Molecular investigation of the origin of Castilleja crista-galli
by Sarah Youngberg Mathews

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
An hypothesis of hybrid origin of Castilleja crista-galli (Scrophulariaceae) was studied. Hybridization and polyploidy are widespread in Castilleja and are often invoked as a cause of difficulty in defining species and as a speciation model. The putative allopolyploid origin of Castilleja crista-gralli from Castilleja miniata and Castilleja linariifolia was investigated using molecular, morphological and cytological techniques. Restriction site analysis of chloroplast DNA revealed high homogeneity among the chloroplast genomes of species of Castilleja and two Orthocarpus. No species of Castilleia represented by more than one population in the analysis was characterized by a distinctive choroplast genome. Genetic distances estimated from restriction site mutations between any two species or between genera are comparable to distances reported from other plant groups, but both intraspecific and intrapopulational distances are high relative to other groups. Restriction site analysis of nuclear ribosomal DNA revealed variable repeat types both within and among individuals. Qualitative species groupings based on restriction site mutations in the ribosomal DNA repeat units do not place Castilleja crista-galli with either putative parent in a consistent manner. A cladistic analysis of 11 taxa using 10 morphological characters places Castilleja crista-galli in an unresolved polytomy with both putative parents and Castilleja hispida. Cytological analyses indicate that Castilleja crista-gralli is not of simple allopolyploid origin. Both diploid and tetraploid chromosome counts are reported for this species, previously known only as an octoploid. Together these data suggest that the origin of Castilleja crista-gralli is a complex event, that within a region, species of Castilleja form an interbreeding system similar to that usually found within populations of a single species, and that little, if any, molecular divergence occurs between hybridization events among species of a region.

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ABSTRACT

An hypothesis of hybrid origin of Castilleja crista-galli (Scrophulariaceae) was studied. Hybridization and polyploidy are widespread in Castilleja and are often invoked as a cause of difficulty in defining species and as a speciation model. The putative allopolyploid origin of Castilleja crista-galli from Castilleja miniata and Castilleja linariifolia was investigated using molecular, morphological and cytological techniques. Restriction site analysis of chloroplast DNA revealed high homogeneity among the chloroplast genomes of species of Castilleja and two Orthocarpus. No species of Castilleja represented by more than one population in the analysis was characterized by a distinctive choroplast genome. Genetic distances estimated from restriction site mutations between any two species or between genera are comparable to distances reported from other plant groups, but both intraspecific and intrapopulational distances are high relative to other groups. Restriction site analysis of nuclear ribosomal DNA revealed variable repeat types both within and among individuals. Qualitative species groupings based on restriction site mutations in the ribosomal DNA repeat units do not place Castilleja crista-galli with either putative parent in a consistent manner. A cladistic analysis of 11 taxa using 10 morphological characters places Castilleja crista-galli in an unresolved polytomy with both putative parents and Castilleja hispida. Cytological analyses indicate that Castilleja crista-galli is not of simple allopolyploid origin. Both diploid and tetraploid chromosome counts are reported for this species, previously known only as an octoploid. Together these data suggest that the origin of Castilleja crista-galli is a complex event, that within a region, species of Castilleja form an interbreeding system similar to that usually found within populations of a single species, and that little, if any, molecular divergence occurs between hybridization events among species of a region.

CHAPTER 1

INTRODUCTION

The genus Castilleja (Scrophulariaceae) comprises approximately 200 species, with the greatest number found in the western United States, and minor representations in eastern North America (two species), Central and Andean South America (about 15 species), and northern Eurasia (about five species; Holmgren, 1984). Castilleja is placed within Pedicularieae, a tribe characterized by a root-hemiparasitic habit and specialized floral features, such as a bilabiate corolla in which the upper lip forms a hood enclosing the four anthers. As with others of the tribe, Castilleja has a prominent place in the western landscape. Taxonomy of the genus is complex due to overlapping variation in nearly every character (Holmgren, 1984), and because species distinctions rely on permutations of a very small character set. Nearly all early workers in the genus encountered difficulty in placing taxa (Gray, 1862; Fernald, 1898; Eastwood, 1909; Pennell, 1935), with later workers ascribing the difficulty to reticulate evolution and polyploidy. Ownbey (1959) invoked hybridization to explain the lack of species with clearly defined limits. Heckard

