that *Castilleja christii* is likely a homoploid hybrid between the sympatric *C. miniata* and *C. linariifolia* (Chapter 2). In this chapter, we determine whether morphological data corroborate these results, whether *C. christii* can be considered a distinct species based on morphology alone, and what morphological characters are important for the identification of all three sympatric species.

A crucial step in studying homoploid hybrid speciation is determining which mechanisms assist in reproductive isolation from paternal types. Here, we determine how the endemic homoploid hybrid species *Castilleja christii* is distinct from its progenitor and widespread sympatric congenerics *C. miniata* and *C. linariifolia* by determining if morphological characters seen in *Castilleja christii* are parental-like, intermediate, or transgressive when compared with traits of parental taxa from populations in sympatry with *C. christii* and outside of the range of *C. christii*.

These data will be compared with molecular, cytological, and ecological data in our broader scale study.

## Methods

Specimens used for morphological measurements were collected at all field sites between July 11 and August 31, 2009. When collecting specimens, a narrow interpretation of each published species' descriptions was taken. If plants of *Castilleja christii* sampled did not meet strict criteria based on its initial description, they were sampled as putative hybrids (Appendix A.1, C.1). Taxonomically informative traits were inferred from species descriptions of *Castilleja* in Holmgren (1984). Above ground portions of plants were collected to minimize disturbance to the habitat of *Castilleja christii*. Between one and three flowering stems from each individual were pressed, dried, frozen, and stored in the Snake River Plains Herbarium (SRP), Boise, ID. When pressing field-collected samples, three flowers were dissected per plant and pressed separately to ease the measurement of these traits in the herbarium. In total, 724 collections were made from within control plots, within plots on Mt. Harrison, or opportunistically to increase sample size.

Morphological traits on all *Castilleja* species were measured in the field from July 11 until August 31, 2009 or using dried herbarium specimens. Traits pertaining to an entire plant were measured in the field and traits that could be conducted with dried plant specimens were measured with pressed samples (Table 3.1). Morphological traits were measured using a Cen-Tech 6" dial caliper. Traits difficult to measure with the naked eye were measured under a Leica S8AP0 dissecting microscope. In total, morphological measurements were obtained from ten plants per species per plot and individuals were randomly selected via 50 randomizations of within plot collection numbers. The order in which plots were measured was also randomized 50 times. In total, 33 taxonomically

informative traits were measured and analyzed (*C. christii* N = 114; *C. miniata* N = 104; *C. linariifolia* N = 50; Table 3.1).

Thirteen continuous traits were measured either in the field or herbarium (*C. christii* N = 114; *C. miniata* N = 104; *C. linariifolia* N = 50; Table 3.1). These traits were first analyzed using one-way analyses of variance (ANOVA, Proc GLM procedure of SAS 9.2, SAS Institute, 2008) to check for normalcy. If data were not normal, they were log transformed. Additionally, MEANS and LSMEANS statements with an SNK option were run to obtain means and standard deviations and to test for mean differences among groups, respectively. Secondly, we ran discriminant functions analyses (DFA) to predict group (species) membership of individual plants, based on eleven continuous and four discrete traits. Additionally, correlation analyses were run on qualitative data to ensure that traits were not highly correlated.

Twenty qualitative traits were measured in the field or herbarium, thirteen of which were analyzed (Table 3.1). We tested for an association between species and the affect of ordinal or nominal data using the Cochran-Mantel-Haenszel (CMH) statistic of general association (CMH; FREQ procedure of SAS 9.2, SAS Institute, 2008). Because data for each species were pooled across plots, we opted to run a CMH test instead of a chi-square or G-test. We also chose CMH over logistic regression, as no logistic regression models sufficiently explained the variation in all of the data (data not shown). Four qualitative traits were also analyzed along with continuous data in DFA analyses.

## Results

Results from ANOVA and means statistics revealed several traits that significantly differed among groups (Table 3.2), and correlation analyses revealed traits measured were not significantly correlated (data not shown). Traits that were significantly different among all species included mean bract length, total raceme height, corolla length, beak length, calyx inner segment length, stem height, and mean leaf width. When control populations were excluded from the analyses, mean bract length, total raceme height, beak length, and calyx inner segment length were found to be significantly different between all groups (Table 3.3). Though each species was distinctive for the aforementioned traits, a few traits were overlapping among species. *Castilleja christii* shared means with *C. miniata* for mean bract width (all populations, Table 3.2) and mean leaf width (control populations only; Table 3.3), and with *C. linariifolia* for calyx outer segment length and mean leaf length (when analyzed with control and all populations). *Castilleja miniata* shared means with *C. linariifolia* for calyx length (all populations; Table 3.2) and corolla length, calyx length, and stem height (Mt. Harrison populations only; Table 3.3).

The CMH statistic for qualitative data separated *Castilleja linariifolia* from other parental species by having lobed leaves and bracts, having no exudate below or within the inflorescence, an absence of glandular hairs below the inflorescence, and usually having significant stem branching (90%; Figures 3.1 and 3. 2). In contrast, *Castilleja christii* and *C. miniata* can be categorized on a gradient, with most individuals exhibiting species-specific traits and others being more intermediate. Traits that are generally associated with *Castilleja christii* include presence of lobes on the bracts, having little to

no exudate below the inflorescence (68%), having glandular hairs below the inflorescence, and usually not branching above the base (62%). *Castilleja miniata* lacked lobed leaves (87%), was usually branching above the base, and was commonly found with exudate both below (73%) and within the inflorescence (98%). Traits that were not significant among the different species and hybrids included bract shape, number of stems, and percent lobed leaves (data not shown).

The first two axes of the discriminant functions analysis delineate the total variation seen in continuous and discrete traits (Table 3.4 a, b; Figure 3.3). By comparing the class means of the canonical variables for each species with the loadings of canonical coefficient functions on each axis, we can obtain an idea of how each trait affects species groupings (Table 3.4 a, b). The first axis clearly separates *Castilleja linariifolia* based on short mean bract length, short calyx inner segment length, and no exudate within the inflorescence. Looking at the second axis, a gradient is apparent between *Castilleja christii* and *C. miniata*, with trait values overlapping in the middle. *Castilleja christii* can be generally distinguished by having a short beak length (10.21 $\pm$ 1.62 mm; Table 3.3), short stem heights (22.89 $\pm$ 6.21 mm; Table 3.2), little exudate within the inflorescence and is unbranching, whereas *C. miniata* is generally found to be opposite these traits with longer stems (39.66 $\pm$ 13.36 mm; Table 3.2), longer beak lengths (12.66 $\pm$ 2.10 mm; Table 3.3), is typically branching above the base, and has exudate almost always present in the inflorescence (Table 3.3- 3.4 a, b; Figures 3.2-3.3).

# Discussion

## Detection of Hybridization Based on Morphology

Hypotheses of natural hybridization events are generally first developed in response to morphological trait intermediacy observed in the field between suspected parents. Researchers in the past have assumed that character expression between species is under quantitative genetic control leading to intermediate character expression in hybrids, and that hybrids could not generate traits novel from their progenitors even in later generations (Wagner, 1969; Stebbins, 1974). Rieseberg and Ellstrand (1993) evaluated these assumptions in a review by analyzing character expression in 46 hybrids from 33 genera that included first generation hybrids, later generation hybrids, and hybrid species. Based on this survey, they concluded that hybrids have an almost equal probability of displaying intermediate and/or parental traits, depending on whether traits were under genic control (leading to parental character expression) or multigenic control (leading to intermediate character expression due to dominant or co-dominant allelic expression; Grant, 1975). Interestingly, they also found hybrids may have a relatively high proportion of novel or "extreme" character trait expression (10%, see review, Rieseberg and Ellstrand, 1993), particularly in later generation hybrids and instances of hybrid speciation. Their findings suggest that novel character expression is not uncommon in hybrids and hybrid species and can be an important evolutionarily creative force (Anderson, 1949; Arnold, 1997; Rieseberg et al., 1993).

In *Castilleja,* morphologically intermediate hybrids have been detected in zones of sympatry between *Castilleja miniata* and *C. linariifolia* (Ownbey, 1959; Heckard and Chuang, 1977; Holmgren, 1984). Ownbey (1959) described *Castilleja crista-galli* as

potentially being of hybrid origin between *C. miniata* and *C. linariifolia*, as this species occurs in areas of sympatry and appears to be morphologically intermediate between the two. Heckard and Chuang (1977) agreed with Ownbey in the putative hybrid origin of *C. crista-galli* and speculated that *C. crista-galli* became reproductively isolated in sympatry with its progenitors via allopolyploidy. More recent research by Mathews and Lavin (1998) has indicated that *Castilleja crista-galli* is not intermediate in morphology between its two putative progenitors, but clusters more closely with *C. miniata* and other closely related *Castilleja* species than with *C. linariifolia*. Further, while the authors retained specific status of *C. crista-galli* due to ecological separation, they are cautious, as *C. crista-galli* did not show any unique combinations of fixed characters. One criterion for recognizing a species is a unique combination of fixed character states (Nixon and Wheeler, 1990; Davis and Nixon, 1992).

In contrast to *Castilleja crista-galli, C. christii* was not originally inferred to be of hybrid origin by Holmgren (1984) due to the presence of species-specific morphological traits (see Results above). Early collections by John Christ in the 1950s at the summit of Mt. Harrison indicate that the zone of sympatry between *Castilleja christii, C. linariifolia,* and *C. miniata* has been present for at least 60 years, as has the potential for hybridization and introgression between these species. Our data do not suggest that *Castilleja christii* is morphologically a hybrid between *C. miniata* and *C. linariifolia* because *C. christii* is not morphologically intermediate between its two progenitors and has multiple unique or novel morphological traits (Table 3.2; 3.3).

Comparison of Morphological Traits in *Castilleja christii* to *C. miniata* and *C. linariifolia*

Though *Castilleja christii* is hybrid in origin (Chapter 2), our DFA analyses revealed that *C. christii* was not intermediate between *C. miniata* and *C. linariifolia* and instead clustered with *C. miniata* while also expressing novel character states (DFA; Tables 3.4 a, b; Figure 3.3; Chapter 2). Species determined to be of hybrid origin in other studies have exhibited morphological traits novel to those of their progenitors (Heiser, 1947, 1949; Grant, 1950, 1954, 1966; Schwarzbach et al., 2001) and the provisional delimitation of species can be determined by the presence of at least one fixed or non-overlapping morphological character (Wiens, 2007). *Castilleja christii* was found to be transgressive for five or six continuous morphological traits when analyzed with individuals from Mt. Harrison only or with control populations of *C. miniata* and *C. linariifolia* according to ANOVA analyses (Tables 3.2-3.3). Qualitatively, *Castilleja christii* also exhibited novel traits such as glandular hairs on the stems and cauline leaves, a lack of stem branching (Figures 3.1 and 3.2), and yellow to orange coloration of the inflorescence bracts (Holmgren, 1973). These characters are non-overlapping and are only found in *Castilleja christii*, which provides sufficient morphological evidence to support *C. christii* as a species, based on Wiens' (2007) delimitation criteria.

When populations of *Castilleja miniata* and *C. linariifolia* collected from areas outside of the range of *C. christii* (i.e., "control" populations) were included in ANOVAs, *C. christii* was found to be transgressive for six continuous traits versus five when compared with parental populations growing in sympatry (Tables 3.2 – 3.3). The increase in number of transgressive traits when *Castilleja christii* was compared with parental populations outside of the range of *C. christii* could be due to potential introgression of

*Castilleja linariifolia* and *C. miniata* into *C. christii* at the summit of Mt. Harrison, which may have gone undetected in our study. Alternatively, these may reflect later generations of *C. christii* X *C. christii* matings that have generated new combinations of genes, as seen in historically controlled hybridization studies involving *Nicotiana* L. (Stebbins, 1969), *Lotus* L. (Harney and Grant, 1964), and *Primula* L. (Haldane, 1959). New combinations of genes derived from novel allelic variation produced during hybridization has also been seen in natural homoploid hybridization studies of *Stepahnomeria* Nutt. (Gallez and Gottlieb, 1982), *Clarkia* Pursh (Gottlieb and Higgins, 1984), *Gossypium* L. (Wendel et al., 1991), and *Helianthus* L. (Rieseberg et al., 1990).

Transgressive or novel phenotypic character expression can be associated with instances of hybrid speciation due to reproductive isolation of the hybrids from parental species, which is maintained via ecological divergence and/or spatial separation from parental populations (Grant, 1975; Rieseberg et al., 1999, 2003; Hardig et al., 2000; Schwarzbach et al., 2001; Rosenthal et al., 2002; Gross et al., 2004) and can also be due to genic and/or chromosomal sterility barriers established during genetic recombination (Grant, 1958; Rieseberg, 1997). Traits found to be transgressive in *Castilleja christii* were generally associated with the length of the bracts or several corolla measurements, which are both floral display characters. Floral traits have been suggested to be under strong pollinator-mediated selection in several other studies (Campbell et al., 1997; Rieseberg et al., 1999, 2003; Rosenthal et al., 2002; Gross et al., 2004; Hersch and Roy, 2007). The combination of pollinator-mediated selection acting on phenotypic expression with that of physical ecological divergence of *Castilleja christii* from other sympatric *Castilleja* species on Mt. Harrison is evidence for speciation.

Species Delimitation in *Castilleja christii*

Our data support *Castilleja christii* as a separate species due to the presence of transgressive traits that have evolved in sympatry with *C. miniata* and *C. linariifolia* for at least the last 60 years. However, though morphological data seem to indicate three distinct taxa, morphological data alone do not preclude a potential hybridization event.

Our findings regarding morphological attributes of *Castilleja christii* are in agreement with the original species description written by Holmgren (1973), with the exception of finding shorter than described leaves (35.14 mm + 9.20 versus 25-50 mm from N. Holmgren, 1973), and bracts that were more shallowly lobed than those described (3.71 $\pm$ 1.17 mm inner segment length versus 2.5-6 mm from N. Holmgren, 1973 and outer segment lengths of 8.45 $\pm$ 1.62 mm versus 7-12 mm from N. Holmgren, 1973). At the summit of Mt. Harrison, it appears that several characters are of value for the differentiation between three *Castilleja* species occurring in sympatry (Tables 3.2 – 3.4; Figures 3.1 – 3.3; Key to species, below). *Castilleja linariifolia* had the shortest bracts of all three species (17.01 $\pm$ 3.68 mm), which were always lobed, was the tallest of all species measured in terms of stem and raceme heights (32.44 $\pm$ 6.88 mm and 8.34$\pm$ 3.02 mm respectively), was highly branching, and had an absence of exudate and gland tipped hairs. Additionally, one trait that is diagnostic for *Castilleja linariifolia* is the differential lobing of the calyx, the front being typically much more deeply lobed than the back (Holmgren, 1984), a character that was significant in our results as well (DFA; Table 3.4 a, b; Figure 3.3). *Castilleja christii* typically has short (20.24 $\pm$ 3.19 mm), lobed bracts, short racemes (5.42 $\pm$ 2.08 mm) and stems (22.89 $\pm$ 6.21 mm), is densely pubescent with hairs gland tipped, lacks exudate, and is typically unbranching (Tables 3.2

– 3.4; Figures 3.1 – 3.3). *Castilleja miniata* was found to have traits opposite to those of *C. christii,* with bracts being lobed and the longest of all three species (22.58 $\pm$ 3.55 mm), had longer racemes and stems than *C. christii* (7.34 $\pm$ 3.04 mm and 39.66 $\pm$ 13.36 mm, respectively), and is sometimes found with glandular hairs on stems and leaves, however hairs were more sparse than those found on *C. christii* (Clay, personal observation). In general, *Castilleja linariifolia* strongly separates morphologically from the other species, whereas *C. christii* and *C. miniata* can be characterized on a gradient (DFA; Table 3.4 a, b; Figure 3.3). Importantly, species are morphologically distinctive, particularly when analyses include control and Mt. Harrison populations (Tables 3.2 – 3.4; Figures 3.1 – 3.3).

