

TANGLED TRIOS?: CHARACTERIZING A HYBRID ZONE IN *CASTILLEJA* (OROBANCHACEAE)¹

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Hybridization and polyploidization are exceedingly important processes because both influence the ecological envelope and evolutionary trajectory of land plants. These processes are frequently invoked for *Castilleja* (Indian paintbrushes) as contributors to morphological and genetic novelty and as complicating factors in species delimitations. Here, we provide a detailed analysis of morphological and genetic evidence for hybridization in a well-characterized hybrid swarm involving three broadly sympatric species (*C. miniata*, *C. rhexiifolia*, *C. sulphurea*) in western Colorado. Field-classified hybrids are present at high frequencies at these sites and show morphological intermediacy to and segregate for chloroplast DNA haplotypes with *C. rhexiifolia* and *C. sulphurea*. Contrarily, DNA content and AFLP variation show that field-classified hybrids are not recent hybrids but a distinctive fourth taxon. Actual hybrids (plants showing admixture >10% for two genotypic groups) comprised 13% of our sample, with most admixture involving *C. rhexiifolia*, *C. sulphurea*, and the unknown taxon. The identity of the field-classified "hybrids" remains unknown; they either represent a stabilized hybrid species or a species with uncharacteristically high diversity for color alleles. This study highlights the importance of examining concordance and discordance between morphology, cytology, and genetic criteria to understand the complex evolutionary history of diverse groups such as *Castilleja*.

Key words: admixture; *Castilleja*; hybridization; introgression; Orobanchaceae; polyploidy; population structure.

Interspecific hybridization and introgression are processes of central importance in population and evolutionary biology due to their impacts on the maintenance of species distinctions, enhancing genetic diversity and diversification, and on rare occasion the formation of new species (Anderson and Stebbins, 1954; Grant, 1981; Barton and Hewitt, 1985; Arnold, 1992, 1997; Rieseberg and Wendel, 1993; Rieseberg and Carney, 1998). The outcome of interspecific mating depends upon the frequency of species contact and the complex interplay between hybrid adaptive fitness and the genetic and the environmental systems that control trait expression (Endler, 1977, 1986; Barton and Hewitt, 1985). Interspecific hybridization among plant species can result in the breakdown or the reinforcement of previously distinct species barriers, increased genetic and phenotypic diversity, introgression of traits across species boundaries, new traits, and/or the formation of new evolutionary lineages (Grant, 1981; Arnold, 1992, 1997; Rieseberg and Wendel, 1993; Rieseberg and Carney, 1998). Plant hybridization also has ecological and evolutionary consequences for their associated communities and ecosystems (Whitham et al., 1999, 2006). For these reasons, interspecific hybridization plays a pivotal role in the evolution and ecology

of many plant taxa, although its importance varies by taxon and location (Ellstrand et al., 1996).

Morphological intermediacy and spatial patterns of elevated trait diversity are often the first cues to developing hypotheses of interspecific hybridization (Anderson, 1949). Nevertheless, hybrids do not always show morphological intermediacy, and hybrid zones are not universally characterized by increased or spatially structured trait diversity (see Rieseberg and Ellstrand, 1993). Furthermore, hybridization can generate novel traits not present in any of the parent species (Rieseberg and Ellstrand, 1993; Rieseberg, 1995); this type of transgressive segregation can further complicate morphology-based assessments of hybridity. The use of morphological traits to infer hybridity can be particularly limiting when hybridization involves closely related species, due to a paucity of trait differences (Avice, 1994), and for polyploid complexes, due to the variable effect of polyploidy on gene and trait expression (Garbutt and Bazzaz, 1983; Segraves and Thompson, 1999; Adams et al., 2003).

In such instances, molecular markers are invaluable for identifying hybrids and for dissecting population genetic processes involving hybridization (Cronn and Wendel, 2004; Hegarty and Hiscock, 2005). For instance, nuclear and cytoplasmic molecular markers can be used together to infer rates of hybridization and introgression (i.e., Arnold, 1993; Brubaker et al., 1993; Welch and Rieseberg, 2002) and to confirm species of hybrid origin (Rieseberg and Soltis, 1991; Wendel et al., 1991; Arnold, 1993; Wolfe et al., 1998; Mallet, 2007). In theory, the genetic architecture of hybrids should reflect the combined effects of gene flow, mutation, pre- and postzygotic isolating barriers, and selection (Endler, 1977; Barton and Hewitt, 1985). Because selection acts on standing genetic variation for fitness (Fisher, 1930) and there are correlations between selectively important phenotypic traits and fitness (Lande and Arnold, 1983), a combination of molecular and morphology-based approaches may reveal complementary facets about the frequency of contact, symmetry of contact, morphological consequence, and adaptive potential of hybridization.

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In this paper, we expand on previous studies of an Indian paintbrush hybrid zone (*Castilleja*, Orobanchaceae; Hersch and Roy, 2007) in the Elk Mountains surrounding Gothic, Colorado, by explicitly examining patterns of morphological and molecular genetic hybridity in field-classified hybrids relative to their parental species. This hybrid zone includes three species, *C. miniata* Dougl. ex Hook., *C. rhexiifolia* Rydb., and *C. sulphurea* Rydb., all of which co-occur in the Rocky Mountains of North America (Heckard and Chuang, 1977). Hybridization is thought to be common among these species, and more generally in *Castilleja*, where it is widely cited as a complicating factor in defining species circumscriptions (Ownbey, 1959; Heckard, 1964, 1968; Heckard and Chuang, 1977; Holmgren, 1984; Egger, 1994; Tank and Olmstead, 2008). As an additional complication, intraspecific ploidy variation is well known in these species (Heckard, 1968; Heckard and Chuang, 1977; Holmgren, 1984), and hybridization between taxa with differing ploidy levels is thought to lead to allopolyploid formation.

Documentation of hybrids in *Castilleja* has primarily relied upon morphology and ploidy variation (Heckard and Chuang, 1977; Anderson and Taylor, 1983; Egger, 1994; but see Mathews and Lavin, 1998), although the use of morphological criteria to detect hybrids in *Castilleja* is complicated by ploidy variation. Morphologically, the most obvious feature indicating hybridization between *C. miniata*, *C. rhexiifolia*, and *C. sulphurea* is floral bract color, which is typically uniform within species (red in *C. miniata*, pink in *C. rhexiifolia*, yellow in *C. sulphurea*), and highly variable and intermediate in putative hybrids (Fig. 1). Individual bracts also exhibit multiple hues along the central lamina and margins in putative hybrids (Fig. 1), a condition that is rare to absent in parental species. Hybridization between these species is likely mediated by pollinators that promote interspecific pollen transfer (Hersch and Roy, 2007) and by weak postzygotic barriers to hybridization (66% of heterospecific hand pollinations resulted in seed production; Hersch, 2007).

To characterize the frequency of hybridity and to understand the symmetry of gene flow and potential barriers to hybrid establishment among these species, we examined morphological traits, molecular variation, and nuclear DNA content in *C. miniata*, *C. rhexiifolia*, *C. sulphurea*, and their field-classified hybrids in a zone of sympatry. We addressed three specific questions: (1) What traits provide the clearest evidence for hybridization? (2) Do molecular markers provide evidence for contemporary hybridization or past introgression between the species, and if so, what are the proportional contributions of each species to the hybrid pool? (3) Finally, do molecular data indicate whether the putative hybrids are F1 hybrids, advanced generation introgressants, or something altogether different?

MATERIALS AND METHODS

Study sites—We primarily examined plants from two basins in the Elk Mountains surrounding Gothic, Colorado, USA (Fig. 2). In the Schofield Pass section of the Hast River basin, the three *Castilleja* species co-occur between 3260–3630 m a.s.l., with no plants having morphologically intermediate/variable bracts (putative hybrids). In Copper Basin, there is considerable spatial structure; between 3060–3160 m a.s.l., *C. miniata* and *C. sulphurea* co-occur, and their putative hybrids can be found starting at 3170 m a.s.l. At approximately 3350 m, *C. rhexiifolia* appears, and at this elevation, all three *Castilleja* species can be found growing in close proximity to the putative hybrids. The putative hybrids are most abundant at higher elevations and mostly occur in spatially discrete populations.

In these basins, plants were sampled from three types of populations: (1) "pure parental" populations, in which only one of the three putative parent spe-

cies occurred within a 20 m radius; (2) "hybrid" populations, in which some or all parent species co-occurred at low frequencies and putative hybrids occurred at high frequencies; and (3) "comingled" populations, in which putative parent species and/or putative hybrids occurred in close proximity (between 0.15 m and 20 m) to each other (Fig. 2). *Castilleja sulphurea* was also sampled from a nearby basin in which no putative hybrids have been observed (location S2" Fig. 2). We sampled the three *Castilleja* species from "pure parental" populations to maximize detection of species-diagnostic traits and markers. At higher elevations in Copper Basin, some *C. sulphurea* were shorter and more pubescent; they are either a high-elevation form of *C. sulphurea*, or they could be a separate species, most notably *C. occidentalis* Torr., with which it can be confused (Heckard and Chuang, 1977). We intentionally included 10 of these plants to determine whether they were different and could be implicated in hybridization (all these plants were collected from the comingled SC populations near Copper Lake, Fig. 2). In most cases, we examined the same plants for morphological, genetic, and nuclear DNA content variation. All statistical analyses were performed with the program JMP IN version 5.1.2 (SAS, 2004).