found that successful crosses could be made between distantly related species (Heckard and Chuang, 1977), between species of different ploidy levels (Heckard,1968), and between species of Castilleja and one of the closely related genus Orthocarpus (Heckard, 1964). He also found polyploidy to be widespread in species of Castilleja (Heckard, 1968; Heckard and Chuang, 1977). He hypothesized that variation resulting from allopolyploidy complicated by additional hybridization between the variants caused the lack of defined species limits, and proposed hybrid origin for taxa which lacked diploid populations (Heckard, 1968). He thus emphasized the role of polyploidy and hybridization in the evolution of Castilleja, as well as in creating taxonomic complexity.

Holmgren (1971), in addressing why development of reproductive barriers has been slow, suggested that speciation in Castilleja has occurred simply by morphological divergence followed by geographical isolation, the latter occurring during uplifts following the Miocene and Pliocene epochs. This explanation alone does not account for speciation in Castilleja however, as this mode is invoked for many plant species not of reticulate origins (examples in Grant, 1971). Although only first generation interspecific hybrids have been observed in the wild (Heckard and Chuang, 1977; Holmgren, 1984), numerous hypotheses of allopolyploid origin of Castilleja species

exist (Ownbey, 1959; Heckard, 1968; Holmgren, 1971; Holmgren, 1973; Heckard and Chuang, 1977; Holmgren, 1984). One of these pertains to the putative origin of Castilleja crista-galli, which is the subject of this analysis.

Castilleja crista-galli was described by Rydberg in 1900 from the Bridger Range of south central Montana. Ownbey (1959) questioned its specific status and suggested a hybrid origin involving C. miniata Douglas ex Hooker and C. linariifolia Bentham as parents, noting its morphological intermediacy between them. Heckard supported the hypothesis when he discovered that C. crista-galli in central Idaho and northwest Wyoming was octoploid, while the putative parents were diploid or tetraploid in the same region.

Castilleja crista-galli is not widely distributed, known presently from central Idaho, northwest Wyoming, and southcentral Montana (Figure 1). Castilleja miniata is a variable, widespread species of the western Cordillera, occurring primarily in wet meadows from Alaska and northwest Canada south to New Mexico, Arizona and southern California. Castilleja linariifolia grows in sagebrush parks and dry woodlands of the Rocky Mountains, Sierra Nevada and Great Basin, from southeast Oregon, southern Idaho and Montana south to southern California, northern Arizona and New Mexico. The range of C. crista-galli is encompassed within that of C. miniata, and often populations of the two are sympatric, with C. miniata inhabiting the more mesic sites

and C. crista-galli found on drier sites. The ranges of C. miniata and C. linariifolia overlap but the latter is allopatric with C. crista-galli.