Species delimitation in *Castilleja* is difficult, as species often overlap in many characters; this morphological complexity has been attributed to high levels of interspecific hybridization and polyploidy within this genus (Ownbey, 1959; Heckard, 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Chuang and Heckard, 1993; Egger, 1994). All three species occurring at the summit of Mt. Harrison were diploid based on chromosome counts (see Chapter 4). Therefore, *Castilleja christii* is of homoploid hybrid origin. *Castilleja christii* also appears to be ecologically distinct from *C. linariifolia* and *C. miniata* based on spatial separation of species at the summit of Mt. Harrison (Clay, personal observation; Chapter 4). Hybrid taxa that are both ecologically divergent and have become reproductively isolated from parental species have been considered species unto themselves (Templeton, 1981; Arnold et al., 1991; Rieseberg, 1991, 1997). Species defined by the general lineage concept (De Quieroz, 1998) are "segments of separately evolving metapopulation lineages where contingent properties

(e.g. morphological character divergence, reciprocal monophyly, niche divergence) can be reached independently though evolutionary time." *Castilleja christii* meets the requirements of a species as defined by De Quieroz based on multiple lines of evidence: (1) The presence of transgressive floral characteristics may indicate that these species are more isolated from sympatric congeners than they seem, despite an initial hybrid origin and common ploidy level, (2) though *Castilleja christii* overlaps with *C. miniata* and *C. linariifolia* for several traits, *C. christii* has several transgressive traits that clearly separate it from other sympatric *Castilleja* species morphologically, (3) our morphological data are in agreement with Holmgren's (1973) initial description for the *C. christii*, and (4) *C. christii* seems to be ecologically divergent at the summit of Mt. Harrison. Based on our data, we conclude that *Castilleja christii* is a homoploid hybrid species, originating from a past hybridization event (or events) involving *C. miniata* and *C. linariifolia*.

In this chapter, morphological data were evaluated for their potential to infer hybridization in *Castilleja* at the summit of Mt. Harrison, Idaho and the potential hybrid speciation of *C. christii*. Based on combined analyses from other chapters, we conclude that *Castilleja christii* is a homoploid hybrid species, originating from a hybridization event involving *C. miniata* and *C. linariifolia*. We have evidenced this through morphological analyses, in which *Castilleja christii* was significantly different for traits found in the parental species including some transgressive character states. Though morphology can be helpful at initially identifying potential hybrid species, a more exhaustive analysis is required to address the mechanisms involved in a hybrid speciation

event. In other chapters of this thesis, we present molecular, ecological, and cytological data to support our hypothesis of the homoploid hybrid origin of *Castilleja christii.*

Key to *Castilleja* species occurring at the summit of Mt. Harrison, ID

1. Inflorescence non-glandular-puberulent; not exceeding 1dm in height; bracts pale yellow with a purplish tinge; flowering just as snow melts for approx. two weeks, late June – mid-July……..……....*C. pallescens var. inverta*

1. Inflorescence with glandular hairs; plants taller than 1dm in height; plants not flowering until mid-July……...……2

2. Plant stems tall 32 ± 7 mm and branching above the base; inflorescence lacks white exudate; primary calyx lobes unequally cleft 2 ± 0.7 mm in front and 8 ± 1.5 mm in back; bracts shorter 17 ± 2.7 mm and always lobed with a deep red coloration….……..….*C. linariifolia*

2. Plants shorter than above; calyx lobes more or less equal……...……3

3. Plants stems shorter, 23 ± 6.2 mm, usually unbranching above the base; stems and leaves densely pubescent with hairs both gland-tipped and puberulent; racemes shorter, 5.4 + 2.1 mm; inflorescence usually without white exudate, if present, less than 25% of inflorescence covered; bracts pale yellow (almost white), yellow or yellow-orange…….……*C. christii*

3. Plant stems taller than above, 31.6 ± 8.7 mm, usually branching above the base; stems and leaves glabrous to hispid, rarely glandular-puberulent; racemes longer, 7.3 ± 3 mm; inflorescence with white exudate; bracts red to reddish-orange, rarely pink…….……...……*C. miniata*

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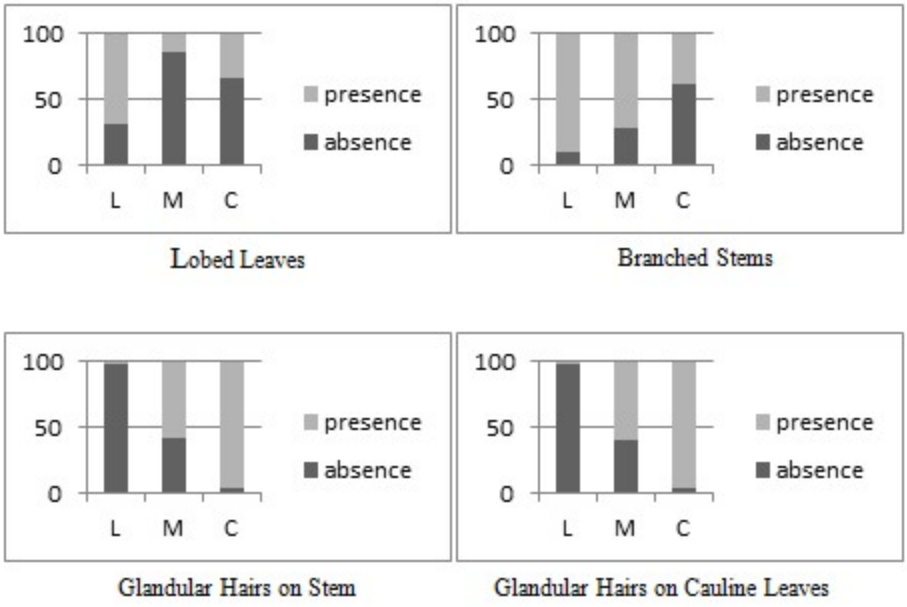


**Figure 3.1 Nominal morphological characters. Presence and absence data on four nominal characters measured on *Castilleja christii* (C), *C. miniata* (M), and *C. linariifolia* (L) obtained from individuals collected at both control and Mt. Harrison sites during the summer of 2009. For all traits shown, $P < 0.001$ (CHM statistic, FREQ procedure in SAS).**

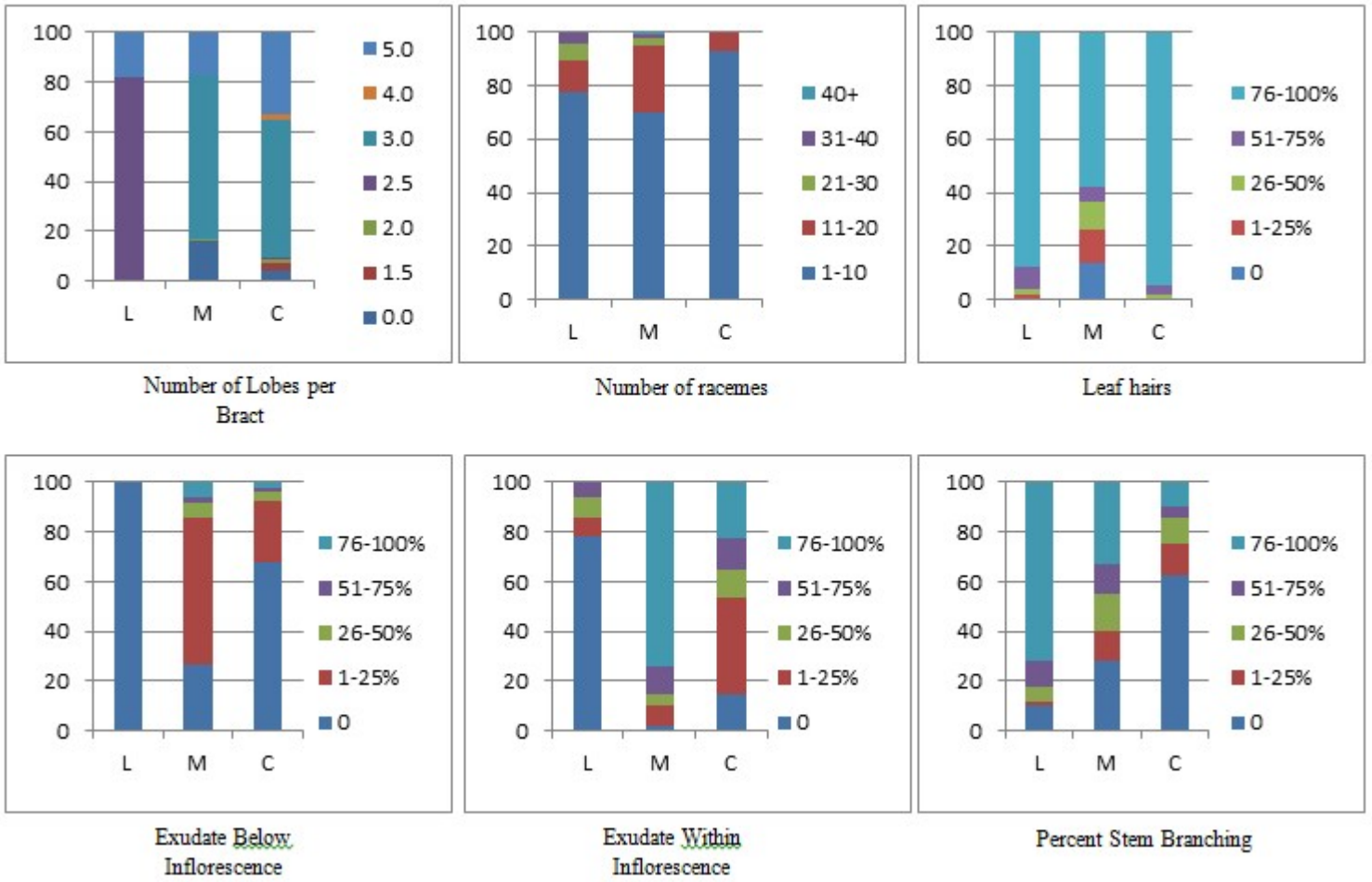


**Figure 3.2 Ordinal Morphological Characters. The mean percentages of five ordinal characters measured on *Castilleja christii* (C), *C. miniata* (M), and *C. linariifolia* (L) from control and Mt. Harrison sites. For all traits shown, $P < 0.001$ (CHM statistic, FREQ procedure in SAS).**

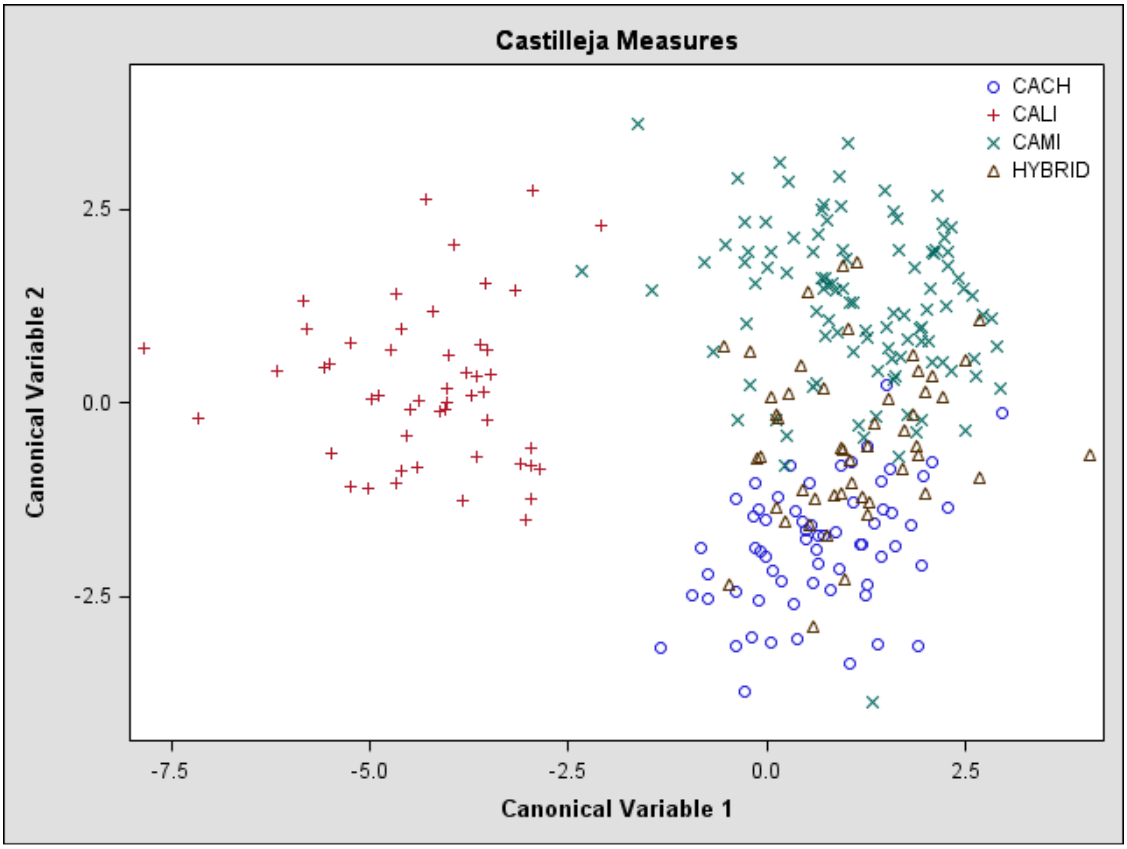


**Figure 3.3 Discriminant Function Analysis. Discriminant Function Analysis showing the first two axes of 15 continuous and discreet morphological variables used to distinguish between *Castilleja christii, C. miniata, C. linariifolia*, and individuals identified in the field as hybrids, obtained from individuals collected from the summit of Mt. Harrison and in control populations during the summer of 2009. All field identified hybrids and individuals identified as *Castilleja christii* in the field shared the genome of both *C. miniata* and *C. linariifolia.* CACH = *Castilleja christii*; CALI = *Castilleja linariifolia*; CAMI=*C. miniata.* Note that *C. linariifolia* (red plus signs) cluster heavily toward the left of the central axis while *C. miniata* (green x) and *C. christii* (blue circles and black triangles) cluster slightly to the right and overlap in the center of their values.**

**Table 3.1 Morphological characters measured on *Castilleja* species. Thirty-three morphological characters measured on *Castilleja miniata* (N=103), *C. christii* (N=114), and *C. linariifolia* (N=50).The first column denotes type of data measured (N=nominal; C=continuous; O=ordinal). The second column indicates where traits were measured (SRP=Snake River Plains Herbarium, Ada Co., Boise, ID; MTH= Mount Harrison, Cassia Co., ID). The third column denotes how data were measured: in the case of ordinal and nominal data, the number of levels is provided. The fourth column indicates how data were analyzed (DFA=discriminant function analysis; CMH= Cochran-Mantel-Haenszel Test; *=data were not analyzed).**

| Morphological Characters: | |
|---|---|
| Bracts, general: | |
| P/A lobed bracts | (N, SRP, 2, *) |
| Bracts, lowest flower on raceme | |
| Mean bract length | (C, SRP, mm, DFA) |
| Mean bract width | (C, SRP, mm, DFA) |
| Bract shape | (N, SRP, 2, CMH) |
| Number of lobes per bract | (N, SRP, 2, CMH) |
| Leaves | |
| Mean leaf length | (C, SRP, mm, DFA) |
| Mean leaf width | (C, SRP, mm, DFA) |
| Percent lobed leaves | (O, SRP, 4, CMH) |
| P/A lobed leaves | (N, SRP, 2, CMH) |
| Leaf hairs | (N, SRP, 4, *) |
| Infl | |
| Number of racemes | (N, MTH, count, DFA/CMH) |
| Total height of racemes | (C, MTH, cm, DFA) |
| Exudate within inflorescence | (O, SRP, 4, DFA/CMH) |
| Exudate below inflorescence | (O, SRP, 4, CMH) |
| Flowers | |
| Corolla length | (C, SRP, mm, DFA) |
| Corolla beak length | (C, SRP, mm, DFA) |
| Calyx length | (C, SRP, mm, DFA) |
| Calyx inner segment length | (C, SRP, mm, DFA) |
| Calyx outer segment length | (C, SRP, mm, DFA) |
| Calyx segment shape | (N, SRP, 4, *) |
| Calyx front | (C, SRP, mm, *) |
| Calyx back | (C, SRP, mm, *) |
| Pubescence | |
| P/A gland hairs on stem | (N, SRP, 2, CMH) |
| P/A gland hairs on cauline leaves | (N, SRP, 2, CMH) |
| Pubescence within inflorescence | (O, SRP, 4, *) |
| Pubescence below inflorescence | (O, SRP, 4, CMH) |
| Hair type below inflorescence | (N, SRP, 4, *) |
| Stems | |
| Stem max height | (C, MTH, cm, DFA) |
| Number of stems | (N, MTH, count, DFA/CMH) |
| Percent stem branching | (O, SRP, 4, DFA/CMH) |
| P/A branched stems | (N, SRP, 2, CMH) |