Morphology—Morphological traits measured in this study include taxonomically informative and objectively measured characters that are used to identify *Castilleja* species (Barrell, 1969; Anderson and Taylor, 1983; Holmgren, 1984; Weber and Wittmann, 2001). All plants were flowering, and measurements were made in July 2005.

Quantitative traits—Twenty-two quantitative characters were measured from 89 *C. miniata*, 87 *C. rhexiifolia*, 77 *C. sulphurea*, and 103 putative hybrids (Table 1). Summary statistics for each of these characters are provided in Appendix S1 (see Supplemental Data with the online version of this article). To reduce redundancy in the data set, we calculated Pearson product-moment correlations between traits to remove highly correlated characters ($R^2 > 0.80$; Table 1). As a result, we analyzed 17 quantitative characters using two different approaches. First, we used principal component analysis (PCA) to examine relationships among the quantitative traits and between individual plants. When necessary, characters were transformed to meet assumptions of normality and homogeneity of variances (Sokal and Rohlf, 2000). Second, also using principal component analysis, we detected five groups of traits that had similar eigenvector loadings, in both magnitude and direction, on the first three axes (Table 2). We used this information to create five new composite traits (flower size, bract size, leaf size, plant size, and hairiness) by standardizing individual traits to have a mean of zero and a standard deviation of one, and then summing all individual traits within a composite trait (Table 2). Summary statistics for each of these composite characters are provided in Appendix S2 (see Supplemental Data with the online version of this article). For each composite trait, we tested for significant differences between the three *Castilleja* species and the putative hybrids using one-way analyses of variances (ANOVA).

Qualitative traits—Six qualitative characters were measured from the same plants as described above (Table 1). We used logistic regressions to examine qualitative trait differences among the plant groups (*C. miniata*, *C. rhexiifolia*, *C. sulphurea*, and putative hybrids). Because tests to detect significant trait differences between plant groups were performed on the same plants, we assumed non-independence among the II tests involved (five tests for the composite quantitative traits and six for the qualitative traits), and we used the conservative sequential Bonferroni procedure (Rice, 1989) with a minimum significance level of 0.005 to correct for type I errors.

Nuclear DNA Content—Nuclear 2C DNA content of 180 plants from the different populations (81 *C. miniata*, 80 *C. rhexiifolia*, 80 *C. sulphurea*, and 90 putative hybrids) was determined using flow cytometry following the procedures of Dart et al. (2004), modified for dry leaf material by P. Kron and B. Husband (University of Guelph, Canada; personal communication). Briefly, we used fresh *Chamerion angustifolium* (Onagraceae) leaf material as an internal standard for each sample (2C DNA content = 0.84 pg; P. Kron and B. Husband, personal communication). Unknowns and internal standards were run simultaneously on a Becton Dickinson Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA) in B. Husband's laboratory at the University of Guelph, Canada. An estimate of nuclear 2C DNA content was determined using Cell Quest Pro software, version 4.0.1 (Becton, Dickinson and Co., San Jose, California, USA). Plant nuclear 2C DNA content for 31 of the 180 plants could not reliably be obtained due to low nuclei counts (<1000) and/or high coefficients of variation (>5.0); these plants were not included in the final analyses. We used a two-way ANOVA on plant nuclear DNA content

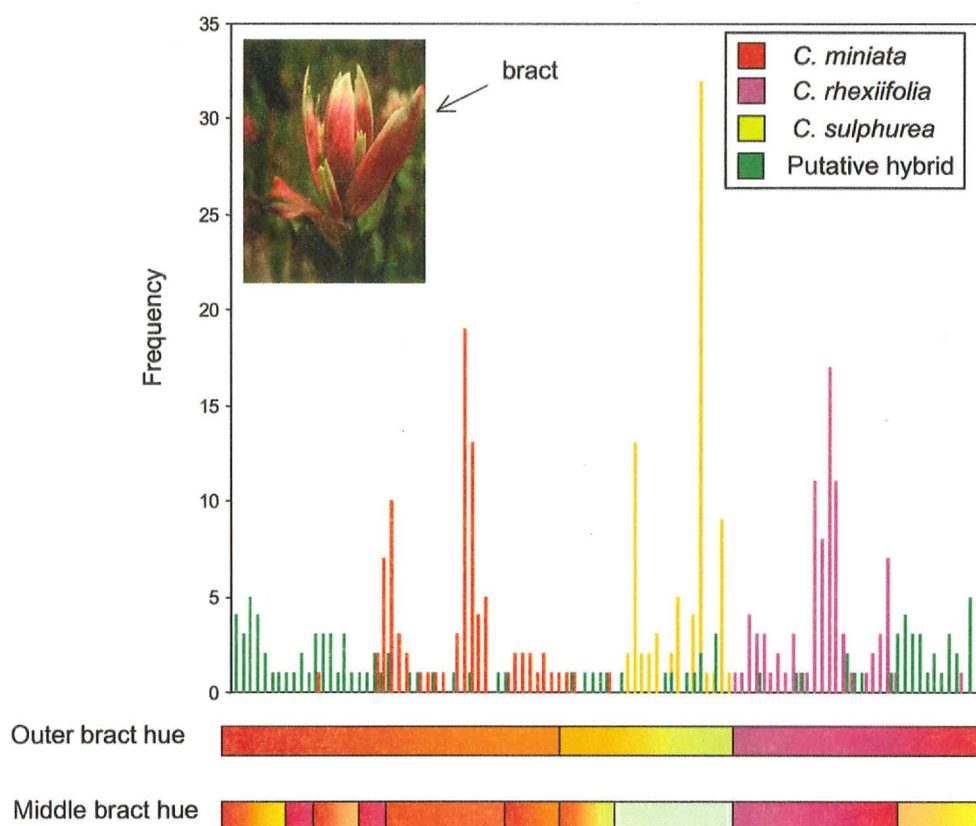


Fig. 1. Frequency histogram of bract color hue for the outer and the middle portion of bracts for three Indian paintbrush species (*Castilleja*) and their putative hybrids. Bract hue color was measured with a color reader (Minolta, Model # CR-11), which measures hue on a circular scale of 0–100 and is depicted as the x-axis. Photograph in the upper left hand corner depicts a putative hybrid; arrow points to a flower bract.

with plant group and site (nested within plants group) treated as fixed factors. Tukey's honestly significant difference (HSD) tests were used for a posteriori comparisons among group means, and data are presented as LS means $\pm$ 1 SE.

DNA analyses—Total genomic DNA from 375 individuals was isolated from silica gel-dried leaf material using the modified CTAB method of McNeal et al. (2006). Quality and quantity of the DNA extracts were verified using 1% agarose/Tris-borate-EDTA (TBE) gels.

Chloroplast DNA—Chloroplast (cp) DNA haplotypes from two chloroplast intergenic spacer regions, *trnL*^{UAA}-*trnF*^{GAA} and *trnH*^{SUG}-*psbA* (Small et al., 1998), were determined from a subset of the samples to infer Matrilines. PCR conditions and cycle sequencing were conducted following Horning and Cronn (2006). Sequences were checked using the program BioEdit version 7.09 (Hall, 1999), alignments constructed using the module CLUSTAL_X (in BioEdit; Thompson et al., 1997) with slight visual modifications, and then concatenated with the program MEGA version 4.0 (Tamura et al., 2007). In total, we obtained 78 concatenated sequences (17 *C. miniata*, 13 *C. rhexiifolia*, 17 *C. sulphurea*, and 31 putative hybrids), available as GenBank accessions FJ765576-FJ765725. Nucleotide diversity (π) was calculated with the program DNAsp version 4.5 (Rozas et al., 2003), and most-parsimonious haplotype networks were created using statistical parsimony (program TCS version 1.2; Clement et al., 2000).