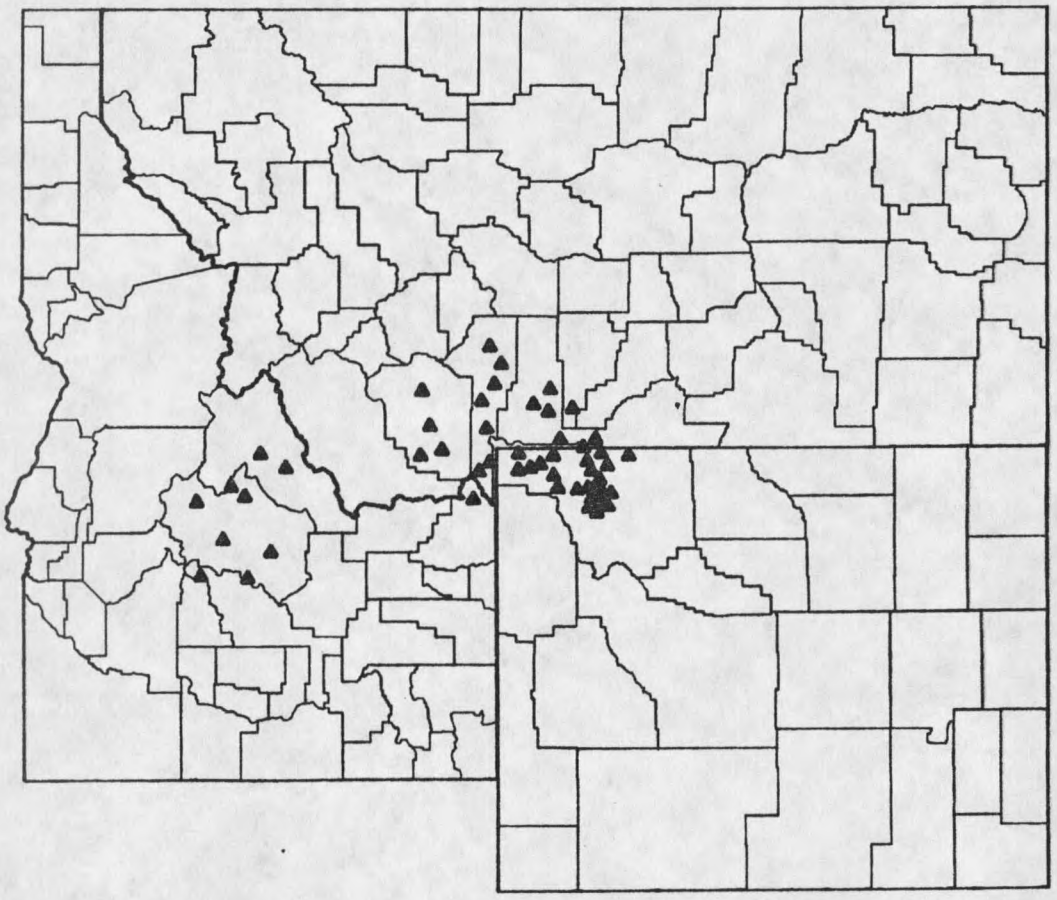

Fig. 1. Distribution of Castilleja crista-galli in Idaho, Montana, and Wyoming.

CHAPTER TWO

MATERIALS AND METHODS

Chloroplast and Nuclear Ribosomal DNA

Leaf material for DNA analyses was collected from populations of C. crista-galli, C. miniata and C. linariifolia from southcentral Montana and adjacent Wyoming (Table 1). To determine if other species might be involved in the origin of C. crista-galli, populations of an additional five species of Castilleja occurring in the region were sampled. Two species of the closely related genus Orthocarpus were sampled to serve as outgroups so that the direction of the evolution of restriction site mutations could be determined. Tissue was kept at 4^0C or preserved at -80^0C until total DNA was isolated following the protocol of Doyle and Doyle (1987). Ninety DNA samples representing forty-nine populations were digested singly, according to manufacturers' specifications, using the following restriction endonucleases: Bam HI, Bgl II, Bst BI, Dra I, Eco RI, Eco RV, Eco 0109I, Hind III, Pvu II, Sst I, Sty I, Xba I and Xho I. Fragments were separated on 1% agarose gels, transferred to nylon membranes and sequentially hybridized with the petunia chloroplast DNA (cpDNA) library

Table 1. Populations of Castilleja and Orthocarpus species analyzed for cpDNA and rDNA variation, chromosome numbers and collection data. Numbers in parentheses indicate individuals sampled per accession. Vouchers are deposited at MONT.