**Table 3.2 Continuous traits measured from both control and Mt. Harrison sites. Means (and standard deviations) of 11 continuous morphological traits measured from individuals collected from both control and Mt. Harrison sites. ** denotes data were log transformed. Means with the same symbol are not statistically different, according to SNK tests.**

| Character | Species F (d.f.= 2) | P value | *C. christii* | *C. linariifolia* | *C. miniata* |
|---|---|---|---|---|---|
| Mean bract length | 45.74 | <0.0001 | 20.24 (3.19) ∗ | 17.10 (3.34) ϕ | 22.58 (3.55) ψ |
| Mean bract width | 23.29 | <0.0001 | 5.92 (1.31) ∗ | 4.28 (0.87) ϕ | 5.75 (1.82) ∗ |
| Total raceme height | 32.20 | <0.0001 | 5.42 (2.08) ∗ | 9.36 (4.31) ϕ | 7.34 (3.04) ψ |
| **Corolla length | 40.39 | <0.0001 | 3.23 (0.15) ∗ | 3.44 (0.11) ϕ | 3.40 (0.17) ψ |
| **Beak length | 81.40 | <0.0001 | 2.31 (0.16) ∗ | 2.65 (0.13) ϕ | 2.49 (0.18) ψ |
| **Calyx length | 24.24 | <0.0001 | 2.88 (0.15) ∗ | 3.04 (0.14) ϕ | 3.00 (0.17) ϕ |
| Calyx inner segment length | 75.09 | <0.0001 | 3.71 (1.17) ∗ | 1.85 (0.74) ϕ | 4.52 (1.54) ψ |
| Calyx outer segment length | 9.48 | <0.0001 | 8.45 (1.62) ∗ | 8.57 (2.06) ∗ | 9.47 (1.86) ψ |
| Stem max height | 79.17 | <0.0001 | 22.89 (6.21) ∗ | 35.82 (9.57) ϕ | 39.66 (13.36) ψ |
| **Mean leaf length | 18.94 | <0.0001 | 3.52 (0.28) ∗ | 3.46 (0.33) ∗ | 3.71 (0.22) ψ |
| Mean leaf width | 26.91 | <0.0001 | 4.41 (1.57) ∗ | 3.12 (1.33) ϕ | 5.39 (2.22) ψ |

**Table 3.3 Continuous traits measured from Mt. Harrison sites only. Means (and standard deviations) of 11 continuous morphological traits measured from individuals collected from Mt. Harrison sites only. Means with the same symbol(s) are not statistically different from each other, according to SNK tests. Log transformation failed to normalize the data.**

| Character | Species F (d.f.= 2) | P value | *C. christii* | *C. linariifolia* | *C. miniata* |
|---|---|---|---|---|---|
| Mean bract length | 30.32 | <0.0001 | 20.24 (3.19) ∗ | 17.01 (2.68) ϕ | 23.05 (3.50) ψ |
| Mean bract width | 7.11 | 0.0003 | 5.92 (1.31) ∗ | 4.67 (0.96) ϕ | 5.88 (1.61) ∗ |
| Total raceme height | 15.79 | <0.0001 | 5.42 (2.08) ∗ | 8.34 (3.02) ϕ | 6.81 (2.75) ψ |
| Corolla length | 30.06 | <0.0001 | 25.72 (3.89) ∗ | 30.66 (3.40) ϕ | 30.23 (4.71) ϕ |
| Beak length | 60.36 | <0.0001 | 10.21 (1.62) ∗ | 13.86 (1.52) ϕ | 12.66 (2.10) ψ |
| Calyx length | 16.87 | <0.0001 | 18.07 (2.64) ∗ | 20.55 (2.40) ϕ | 20.53 (3.56) ϕ |
| Calyx inner segment length | 32.19 | <0.0001 | 3.71 (1.17) ∗ | 1.89 (0.79) ϕ | 4.38 (1.37) ψ |
| Calyx outer segment length | 13.18 | <0.0001 | 8.45 (1.62) ∗ | 7.89 (1.39) ∗ | 9.63 (1.82) ψ |
| Stem max height | 37.05 | <0.0001 | 22.89 (6.21) ∗ | 32.44 (6.88) ϕ | 31.57 (8.71) ϕ |
| Mean leaf length | 8.79 | 0.0025 | 35.14 (9.20) ∗ | 36.97 (10.52) ∗ | 41.12 (8.51) ψ |
| Mean leaf width | 6.17 | <0.0001 | 4.41 (1.57) ∗ | 3.32 (1.37) ϕ | 4.86 (2.01) ∗ |

**Table 3.4a Standardized Canonical Discriminant Function Coefficients for the three canonical axes for 15 continuous or discreet variables used to distinguish between *Castilleja christii*, *C. miniata,* and *C. linariifolia* collected from the summit of Mt. Harrison and in control populations during the summer of 2009. An 'l' before the variable denotes data have been log transformed.**

| Variable | Function 1 | Function 2 |
|---|---|---|
| Percent Variation | 75.3 | 24.7 |
| lMean_bract_length | 0.5757600473 | -.0158724211 |
| Mean_bract_width | 0.0255908647 | -.2827782951 |
| lMean_leaf_length | 0.1812173569 | 0.0773222354 |
| Mean_leaf_width | 0.3214660066 | 0.0723235705 |
| Total_height_racemes | -.2895702919 | -.1852385871 |
| lCo_length | 0.2277298547 | -.4850532652 |
| lCo_galea_length | -.5425444784 | 0.7254350190 |
| lCa_length | -.4195814479 | 0.0975361298 |
| Ca_in_seg_length | 0.4976408288 | 0.1100669724 |
| Ca_out_seg_length | -.1317302589 | 0.1016185811 |
| Stem_max_height | -.0502970372 | 0.8038468913 |
| Num_racemes | -.1819998279 | -.2220084399 |
| Num_stems | 0.1003524053 | 0.1732361707 |
| Exudate_within_infl | 0.6000295148 | 0.3798330917 |
| Percent_stem_branching | -.2994488438 | 0.1380515917 |

**Table 3.4b Class means on Canonical Variables from Discriminant Functions Analysis for the first three canonical axes for 15 continuous or discreet variables used to distinguish between *Castilleja christii*, *C. miniata,* and *C. linariifolia* collected from the summit of Mt. Harrison and in control populations during the summer of 2009. Comparing the mean of a species with the corresponding function coefficients (Table 4a) provides insight into how traits are represented phenotypically within the sample and among species.**

| Species | Function 1 | Function 2 |
|---|---|---|
| *C. christii* | 0.73 | -1.20 |
| *C. linariifolia* | -3.98 | 0.20 |
| *C. miniata* | 1.11 | 1.22 |

# CHAPTER 4: PHENOLOGICAL, ECOLOGICAL, AND SPATIAL DIFFERENCES CONTRIBUTING TO THE EVOLUTIONARY SUCCESS OF THE HOMOPLOID HYBRID *CASTILLEJA CHRISTII* (OROBANCHAEAE)

## Introduction

Hybridization and introgression are considered important evolutionary forces contributing to speciation in plants (Stebbins, 1969; Grant, 1981; Arnold, 1992). These processes are driven by gene flow between species, which can result in uni- or bi-directional genetic exchange and blurred species boundaries, thereby creating either unstable tension zones or, if conditions are adaptive, may lead to speciation in hybrid or introgressed populations (Barton and Hewitt, 1985; Rieseberg, 1997). Well-documented cases of plant speciation exist for hybrids that experience genome duplication (allopolyploidy), which results in effective reproductive isolation from parental species (reviewed in Soltis and Soltis, 2009). Hybrid speciation without genome duplication (homoploid hybrid speciation) is consistently more rare in nature, because hybrids must become reproductively isolated from progenitor species in sympatry or parapatry and must develop other isolating mechanisms (i.e., chromosomal or genetic incompatibilities and/or ecological and spatial isolation) to prevent genetic swamping from parental species and to effectively compete for space and resources (Grant, 1981; McCarthy et al., 1995; Rieseberg, 1997; Buerkle et al., 2000; Barton, 2001; Abbott et al., 2003).

Niche separation between hybrid and parent species is crucial to avoid potential genetic swamping or competition from parental species (Lewonton and Birch, 1966; Grant, 1981; Templeton, 1981; Schulter, 1998; Buerkle et al., 2000). In empirically tested cases of homoploid hybrid speciation, most stabilized hybrid species were found to be ecologically or spatially distinct from their progenitors, thus minimizing the potential of genetic swamping and reinforcing reproductive isolation (Abbott, 1992; Arnold, 1997; Rieseberg, 1997; Brennan et al., 2009). Discovering how homoploid hybrid species achieve ecological divergence and which adaptations allow recruitment outside of parental niches involves a complex series of questions and examinations that make studying homoploid hybrid speciation a difficult task (Rieseberg, 1997).

The importance of ecological selection in the reproductive isolation of homoploid hybrid species has been modeled to test the role of ecological or spatial divergence for the establishment of homoploid hybrid species' reproductive isolation. Buerkle et al. (2000) built a spatial model that incorporated habitats for two parental species and a third unoccupied, novel habitat. The model demonstrated that as the strength of ecological selection against parental types in the unoccupied habitat increased, and when hybrids had high levels of fertility, the frequency of hybrid speciation also increased. When the unoccupied habitats were removed from the model, hybrid speciation was rare or unstable, the hybrids being subject to eradication through genetic swamping (Buerkle et al., 2003). Similarly, McCarthy et al. (1995) constructed a model to examine if chromosomal rearrangements alone could theoretically lead to hybrid speciation. Their model demonstrated that in the absence of ecological selection, chromosomal rearrangements alone were not sufficient to lead to hybrid speciation. From these

theoretical models, the importance of ecological selection and the availability of novel habitats for colonization in the promotion of new hybrid species are substantiated.

In nature, ecological or spatial divergence has been documented in almost all examples of homoploid hybrid speciation (Gross and Rieseberg, 2005). An intermediate habitat between parental types may allow a hybrid derivative to easily colonize such a location. This scenario has been documented in the homoploid hybrid *Iris nelsonii*, which occupies a divergent habitat that combines features from all three parental progenitors: *Iris fulva* occupies shady, shallow riparian areas surrounding bayous; *I. hexagona* inhabits sunnier, deeper swamp waters; and *I. brevicaulis* inhabits drier upland pastures and forests. *Iris nelsonii* combines these features and is found in areas of shady, deep water cypress swamps (Arnold, 1993). Alternatively, hybrid species may develop transgressive traits though chromosomal recombinations, enabling hybrid species to escape the hybrid zone and to occupy niches that are novel to their progenitor species, as in the case of the homoploid *Helianthus* species, *H. anomalus, H. deserticola*, and *H. paradoxus* (Rieseberg et al., 1993).

Within the genus *Castilleja,* the combination of allopolyploidy and interspecific hybridization has significantly contributed to the evolution of the genus (Ownbey, 1959; Heckard, 1968; Heckard and Chuang, 1977; Hersch-Green and Cronn, 2009; Tank and Olmstead, 2009). Hybrids may become genetically isolated from parental species via polyploidy, which seems to be the only barrier to hybridization in zones of sympatry (Holmgren, 1984) other than contextual pollinator-mediated selection (Hersch and Roy, 2007). Hybridization events within the genus have largely been inferred from morphological observations in the field and verified via cytological studies in the lab or

with molecular markers (Ownbey, 1959; Holmgren, 1984; Heckard, 1968; Heckard and Chuang, 1977; Egger, 1994; Hersch and Roy, 2007; Hersch-Green and Cronn, 2009).

On 80 hectares at the summit of Mt. Harrison (Cassia county, Idaho), the rare diploid endemic Christ's paintbrush (*Castilleja christii* N. Holmgren) occurs in sympatry with *Castilleja miniata* Gray and *C. linariifolia* Benth. *Castilleja christii* is critically imperiled and at high risk of extinction due to extreme rarity, earning it a G1 global rank (CPC, 2005). Additionally, *Castilleja christii* is considered a candidate species for Federal Endangered Species Status by the U.S. Fish and Wildlife Service (CPC, 2005). In contrast, *Castilleja miniata* and *C. linariifolia* have widespread distributions and multiple ploidy levels (Heckard and Chuang, 1977).

*Castilleja christii* was first described by Holmgren (1973), who noted the bracts of this species were yellow to yellow-orange, and are different than bracts of *C. miniata* and *C. linariifolia,* which are generally red or reddish orange (Holmgren, 1984). Field-based observations of intermediate forms between *Castilleja christii* and other congeneric species led us to explore putative hybridization events occurring on Mt. Harrison. Analyses of molecular and morphological data have indicated that *C. christii* is a homoploid hybrid between the sympatric *C. miniata* and *C. linariifolia* based on the absence of unique alleles (Chapter 2) and transgressive morphological character states in *C. christii* (Chapter 3).

In this chapter, we determine whether: (1) *Castilleja christii* is ecologically and/or spatially distinct from *C. miniata* and *C. linariifolia* in an area of sympatry and, if so, what factors contribute to isolation from parental species?; (2) flowering times contribute to pre-zygotic reproductive isolation of the homoploid hybrid *Castilleja christii*; (3)

ploidal differences among *Castilleja* species at the summit provide a barrier to reproduction among species; and (4) *Castilleja* species on Mt. Harrison produce viable offspring through seed germination trials. These data will be compared with molecular and morphological data in our broader scale study.

## Methods

### Field Site

The single field site for sampling phenological, ecological, and spatial differences between *Castilleja christii, C. linariifolia*, and *C. miniata* was in an area of sympatry on 80 hectares at the summit of Mt. Harrison, ID (Cassia Co.; Figure 4.1 and 4.2) and is inclusive of the entire range of *C. christii* (Figure 4.1 and 4.2).

### Plot Establishment

Plots at the summit of Mt. Harrison were circular and 25 meters in diameter. A center point was established at each plot and its coordinates determined using a GPS. A wooden stake was driven into the ground at each center point and was flagged. Elevation, slope, aspect, soil type, weather (e.g., cloudy, sunny, precipitation), and a brief description of plant cover within the plot were recorded.

Three types of plots were established at the Mt. Harrison site, with number of plots following each type: (A) plots containing only *C. christii* (n = 4); (B) plots containing only *C. miniata* (n = 4); and (C) plots containing only *C. linariifolia* (n = 2). In total, 10 plots on Mt. Harrison were established. To sample as widely as possible throughout the range of *Castilleja christii,* plots were established at least 100 m apart. Equal sample sizes at the Mt. Harrison site for both *Castilleja linariifolia* and *C. miniata*

were unobtainable because *C. linariifolia* was only found in two small populations at the summit.

Microplots and Ecological Sampling

Five, one meter square micro-plots were established per plot, one at center point and the others 12 meters from center point in each cardinal direction to estimate percent cover of woody plants, forbs, and graminoids and to assess differences in structural characteristics between plots. Coverage values were obtained using ocular estimations by D. Clay. Ocular estimations were calibrated by practicing quantification of cover by first estimating cover of several species within a microplot by sight and then using a one percent square to measure the coverage of those species within the microplot manually. Within all microplots, percent cover estimates exceeded 100% to account for overlap of vegetation and/or ground coverage.