AFLP fingerprinting—Amplified fragment length polymorphism (AFLP) analyses were performed as described by Vos et al. (1995) with minor modifications (Hersch, 2007). From an initial screen of 20 primer combinations, we selected the four most variable combinations (*EcoRI*-*AAC*/*MseI*-*GAA*, *EcoRI*-*AAC*/*MseI*-*GAT*, *EcoRI*-*AGC*/*MseI*-*GAA*, *EcoRI*-*ACA*/*MseI*-*GAA*). Selectively amplified PCR products were resolved on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, California, USA) at the Oregon

State University Center for Gene Research and Biotechnology (Corvallis, Oregon, USA; <http://www.cgrb.orst.edu/>) using GS500 ROX (Applied Biosystems, Foster City, California, USA) internal lane standards. Bands were scored using Genotyper version 3.7 NT software (Applied Biosystems), and multi locus AFLP profiles were scored for band presence (1) or band absence (0) for unambiguous bands between 90 and 500 bases. To evaluate band repeatability, we repeated the procedure (including DNA extraction) for three individuals at each step in the process, yielding four complete replicates for three individuals. Comparison of the AFLP profiles from independent runs indicated a cumulative error rate of 6%. Nonreproducible bands, redundant bands, bands fixed in all samples, and three plant samples that were not amplified were excluded from the data matrix. In total, we analyzed 372 individuals, including 90 *C. miniata*, 89 *C. rhexiifolia*, 95 *C. sulphurea*, and 98 putative hybrids.

We used several methods to analyze population divergence and structure. First, we used an analysis of molecular variance model (AMOVA; Excoffier et al., 1992), as implemented by the program ARLEQUIN version 3.1 (Schneider et al., 2000; Excoffier et al., 2005), to examine levels of variation in AFLP banding patterns among plant groups (*C. miniata*, *C. rhexiifolia*, *C. sulphurea*, and putative hybrids), among populations within plant groups, and within populations.

Second, genetic distances among individuals were examined with principal coordinate analysis (PCoA) based on squared Euclidean distances (program NTSYS-PC version 2.02; Rohlf, 1997). We chose squared Euclidean distances as opposed to other metrics (i.e., Dice or Jaccard coefficients) because (1) squared Euclidean distance equally weights shared presences and absences of bands, which seems warranted because inheritance patterns of dominant markers in polyploid organisms is unknown, and (2) post hoc goodness of fit tests revealed that PCoA based on squared Euclidean distances performed slightly better than either Dice or Jaccard distances. Differences between the four plant groups on the first four axes were tested with one-way analyses of variances.

Finally, we examined population structure and admixture with a Bayesian-model-based clustering method implemented in the program Structure

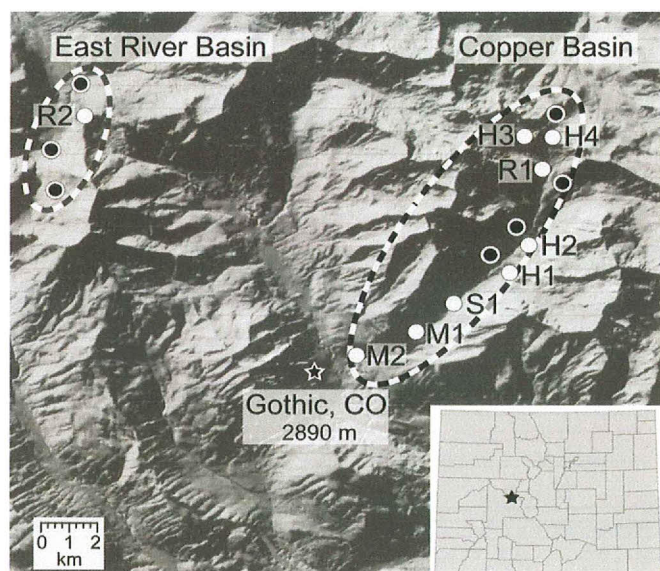


Fig. 2. Map showing the location of populations from which parental and putative hybrid plants were sampled: M1 and M2 = *C. miniata*; R1 and R2 = *C. rhexiifolia*; S1 = *C. sulphurea*; H1 and H2 = putative hybrids. Plants were sampled from two basins, East River (~39°N107°W, 3265 m a.s.l.; sampling sites in this basin encircled by a dashed line) and Copper (~39°N106°W, 3450 m a.s.l.; sampling sites encircled by a dashed line). One additional *C. sulphurea* population (S2) was collected near the town-site of Irwin, Colorado (~38°N107°W, 3230 m a.s.l.). Plants were also sampled from comingled populations in each basin; filled circles designate comingled sites from East River basin (ME, RE, SE) and Copper basin (HC, MC, RC, SC).

version 2.2 (Pritchard et al., 2000; Falush et al., 2003, 2007). This program uses allele frequencies at each locus and implements a model-based clustering algorithm to assign individuals to a parameterized number of predetermined groups (K). We examined the probabilities of obtaining the data $\text{Ln}(K/D)$ for $K = 1$ to 10. All analyses used an admixture ancestry model with independent allele frequencies and burn-in and run Markov chain Monte Carlo lengths of 10^4 and 10^5 respectively. We performed 10 separate runs of each K and used the program CLUMPP (Jakobsson and Rosenberg, 2006) to obtain average estimates of individual admixture for each K . To select the best estimate of K , we used a post hoc quantity $\hat{K}$, because simulations by Evano et al. (2005) showed that the true value of K corresponds to the maximum when $\hat{K}$ is plotted against K .

RESULTS

Morphological variation—The first three principal component (PC) axes accounted for 64.6% of the total variation in the quantitative traits (Table 2). The three *Castilleja* species and the putative hybrids separated along the first two principal component axes (Fig. 3) but not along the third axis (not shown). Overall, the putative hybrids formed a morphologically coherent assemblage and occupied an intermediate position between the three *Castilleja* species on PC I (Fig. 3).

There were significant differences among the four plant groups for all of the composite quantitative traits (Fig. 4). Relative to the three *Castilleja* species, the putative hybrids had intermediate trait values and, as a group, were not transgressive for any trait (Fig. 4). For three of the traits, putative hybrids appeared indistinguishable from two of the potential parent species, with alternate affinities to *C. rhexiifolia* (plant size,

TABLE 1. Morphological characters measured on *Castilleja*. The first entry following each character reflects the data type: continuous (C), nominal (N), or ordinal (O); the second entry reflects the measurement scale or formula for continuous data or the number of levels for nominal or ordinal data; and the third entry specifies the type of data analysis: PCA (P), logistic regression (L), not analyzed (*). Means were taken from four separate measurements. The number of plants that were measured include: 30, 33, and 40 putative hybrids from populations H1, H2, and HC, respectively, 27, 22, 20, and 20 *C. miniata* from populations M1, M2, MC, and MS, respectively, 20, 27, 20, and 20 *C. rhexiifolia* from populations R1, R2, RC, and RS, respectively, and 27, 10, 20, and 20 *C. sulphurea* from populations S1, S2, SC, and SS, respectively.

Morphological characters

Bracts: general

Percentage lobed bracts: C, number, *
Presence/Absence lobed bracts: N, 2, L
Mean number of bract hairs: C, number per 0.5 mm, P

Bracts: lower flower on raceme

Mean bract width: C, mm, P
Mean bract length: C, mm, P
Presence/Absence color shift between middle and upper part of bract: N, 2, L

Inflorescence Measures

Number of racemes: C, number, P
Total height of racemes: C, cm, P
Pubescence within inflorescence: O, 11, *
Pubescence below inflorescence: O, 11, P

Flower Measures

Corolla tube length: C, mm, P
Corolla galea length: C, mm, P
Calyx length: C, mm, P
Calyx inner segment length: C, mm, P
Calyx outer segment length: C, mm, P
Calyx segment shape: N, 3, L
Stamen mean length of lower pair: C, mm, *
Stamen mean length of upper pair: C, mm, P

Leaf Measures:

Mean leaf length: C, mm, P
Mean leaf width: C, mm, P
Percentage lobed leaves: C, number, *
Presence/Absence lobed leaf: N, 2, L
Mean number of leaf hairs: C, number per 0.5 mm, P

Overall Plant Measures

Stem maximum height: C, cm, *
Stem diameter: C, cm, *
Number of stems: C, number, P
Percentage stem branching: C, number, *
Presence/Absence branched stems: N, 2, L
Stem color: N, 3, L
Plant_size_cone: C, $1/3\pi(\text{stem radius})^2 \times \text{stem height}$, P

hairiness) and *C. sulphurea* (leaf size). The remaining two traits (flower size, bract size) in the putative hybrids were distinctive but intermediate to those in the three parent species (Fig. 4). There were also significant qualitative trait differences among the plants (Fig. 4). Relative to the three *Castilleja* species, putative hybrids were intermediate in trait values for lobed leaves, stem branching, calyx shape, and stem color (Fig. 4), but they were transgressive from the three *Castilleja* species for the presence/absence of bract color shifts and for the presence/absence lobed bracts (Fig. 4).

DNA content variation—Each plant group examined was nearly fixed for a different nuclear DNA content (Fig. 5), and the three species and putative hybrids differed from each other in mean nuclear 2C DNA content ($F_{3,287} = 563.82$, $P < 0.0001$),

TABLE 2. Factor loadings on the first three principal components for 17 morphological characters for *Castilleja*. Characters followed by similar letters were standardized and then summed together to create new standardized composite traits for subsequent ANOVA's.