Species and Haploid Number	Collection Data
Castilleja crista-galli Rydberg	
n=24	Montana: Madison Co., *Mathews s.n.*(2)
nc	Montana: Gallatin Co., *Mathews 280*(2)
nc	Montana: Gallatin Co., *Mathews 148*(3)
n=24	Montana: Gallatin Co., *Mathews 035*(2)
n=24	Montana: Gallatin Co., *Mathews 010*(2)
nc	Montana: Gallatin Co., *Lavin 6263*(1)
nc	Montana: Gallatin Co., *Lavin 6245*(1)
n=48	Montana: Park Co., *Mathews 140*(2)
n=12,n=24	Montana: Park Co., *Mathews 184*(2)
nc	Montana: Park Co., *Mathews 192*(2)
nc	Wyoming: Park Co., *Mathews 033*(2)
nc	Wyoming: Park Co., *Mathews 036*(2)
n=24	Montana: Park Co., *Mathews 295*(2)
Castilleja linariifolia Bentham	
nc	Montana: Carbon Co., *Mathews 290*(2)
nc	Montana: Carbon Co., *Mathews 291*(1)
nc	Wyoming: Park Co., *Mathews 292*(3)
nc	Wyoming: Park Co., *Mathews 293*(1)
nc	Montana: Carbon Co., *Lavin 6268*(2)
nc	Wyoming: Park Co., *Lavin 6270*(1)
n=12,n=18	Montana: Carbon Co., *Mathews 070*(3)
Castilleja miniata Bentham ex Hooker	
n=9	Montana: Madison Co., *Mathews s.n.*(2)
nc	Montana: Gallatin Co., *Mathews 281*(2)
nc	Montana: Gallatin Co., *Mathews 274*(2)
nc	Montana: Gallatin Co., *Mathews 151*(3)
nc	Montana: Gallatin Co., *Lavin 6265*(1)
nc	Montana: Gallatin Co., *Lavin 6246*(1)
n=12	Montana: Park Co., *Mathews 141*(2)
nc	Montana: Park Co., *Mathews 240*(2)
n=12	Montana: Park Co., *Mathews 193*(2)
n=12	Wyoming: Park Co., *Mathews 047*(2)
nc	Montana: Park Co., *Mathews 294*(2)
n=12	Montana: Park Co., *Mathews 056*(2)
nc	Montana: Mineral Co., *Mathews 298*(1)
nc	Montana: Fergus Co., *Siebert s.n.*(1)
Castilleja flava S. Wats.	
nc	Montana: Gallatin Co., *Mathews 272*(3)

Table 1. Continued.

nc	Montana: Gallatin Co., *Lavin 6264*(1)
n=12	Montana: Meagher Co., *Mathews 337*
Castilleja pallescens (A. Gray) Greenman	
n=24	Montana: Gallatin Co., *Mathews 300*(3)
nc	Montana: Meagher Co., *Siebert s.n.*(1)
Castilleja pulchella Rydberg	
nc	Montana: Park Co., *Mathews 288*(5)
Castilleja rhexifolia Rydberg	
nc	Montana: Sweet Grass Co., *Mathews 266*(4)
nc	Montana: Gallatin Co., *Lavin 6266*(3)
nc	Montana: Madison Co., *Mathews 080*(1)
n=12	Montana: Park Co., *Mathews 051*(4)
Castilleja gracillima Rydberg	
nc	Montana: Gallatin, *Mathews 275*(3)
n=12,24	Montana: Park Co., *Mathews 040*(2)
Orthocarpus luteus Nuttall	
nc	Wyoming: Park Co., *Mathews 296*(2)
nc	Montana: Carbon Co., *Rumely and Lavin s.n.*(2)
Orthocarpus tenuifolius (Pursh) Bentham	
nc	Montana: Sanders Co., *Mathews s.n.*(1)

(courtesy of J. D. Palmer) and with the ribosomal DNA (rDNA) clone pGmr1 (courtesy of E. Zimmer) following Palmer (1986). Nick translation of the petunia and rDNA clones with ^{32}P followed Maniatis et al. (1982). Three of the ninety-one DNAs were doubly digested in an attempt to map their rDNA repeat units. The membranes generated by this experiment were sequentially hybridized with pGB28s (courtesy of S.K. Davis) and pFH84 (courtesy of N. Arnheim) in addition to pGmr1.

Cladistic analysis of restriction site data was performed using computer programs PAUP (by D. Swofford), PHYLIP (by J. Felsenstein) and CLADOS (by K.C. Nixon).

Restriction site mutations were polarized using Orthocarpus as the outgroup. Species of Orthocarpus section Castillejoides are interfertile with, and morphologically most similar to Castilleja species (Heckard, 1964; Holmgren, 1971).