Spatial Differences among *Castilleja* Species on Mt. Harrison

Geographical positioning system (GPS) points were taken at the center of each plot. These were projected in ArcGIS and placed onto a spatial layer that separates each aspect (North, South, East, West) by color. This is used to address spatial differences between the three sympatric *Castilleja* species.

Phenology

The number of open flowers per inflorescence were counted on each *Castilleja* individual sampled for all three species. These counts were then plotted graphically by species and date to assess phenological differences within and among sites. To obtain counts of flowers open per inflorescence from twenty-five individual plants per plot, belt

transects one meter in width were run from center point in random directions, extending out 25 meters. Only one *Castilleja* species per transect was sampled, and transects radiated from the center point. Flowers of each *Castilleja* individual within the one meter belt along a transect were counted until 25 individuals had been sampled. If at the end of a transect 25 individuals were not sampled, another randomized transect was run.

Infructescence Collection for Seed Germination Studies

To assess seed germination differences in all three *Castilleja* species, two plants were marked along one transect per plot for infructescence collection, which were collected in fall of 2009. These were flagged and marked with a metal tag inscribed with a unique number at the base of the plant during the field season from the second and the $24^{th}$ individual collected within each plot for a particular species.

Fifty seeds from each species were subjected to cold-moist stratification. Seeds were placed in multiples of 10 in five petri dishes in between moist filter paper and placed in refrigerator at 1° - 2° C for approximately 150 days (Luna, 2005). Once seeds germinated and cotyledons were present and green, they were counted, removed from the petri dishes in the refrigerator, and were transferred to pots containing *Lupinus* sp. in standard potting medium. *Castilleja* is hemiparasitic and successful establishment is better when grown with a host species such as *Lupinus spp.* (Luna, 2005).

Cytology and Pollen Mother Cell Collection

To evaluate potential differences in ploidy between parental taxa and hybrids, unopened inflorescences containing immature floral buds were collected during the summer of 2009 for use in cytological chromosome counts. On the top of Mt. Harrison,

two *Castilleja* plants from each plot were marked with flagging for the collection of pollen mother cells later in the season. In August of 2009, when all taxa were fully in bloom, plots were revisited and inflorescences containing immature floral buds were placed in small Nalgene bottles containing Farmer's solution (3 anhydrous ethanol: 1 glacial acetic acid, v/v) to fix the tissues, preserving them for future use in the lab (see Chuang and Heckard, 1993). The samples were placed on ice for transport in the field and refrigerated in the laboratory until needed.

Individuals from each species collected for cytological analyses were verified through genetic analysis (see Chapter 2). For chromosome counts, one inflorescence was placed in a petri dish under a dissecting microscope. Samples were kept in Farmer's solution on ice while they were out of the refrigerator. *Castilleja* inflorescences are indeterminate, therefore the lowest unopened (oldest) flower from the inflorescence was used because pollen cells had likely undergone meiosis, resulting in condensed chromosomes and more reliable counts. Occasionally, flowers from the middle of the inflorescence were used; these inflorescences usually contained pollen cells in various stages of meiosis. The calyx and corolla were cut transversely and dissected away, leaving the androecium and gynoecium. The anthers were removed from the filaments and were carefully placed onto a slide in a petri dish. The anthers were soaked in 100 ul room temperature MAA (3 methanol : 1 glacial acetic acid, v/v), until the MAA evaporated, at which point 100 ul more MAA was pipetted onto the anthers, soaking them in MAA for a total of 10 minutes. After 10 minutes, any remaining MAA was wicked away using a kimwipe. To the anthers on the slide, one drop of iron aceto-carmine was added. The petri dish containing the slide with the anthers was moved under

the dissecting scope and using tweezers and a dissecting needle, anthers were carefully cut transversely, and the pollen grains were carefully squeezed out of the anthers into the iron aceto-carmine. Once all anthers were devoid of pollen, anther walls were carefully removed and a glass coverslip was placed over the drop of aceto-carmine containing the pollen grains. The slide was then gently heated to just before boiling using a gas flame (approximately 5-10 seconds).

Pollen grain development was verified using a Nikon Eclipse 80 phase contrast microscope (Nikon, Inc. Melville, NY) under 60x magnification. The slide was then sealed using clear nailpolish around the coverslip to preserve the slide. Nail polish was allowed to set overnight. For chromosome counts, pollen grains were viewed with a 100X oil immersion lens in either the Nomarski or the contrast "Phase 3" light setting using the Nikon Eclipse 80 phase contrast microscope. Pictures were taken using a Spot Insight Color camera (Spot Imaging Solutions, Sterling Heights, MI) attached to the scope, using the Active Image software program.

Chromosomes from as many cells as possible were counted (generally between 2 – 5 cells per flower) and averaged for a final count per individual (data not shown). Up to three flowers per individual were analyzed. Some counts were dysploid due to the lack of clarity of the meiotic cells; when this occurred, we determined the ploidy level to be the nearest multiple of 12 (in *Castilleja* N = 12; Heckard and Chuang, 1977).

Data Analysis

Coverage values for plant species and bare ground within each of the five microplots were pooled (averaged among plots of the same species) for each plot. Pooled coverage values were used to evaluate potential differences in plant cover diversity and

evenness among plots of the three *Castilleja* species using three diversity indices. The Shannon-Wiener Index of Diversity (Shannon, 1948) is defined as:

$$H = -\sum p_i (\ln p_i).$$

where: $p_i$ = the proportion of individuals in the $i$th species. This index was used to measure the 'total' diversity within plots at the summit of Mt. Harrison, ID. The Shannon-Wiener index increases with the number of species in the community and is an ordinal scale; an index of 2 does not suggest that community is twice as diverse as a community with an index of 1. Because Shannon-Wiener index is influenced by the number of species in each plot, we calculated an evenness (J) value:

$$J = H' / H_{max}$$

where $H_{max}$ is the maximum value of diversity for the number of species present ($S$); ($H_{max} = \ln S$) (Pielou, 1975).

Richness (the number of species within each microplot) within plots was averaged from among all five microplots, thereby obtaining one value for each plot. Plots of each *Castilleja* species were further averaged to obtain one value for each plot.

Plots containing one *Castilleja* species (either *C. christii, C. miniata* or *C. linariifolia*) were evaluated for their similarity in vegetation composition using Sørensen's (1948) similarity index:

$$QS = 2C / (A + B)$$

where $QS$ is the index of community similarity, $A$ is the number of species in plot 1, $B$ is the number of species in plot 2, and $C$ is the number of species the two plots have in common.

All of the aforementioned metrics were analyzed for differences using an analysis of variance (ANOVA), with subsequent Student-Newman-Kewls (SNK) means comparisons.

## Results

Ecological Sampling

Plots containing *Castilleja linariifolia* had the most diversity among plots of all *Castilleja* species occurring at the summit of Mt. Harrison, according to the Shannon-Weiner diversity index (H' = 2.39; Table 4.1). None of the averaged Shannon-Weiner values estimating coverage were significantly different among plots containing each of the three *Castilleja* species, according to ANOVA test statistics ($P = 0.0593$; Table 4.1). *Castilleja miniata* and *C. christii* plots were not significantly different from each other according to SNK tests, had similar Shannon-Weiner diversity values of 1.88 and 1.84, respectively (Table 4.1), and the diversity within each plot was similar.

Evenness values among all three sympatric *Castilleja* species were not significantly different (ANOVA; $P = 0.1449$; Table 4.1). Evenness increases as the value $J$ increases; high diversity and less evenness within a population occurs when $J$ is closer to zero (Pielou, 1975). Evenness values within plots containing each *Castilleja* species were relatively high and ranged from 0.61 - 0.73, indicating more variation within plots and a relatively diverse plant community among and within plots for plots containing any given *Castilleja* species (Table 4.1).

Plot richness varied between *Castilleja* plots, with *Castilleja linariifolia* plots being on average the most species rich (average number of species per plot = 27; Table

4.1), *C. miniata* had the smallest number of species per plot (15.5; Table 4.1), and *C. christii* plots were found to be intermediate (average number of species per plot = 22.2). When species richness for all plots of each *Castilleja* species were pooled, *Castilleja christii* plots were the most species rich, with 40 total species across four plots, however many of these species were uncommon to rare (Table 4.2). In comparison, *Castilleja linariifolia* plots contained 36 species across four plots and *C. miniata* plots contained 26 species total. ANOVA test statistics between plots of all three species for species richness were not significant ($P = 0.1167$; Table 4.1).

When comparing Sørensen's (1948) index of similarity values, coverage of plant species and inorganic coverage presence within all species of *Castilleja* plots, *Castilleja christii* plots were most similar to *Castilleja miniata* (62.69% similarity; Table 4.3), however *C. christii* also had relatively high similarity with *C. linariifolia* plots (57.14%, Table 4.2). Plots of both parental species had a similarity value of 48.84% (Table 4.3).

Though the Shannon-Weiner index, evenness and richness values were not significantly different between the three species according to ANOVA and SNK tests (with the exception of *Castilleja linariifolia* for Shannon-Weiner), and plant species similarity compared among the different *Castilleja* species' plots was high, differences in community composition were observed among each of the different *Castilleja* plots when comparing pooled lists of the top species' coverage within respective plots (Table 4.2). The highest coverage values in *Castilleja linariifolia* plots were *Artemisia tridentata subsp. vaseyana* (24.1%), *Phlox multiflora* (14.5%), and rock (10.5 %). In comparison, the top values pooled from all plots for *Castilleja christii* include bare ground (37.07%), *Festuca idahoensis* (16.70%), *Artemisia tridentata subsp. vaseyana* (10.30%), and

*Solidago multiradiata* (6.60%); and for *C. miniata* plots: bare ground (31.42%), *Symphyotrichum foliaceum* (14.17%), *Penstemon rydbergii* (10.05%), and *Lupinus argenteus* (8.55%) (Table 4.2). When comparing coverage values between *Castilleja christii* plots and plots of its parents, *C. christii* plots tend to have a wide range of species when pooled across the plots (40 total), with some of the top species being unique (*Festuca idahoensis* and *Solidago multiradiata*) and some species being shared among plots of its parental types (*Artemisia tridentata* subsp. *vaseyana* and bare ground). Therefore, although the percent similarity among plots for each species was high, the top species represented within plots of a particular *Castilleja* species differed.

Phenology

*Castilleja christii* bloomed as early as July 5, 2009, earlier than the other two *Castilleja* species at the summit of Mt. Harrison (D. Clay, pers. obs.). By July 21, *Castilleja christii* was in full bloom in areas of the summit that were not covered by snow (Table 4.4). *Castilleja miniata* was also flowering on and a few days after July 21, however *C. christii* had fewer flowers open during the earlier part of July, with most of the flowers blooming later in early August (Table 4.4). *Castilleja christii* also had some plots nearer to the summit ridgeline (plot 16, Figure 4.1) that bloomed later (late July, early August) as they were released from snow (Table 4.4). *Castilleja linariifolia* did not bloom until mid-August (Table 4.4).

Seed Germination

Of the 50 seeds per species subjected to cold-moist stratification: 22% (n = 11) of *Castilleja miniata* seeds germinated; however, no seedlings reached maturity (i.e., none

survived transplanting to soil) 26% (n = 13) of *Castilleja linariifolia* seeds germinated and no seedlings reached maturity, and 80% (n = 40) of *Castilleja christii* seeds germinated. Some *Castilleja christii* seedlings persisted for a few weeks in the greenhouse before dying, however five individuals produced multiple true leaves and one individual survived to flowering.

Cytology

All species at the summit were found to be diploid ($n$ = 12) based on chromosome counts from pollen mother cells, with the exception of one small population of *Castilleja miniata,* which was tetraploid ($n$ = 24; Table 4.5). This was not surprising, because this population of *Castilleja miniata* was morphologically different than other *C. miniata* populations on Mt. Harrison: instead of having green stems and red bracts and being relatively tall (approximately 3.1 decimeters; Chapter 2), these plants were shorter (less than described), had purple stems and darker green leaves, and the bracts were pinkish purple.

Spatial Variation

All three *Castilleja* species found at the summit of Mt. Harrison were spatially distinct (Figure 4.2). *Castilleja miniata* plots were found on North or East facing slopes, *C. linariifolia* were found on south or southwest facing slopes, and *C. christii* plots were located on north or west facing slopes. *Castilleja christii* had a broader distribution at the summit than either of the parental species, which were more restricted in their locations (Figure 4.1).

## Discussion

The combination of reproductive isolation and ecological and/or niche divergence is paramount in almost all cases of homoploid hybrid speciation. When chromosomal isolation does not prevent genetic swamping of a hybrid neospecies with the genome of progenitor species, reproductive and ecological divergence ensures that a hybrid neospecies would not be outcompeted by the parental species (Templeton, 1981). Due to chromosomal rearrangements during hybridization, hybrid species often have morphological traits that are novel or transgressive from their progenitors, which may assist them in escape from, and establishment outside, of the hybrid zone (Rieseberg, 1997; Gross and Rieseberg, 2005). Based on genetic and morphological data, *Castilleja christii* is of hybrid origin and has morphological traits that are novel and transgressive from those of its progenitors, *C. miniata* and *C. linariifolia* (Chapters 2 and 3, respectively). However, these data do not explain the persistence and maintenance of *Castilleja christii* over time, which is likely due to reproductive barriers enforced via ecological and spatial differences between *C. christii* and its progenitors, despite *C. christii* sharing a common ploidy level.

### Ecological Divergence in *Castilleja christii*: Habitat, Temporal and Floral Differences Contribute to Reproductive Isolation

Most cases of homoploid hybrid species have been shown to occupy ecologically divergent habitats, have divergent floral or pollinator assemblages, and phenological attributes that differ when compared with their parents (see examples in Gross and Rieseberg, 2005). Homoploid hybrids may succeed in habitats that feature a combination of parental attributes, (i.e. *Iris*; Arnold, 1993), may have colonized a habitat extreme from

their progenitors (i.e., *Helianthus*; Rieseberg, 1991), or have developed transgressive floral traits novel to their progenitors, allowing them to shift pollinator strategies (i.e., *Penstemon*; Straw, 1956). *Castilleja christii* was found in habitats and spatial aspects that displayed attributes of both *C. miniata* and *C. linariifolia* at the summit, while also displaying morphological traits that were either intermediate or transgressive from parental types, suggesting a potential pollinator shift (Chapter 3).

Spatially, *Castilleja* species are allopatric at the summit, with *C. christii* having a relatively wide range, occupying a northwest ridgeline in between the true and false summits of Mt. Harrison, ID, and encompassing approximately 80 hectares (Figure 4.1). *Castilleja christii* tended to be found on north or west-facing aspects (Figure 4.1). *Castilleja miniata* has a smaller range than *C. christii* at the summit, is generally restricted to a northeast ridgeline and tends to be found on north or east-facing slopes, encompassing only between 20 - 30 hectares (Figure 4.1; Figure 4.2). *Castilleja linariifolia* had the smallest distribution at the summit, and is known from only two small populations no larger than 25 meters square (Figure 4.1). In fact, we did not find *C. linariifolia* growing until it flowered in early August. This species tended to be found on south-facing slopes near the summit (Figure 4.2), however this species was more abundant at lower elevations on Mt. Harrison. The spatial distribution of the three *Castilleja* species at the summit of Mt. Harrison is not unlike those of Arnold's (1993), irises where the hybrid neospecies, *Iris nelsonii*, occupies a divergent habitat that combined features from all three parental progenitors: *Iris fulva* occupies shady, shallow riparian areas surrounding bayous; *I. hexagona* inhabits sunnier, deeper swamp waters;

and *I. brevicaulis* inhabits drier upland pastures and forests. *Iris nelsonii* combines these features and is found in areas of shady, deep water cypress swamps (Arnold, 1993).