Character	PC1	PC2	PCA3
Percentage variation	31.2722	21.3483	11.9917
Tube length ^a	0.38399	0.09574	-0.04074
Galca length ^a	0.35469	0.11409	-0.06391
Calyx length ^a	0.38593	0.10966	-0.06529
Inner segment length ^a	0.31552	0.06546	-0.01822
Outer segment length ^a	0.35595	0.10329	-0.08543
Stamen length upper ^a	0.35054	0.09421	-0.02772
Average bract length ^b	0.20217	0.26661	0.21880
Average bract width ^b	-0.11412	0.31429	0.40271
Leaf length ^c	0.28277	0.02407	0.23885
Leaf width ^c	-0.08719	0.16544	0.50974
Plant size ^d	0.09827	-0.34092	0.28887
Log_Raceme height ^d	0.14954	-0.29721	0.35445
Log_Number of racemes ^d	0.09288	-0.38166	0.31205
Log_Number of stem ^d	0.06061	-0.39419	0.17146
Raceme hairs below ^e	0.00347	0.27152	0.29582
Average number of bract hairs ^e	-0.13807	0.27024	0.15982
Average number of leaf hairs ^e	-0.15432	0.30297	0.07591

^a Flower size, ^b bract size, ^c leaf size, ^d plant size, ^e hairiness

with no effect of population nested within plant group ($F_{10, 287} = 0.31$, $P = 0.3080$). Rare DNA content variation was evident within species and hybrids (i.e., three *C. miniata*, one *C. rhexiifolia*, four *C. sulphurea*, and four putative hybrids, see Fig. 5) and is likely attributable to within-taxon ploidy variation. After excluding these 12 outliers, mean nuclear DNA content was $5.46 \text{ pg/2C} \pm 0.03$ for *C. miniata*, $3.14 \text{ pg/2C} \pm 0.03$ for *C. rhexiifolia*, $1.52 \text{ pg/2C} \pm 0.03$ for *C. sulphurea*, and $1.74 \text{ pg/2C} \pm 0.03$ for the putative hybrids. Correlations relating chromosome count data (following the protocol of Heckard and Chuang, 1977) from one *C. miniata* plant, five *C. rhexiifolia* plants and three *C. sulphurea* plants with nuclear 2C DNA values (Hersch, 2007) show that *C. miniata* are primarily octaploid ($2n = 8x = 96 = 5.38 \text{ pg/2C}$), *C. rhexiifolia* are primarily tetraploid ($2n =$

$4x = 48 = 2.96 \text{ pg/2C} \pm 0.05$), and *C. sulphurea* are primarily diploid ($2n = 2x = 24 = 1.55 \text{ pg/2C} \pm 0.04$). Chromosome counts could not be obtained for the putative hybrids, but their nuclear 2C values indicate that they are likely diploids.

Chloroplast DNA variation- Sequences from the different plant groups showed high similarity to each other, with only 13 variable sites in 881 total sites, generating 10 unique haplotypes (not including indels). High uniformity is also depicted by overall low nucleotide diversity ($\pi = 0.0017$). The three *Castilleja* species and putative hybrids differed in the number and identity of haplotypes (Fig. 6), which is also reflected by individual differences in nucleotide diversity for *C. miniata* (two haplotypes; $\pi = 0.0004$), *C. rhexiifolia* (five haplotypes; $\pi = 0.0027$), *C. sulphurea* (four haplotypes; $\pi = 0.0016$), and the putative hybrids (four haplotypes; $\pi = 0.0022$). In our sample, *C. rhexiifolia*, *C. sulphurea*, and the putative hybrids segregate for two haplotypes (IV, IX), while haplotypes for *C. miniata* are private and more divergent (Fig. 6). Private haplotypes were found in all of the putative parental species (*C. miniata* = 2; *C. rhexiifolia* = 3; *C. sulphurea* = 1), and none were observed in the field-classified hybrids, supporting the hypothesis that the hybrids share a recent matrilineage coancestry with at least *C. rhexiifolia* and/or *C. sulphurea*.

AFLP variation- The four primer combinations yielded 329 polymorphic, repeatable bands. We found no diagnostic, species-specific alleles and only six private, species-specific alleles (four in *C. rhexiifolia*, two in *C. sulphurea*). These private alleles were also found in the putative hybrids, but they are unlikely to be informative because they occurred at low frequencies in the *Castilleja* species and putative hybrids (<6%).

AMOVA revealed that most of the total genetic variation (>78%) was found among individuals within populations, and <18% was found among the different plant taxa (Table 3). Only modest genetic variation was found among populations within plant taxa (Table 3).

Principal coordinate analysis (PCoA) revealed that the 372 individual plants clustered into four distinct groups corresponding to *C. miniata*, *C. rhexiifolia*, *C. sulphurea*, and putative hybrid plants (Fig. 7). The first three axes accounted for 31.8% of the total variance in the data set, with the first, second, and third axis explaining 14.1%, 10.1%, and 7.6% of the variation, respectively (Fig. 7). The first three axes also clearly separated the taxonomic groups (axis 1: $F_{3,368} = 804.13$, $P < 0.0001$, axis 2: $F_{3,368} = 816.57$, $P < 0.0001$, axis 3: $F_{3,368} = 575.60$, $P < 0.0001$). Remarkably, the axis accounting for the largest proportion of the total variation (PCol) separates the putative hybrids from all three species. Restated, the putative hybrids are more divergent from the three known species than any of these species are from each other, despite the distinctiveness of the three species in regards to 2C DNA content ($2x$, $4x$, $8x$), and divergence in chloroplast haplotypes. In the second and third axes, the putative hybrids resolved intermediate to all three *Castilleja* species (Fig. 7; PCoA axis 3 not shown). Noteworthy, different populations of the same taxon clustered together, mirroring AMOVA results showing relatively low (3.9%) among population, within plant group variation (Table 3).

Posterior probabilities of the models given the data showed that the peak of $\Delta K/K$ was at $K = 4$. In addition to this important level of genetic structure, we evaluated a range of values for K from 2 through 6 (Fig. 8). Bar plots of posterior probabilities of group membership reveal that the three *Castilleja* species were

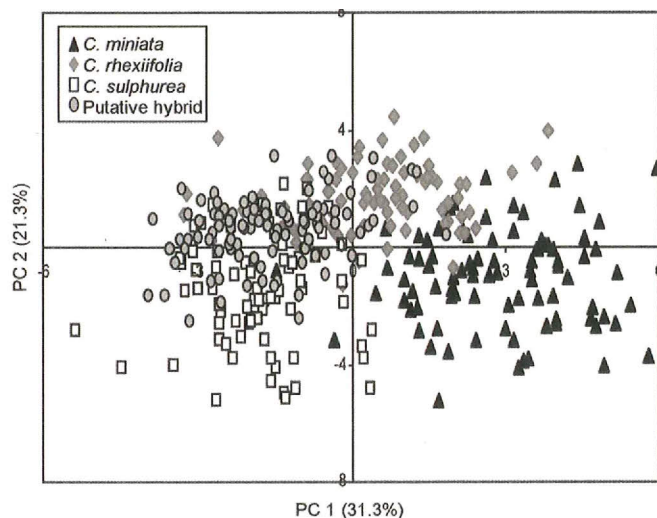


Fig. 3. Principal component analysis of 17 quantitative traits for the three *Castilleja* species and their putative hybrids showing the principal component (PC) scores for PC1 vs. PC2. The percentage variation explained by each axis is given in parentheses.