Genetic distances were estimated from restriction site data (Nei and Li, 1979; Nei, 1987) using the program UENZYME (by K.C. Nixon). Estimates were obtained from two different surveys, each using a slightly different battery of enzymes, and comprising a different set of individuals. The two matrices of divergence values (Table 4) output by UENZYME were subject to cluster analysis using the FITCH algorithm of PHYLIP because it makes no assumptions regarding rates of evolution.

Cytological Analysis

Floral buds for chromosome counts were collected in the field or greenhouse and fixed in Farmer's solution (1 part glacial acetic acid : 3 parts 95% ethanol). Buds were transferred to 70% ethanol after fixation periods of 4-24 hours. Immature anthers dissected from floral buds were squashed in 45% aceto-orcein and examined under 400x magnification using a Wilde M20 phase-contrast microscope.

Morphological Analysis

All morphological characters potentially bearing on the

relationships of Castilleja crista-galli are here enumerated as a preliminary assessment. The appendix contains citations of C. crista-galli specimens examined. Subgeneric categories chosen for analysis, although poorly characterized, have been recognized by two or more authors (Pennell, 1951; Ownbey, 1959; Holmgren, 1984). This assemblage of species-groups consists mostly of lineages distributed in North America. More southerly lineages included in this analysis are represented by Castilleja coccinea (sect. Euchroma Bentham) and Castilleja linariifolia. The latter is placed by Holmgren (1976) in his section Castilleja. It mainly comprises Mexican and Central American species and is considered to contain the most derived members of the genus (Holmgren, 1976).

A matrix of ten binary and multistate characters by eleven taxa is presented in Table 2. All characters were unordered during analysis, but were subsequently polarized on the cladograms using Orthocarpus as the outgroup. Of special concern in this analysis is reticulate evolution, as is hypothesized in Castilleja, because it confounds homology decisions. For example, a trait shared by two taxa may be due to introgression rather than common ancestry. It is preferable first to analyze divergent diploid taxa (eg. Kellogg, 1989), superimposing hybrid taxa after the initial assessment of character homology is made. This avenue was not available for Castilleja, as diploid species are as

likely the result of hybridization as are polyploids. But in order to make a preliminary assessment of morphological data that could then be analyzed in light of hypotheses of hybridization, the matrix was analyzed cladistically. It is possible that because characters chosen mark taxa above the species level, they are less prone to the confounding effects of reticulate evolution.

Discussion of Characters

1. Duration. Of the 200 species of Castilleja, only six are herbaceous annuals. The rest are herbaceous perennials or shrubs. The shrubby species are found in Mexico, Central and South America, while only herbaceous species are found in North America. In 1927, Keck removed all perennials from Orthocarpus and placed them in Castilleja, leaving the former a more homogenous group. Because Orthocarpus retains the ancestral floral type (Holmgren, 1971), and because its connection with Castilleja is through Castilleja sect. Pilosae, members of which are considered least derived and which have an Orthocarpus-like flower, it is used as the outgroup. While the annual habit is considered derived in the Pedicularieae, and the outgroup was originally scored as displaying the derived condition, results indicate that it is more parsimonious to score the annual habit as ancestral.

2. Leaf Margin. Upper leaves in most Castilleja

species are dissected or cleft, a few displaying the entire condition, all of which Holmgren (1971) considers to be derived from a pinnately lobed leaf typical of Pedicularis. The prominent condition in Orthocarpus is linear to digitate. Taxa with digitate leaves are coded as plesiomorphic, those with entire leaves as derived.

3. Bract Margin. Margins of the floral bracts of both genera vary from digitate or cleft to entire. Because floral bracts are homologous with leaves, their margins often reflect those of the upper leaves (character 2). This relationship does not necessarily hold, however, and the two characters are scored separately. Orthocarpus section Castillejoides is considered among the least specialized in the genus and of its five species, four have bracts which are digitate, with the fifth having cleft bracts. Therefore, cleft to entire was scored as derived, digitate as ancestral. The most parsimonious distribution requires reversing the polarity of this character (see Chap. 3).

4. Pigment Location. Floral bracts of Castilleja and Orthocarpus are green or pigmented. Green bracts subtending highly colored flowers is considered ancestral in the Pedicularieae, with more derived members in Castilleja and Orthocarpus having green corollas subtended by highly colored bracts. Bentham (1846) used location of pigmentation when originally distinguishing major groups

within the genus. Pigmented flowers are scored as zero, green flowers as one.