*Castilleja linariifolia* had the highest diversity according to Shannon-Weiner indicies, as compared with *C. christii* and *C. miniata*, which were not significantly different (Table 4.1; 4.3); however, plant species cover and substrate composition were different among the three *Castilleja* species (Table 4.2). As with spatial distributions, *Castilleja christii* shared a combination of plant species and cover types that were found in parental plots, while also co-occurring with species that were not found growing with either *C. miniata* or *C. linariifolia* (Table 4.2). Ecological and spatial divergence from parental types may provide reproductive isolation between sympatric congenerics at the summit. As models of homoploid hybrid speciation have shown (e.g., Buerkle et al., 2000), these two factors are important tenets for the success of homoploid hybrid species. Several examples of this model exist in nature as well, where homoploid hybrid species are maintained through a combination of chromosomal and ecological or spatial barriers, providing effective reproductive isolation (e.g., Gallez and Gottlieb, 1982; Rieseberg 1991; DeMarais et al., 1992; Arnold, 1993; Sang et al., 1995, 1997; Wolfe et al., 1998; Sang and Zhang, 1999; Brochmann et al., 2000; Ferguson and Sang, 2001; Maki and Murata, 2001; Wolfe and Randle, 2001; Hardig et al., 2002; Wang et al., 2001 Lowe and Abbott, 2004; James and Abbott, 2005).

Transgressive Morphological Traits Derived via Hybridization May Be Responsible for Reproductive Isolation, Niche Divergence and Speciation

Recombination during meiosis may result in novel chromosomal configurations that may manifest in novel or transgressive morphological and ecophysiological changes

in a hybrid derivative. These changes can assist the hybrid neospecies in escaping from the hybrid zone, thus ensuring its success as a species and eliminating the potential for genetic swamping (Rieseberg, 1997; Gross and Rieseberg, 2005). The adaptation of transgressive traits in hybrid species have been explained by studies using quantitative trait loci (QTL; reviewed in Rieseberg et al., 1999), which have indicated that genetically divergent lineages have adapted transgressive phenotypes through hybridization, and these phenotypes provide a means by which the hybrid species may achieve evolutionary independence (Lai et al., 2005). It is likely that *Castilleja christii* is chromosomally admixed, due to the presence of one of each parental copy of the *waxy* gene in each *C. christii* analyzed: the presence of both parental alleles in *Castilleja christii* may have become fixed within the genome as a homozygotic condition, with all *Castilleja christii* individuals from intraspecific crosses inheriting both parental "alleles" (now paralogs) regularly (Chapter 2). Genetic recombination may be the reason why *Castilleja christii* has traits that are novel and transgressive from its progenitors (Chapter 3). Traits found to be transgressive in *Castilleja christii* were generally associated with the length of the bracts or several corolla measurements, which are both floral display characters (Chapter 3). *Castilleja christii* generally has yellow to yellow-orange bracts, while the parental species generally have red colored bracts (Holmgren, 1973, 1984). Floral traits have been suggested to be under strong pollinator-mediated selection in several other studies (Campbell et al., 1997; Rieseberg et al., 1999, 2003; Rosenthal et al., 2002; Gross et al., 2004; Hersch and Roy, 2007) and the difference in the color of bracts may indicate a potential pollinator shift between the hybrid derivative *Castilleja christii* and its progenitors (e.g., yellow flowers tend to be pollinated by *Bombus spp.*, while red flowers

are generally pollinated by hummingbirds; Duffield, 1972). Our evidence suggests that *Castilleja christii* flowers for a longer period of time than either *C. miniata* or *C. linariifolia*: the former had the most overlap in flowering time with *C. christii* and the latter flowered much later than *C. christii* (Table 4.4). The combination of pollinator-mediated selection acting on phenotypic expression and flowering time with that of physical ecological divergence due to spatial differences and differences in plant community composition of *Castilleja christii* from other sympatric *Castilleja* species on Mt. Harrison is evidence for speciation.

Potential Selection at the Summit in Favor of *Castilleja christii*

Seed germination studies from collections of all three *Castilleja* species at the summit of Mt. Harrison suggest that *C. christii* has a higher germination rate and seedling survival (80%; seedlings were robust and one seedling was raised to flowering) than those of *C. miniata* and *C. linariifolia*, which were lower (~25%; seedlings were delicate and none survived). These tests were carried out in a controlled laboratory environment. If the germination rates of *Castilleja christii* seedlings were comparable on Mt. Harrison to those in the lab, *C. christii* seedlings may have a selective advantage at the summit of Mt. Harrison over parental species, potentially due to higher allelic variation as it was observed for the waxy gene. Additionally, the combination of alleles from both parents in *Castilleja christii* may be what restricts this species to the summit of Mt. Harrison and why it is not found elsewhere. Several other species of *Castilleja* are endemic to mountaintops throughout the genus' range. It would be interesting to explore the evolution of these species to see what factors limit their range and to compare their modes of speciation with our analysis of *Castilleja christii*

Suggestions for Further Research

Taken together, our data provide evidence to support *Castilleja christii* as a hybrid species, which is a novel discovery within the genus (Chapters 2 – 4). Speciation and diversification in *Castilleja* is generally attributed to the ease with which species hybridize and experience subsequent genome duplication (allopolyploidy; Ownbey, 1959; Heckard, 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Chuang and Heckard, 1993). To further shed light on the mechanisms of hybrid speciation in *Castilleja christii* and to gain insights into the evolution of the genus *Castilleja,* our initial findings must be tested with other lines of evidence. In general, all analyses from this chapter should be replicated in future years because one year of ecological data is not sufficient to draw definitive conclusions. Furthermore, a study examining the pollination systems in *Castilleja* at the summit would clarify our initial findings regarding differences in floral structure and phenology, which may provide a barrier to hybridization among sympatric congeners. In addition, a more rigorous study involving flowering times would elucidate the potential for gene exchange among *Castilleja* species at the summit. Seed germination trials at the summit would also provide an idea of any selective advantage of one *Castilleja* species over another. Also, a more rigorous study of plant species community composition and its relationship to putative soil differences could be undertaken at the summit between the three *Castilleja* species. Along these lines, including comparisons of plant community composition outside of the range of *C. christii* would perhaps assist in our understanding of the endemic nature of *C. christii.*

## Conclusion

The rare endemic *Castilleja christii* was found to be of hybrid origin, based on the presence of additive parental genomes of both *C. miniata* and *C. linariifolia* (Chapter 2). Though *Castilleja christii* shares a common ploidy level with its progenitors, it is spatially and ecologically isolated from them due to differences in plant community composition, aspects at the summit on which each *Castilleja* species is found, and may further be isolated due to differences in flowering times and seed germination rates. *Castilleja christii* has also been found to have morphological traits that are novel or transgressive from progenitor species (Chapter 3). Therefore, there is strong evidence to support *Castilleja christii* as a homoploid hybrid species.

The discovery of *Castilleja christii* as a homoploid hybrid species is exciting because it provides the opportunity for chronicling the continued ecological and genetic differentiation of *C. christii* from *C. linariifolia* and *C. miniata.* Further, by studying the speciation process of *Castilleja christii,* we gain insight into the continued speciation and diversification within *Castilleja*, while furthering our knowledge of the mechanisms and processes involved in homoploid hybrid speciation, of which only a handful of empirically tested examples exist in the literature.

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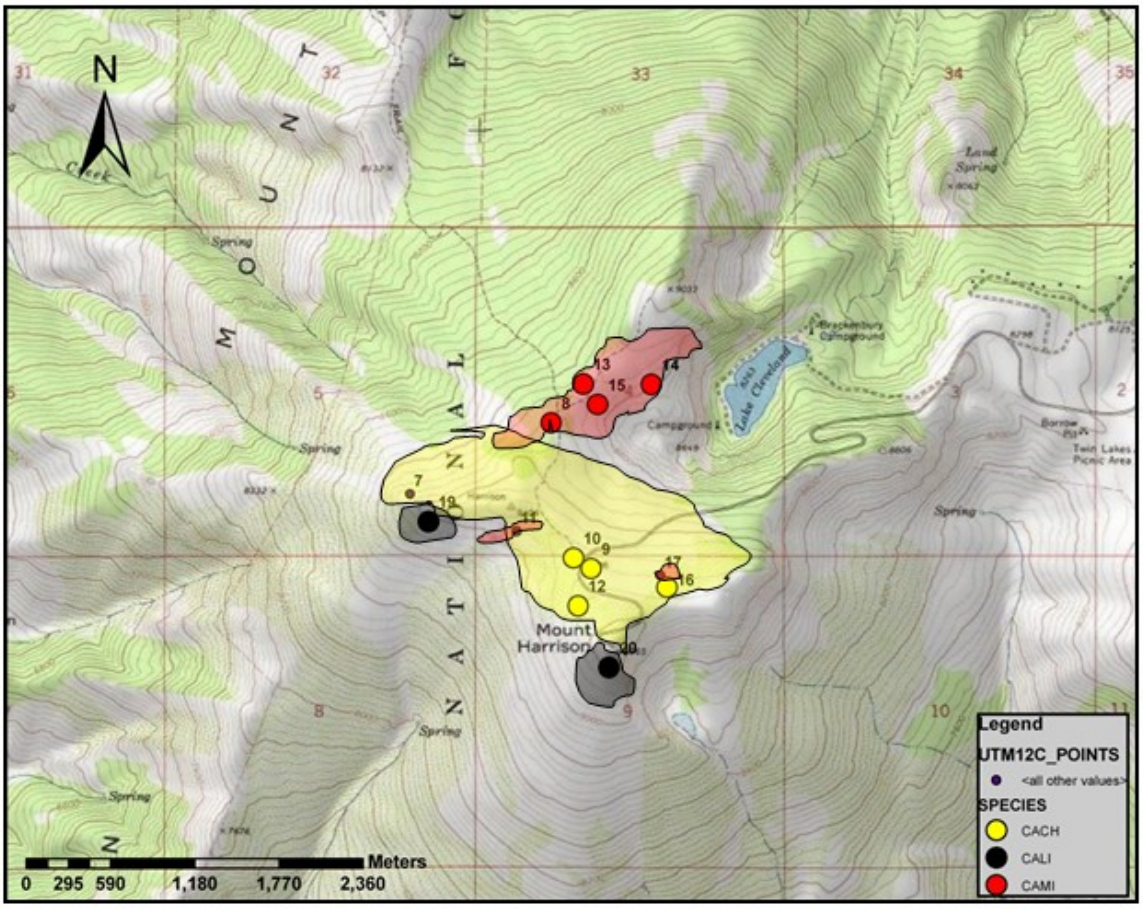


**Figure 4.1 Ranges of the three *Castilleja* species in this study, Mt. Harrison, ID. Relative ranges of three *Castilleja* species in an area of sympatry on Mt. Harrison, ID. Yellow = CACH = *Castilleja christii*; Red = CAMI = *C. miniata*; Black = CALI = *C. linariifolia*; Purple dots = all other values = *C. christii* and *C. miniata* plots.**

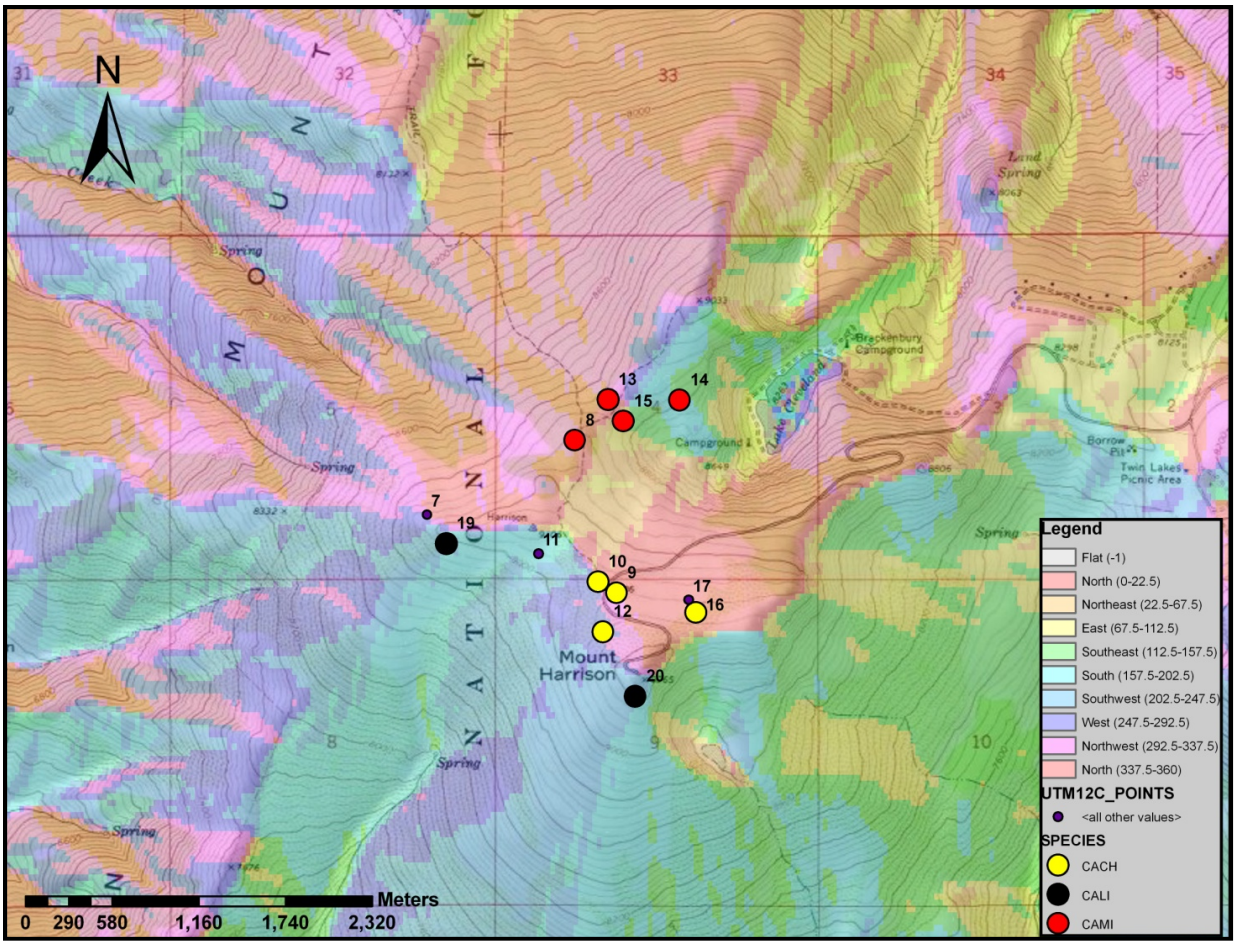


**Figure 4.2 Spatial map of plots located at the Mt. Harrison Site. Yellow circles = CACH = *Castilleja christii* plots; Red circles = CAMI = *C. miniata* plots; Black circles = CALI = *C. linariifolia* plots; Purple dots = all other values = *C. christii* and *C. miniata* plots. Dots are not representative of plot size; they have been enlarged for clarity. *Castilleja miniata* plots tend to be located on north, northeast or eastern slopes at the summit; *C. linariifolia* is found only from two small populations at the summit, on either south or southwest-facing slopes; *C. christii* is located in a large area in between the two parental populations, and tends to be located on north or west-facing slopes.**

**Table 4.1 Richness and Evenness values for three sympatric *Castilleja* species. Richness, Shannon-Weiner diversity (H'), and Pielou's (1975) Evenness (J) for three sympatric *Castilleja* species at the summit of Mt. Harrison, ID. CACH = *Castilleja christii*; CALI = *C. linariifolia;* CAMI = *C. miniata*. Total N refers to counts of plant species found within all microplots within all plots sampled for each *Castilleja* species. ANOVA means were not significantly different among species for any of the diversity indices (Richness: *P* = 0.1167; Shannon-Weiner: *P* = 0.0593; Evenness: *P* = 0.1449). Shannon-Weiner means for *Castilleja linariifolia* plots were significantly different than means of *C. miniata* and *C. christii* plots, according to Student-Newman-Keuls (SNK) tests.**

| Plot | Species | Richness | H' | J |
|---|---|---|---|---|
| 9 | CACH | 15 | 1.37 | 0.51 |
| 10 | | 24 | 1.86 | 0.58 |
| 12 | | 20 | 2.24 | 0.75 |
| 16 | | 30 | 1.90 | 0.56 |
| **Average** | | **22.2** | **1.84** | **0.60** |
| **Total N (pooled)** | **40** | | | |
| **SNK** | | **A** | **B** | **A** |
| 19 | CALI | 28 | 2.30 | 0.69 |
| 20 | | 26 | 2.49 | 0.76 |
| **Average** | | **27** | **2.39** | **0.73** |
| **Total N (pooled)** | **36** | | | |
| **SNK** | | **A** | **A** | **A** |
| 8 | CAMI | 12 | 1.82 | 0.73 |
| 13 | | 13 | 1.77 | 0.69 |
| 14 | | 14 | 1.85 | 0.70 |
| 15 | | 23 | 2.10 | 0.70 |
| **Average** | | **15.5** | **1.88** | **0.70** |
| **Total N (pooled)** | **26** | | | |
| **SNK** | | **A** | **B** | **A** |