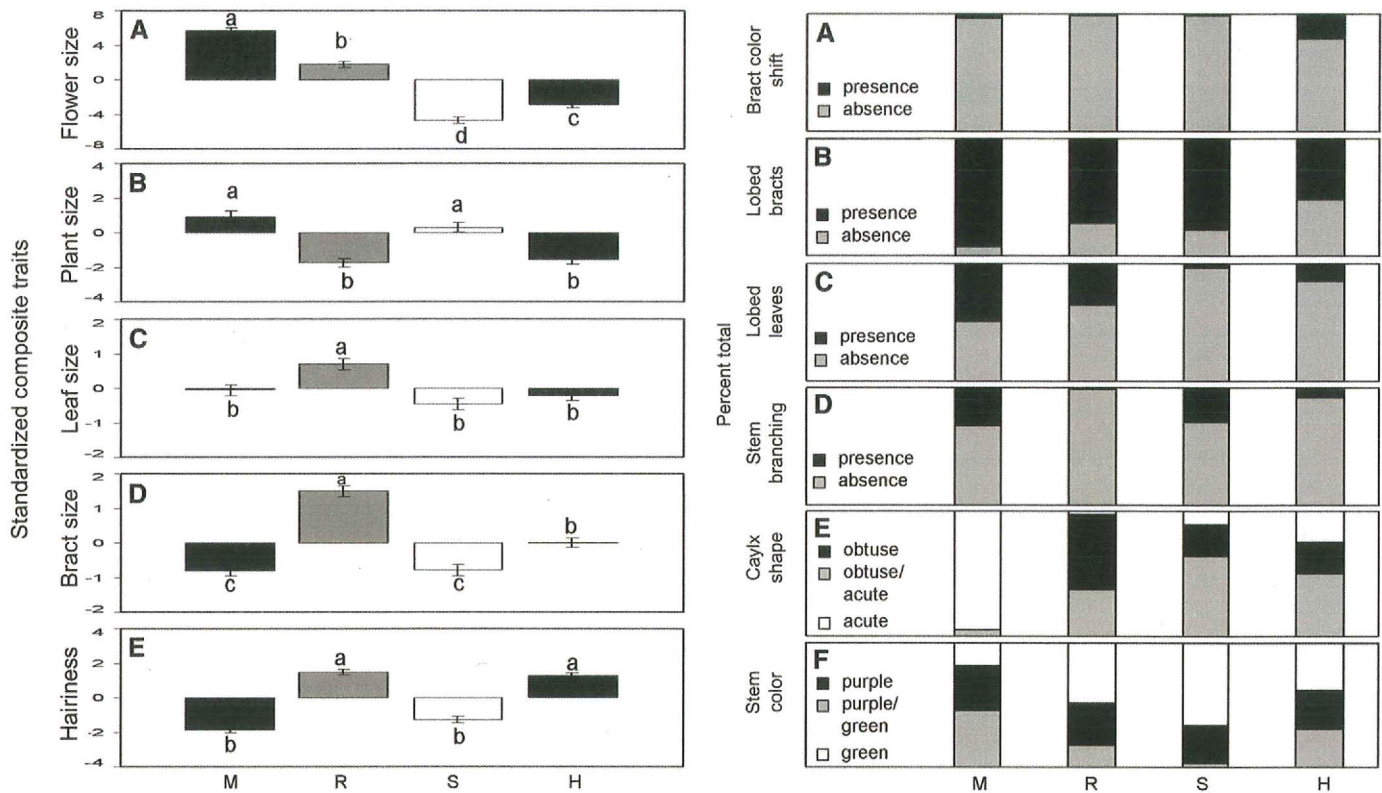


Fig. 4. Variability in quantitative and qualitative traits between *Castilleja* species and their putative hybrids. (A) ANOVA results for standardized composite morphological quantitative trait differences for flower size: $F_{3,352} = 183.83$, $P < 0.0001$, plant size: $F_{3,352} = 29.23$, $P < 0.0001$, leaf size: $F_{3,351} = 9.64$, $P < 0.0001$, bract size: $F_{3,352} = 51.20$, $P < 0.0001$, and hairiness: $F_{3,352} = 93.90$, $P < 0.0001$. For composite traits, different lower case letters represent statistically significant differences in trait values. (B) Logistic regression for qualitative morphological trait differences in bract color shift: $\chi^2 = 35.06$, $df = 1$, $P < 0.0001$, lobed bracts: $\chi^2 = 41.66$, $df = 1$, $P < 0.0001$, lobed leaves: $\chi^2 = 60.00$, $df = 1$, $P < 0.0001$, stem branching: $\chi^2 = 51.00$, $df = 1$, $P < 0.0001$, calyx shape: $\chi^2 = 252.64$, $df = 1$ and stem color: $\chi^2 = 66.70$, $df = 1$, $P < 0.0001$. M = *C. miniata*, R = *C. rhexiifolia*, S = *C. sulphurea*, H = putative hybrids.

genetically more similar to each other than they were to the putative hybrids ($K = 2$, Fig. 8), mirroring the distance-based analyses (PCoA axis 1; Fig. 7). At $K = 3$, the hybrids and *C. sulphurea* appear as distinct groups, while *C. rhexiifolia* and *C. miniata* remain indistinct. Finally, at values of $K = 4$ or higher, the three *Castilleja* species and the putative hybrids each resolve into distinct groups (Fig. 8).

For a small number of plants, principal coordinate analyses and Bayesian clustering at $K < 4$ revealed evidence of admixture (potential hybridity); these plants deviated from their morphologically assigned identity. In particular, 48 plants (13% of the total sample) had coefficients of membership (Q) less than 90% of their field-classified taxon. These included plants classified as "hybrids" ($N = 16$), "*C. miniata*" ($N = 10$), "*C. rhexiifolia*" ($N = 7$), and "*C. sulphurea*" ($N = 15$). Fourteen of these plants had 2C nuclear DNA estimates that were unusual for the taxon they resembled. For example, two plants classified as "*C. miniata*" (most frequently octaploid in our sample) had 2C nuclear DNA content of $1.46 \text{ pg/2C} \pm 0.14$ and a genetic architecture resembling diploid *C. sulphurea*. Likewise, four putative plants classified as "hybrids" and two classified as "*C. sulphurea*" (most frequently diploid) had a mean 2C DNA content of $3.11 \text{ pg/2C} \pm 0.03$ and a genetic architecture resembling tetraploid *C. rhexiifolia*. Interestingly, the two *C. sulphurea* were noted in the field as "potential hybrids" because they displayed bract color features indicative of *C. rhexiifolia*. Finally, six

putative hybrids with genetic architectures resembling *C. sulphurea* had a mean nuclear 2C DNA content of $1.59 \text{ pg/2C} \pm 0.03$, which is intermediate to the putative hybrid and *C. sulphurea* means ($1.74 \text{ pg/2C} \pm 0.03$ and $1.52 \text{ pg/2C} \pm 0.03$, respectively). In 30 cases, we found no apparent relationship between admixture and odd nuclear DNA content, and in four cases nuclear DNA content was not available. In summary, genetically admixed plants ($N = 48$) are far less frequent than we would have

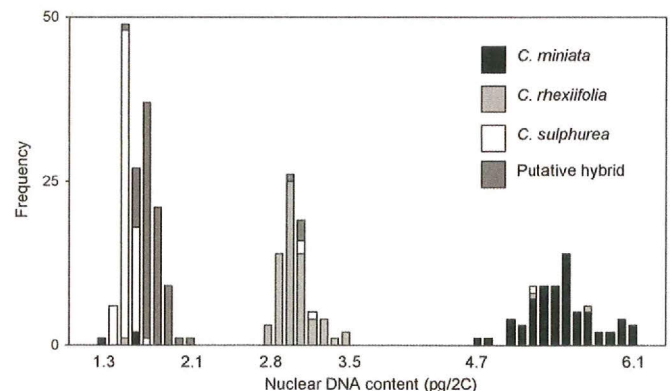


Fig. 5. Histogram showing nuclear 2C DNA content variation for *C. miniata*, *C. rhexiifolia*, *C. sulphurea*, and the putative hybrids.

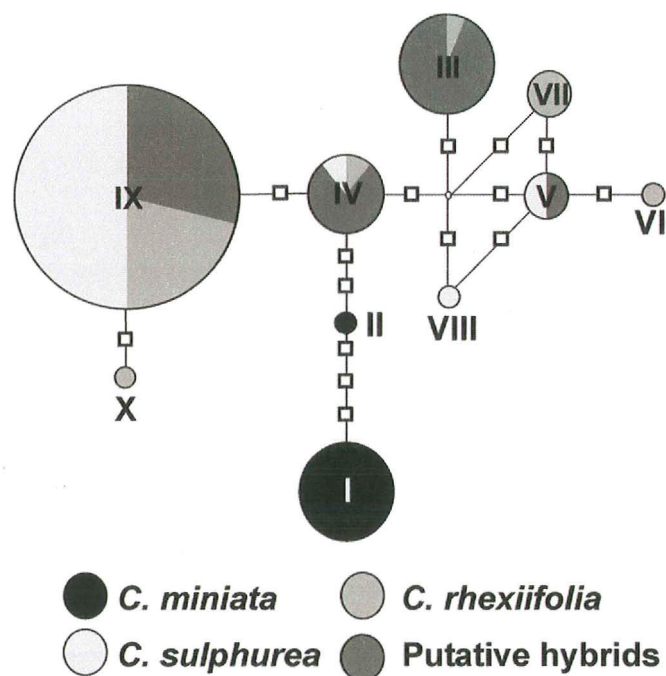


Fig. 6. Haplotype networks derived from chloroplast nucleotide data for the three *Castilleja* species and the putative hybrids. Pie diagrams represent the different haplotypes and are numbered for reference purposes. The size of each pie is proportional to the frequency of each haplotype, and the size of the pie slices depicts the proportion of each plant group belonging to that haplotype. Nucleotide substitutions (□) are traced along branches.

predicted based on a sample of 98 putative "hybrids". The frequency distribution of admixed individuals in these four taxa are not different than those expected from random sampling proportions ($G = 2.370$; $P = 0.499$), indicating that the field-classified hybrids show no more frequent evidence of genomic hybridity than do individuals classified as "*C. miniata*", "*C. rhexiifolia*", or "*C. sulphurea*".