5. Pigment Type. Populations of a given Castilleja species may exhibit a wide range of color variation. A distinction is possible however, between taxa with predominantly creamy white or yellow inflorescences and those that vary in the red, pink and orange shades. A condition of uniform yellow or white, resulting from carotenoids or flavenoids, respectively (Harborne 1988), is scored as zero, one of red or orange resulting from anthocyanins as one.

6. Calyx Clefts. The degree and pattern of calyx clefts is considered one of the most reliable characters for diagnosis of Castilleja species. The calyx of Castilleja section Pilosae with four equal segments is considered the ancestral type in the Pedicularieae (Pennell, 1935). Most species of Castilleja and Orthocarpus have calyces which are deeply cleft medianly and more shallowly cleft to entire laterally. This results in a calyx with two primary segments and two to four secondary segments. Species in section Castilleja have calyces much more deeply cleft in front than behind. A calyx of four equal segments was originally given a score of zero, one with subequal median clefts a score of one, and one which is more deeply cleft in front than behind a score of two. Cladistic analyses

revealed that a more parsimonious distribution is obtained when subequal bracts are scored as zero, equal as one, and unequal as two (see Chap. 3).

7. Calyx Secondary Segments. In addition to the pattern of calyx clefts, shape of the secondary segments is diagnostic. Taxa with four subequal clefts (as opposed to four equal clefts) may have obtuse, acute or truncate secondary segments, or in a calyx with just a vestige of lateral clefts, the primary segments are merely emarginate. Thus character 6 deals with the trend of deepening median clefts, character 7 with the trend of the lateral clefts to disappear, and the conconmitant change of shape in secondary segments. Acute or obtuse secondary segments separated by several millimeters of lateral cleft predominates in North American groups and is considered ancestral. The tendency for lateral clefts to be shallow or absent is observed in Central and South American groups and is scored as derived.

8. Length of Galea. Corollas of genera in the Pedicularieae are tubular and bilabiate, with the upper lip forming a hood (galea) enclosing the anthers. In Orthocarpus, the galea does not much exceed the lower, pouched lip. In Castilleja, a continuous range is found from galeas equalling the lower lip to those much exceeding it and equalling the tube in length. A pouched lower lip serves as a landing platform for insect pollinators, as does

the lower lip of most other genera in the Scrophulariaceae. In long galeate Castilleja species, there is often a concomitant reduction of the lower lip, resulting in a flower which is visited by hovering insects and hummingbirds. A division is made between taxa with short galeas (3-10 mm), and those with long galeas (10-20 mm). Groups used in this analysis fall easily into one category or the other, those with short galeas being coded as zero, and those with the long galeas as one.

9. Degree of Lip Reduction. In Orthocarpus, the lower lip is petaloid and more or less saccate-inflated. In Castilleja, the lip varies from a petaloid-pouched condition to being a much reduced, thickened green lip of three incurved teeth, just one to a few millimeters long. As mentioned above, the lip reduction frequently accompanies the lengthening of the galea and the question of whether the characters are independent arises. Long galeas do occur in combination with lips only partially reduced, for example in bee-pollinated C. rhexifolia, and so the two characters are scored separately. A petaloid lip is given a score of zero, a lip of intermediate reduction a score of one, and a much reduced lip a score of two.

10. Stigma. Stigmas in Orthocarpus are entire and scored as ancestral, those in Castilleja are capitate or bifid and scored as derived.

Table 2. Data matrix of morphological characters, with polarization against the outgroup Orthocarpus. Sectional placement of C. pilosa follows Pennell (1951), that of the last 6 taxa follows Ownbey (1959).