**Table 4.2. Within plot mean coverage values for common plant species. Mean percent cover of most common species within plots of three sympatric Castilleja species at the summit of Mt. Harrison, ID. Only species above one percent cover are listed; those that are below are displayed as <1. Percent cover of Castilleja linariifolia is not shown, as this species did not exceed an average of one percent in any plots.**

| | *Castilleja christii* N = 4 | *Castilleja miniata* N = 4 | *Castilleja linariifolia* N = 2 |
|---|---|---|---|
| Species | | | |
| Bare ground | 37.1 | 31.4 | 3.8 |
| *Festuca idahoensis* | 13.4 | 0 | < 1 |
| *Artemisia tridentata subsp. vaseyana* | 10.3 | 4.7 | 24.1 |
| *Solidago multiradiata* | 6.6 | 0 | 0 |
| Litter | 4.9 | 6.2 | 7.0 |
| *Achillea millefolium* | 3.5 | 0 | < 1 |
| *Lupinus argenteus* | 3.5 | 8.5 | 5.5 |
| Rock | 2.8 | 5.7 | 10.5 |
| *Arenaria congesta* | 1.7 | 0 | 2.5 |
| *Castilleja christii* | 2.6 | 0 | 0 |
| *Eridgeron perigrinus* | 1.7 | 0 | < 1 |
| *Pedicularis contorta* | < 1 | 0 | 1.0 |
| *Penstemon rydbergii* | 0 | 10.0 | 1.5 |
| *Symphyotrichum foliaceum* | < 1 | 14.2 | 0 |
| *Artemisia ludovisciana* | 0 | 5.9 | 0 |
| *Ligusticum porterii* | 0 | 5.7 | 0 |
| *Castilleja miniata* | 0 | 3.1 | 0 |
| *Pseudostellaria jamesiana* | < 1 | 1.7 | 0 |
| *Polygonum bistoroides* | < 1 | 0 | 0 |
| *Epilobium alpinum* | < 1 | < 1 | < 1 |
| moss | < 1 | < 1 | 2.3 |
| *Cymopterus davisii* | < 1 | < 1 | 0 |
| *Phlox multiflora* | 0 | 0 | 14.5 |
| *Poa secunda* | 0 | 0 | 7.4 |
| *Arenaria capillaris* | 0 | 0 | 6.0 |
| *Eriogonum sp.* | 0 | 0 | 4.0 |
| *Trisetum spicatum* | < 1 | < 1 | 3.2 |

**Table 4.3 Index of similarity among *Castilleja* species on Mt. Harrison. Sørensen's (1948) index of similarity between plots from three *Castilleja* species at the summit of Mt. Harrison, ID. While the homoploid hybrid *Castilleja christii* has higher similarity of overall plot community composition when compared with both parental progenitors, when parental plots were compared with each other, they also shared a similar plot community composition.**

| Species | QS | Percent similarity |
|---|---|---|
| CACH vs. CAMI | 0.626865672 | 62.69% |
| CACH vs. CALI | 0.571428571 | 57.14% |
| CAMI vs. CALI | 0.483870968 | 48.84% |

**Table 4.4 Phenological data obtained from *Castilleja* measured in 2009. Phenological data obtained between July 21 and August 9 from three *Castilleja* species in an area of sympatry at the summit of Mt. Harrison, Cassia Co., ID. CAMI = *Castilleja miniata*; CALI = *Castilleja linariifolia;* CACH = *Castilleja christii*. The number of flowers open on a single inflorescence was averaged for each species on a single day. *Castilleja christii* had flowers senesce earlier in the season than the other two sympatric species; however, *C. christii* and *C. miniata* were flowering at the same time throughout this period. *Castilleja linariifolia* was located at the summit on August 4th, however did not flower until August 8th. An asterisk (*) means that no data were obtained for that day for a given species.**

| Average Number of Flowers Open per Species | | | |
|---|---|---|---|
| **Date** | **CACH** | **CAMI** | **CALI** |
| 7/21/2009 | 8.7 | 10.4 | 0 |
| 7/23/2009 | 6.7 | 8.4 | 0 |
| 7/25/2009 | 7.7 | * | 0 |
| 7/26/2009 | 9.0 | * | 0 |
| 7/28/2009 | 7.8 | * | 0 |
| 7/29/2009 | * | 11.8 | 0 |
| 8/4/2009 | 6.6 | 13.2 | * |
| 8/5/2009 | * | 10.4 | * |
| 8/6/2009 | 6.5 | * | * |
| 8/7/2009 | 10.8 | 9.5 | * |
| 8/8/2009 | * | * | 9 |
| 8/9/2009 | * | * | 8.8 |

**Table 4.5 Chromosome counts of three sympatric *Castilleja* species at the summit of Mt. Harrison, ID. The base chromosome number for the genus is n = 12 (Heckard and Chuang, 1977). *Castilleja miniata* and *C. linariifolia* are known to have multiple ploidy levels (*C. miniata* n = 12, 24, 48, 60; *C. linariifolia* n = 12, 24). This is the first documented chromosome count for *Castilleja christii,* which is a diploid, as were all other *Castilleja* species sampled at the summit, which were also diploid with the exception of one *C. miniata* population, which was tetraploid.**

| Species | Coll_num | Plot | Number of flowers counted | Actual Count | Average Count | Ploidy |
|---|---|---|---|---|---|---|
| *C. christii* | 247 | 9 | 1 | 12 | 12 | 2N |
| *C. christii* | 461 | 12 | 1 | 10 | 10 | 2N |
| *C. christii* | 338 | 10 | 1 | 12 | 12 | 2N |
| *C. linariifolia* | 654a | 19 | 2 | 12 | 12 | 2N |
| | 654b | 19 | | 12 | | 2N |
| *C. linariifolia* | 692 | 20 | 1 | 9 | 9 | 2N |
| *C. miniata* | 504a | 14 | 2 | 12 | 11.5 | 2N |
| | 504b | 14 | | 11 | | 2N |
| *C .miniata* | 417 | 11 | 1 | 10 | 10 | 2N |
| *C. miniata* | 597 | 17 | 1 | 22 | 22 | 4N |

# APPENDIX A

## Table of Collection Locations for Samples of All Three *Castilleja* Species in This Study

**Table A.1 Table of *Castilleja* collection locations. Table of locations made for collections of *Castilleja miniata* and *C. linariifolia*, occurring outside of the range of *C. christii* and at the top of Mt. Harrison, ID. Because *Castilleja christii* is endemic to the summit of Mt. Harrison, all collections of *C. christii* occurred there. Universal Transverse Mercators (UTM) were recorded in the North American Datum 83. Those collections in the Boise National Forest are in zone 11; those in Cassia Co. are in zone 12. Whether each collection was analyzed for morphological (Morpho) and/or molecular analyses are noted in the far-right columns.**