DISCUSSION

Published accounts based on morphology and cytology suggest that *Castilleja miniata*, *C. rhexiifolia*, and *C. sulphurea* hybridize across a broad zone of sympatry in the Rocky Mountains of North America (Heckard and Chuang, 1977; Anderson and Taylor, 1983; Chuang and Heckard, 1993; Egger, 1994). In addition, experimental pollination arrays show that interspecific pollen transfer occurs frequently among these species and that pollinator constancy is diminished in areas where the species co-occur with morphologically intermediate, putatively hybrid

plants (Hersch and Roy, 2007). These observations led us to examine a "hybridization hotspot" in detail to estimate frequency of interspecific gene flow and the contribution of each species to the hybrid swarm. While quantitative phenotypic trait analyses confirm that field-classified "hybrid" plants are intermediate to the parental species for nearly all traits, cytogenetic and AFLP data suggest a more complicated, less intuitive relationship between these entities than might be inferred from morphology alone.

Relationships based upon morphological and molecular evidence—The three putative parent species are readily discriminated on the basis of principal component analysis (Fig. 3) and variation in composite traits (Fig. 4). Species distinction is a critical requirement for identifying hybrids, as a lack of readily diagnosable characters separating parental species would make it difficult to identify putative hybrids and their hypothetical parentage. In our sample, *C. rhexiifolia* and *C. sulphurea*—taxa that are closely related and have been treated as conspecific by some authorities [e.g., *C. rhexiifolia* Rydb. var *sulphurea* (Rydb.) N. D. Atwood]—differed significantly in all five composite traits.

Field-classified hybrids had intermediate or parent-type expression for nine of the 11 morphological traits, with trait values more similar to *C. rhexiifolia* and *C. sulphurea* than to *C. miniata* (Figs. 3–5). In early-generation hybrids, the expression of intermediate or parent-type trait values is common, but similar expression can also arise from other evolutionary phenomena (see Rieseberg and Ellstrand, 1993). In hybrid species, transgressive segregation and novel phenotypic expression can be common, and these traits are often associated with ecological divergence (Grant, 1975; Rieseberg et al., 1999, 2003; Rosenthal et al., 2002; Gross et al., 2004). It is interesting that the two traits for which the putative hybrids showed extreme character expression—bract color shift and presence/absence of lobed bracts—are associated with flower display. These traits may be important for pollinator-mediated selection and introgression in *Castilleja*, just as floral display traits are under strong selection by pollinators in other plant groups (see for example, Grant, 1949; Campbell et al., 1997; Melendez-Ackerman and Campbell, 1998). On the basis of morphology alone, the *Castilleja* hybrids could easily be interpreted as the hybrid product of a "tangled trio" of species, displaying trait variation that matches alternative parents (e.g., plant size is indistinguishable from *C. sulphurea*, leaf size is indistinguishable from *C. rhexiifolia* and bract coloration that reflects the contributions of *C. rhexiifolia* and *C. sulphurea*, and *C. miniata*).

As with morphological traits, putative parent species were readily discriminated using DNA content (a proxy for chromosome number; Fig. 6) and multivariate or Bayesian clustering of AFLP loci (Fig. 8). Discrimination by cpDNA variation was less complete; *C. miniata* possessed private haplotypes that were divergent from *C. rhexiifolia* and *C. sulphurea*, but the

TABLE 3. Analysis of molecular variance (AMOVA) for AFLP variation among and within the three *Castilleja* species and the putative hybrids.

Source of variation	df	Variance components	% Total ^a	P value ^b
Among groups	3	8.69	17.11	<0.0001
Among populations within groups	10	1.98	3.90	<0.0001
Within populations	358	40.12	78.99	<0.0001

^a Percentage of total molecular variance.

^b Probability of obtaining a larger variance component estimate. The number of permutations = 1023.

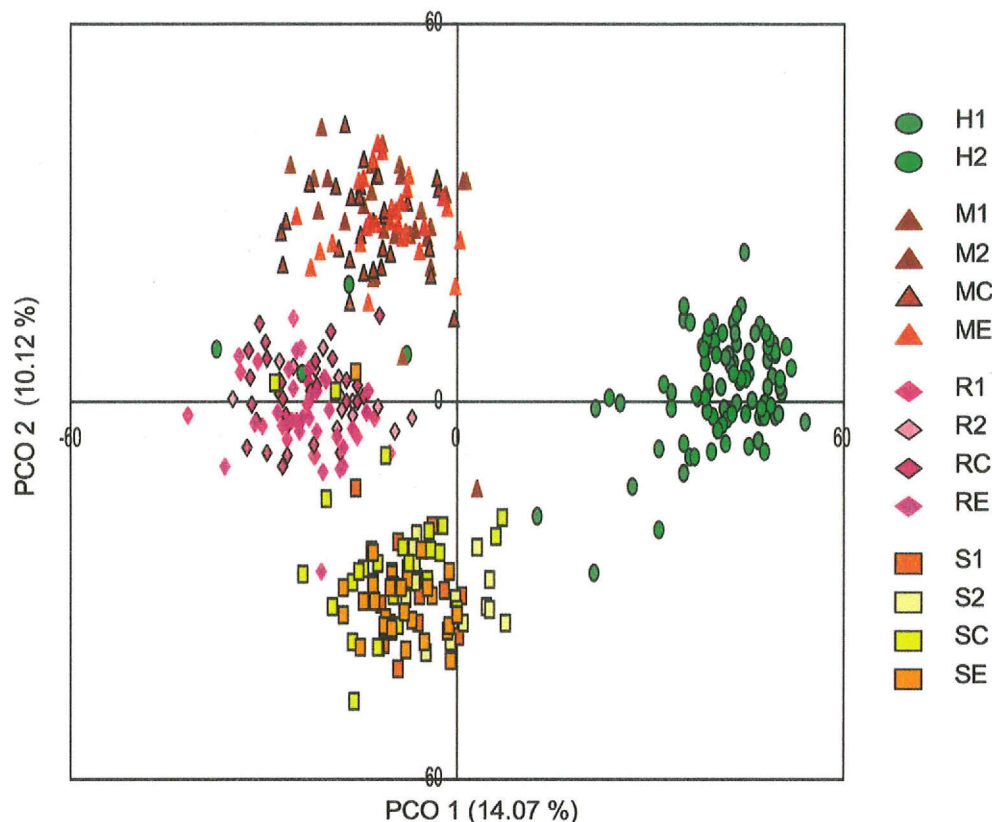


Fig. 7. Principal coordinate (PCo) scores of the AFLP banding patterns for the three *Castilleja* species and their putative hybrids along (A) PCo 1 vs. PCo 2. The percentage variation explained by each axis is given in parentheses. Different symbols depict which population of origin (see Fig. 2).

latter two species segregated for common haplotypes (confirming their recent cytoplasmic coancestry).

In contrast to morphological analyses, however, the four taxa formed four distinct genetic groups corresponding to the three *Castilleja* species and their putative hybrids. A different nuclear DNA content characterizes each group; generally, *C. miniata* is octaploid, *C. rhexiifolia* is tetraploid, *C. sulphurea* is diploid, and the putative hybrids are probably diploid (Hersch, 2007), with a slightly larger DNA content than *C. sulphurea* (Fig. 6). The four groups show sufficiently distinct AFLP profiles that the majority of individuals from the same taxon formed cohesive groups in principal coordinate and Bayesian clustering analyses. In general, molecular variation among different populations within a taxon was low (3.9%) relative to the magnitude of variation among taxa (17.1%). Cumulatively, molecular data points to a pattern of distinctiveness-not hybridity-in the putative hybrids because they are as genetically distinct from *C. miniata*, *C. rhexiifolia*, or *C. sulphurea* as any of the three species are from each other.

Hybrids in Castilleja: Are they tangled trios, deceptive duos, or something different?—Tangled trios—From molecular evidence, it seems unlikely that the field-classified hybrids are recent hybrids involving the three *Castilleja* species. In a "tangled trio" (or tri-hybrid) scenario, admixture proportions in hybrids should be highly variable depending on the individual hybrid parentage, ranging from roughly equal proportions of parental-type admixture in the case of F1 hybrids (Arnold, 1993; Dodd and Afzal-Rafii, 2004; Tung et al., 2008), to increasing

admixture toward a backcross parent genome with each successive backcross generations (Dodd and Afzal-Rafii, 2004; Van Droogenbroeck et al., 2006; Tung et al., 2008). Because the field-classified hybrids form a genetic cluster distinct from all putative parental species, they likely share a similar evolutionary origin, one that does not include widespread recurrent hybridization with one or more of the sympatric *Castilleja* species. Last, hybridization between these *C. miniata*, *C. rhexiifolia*, and *C. sulphurea* should result in F1 hybrids of intermediate or odd ploidy (but see Price et al., 1983; Brochmann et al., 1993; Bures et al., 2004; Lowe and Abbott, 2004; Tel-Zur et al., 2004), and later generation hybrids of variable ploidy levels (Price et al., 1983; Bures et al., 2004). If the putative hybrids resulted from recent and ongoing hybridization, they should show intermediate and/or variable ploidy levels; we found no evidence for either of these patterns.

Deceptive duos—A second possibility is that the field-classified hybrids are hybrid in origin, but that we failed to sample one of the parental species. In a "deceptive duos" scenario, one of the local species (*C. miniata*, *C. rhexiifolia*, or *C. sulphurea*) could have hybridized with a second unsampled species. Because the field-classified hybrids were believed to represent early generation hybrids, we expected that such a history would be evident in admixture analysis. Nevertheless, many generations of backcrossing between hybrids and an unsampled species could eliminate evidence of admixture, making it difficult to detect an introgressive, hybrid ancestry. Because this scenario (namely, a long history of backcrossing to an unsampled

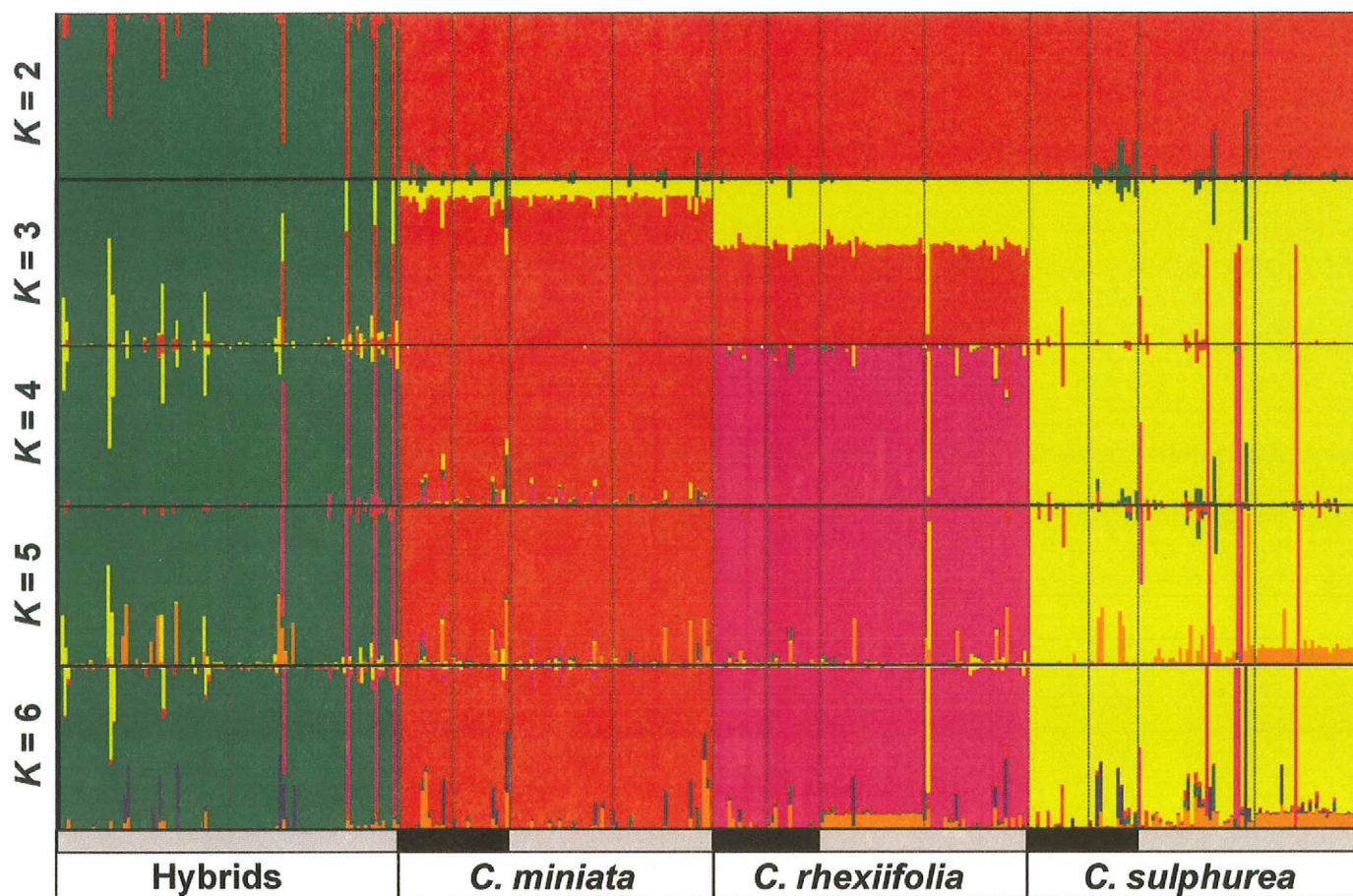


Fig. 8. Population structure and individual admixture for *Castilleja* as revealed by AFLP banding patterns under different assumptions of K (the number of clusters) from 2 to 6. Each individual is represented by a line that is partitioned into sections that depict the individual's estimated membership into each of the K clusters. Samples from "pure parental" populations are indicated by a black bar above the species name; samples from "comingled" and "hybrid" populations are indicated by a gray bar.

species) is possible, we can only confidently conclude that the field-classified hybrids aren't recent hybrid derivatives from a second, unsampled species.

Something different, part 1: Simply another Castilleja species-A third possibility is that field-classified "hybrids" are a previously described *Castilleja*. Two other *Castilleja* occur in these basins, *C. linariifolia* Benth and *C. occidentalis* Torr. The field-classified hybrids are certainly not the former; red-bracted *C. linariifolia* occurs at lower elevations, is restricted to drier sagebrush locations, and has distinctively different floral, bract, and leaf morphology than the taxa under consideration. On the other hand, yellow-bracted *C. occidentalis* occurs at high elevations, can occur at diploid and tetraploid levels, has a similar floral, bract, and leaf morphology as *C. sulphurea*, and has been reported to hybridize with *C. sulphurea* (Heckard and Chuang, 1977). Morphological discrimination between these species is difficult, but we believe that they argue against the field-classified hybrids being *C. occidentalis*. For example, *C. occidentalis* is described as a dwarf, decumbent form of *C. sulphurea* that has floral bracts most frequently pale yellow to rarely brown-purple (Holmgren, 1984). Heckard and Chuang (1977) also described *C. occidentalis* from Colorado as primarily tetraploid, but with diploid forms that converged on the

morphology of *C. sulphurea*. Our field-classified hybrids were erect, substantially taller (mean = 3.3 dm; Appendix S1, see online Supplemental Data), and statistically indistinguishable from *C. rhexiifolia* (Fig. 4), which has been described as considerably larger (2.5-6 dm reported by Holmgren, 1984; mean = 3.4 dm in our sample) than *C. occidentalis* (0.8-1.5 dm high; Holmgren, 1984). While many of our field-classified hybrids possessed pale yellow bracts, the pink-red end of the spectrum was most frequent and brown-purple pigmentation was not observed. Importantly, our plants are almost exclusively diploid (86 of 90 samples), indicating that tetraploidy was rare at these locations.

Chloroplast DNA evidence also provides a measure of support for our assertion. Chloroplast *trnL^{UAA}-trnF^{GAA}* sequences in GenBank for *C. sulphurea* (AF479008) and *C. occidentalis* (EF 103859) were compared to our 78 sequences. This comparison shows that *C. sulphurea* AF479008 is genetically identical to our 28 samples of field-classified hybrids, and that it has nearly identical pairwise P-distances to *C. sulphurea* (0.0002) and *C. rhexiifolia* (0.0004). In contrast, *C. occidentalis* EF 103859 is almost 20-fold more divergent from our samples, showing P-distances ranging from 0.0080 to the field-classified hybrids, up to 0.0083 for *C. rhexiifolia* (data not shown). Given the absence of intraspecific sampling for *C. occidentalis* in

GenBank, this comparison is not definitive. Nevertheless, it seems improbable that haplotype diversity within *C. occidentalis* is so great as to include the field-classified hybrids because their high pairwise divergence (-0.8%) at this spacer region exceeds our highest pairwise P-distance between the most divergent chloroplast types (e.g., 0.0053 between *C. miniata* haplotype I and *C. rhexiifolia* haplotype VI; Fig. 6).

Something different, part 2: A previously unrecognized Castilleja species-A final possibility is that field-classified hybrids represent a distinct, reproductively isolated species that is so morphologically similar to *C. rhexiifolia* and *C. sulphurea* that it has defied classification and typification. If the field-classified hybrids are a reproductively distinct entity, this could explain their high genetic similarity as a group and their rare admixture with other *Castilleja* species. Because the most variable trait in this taxon is floral bract color, it is possible that variation in color alleles was acquired via introgression. Because low frequency hybridization does occur among these taxa (Fig. 8), even modest introgression could transfer such traits.