| Species | Col_Num | Plot | UTM_X | UTM_Y | Location | Morpho | Molecular |
|---|---|---|---|---|---|---|---|
| *C. miniata* | 1 | 1 | 734233 | 4858488 | Wildhorse Creek Campground, Mt. Borah, ID | Yes | No |
| *C. miniata* | 5 | 1 | | | | Yes | No |
| *C. miniata* | 11 | 1 | | | | Yes | No |
| *C. miniata* | 12 | 1 | | | | Yes | No |
| *C. miniata* | 13 | 1 | | | | Yes | No |
| *C. miniata* | 18 | 1 | | | | Yes | No |
| *C. miniata* | 19 | 1 | | | | Yes | No |
| *C. miniata* | 23 | 1 | | | | Yes | Yes |
| *C. miniata* | 24 | 1 | | | | Yes | Yes |
| *C. miniata* | 25 | 1 | | | | Yes | No |
| *C. linariifolia* | 26 | 2 | 289915 | 4690170 | Lower slopes of Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. linariifolia* | 28 | 2 | | | | Yes | No |
| *C. linariifolia* | 29 | 2 | | | | Yes | No |
| *C. linariifolia* | 30 | 2 | | | | Yes | No |
| *C. linariifolia* | 32 | 2 | | | | Yes | No |
| *C. linariifolia* | 37 | 2 | | | | Yes | No |
| *C. linariifolia* | 38 | 2 | | | | Yes | No |
| *C. linariifolia* | 45 | 2 | | | | Yes | No |
| *C. linariifolia* | 46 | 2 | | | | Yes | No |
| *C. linariifolia* | 442 | 2 | | | | Yes | No |
| *C. linariifolia* | 51 | 3 | 276723 | 4680329 | Mt. Independence, Cassia Co., ID | No | Yes |
| *C. linariifolia* | 52 | 3 | | | | No | Yes |
| *C. linariifolia* | 53 | 3 | | | | No | Yes |
| *C. linariifolia* | 56 | 3 | | | | Yes | No |
| *C. linariifolia* | 58 | 3 | | | | Yes | No |
| *C. linariifolia* | 59 | 3 | | | | Yes | No |
| *C. linariifolia* | 60 | 3 | | | | Yes | No |
| *C. linariifolia* | 63 | 3 | | | | Yes | No |
| *C. linariifolia* | 64 | 3 | | | | Yes | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. linariifolia* | 65 | 3 | | | | Yes | No |
| *C. linariifolia* | 68 | 3 | | | | Yes | No |
| *C. linariifolia* | 70 | 3 | | | | Yes | No |
| *C. linariifolia* | 163 | 3 | | | | Yes | No |
| *C. miniata* | 76 | 4 | 227124 | 4678353 | Mt. Independence, Cassia Co., ID | Yes | Yes |
| *C. miniata* | 77 | 4 | | | | No | Yes |
| *C. miniata* | 78 | 4 | | | | Yes | Yes |
| *C. miniata* | 82 | 4 | | | | Yes | No |
| *C. miniata* | 83 | 4 | | | | Yes | No |
| *C. miniata* | 84 | 4 | | | | Yes | Yes |
| *C. miniata* | 91 | 4 | | | | Yes | No |
| *C. miniata* | 95 | 4 | | | | Yes | Yes |
| *C. miniata* | 96 | 4 | | | | Yes | Yes |
| *C. miniata* | 151 | 4 | | | | Yes | No |
| *C. miniata* | 157 | 4 | | | | Yes | No |
| *C. miniata* | 101 | 5 | 610117 | 4868673 | Bogus Basin Ski Area | Yes | Yes |
| *C. miniata* | 102 | 5 | | | Boise National Forest, Boise Co., ID | No | Yes |
| *C. miniata* | 103 | 5 | | | | No | Yes |
| *C. miniata* | 104 | 5 | | | | Yes | Yes |
| *C. miniata* | 105 | 5 | | | | No | Yes |
| *C. miniata* | 106 | 5 | | | | Yes | Yes |
| *C. miniata* | 107 | 5 | | | | Yes | No |
| *C. miniata* | 108 | 5 | | | | Yes | Yes |
| *C. miniata* | 111 | 5 | | | | Yes | Yes |
| *C. miniata* | 113 | 5 | | | | Yes | No |
| *C. miniata* | 116 | 5 | | | | Yes | Yes |
| *C. miniata* | 118 | 5 | | | | Yes | No |
| *C. miniata* | 124 | 5 | | | | Yes | No |
| *C. miniata* | 126 | 6 | 572999 | 4847308 | Whoop-em-up Campground, | Yes | Yes |
| *C. miniata* | 127 | 6 | | | Boise National Forest, Boise Co., ID | Yes | Yes |
| *C. miniata* | 128 | 6 | | | | Yes | Yes |
| *C. miniata* | 129 | 6 | | | | Yes | Yes |
| *C. miniata* | 130 | 6 | | | | Yes | Yes |
| *C. miniata* | 131 | 6 | | | | Yes | No |
| *C. miniata* | 132 | 6 | | | | Yes | No |
| *C. miniata* | 133 | 6 | | | | Yes | No |
| *C. miniata* | 134 | 6 | | | | Yes | No |
| *C. miniata* | 139 | 6 | | | | Yes | No |
| *C. miniata* | 146 | 6 | | | | Yes | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 170 | 7 | 279814 | 4688350 | Mt. Harrison, Cassia Co., ID | No | Yes |
| *C. christii* | 171 | 7 | | | | No | Yes |
| *C. christii* | 173 | 7 | | | | Yes | Yes |
| *C. christii* | 174 | 7 | | | | Yes | No |
| *C. christii* | 178 | 7 | | | | Yes | No |
| *C. christii* | 180 | 7 | | | | No | Yes |
| *C. christii* | 181 | 7 | | | | No | Yes |
| *C. christii* | 182 | 7 | | | | No | Yes |
| *C. christii* | 183 | 7 | | | | Yes | Yes |
| *C. christii* | 184 | 7 | | | | No | Yes |
| *C. christii* | 185 | 7 | | | | No | Yes |
| *C. miniata* | 186 | 7 | | | | Yes | No |
| *C. christii* | 187 | 7 | | | | Yes | Yes |
| *C. christii* | 188 | 7 | | | | Yes | No |
| *C. miniata* | 192 | 7 | | | | Yes | Yes |
| *C. miniata* | 193 | 7 | | | | No | Yes |
| *C. miniata* | 195 | 7 | | | | Yes | Yes |
| *C. miniata* | 196 | 7 | | | | Yes | No |
| *C. miniata* | 197 | 7 | | | | Yes | No |
| *C. miniata* | 198 | 7 | | | | Yes | Yes |
| *C. miniata* | 199 | 7 | | | | No | Yes |
| *C. miniata* | 200 | 7 | | | | Yes | No |
| *C. miniata* | 201 | 7 | | | | Yes | No |
| *C. christii* | 203 | 7 | | | | Yes | No |
| *C. christii* | 204 | 7 | | | | Yes | No |
| *C. christii* | 205 | 7 | | | | No | Yes |
| *C. christii* | 206 | 7 | | | | No | Yes |
| *C. christii* | 208 | 7 | | | | Yes | No |
| *C. christii* | 209 | 7 | | | | No | Yes |
| *C. christii* | 214 | 7 | | | | Yes | No |
| *C. miniata* | 267 | 8 | 280557 | 4688697 | Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. miniata* | 268 | 8 | | | | Yes | No |
| *C. miniata* | 271 | 8 | | | | Yes | Yes |
| *C. miniata* | 272 | 8 | | | | Yes | No |
| *C. miniata* | 277 | 8 | | | | Yes | No |
| *C. miniata* | 278 | 8 | | | | Yes | Yes |
| *C. miniata* | 283 | 8 | | | | Yes | No |
| *C. miniata* | 288 | 8 | | | | Yes | Yes |
| *C. miniata* | 290 | 8 | | | | Yes | No |
| *C. miniata* | 291 | 8 | | | | Yes | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 292 | 8 | | | | No | Yes |
| *C. christii* | 295 | 8 | | | | Yes | No |
| *C. christii* | 296 | 8 | | | | No | Yes |
| *C. christii* | 297 | 8 | | | | Yes | No |
| *C. christii* | 300 | 8 | | | | Yes | Yes |
| *C. christii* | 301 | 8 | | | | No | Yes |
| *C. christii* | 302 | 8 | | | | No | Yes |
| *C. christii* | 303 | 8 | | | | Yes | No |
| *C. christii* | 304 | 8 | | | | No | Yes |
| *C. christii* | 306 | 8 | | | | Yes | No |
| *C. christii* | 307 | 8 | | | | Yes | No |
| *C. christii* | 308 | 8 | | | | Yes | Yes |
| *C. christii* | 310 | 8 | | | | Yes | Yes |
| *C. christii* | 311 | 8 | | | | No | Yes |
| *C. christii* | 312 | 8 | | | | Yes | Yes |
| *C. christii* | 313 | 8 | | | | No | Yes |
| *C. christii* | 314 | 8 | | | | Yes | Yes |
| *C. christii* | 316 | 8 | | | | No | Yes |
| *C. christii* | 215 | 9 | 280741 | 4687937 | Mt. Harrison, Cassia Co., ID | No | Yes |
| *C. christii* | 216 | 9 | | | | Yes | No |
| *C. christii* | 221 | 9 | | | | No | Yes |
| *C. christii* | 224 | 9 | | | | Yes | No |
| *C. christii* | 225 | 9 | | | | Yes | No |
| *C. christii* | 226 | 9 | | | | No | Yes |
| *C. christii* | 227 | 9 | | | | No | Yes |
| *C. christii* | 228 | 9 | | | | No | Yes |
| *C. christii* | 229 | 9 | | | | No | Yes |
| *C. christii* | 231 | 9 | | | | Yes | Yes |
| *C. christii* | 233 | 9 | | | | Yes | No |
| *C. christii* | 234 | 9 | | | | No | Yes |
| *C. christii* | 237 | 9 | | | | No | Yes |
| *C. christii* | 242 | 9 | | | | No | Yes |
| *C. christii* | 243 | 9 | | | | No | Yes |
| *C. christii* | 244 | 9 | | | | Yes | No |
| *C. christii* | 245 | 9 | | | | No | Yes |
| *C. christii* | 246 | 9 | | | | No | Yes |
| *C. christii* | 248 | 9 | | | | No | Yes |
| *C. christii* | 249 | 9 | | | | No | Yes |
| *C. christii* | 250 | 9 | | | | Yes | Yes |
| *C. christii* | 251 | 9 | | | | No | Yes |
| *C. christii* | 252 | 9 | | | | No | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 253 | 9 | | | | Yes | Yes |
| *C. christii* | 254 | 9 | | | | Yes | Yes |
| *C. christii* | 255 | 9 | | | | No | Yes |
| *C. christii* | 256 | 9 | | | | No | Yes |
| *C. christii* | 257 | 9 | | | | No | Yes |
| *C. christii* | 258 | 9 | | | | No | Yes |
| *C. christii* | 259 | 9 | | | | No | Yes |
| *C. christii* | 260 | 9 | | | | No | Yes |
| *C. christii* | 261 | 9 | | | | No | Yes |
| *C. christii* | 262 | 9 | | | | No | Yes |
| *C. christii* | 263 | 9 | | | | No | Yes |
| *C. christii* | 264 | 9 | | | | No | Yes |
| *C. christii* | 265 | 9 | | | | No | Yes |
| *C. christii* | 266 | 9 | | | | Yes | Yes |
| *C. christii* | 317 | 10 | 280650 | 4687994 | Mt. Harrison, Cassia Co., ID | Yes | Yes |
| *C. christii* | 318 | 10 | | | | Yes | Yes |
| *C. christii* | 319 | 10 | | | | No | Yes |
| *C. christii* | 320 | 10 | | | | No | Yes |
| *C. christii* | 321 | 10 | | | | No | Yes |
| *C. christii* | 322 | 10 | | | | No | Yes |
| *C. christii* | 323 | 10 | | | | No | Yes |
| *C. christii* | 324 | 10 | | | | Yes | Yes |
| *C. christii* | 325 | 10 | | | | No | Yes |
| *C. christii* | 326 | 10 | | | | No | Yes |
| *C. christii* | 327 | 10 | | | | Yes | Yes |
| *C. christii* | 328 | 10 | | | | No | Yes |
| *C. christii* | 329 | 10 | | | | Yes | Yes |
| *C. christii* | 330 | 10 | | | | No | Yes |
| *C. christii* | 331 | 10 | | | | Yes | Yes |
| *C. christii* | 332 | 10 | | | | No | Yes |
| *C. christii* | 333 | 10 | | | | No | Yes |
| *C. christii* | 334 | 10 | | | | Yes | No |
| *C. christii* | 335 | 10 | | | | No | Yes |
| *C. christii* | 336 | 10 | | | | Yes | Yes |
| *C. christii* | 337 | 10 | | | | No | Yes |
| *C. christii* | 338 | 10 | | | | Yes | Yes |
| *C. christii* | 339 | 10 | | | | Yes | No |
| *C. christii* | 340 | 10 | | | | No | Yes |
| *C. christii* | 345 | 10 | | | | Yes | Yes |
| *C. christii* | 346 | 10 | | | | No | Yes |
| *C. christii* | 348 | 10 | | | | No | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 349 | 10 | | | | Yes | No |
| *C. christii* | 350 | 10 | | | | Yes | Yes |
| *C. christii* | 351 | 10 | | | | Yes | Yes |
| *C. christii* | 352 | 10 | | | | Yes | Yes |
| *C. christii* | 353 | 10 | | | | Yes | No |
| *C. christii* | 354 | 10 | | | | No | Yes |
| *C. christii* | 355 | 10 | | | | Yes | Yes |
| *C. christii* | 357 | 10 | | | | Yes | No |
| *C. christii* | 360 | 10 | | | | No | Yes |
| *C. christii* | 361 | 10 | | | | Yes | Yes |
| *C. christii* | 362 | 10 | | | | No | Yes |
| *C. christii* | 364 | 10 | | | | No | Yes |
| *C. christii* | 365 | 10 | | | | No | Yes |
| *C. christii* | 366 | 10 | | | | Yes | Yes |
| *C. christii* | 722 | 10 | | | | No | Yes |
| *C. christii* | 367 | 11 | 280361 | 4688141 | Mt. Harrison, Cassia Co., ID | Yes | Yes |
| *C. christii* | 368 | 11 | | | | Yes | Yes |
| *C. christii* | 369 | 11 | | | | No | Yes |
| *C. christii* | 370 | 11 | | | | No | Yes |
| *C. christii* | 371 | 11 | | | | No | Yes |
| *C. christii* | 372 | 11 | | | | No | Yes |
| *C. christii* | 373 | 11 | | | | Yes | Yes |
| *C. christii* | 374 | 11 | | | | Yes | Yes |
| *C. christii* | 375 | 11 | | | | Yes | Yes |
| *C. christii* | 376 | 11 | | | | Yes | Yes |
| *C. christii* | 377 | 11 | | | | Yes | Yes |
| *C. christii* | 378 | 11 | | | | No | Yes |
| *C. christii* | 379 | 11 | | | | No | Yes |
| *C. christii* | 380 | 11 | | | | Yes | No |
| *C. christii* | 381 | 11 | | | | Yes | Yes |
| *C. christii* | 382 | 11 | | | | No | Yes |
| *C. christii* | 383 | 11 | | | | No | Yes |
| *C. christii* | 384 | 11 | | | | No | Yes |
| *C. christii* | 385 | 11 | | | | Yes | Yes |
| *C. christii* | 386 | 11 | | | | No | Yes |
| *C. christii* | 387 | 11 | | | | No | Yes |
| *C. christii* | 388 | 11 | | | | No | Yes |
| *C. christii* | 389 | 11 | | | | No | Yes |
| *C. christii* | 391 | 11 | | | | No | Yes |
| *C. christii* | 392 | 11 | | | | No | Yes |
| *C. christii* | 393 | 11 | | | | Yes | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 394 | 11 | | | | No | Yes |
| *C. christii* | 395 | 11 | | | | No | Yes |
| *C. christii* | 396 | 11 | | | | No | Yes |
| *C. christii* | 397 | 11 | | | | No | Yes |
| *C. christii* | 398 | 11 | | | | No | Yes |
| *C. christii* | 399 | 11 | | | | No | Yes |
| *C. christii* | 400 | 11 | | | | No | Yes |
| *C. christii* | 401 | 11 | | | | Yes | Yes |
| *C. christii* | 402 | 11 | | | | No | Yes |
| *C. christii* | 404 | 11 | | | | No | Yes |
| *C. christii* | 406 | 11 | | | | No | Yes |
| *C. christii* | 407 | 11 | | | | No | Yes |
| *C. christii* | 408 | 11 | | | | No | Yes |
| *C. christii* | 409 | 11 | | | | Yes | Yes |
| *C. christii* | 410 | 11 | | | | Yes | Yes |
| *C. christii* | 411 | 11 | | | | No | Yes |
| *C. christii* | 412 | 11 | | | | No | Yes |
| *C. christii* | 413 | 11 | | | | Yes | No |
| *C. christii* | 414 | 11 | | | | No | Yes |
| *C. christii* | 415 | 11 | | | | No | Yes |
| *C. christii* | 416 | 11 | | | | No | Yes |
| *C. miniata* | 419 | 11 | | | | Yes | Yes |
| *C. miniata* | 423 | 11 | | | | Yes | Yes |
| *C. miniata* | 426 | 11 | | | | Yes | Yes |
| *C. miniata* | 427 | 11 | | | | Yes | No |
| *C. miniata* | 433 | 11 | | | | Yes | Yes |
| *C. christii* | 447 | 12 | 280667 | 4687747 | Mt. Harrison, Cassia Co., ID | No | Yes |
| *C. christii* | 448 | 12 | | | | No | Yes |
| *C. christii* | 449 | 12 | | | | Yes | Yes |
| *C. christii* | 450 | 12 | | | | No | Yes |
| *C. christii* | 451 | 12 | | | | Yes | Yes |
| *C. christii* | 452 | 12 | | | | No | Yes |
| *C. christii* | 453 | 12 | | | | No | Yes |
| *C. christii* | 454 | 12 | | | | No | Yes |
| *C. christii* | 455 | 12 | | | | No | Yes |
| *C. christii* | 456 | 12 | | | | Yes | Yes |
| *C. christii* | 457 | 12 | | | | Yes | Yes |
| *C. christii* | 458 | 12 | | | | No | Yes |
| *C. christii* | 459 | 12 | | | | No | Yes |
| *C. christii* | 460 | 12 | | | | No | Yes |
| *C. christii* | 461 | 12 | | | | No | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 462 | 12 | | | | No | Yes |
| *C. christii* | 463 | 12 | | | | Yes | Yes |
| *C. christii* | 464 | 12 | | | | Yes | Yes |
| *C. christii* | 465 | 12 | | | | Yes | Yes |
| *C. christii* | 466 | 12 | | | | Yes | Yes |
| *C. christii* | 467 | 12 | | | | No | Yes |
| *C. christii* | 468 | 12 | | | | No | Yes |
| *C. christii* | 469 | 12 | | | | Yes | Yes |
| *C. christii* | 470 | 12 | | | | Yes | Yes |
| *C. christii* | 471 | 12 | | | | No | Yes |
| *C. miniata* | 472 | 13 | 280729 | 4688893 | Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. miniata* | 474 | 13 | | | | Yes | No |
| *C. miniata* | 477 | 13 | | | | Yes | No |
| *C. miniata* | 483 | 13 | | | | Yes | No |
| *C. miniata* | 485 | 13 | | | | Yes | No |
| *C. miniata* | 486 | 13 | | | | Yes | No |
| *C. miniata* | 487 | 13 | | | | Yes | No |
| *C. miniata* | 488 | 13 | | | | Yes | No |
| *C. miniata* | 490 | 13 | | | | Yes | No |
| *C. miniata* | 494 | 13 | | | | Yes | No |
| *C. miniata* | 497 | 14 | 281084 | 4688879 | Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. miniata* | 499 | 14 | | | | Yes | Yes |
| *C. miniata* | 500 | 14 | | | | Yes | Yes |
| *C. miniata* | 507 | 14 | | | | Yes | Yes |
| *C. miniata* | 508 | 14 | | | | Yes | No |
| *C. miniata* | 513 | 14 | | | | Yes | Yes |
| *C. miniata* | 518 | 14 | | | | Yes | No |
| *C. miniata* | 519 | 14 | | | | Yes | No |
| *C. miniata* | 520 | 14 | | | | Yes | Yes |
| *C. miniata* | 527 | 14 | | | | Yes | No |
| *C. miniata* | 531 | 15 | 280801 | 4688783 | Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. miniata* | 536 | 15 | | | | Yes | No |
| *C. miniata* | 539 | 15 | | | | Yes | No |
| *C. miniata* | 542 | 15 | | | | Yes | No |
| *C. miniata* | 545 | 15 | | | | Yes | No |
| *C. miniata* | 546 | 15 | | | | Yes | No |
| *C. miniata* | 547 | 15 | | | | Yes | No |
| *C. miniata* | 549 | 15 | | | | Yes | No |
| *C. miniata* | 550 | 15 | | | | Yes | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. miniata* | 552 | 15 | | | | Yes | No |
| *C. christii* | 553 | 16 | 281130 | 4687827 | Mt. Harrison, Cassia Co., ID | Yes | Yes |
| *C. christii* | 554 | 16 | | | | Yes | Yes |
| *C. christii* | 555 | 16 | | | | No | Yes |
| *C. christii* | 556 | 16 | | | | No | Yes |
| *C. christii* | 557 | 16 | | | | Yes | Yes |
| *C. christii* | 558 | 16 | | | | No | Yes |
| *C. christii* | 559 | 16 | | | | Yes | Yes |
| *C. christii* | 560 | 16 | | | | Yes | Yes |
| *C. christii* | 561 | 16 | | | | Yes | Yes |
| *C. christii* | 562 | 16 | | | | Yes | Yes |
| *C. christii* | 563 | 16 | | | | Yes | Yes |
| *C. christii* | 565 | 16 | | | | No | Yes |
| *C. christii* | 566 | 16 | | | | No | Yes |
| *C. christii* | 567 | 16 | | | | No | Yes |
| *C. christii* | 568 | 16 | | | | Yes | Yes |
| *C. christii* | 569 | 16 | | | | Yes | Yes |
| *C. christii* | 570 | 16 | | | | Yes | Yes |
| *C. christii* | 571 | 16 | | | | Yes | Yes |
| *C. christii* | 572 | 16 | | | | Yes | Yes |
| *C. christii* | 573 | 16 | | | | Yes | Yes |
| *C. christii* | 574 | 16 | | | | Yes | Yes |
| *C. christii* | 575 | 16 | | | | Yes | Yes |
| *C. christii* | 576 | 16 | | | | Yes | Yes |
| *C. christii* | 577 | 16 | | | | Yes | Yes |
| *C. christii* | 613 | 16 | | | | No | Yes |
| *C. christii* | 614 | 16 | | | | No | Yes |
| *C. christii* | 615 | 16 | | | | No | Yes |
| *C. christii* | 616 | 16 | | | | No | Yes |
| *C. christii* | 617 | 16 | | | | No | Yes |
| *C. christii* | 618 | 16 | | | | No | Yes |
| *C. christii* | 619 | 16 | | | | No | Yes |
| *C. christii* | 620 | 16 | | | | No | Yes |
| *C. christii* | 621 | 16 | | | | Yes | No |
| *C. christii* | 622 | 16 | | | | No | Yes |
| *C. christii* | 623 | 16 | | | | No | Yes |
| *C. christii* | 624 | 16 | | | | No | Yes |
| *C. christii* | 625 | 16 | | | | No | Yes |
| *C. christii* | 714 | 16 | | | | No | Yes |
| *C. christii* | 717 | 16 | | | | No | Yes |
| *C. christii* | 718 | 16 | | | | No | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. christii* | 578 | 17 | 281097 | 4687890 | Mt. Harrison, Cassia Co., ID | Yes | Yes |
| *C. christii* | 579 | 17 | | | | Yes | Yes |
| *C. christii* | 580 | 17 | | | | Yes | Yes |
| *C. christii* | 581 | 17 | | | | Yes | Yes |
| *C. christii* | 582 | 17 | | | | Yes | Yes |
| *C. christii* | 583 | 17 | | | | Yes | Yes |
| *C. christii* | 584 | 17 | | | | Yes | No |
| *C. christii* | 585 | 17 | | | | Yes | Yes |
| *C. christii* | 586 | 17 | | | | Yes | Yes |
| *C. christii* | 587 | 17 | | | | Yes | Yes |
| *C. miniata* | 588 | 17 | | | | Yes | No |
| *C. miniata* | 589 | 17 | | | | No | Yes |
| *C. miniata* | 590 | 17 | | | | Yes | No |
| *C. miniata* | 591 | 17 | | | | Yes | Yes |
| *C. miniata* | 598 | 17 | | | | Yes | No |
| *C. miniata* | 600 | 17 | | | | Yes | No |
| *C. miniata* | 605 | 17 | | | | Yes | No |
| *C. miniata* | 606 | 17 | | | | Yes | No |
| *C. miniata* | 607 | 17 | | | | Yes | No |
| *C. miniata* | 608 | 17 | | | | Yes | No |
| *C. miniata* | 611 | 17 | | | | Yes | No |
| *C. christii* | 707 | 17 | | | | Yes | Yes |
| *C. christii* | 708 | 17 | | | | Yes | Yes |
| *C. christii* | 709 | 17 | | | | Yes | Yes |
| *C. christii* | 710 | 17 | | | | Yes | Yes |
| *C. christii* | 711 | 17 | | | | Yes | Yes |
| *C. christii* | 712 | 17 | | | | Yes | Yes |
| *C. christii* | 713 | 17 | | | | Yes | Yes |
| *C. christii* | 714 | 17 | | | | Yes | No |
| *C. christii* | 715 | 17 | | | | Yes | Yes |
| *C. christii* | 716 | 17 | | | | Yes | Yes |
| *C. linariifolia* | 626 | 18 | 295468 | 4691140 | Cottrelle Mountains, Cassia Co., ID | No | Yes |
| *C. linariifolia* | 627 | 18 | | | | Yes | No |
| *C. linariifolia* | 628 | 18 | | | | Yes | No |
| *C. linariifolia* | 629 | 18 | | | | No | Yes |
| *C. linariifolia* | 630 | 18 | | | | Yes | Yes |
| *C. linariifolia* | 631 | 18 | | | | Yes | No |
| *C. linariifolia* | 633 | 18 | | | | Yes | No |
| *C. linariifolia* | 637 | 18 | | | | Yes | No |
| *C. linariifolia* | 638 | 18 | | | | Yes | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *C. linariifolia* | 641 | 18 | | | | Yes | No |
| *C. linariifolia* | 642 | 18 | | | | Yes | No |
| *C. linariifolia* | 649 | 18 | | | | Yes | No |
| *C. linariifolia* | 651 | 19 | 279905 | 4688206 | Mt. Harrison, Cassia Co., ID | Yes | No |
| *C. linariifolia* | 653 | 19 | | | | Yes | Yes |
| *C. linariifolia* | 654 | 19 | | | | No | Yes |
| *C. linariifolia* | 655 | 19 | | | | Yes | Yes |
| *C. linariifolia* | 657 | 19 | | | | Yes | No |
| *C. linariifolia* | 661 | 19 | | | | Yes | No |
| *C. linariifolia* | 662 | 19 | | | | Yes | No |
| *C. linariifolia* | 669 | 19 | | | | Yes | No |
| *C. linariifolia* | 671 | 19 | | | | Yes | No |
| *C. linariifolia* | 672 | 19 | | | | Yes | No |
| *C. linariifolia* | 674 | 19 | | | | Yes | No |
| *C. linariifolia* | 682 | 20 | 280819 | 4687423 | Mt. Harrison, Cassia Co., ID | No | Yes |
| *C. linariifolia* | 683 | 20 | | | | Yes | Yes |
| *C. linariifolia* | 684 | 20 | | | | Yes | No |
| *C. linariifolia* | 685 | 20 | | | | Yes | No |
| *C. linariifolia* | 687 | 20 | | | | Yes | No |
| *C. linariifolia* | 688 | 20 | | | | Yes | No |
| *C. linariifolia* | 693 | 20 | | | | Yes | No |
| *C. linariifolia* | 697 | 20 | | | | Yes | No |
| *C. linariifolia* | 699 | 20 | | | | Yes | No |
| *C. linariifolia* | 701 | 20 | | | | Yes | No |
| *C. linariifolia* | 702 | 20 | | | | Yes | No |

APPENDIX B

**Information for Direct Sequenced *Castilleja* Individuals**

**Table B.1 Table of all direct-sequenced *Castilleja* individuals. Table of all direct-sequenced *Castilleja* individuals from both control and Mt. Harrison plots, using the *waxy* primer regions 11F-12R. The headings 'CACH' (*Castilleja christii*), 'CAMI' (*C. miniata*), and 'CALI' (*C. linariifolia*) correspond to identifying field-identified individuals based on alignments of cloned sequences of all three species and accessions of this region accessed from GenBank. A denotation of 'yes' indicates that the individual met the genetic criteria for inclusion into that group. All field-identified hybrids had the same genetic signature at this region as *Castilleja christii*. Some *Castilleja miniata* individuals had *C. linariifolia waxy* sequences. While most of these were found at the summit of Mt. Harrison, some were from adjacent Mt. Independence populations. These may be F1 or introgressed hybrids with *C. linariifolia* in these areas that have gone undetected prior to this study and are denoted by 'No', meaning they were not pure 'miniata.'**

| Species: Molec | Species: Field | Col_Num | Plot | Location | Potential intro? | CACH | CAMI | Miniata A Or B | CALI |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 368 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 379 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 378 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 377 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 376 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 724 | | Harrison | No | Yes | No | | No |
| CACH | CACH | 374 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 372 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 371 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 334 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 369 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 383 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 367 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 341 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 340 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 339 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 338 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 337 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 336 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 459 | 12 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 370 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 448 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 458 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 457 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 456 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 455 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 454 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 453 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 452 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 451 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 381 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 449 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 382 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 447 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 391 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 389 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 388 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 387 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 386 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 385 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 384 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 333 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 450 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 250 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 260 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 259 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 258 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 257 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 256 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 255 | 9 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 254 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 253 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 335 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 251 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 263 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 249 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 248 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 246 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 245 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 243 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 242 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 228 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 215 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 252 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 322 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 332 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 331 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 330 | 10 | Harrison | Yes | Yes | No | | No |
| CACH | CACH | 329 | 10 | Harrison | Yes | Yes | No | A | No |
| CACH | CACH | 328 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 327 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 326 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 325 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 261 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 323 | 10 | Harrison | Yes | Yes | No | | No |
| CACH | CACH | 262 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 321 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 320 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 319 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 318 | 10 | Harrison | No | Yes | No | A | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 317 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 266 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 265 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 264 | 9 | Harrison | No | Yes | No | | No |
| CACH | CACH | 375 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 324 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 567 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 556 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 577 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 576 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 575 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 574 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 573 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 572 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 571 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 570 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 579 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 568 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 580 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 566 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 565 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 563 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 562 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 561 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 560 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 559 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 558 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 557 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 569 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 616 | 16 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 722 | 10 | Harrison | No | Yes | No | | No |
| CACH | CACH | 718 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 717 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 625 | 16 | Harrison | Yes | Yes | No | | No |
| CACH | CACH | 624 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 623 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 622 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 620 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 619 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 578 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 373 | 11 | Harrison | No | Yes | No | | No |
| CACH | CACH | 615 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 460 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 614 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 613 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 587 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 586 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 585 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 583 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 582 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 581 | 17 | Harrison | No | Yes | No | | No |
| CACH | CACH | 618 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 467 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 461 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 462 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 463 | 12 | Harrison | Yes | Yes | No | | No |
| CACH | CACH | 464 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 617 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 466 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 555 | 16 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | CACH | 468 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 469 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 470 | 12 | Harrison | Yes | Yes | No | | No |
| CACH | CACH | 471 | 12 | Harrison | No | Yes | No | | No |
| CACH | CACH | 554 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 553 | 16 | Harrison | No | Yes | No | | No |
| CACH | CACH | 465 | 12 | Harrison | No | Yes | No | | No |
| CALI | CALI | 51 | 3 | Mt. Independence | No | No | No | | Yes |
| CALI | CALI | 52 | 3 | Mt. Independence | No | No | No | | Yes |
| CALI | CALI | 53 | 3 | Mt. Independence | No | No | No | | Yes |
| CALI | CALI | 683 | 20 | Harrison | No | No | No | | Yes |
| CALI | CALI | 682 | 20 | Harrison | No | No | No | | Yes |
| CALI | CALI | 655 | 19 | Harrison | No | No | No | | Yes |
| CALI | CALI | 653 | 19 | Harrison | No | No | No | | Yes |
| CALI | CALI | 630 | 18 | Cotterell Mts. | No | No | No | | Yes |
| CALI | CALI | 629 | 18 | Cotterell Mts. | No | No | No | | Yes |
| CALI | CALI | 626 | 18 | Cotterell Mts. | No | No | No | | Yes |
| CALI | CALI | 654 | 19 | Harrison | No | No | No | | Yes |
| CAMI | CAMI | 591 | 17 | Harrison | No | No | Yes | A | No |
| F1? | CAMI | 423 | 11 | Harrison | Yes | No | No | | No |
| CAMI | CAMI | 426 | 11 | Harrison | No | No | Yes | A | No |
| F1? | CAMI | 499 | 14 | Harrison | Yes | No | No | | No |
| CAMI | CAMI | 128 | 6 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 129 | 6 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 130 | 6 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 589 | 17 | Harrison | No | No | Yes | A | No |
| F1? | CAMI | 193 | 7 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 433 | 11 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 195 | 7 | Harrison | Yes | No | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| F1? | CAMI | 288 | 8 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 278 | 8 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 271 | 8 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 198 | 7 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 500 | 14 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 507 | 14 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 513 | 14 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 199 | 7 | Harrison | Yes | No | No | | No |
| F1? | CAMI | 192 | 7 | Harrison | Yes | No | No | | No |
| CAMI | CAMI | 102 | 5 | Boise National Forest | No | No | Yes | A | No |
| F1? | CAMI | 96 | 4 | Mt. Independence | Yes | No | No | | No |
| F1? | CAMI | 84 | 4 | Mt. Independence | Yes | No | No | | No |
| F1? | CAMI | 78 | 4 | Mt. Independence | Yes | No | No | | No |
| F1? | CAMI | 77 | 4 | Mt. Independence | Yes | No | No | | No |
| CAMI | CAMI | 127 | 6 | Control | No | No | Yes | A | No |
| F1? | CAMI | 76 | 4 | Mt. Independence | Yes | No | No | | No |
| F1? | CAMI | 419 | 11 | Harrison | Yes | No | No | | No |
| CAMI | CAMI | 101 | 5 | Boise National Forest | No | No | Yes | A | No |
| F1? | CAMI | 95 | 4 | Mt. Independence | Yes | No | No | | No |
| F1? | CAMI | 520 | 14 | Harrison | Yes | No | No | | No |
| CAMI | CAMI | 103 | 5 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 104 | 5 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 106 | 5 | Boise National Forest | No | No | Yes | A | No |
| F1? | CAMI | 108 | 5 | Boise National Forest | Yes | No | No | A | No |
| CAMI | CAMI | 111 | 5 | Boise National Forest | No | No | Yes | A | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CAMI | CAMI | 116 | 5 | Boise National Forest | No | No | Yes | A | No |
| CAMI | CAMI | 126 | 6 | Boise National Forest | No | No | Yes | A | No |
| CACH | HYBRID | 227 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 234 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 237 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 208 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 209 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 400 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 226 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 366 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 229 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 231 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 206 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 171 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 221 | 9 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 205 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 187 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 185 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 184 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 183 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 182 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 181 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 716 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 173 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 707 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 170 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 399 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 398 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 397 | 11 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | HYBRID | 180 | 7 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 408 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 314 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 316 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 393 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 394 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 395 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 416 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 415 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 414 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 413 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 412 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 411 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 709 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 409 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 311 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 407 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 406 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 404 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 402 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 401 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 345 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 346 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 348 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 350 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 351 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 352 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 410 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 355 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 714 | 16 | Harrison | No | Yes | No | | No |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CACH | HYBRID | 713 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 712 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 711 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 710 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 708 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 353 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 365 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 364 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 362 | 10 | Harrison | Yes | Yes | Yes | | No |
| CACH | HYBRID | 361 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 313 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 396 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 312 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 354 | 10 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 392 | 11 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 292 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 293 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 296 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 300 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 301 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 302 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 304 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 308 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 310 | 8 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 715 | 17 | Harrison | No | Yes | No | | No |
| CACH | HYBRID | 360 | 10 | Harrison | No | Yes | No | | No |
| | | | | | | | | | |

**Table B.2 Table of GenBank sequences referenced during this study.**

| Taxon | Genbank accession number | G.I. number | Authors |
|---|---|---|---|
| *C. linariifolia* Benth. | FJ939154.1 | 270312330 | Tank and Olmstead, 2009 |
| *C. miniata* Douglas ex Hook. | FJ939164.1 | 270312346 | Tank and Olmstead, 2009 |

**Table B.3 Direct-sequenced *Castilleja* miniata individuals, showing possible introgression. Table of all direct-sequenced *Castilleja miniata* (CAMI) individuals from both control and Mt. Harrison plots, using the *waxy* primer regions 11F-12R. The headings denote what the individual was identified as in the field, and what the molecular genome indicated during our analyses. The heading 'miniata' corresponds to individuals that were deemed to be this species based on alignments of cloned sequences of *Castilleja miniata, C. linariifolia*, and *C. christii* and accessions accessed from GenBank; a denotation of 'yes' indicates that the individual met the genetic criteria for inclusion into that group. Some *Castilleja miniata* individuals had *C. linariifolia waxy* sequences; while most of these were found at the summit of Mt. Harrison, some were from adjacent Mt. Independence populations. These may be F1 or introgressed hybrids with *C. linariifolia* in these areas that have gone undetected in this study and are denoted by 'No,', meaning they were not pure 'miniata.'**

| Species:Field | Species:Molec | Location | Miniata | Count |
|---|---|---|---|---|
| *C. miniata* | *C. miniata* | Boise National Forest | Yes | 8 |
| *C. miniata* | *C. miniata* | Boise National Forest | Yes | 5 |
| *C. miniata* | *C. miniata* | Harrison | Yes | 1 |
| *C. miniata* | *C. miniata* | Harrison | Yes | 2 |
| *C. miniata* | Poss Introgression | Mt. Independence | No | 6 |
| *C. miniata* | Poss Introgression | Harrison | No | 4 |
| *C. miniata* | Poss Introgression | Harrison | No | 3 |
| *C. miniata* | Poss Introgression | Harrison | No | 3 |
| *C. miniata* | Poss Introgression | Harrison | No | 5 |
| *C. miniata* | Poss Introgression | Harrison | No | 1 |

APPENDIX C

## Morphological Criteria Used to Distinguish *Castilleja* Species in the Field

**Table C.1 Morphological criteria used in field identification of *Castilleja* species. Table outlining criteria used during the summer of 2009 field season to determine *Castilleja* individual group (species) membership based on morphological characters. When these criteria were not met for species inclusion for any individual, that individual was treated as a putative hybrid. Information on informative morphological characters for identifying *Castilleja* species in the field was obtained from Holmgren, 1983 and from M. Eggers, pers. comm.**

| Species | Characters used for field identification |
|---|---|
| *C. christii* | - Yellow bract coloration within inflorescence<br>- Stems w/stipitate glandular hairs and puberulent-villious pubescence<br>- No exudate within inflorescence or anywhere on plant<br>- Unbranching |
| *C. linariifolia* | - Red to red-orange bract coloration within inflorescence<br>- Lvs linear to filiform<br>- Calyx decidedly unequally cleft (front very deep at 10-19 mm, behind only 2-8 mm). |
| *C. miniata* | - Red to red-orange to purple bract coloration within inflorescence<br>- Stems glabrous (did make exceptions on Mt. Harrison, as most *C. miniata* individuals had some pubescence below inflorescence)<br>- Lvs. broadly linear to ovate and large<br>- Has exudate within and/or below infl<br>- Has at least one stem with branching |