While field-classified hybrids are clearly not recent hybrids between the three *Castilleja* species, their pattern of hybrid-to-transgressive morphology and distinctive molecular variation is suggestive of the pattern expected from a stabilized hybrid species, perhaps one descended from *C. rhexiifolia* and *C. sulphurea*. Despite the rarity of this incredible feat, hybrid speciation does occur, and often recurrently within the same group of hybridizing species (Rieseberg et al., 1990; Rieseberg, 1991; Abbott, 1992; Soltis and Soltis, 1993; Schwarzbach and Rieseberg, 2002). The biggest hurdle faced by a sexually reproducing hybrid lineage (such as the *Castilleja* in this study; Hersch, 2007) is achieving reproductive isolation from the sympatric progenitor species and reproductive isolation requires either genetic reorganization (chromosome and/or gene) and/or ecological selection (Grant, 1981).

In sexually reproducing plants, differences in ploidy can promote isolation (Grant, 1981; Rieseberg, 1997). Permanent odd ploidy and allopolyploidy involves a change in hybrid chromosome number relative to progenitors and can occur from hybridization between species at equal or different ploidy levels. In both instances, neopolyploids are instantaneously reproductively isolated from their progenitors due to intercytotype mating barriers (Stebbins, 1950; Ramsey and Schemske, 1998). In *Castilleja*, hybridization and polyploidy are inextricably linked, because allopolyploidy is cited as a major mechanism of speciation (Heckard, 1964; Heckard and Chuang, 1977; Heckard et al., 1980; Egger, 1994). Interploidy mating can occur in *Castilleja* (unpublished studies cited in Heckard et al., 1980) and has been shown to occur between octaploid *C. miniata*, tetraploid *C. rhexiifolia*, and diploid *C. sulphurea* (Hersch, 2007). However, the uniform nuclear DNA content and apparent diploidy of the field-classified hybrids argues against permanent odd ploidy or allopolyploidy as a mechanism of stabilization.

It's also possible that the field-classified hybrids arose from interploidy mating that precipitated rearrangements, followed by a return to a diploid state (Cornai, 2005). Putative hybrids between sympatric *Castilleja* species are reported to retain the same chromosome number as one of their chromosomally divergent putative parents, suggesting segregation and concurrent losses of whole chromosome sets (cited in Heckard and Chuang, 1977). Chromosome reductions may explain the diploidy and unique 2C DNA content of the field-classified hybrids, but it

does not account for the absence of evidence for admixture in the hybrids.

In addition to chromosome number changes, hybridization can enhance chromosome rearrangements in hybrids, reinforcing the process of isolation and establishment of new hybrid species (Grant, 1981; Song et al., 1995; Rieseberg, 1997; but see Ainouche et al., 2003; Rieseberg, 2006). Chromosome rearrangements could have contributed to hybrid stabilization, and they might explain the genetic uniformity and apparent lack of admixture in the field-classified *Castilleja* hybrids. For instance, AFLP-based phenetic intermediacy is an expected pattern that can be recovered in multivariate analysis of F1 hybrids (Van Droogenbroeck et al., 2006). In PCoA analysis, the putative hybrids fall intermediate to *C. rhexiifolia* and *C. sulphurea* *C. miniata* on PCoA axis 3 (data not shown); this axis could provide an underlying signature of ancient hybridity. Also, the Bayesian clustering method we used to infer population admixture minimizes Hardy-Weinberg and linkage disequilibrium between groups (Pritchard et al., 2000). Because the three *Castilleja* species are closely related and likely share large linkage blocks, Bayesian clustering should group the three species together relative to a genome with different linkage arrangements. If the field-classified hybrids are hybrid products of two of these species, genomic restructuring could perturb linkage blocks, causing the hybrids to be grouped separately by Bayesian clustering in a manner similar to what we observe (Fig. 8). Characterization of species-specific linkage groups in the field-classified hybrids would be a logical step to test this hypothesis, although reduction in the size of parental linkage blocks with time via recombination could obscure cryptic hybridity (Baird, 1995; Ungerer et al., 1998).

If the field-classified hybrids in this study do represent homoploid hybrid species, such a case would represent a relatively novel finding for *Castilleja* because homoploid hybrid speciation has been only tentatively suggested once before in *Castilleja* (Mathews and Lavin, 1998). In this instance, the hypothesized allopolyploid origin of *Castilleja crista-galli* was evaluated using chloroplast and nuclear ribosomal haplotype variation. The authors found that the molecular markers and diploid chromosome number of *C. crista-galli* did not support an allopolyploid origin, although morphological variation was not at odds with a homoploid hybridization/speciation hypothesis (Mathews and Lavin, 1998). In our system, if homoploid hybridization and subsequent speciation did occur, ecological exigencies may be operative because the field-classified hybrids appear to occupy a distinct soil niche that is more alkaline and more carbon rich than soils of the other species (Hersch, 2007). It should be noted that Mathews and Lavin (1998) also suggested an equally parsimonious conclusion, namely, that the overlapping morphological variation was the result of rapid radiation and the retention of ancestral polymorphism in *Castilleja*. This suggestion mirrors the findings of Tank and Olmstead (2008) and our study and highlights the fact that morphological intermediacy alone is an unreliable indicator of "hybridity" or hybrid origin in *Castilleja*, perhaps as a consequence of the recency of common ancestry of this group.

Assessing the frequency of recent hybridization in Castilleja—In our exploration of the field-classified hybrids, we detected 48 cases where contemporary hybridization has led to detectable admixture. For example, four field-classified hybrids appear to be recent hybrids involving *C. rhexiifolia* with either *C. sulphurea* or *C. miniata*, as is supported by their resolution

with *C. rhexiifolia* along the PCo 1 and at Bayesian clustering using $K = 4$. These plants came from populations spatially close to *C. rhexiifolia* populations, have reddish-pink or yellowish-pink floral bracts, and have tetraploid-like nuclear DNA contents. Hybridization between parental species was also apparent, resulting in the misclassification of plants solely on the basis of morphology. A few plants identified as *C. rhexiifolia* or *C. sulphurea* in the field were almost completely admixed toward another species; these plants may be advanced generation backcrosses that have experienced trait introgression. For instance, two field-classified *C. sulphurea* showed significant genomic contributions from *C. rhexiifolia*, had pinkish bracts, were tetraploid, and were collected close to site H2. Two other *C. sulphurea* showed significant genomic contributions from either *C. rhexiifolia* or from *C. miniata* and were collected from comingling populations.

Conclusions—Our study shows that hybridization *does* occur in populations of *Castilleja* containing sympatric species, and the resulting hybrids can comprise a surprisingly high proportion of the population (up to 13% in mixed populations). True early-generation hybrids are difficult to identify in the context of such diversity (*C. sulphurea*, *C. rhexiifolia*, *C. miniata*) and in the presence of a cryptic taxon (field-classified hybrids) that blurs such modest morphological discontinuities. Although there are no obvious morphological characters that readily delineate or distinguish the hybrids nor this cryptic taxon, there are a suite of features that can be used, including: blended bract colors (i.e., not pure red, yellow, or pink, as shown in Fig. 1), high bract and leaf pubescence, and relatively small flower and stature in comparison to average leaf and bract sizes. A search for cryptic morphological characters would be worthwhile. The high genetic and morphological similarity among *Castilleja* species complicates studies of hybridization, and it adds to the difficulty in convincingly demonstrating admixture.

In light of the discussion, we believe that hybridization, followed by genomic reorganization, provides a likely explanation for the unique and uniform genetic identity of the field-classified hybrids. It is at least as parsimonious as the alternative, namely, that field-classified hybrids represent a species of nonhybrid origin that has maintained multiple color alleles over time. The latter scenario would require variation in color alleles be selectively maintained or linked to other adaptive traits. Molecular data from neutral and nonneutral markers (i.e., *Antirrhinum* color pigmentation genes, Whibley et al., 2006) and from more *Castilleja* species that occur in this region of Colorado are needed to better assess the origin of these field-classified hybrids. If our hypotheses are correct, the parentage of field-classified hybrids seem most likely to include plants similar to *C. rhexiifolia* and *C. sulphurea*, with little contribution from *C. miniata*.

With the advent of whole-genome sequencing technologies, new opportunities exist for characterizing divergence, diversity, and perhaps hybridity at a level of resolution that was unthinkable at the time this work was initiated. The genetic basis of bract color variation may be experimentally tractable using information derived from model relatives and EST-based transcriptome sequencing. Now that "next-generation" approaches are becoming increasingly useful for lower-level studies (Cronn et al., 2008), the challenging questions concerning species delimitation and hybridization in *Castilleja* poses new grist for evolutionary biologists. Given the extreme variations in morphology, polyploidy, hybridization, and allopolyploidy in this

group, detailed genomic examination may show that hybridization and introgression is even more frequent and important than morphological traits suggest. Discerning the evolutionary history of complex groups, such as *Castilleja*, requires a multifaceted approach that includes an investigation into species distribution patterns, as well as patterns of morphological, cytogenetic, and genetic variation.

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