Sect. Monosaccus (Orthocarpus luteus)	00000 00000
Sect. Monosaccus (Orthocarpus tenuifolius)	00011 00000
Sect. Euchroma (Castilleja coccinea)	00111 01011
Sect. Castilleja (Castilleja linariifolia)	10101 20121
Sect. Pilosae (Castilleja pilosa)	10100 10001
Sect. Pallescentes (Castilleja pallescens)	10110 00001
Sect. Chrysanthe (Castilleja pulchella)	10010 00001
Sect. Flavae (Castilleja flava)	10110 20011
Sect. Septentrionales (Castilleja miniata)	11011 00121
Sect. Parviflorae (Castilleja hispida)	10111 00121
Castilleja crista-galli (aff. Sect. Parviflorae)	10111 00121

Characters Examined but Not Used in Analyses

Phenology. Closely related species of Castilleja are often separated temporally. While most populations may be found in anthesis four to six weeks, anthesis of sympatric species often does not overlap. The character is too variable within the taxa analyzed to score the hypothetical ancestral condition for each. Therefore, it was deleted from analyses. Its utility in limiting gene flow will be discussed later.

Seed Testa. Seed testa of Castilleja, Cordylanthus and Orthocarpus, all genera of the Pedicularieae, are prominently reticulate, a result of the collapsing of the outer tangential walls of cells of the epidermal layer. The pattern of reticulation, overall seed shape and secondary

thickenings characterizing the reticulations identified by scanning electron microscopy have been used to identify sections and subsections within Cordylanthus and Orthocarpus. In Cordylanthus, seed morphology has been used to characterize subgenera, as a basis for retaining species within the genus and as a basis for referring to it the monotypic genus Dicranostegia (Chuang and Heckard, 1972). In Orthocarpus, subgeneric groups are characterized by seed morphology, but the extreme variation found among subgenera suggests the genus is polyphyletic (Chuang and Heckard, 1983). Examination by light microscopy of Castilleja seed coats revealed as much variation within species and groups as between them, so the character was not used for phylogenetic analyses.

Inflorescence Indumentum. Degree of pubescence is often variable within populations and species of Castilleja, but type is more likely to be species-specific. A combination of short and long trichomes is usually found and in the taxa dealt with here, the former may be glandular or not. Long trichome type and presence versus absence of glands on the short trichomes were originally scored for analyses, but both were deleted from final analyses due to variability within the Castilleja sections.

CHAPTER THREE

RESULTS

Chloroplast DNA

Restriction Site Mutations

Sixteen restriction site mutations were identified in this analysis, six of which separated Orthocarpus from Castilleja. No species of Castilleja was characterized by a distinctive chloroplast genome with the exception of C. pulchella, which was represented by only one population. Those mutations occurring only in Castilleja (less than half of those detected), distinguished only individuals within species, and sometimes grouped a few populations of one species with one or two of another. For example, a set of four populations of C. linariifolia was marked by six restriction site mutations, numbers 2, 5, 6, 7, 8 and 12, three of which also marked the chloroplast genome of a population of C. flava. Identical chloroplast genomes identified in this analysis are as follows (Acronyms of species are those of the cladogram and the consensus tree in figures 2 and 3): ORLU: O. luteus, *Mathews 296, Rumely and Lavin s.n.*; ORTE: O. tenuifolius, *Mathews s.n.*; CACR3: C.

crista-galli, *Mathews 280; Mathews 036*; CACR4: C. crista-galli, *Mathews 280; Lavin 6245*; CALI: C. linariifolia, *Mathews 292, Mathews 293, Lavin 6268, Lavin 6270*; CAMINW: C. miniata, *Mathews 298, Siebert s.n.*; CAFL1: C. flava, *Mathews 272*; CAFL2: C. flava, *Lavin 6264* and C. pallescens, *Siebert s.n.*; CAPU: C. pulchella, *Mathews 288*; CASP: This designation refers to all other accessions listed in Table 1 and includes members of C. crista-galli, C. miniata, C. linariifolia, C. pallescens, C. rhexifolia and C. gracillima.

Congruent most parsimonious cladograms were found using the branch-and-bound search option of PAUP and the Mixed Parsimony and Dollo Parsimony algorithms of PHYLIP. All trees required 19 steps, with a consistency index of 0.789 (including autapomorphies). Topologies of the terminal branches varied among the trees according to whether parallel gains or reversals were being maximized. The most parsimonious cladogram (Figure 2) reflects Dollo parsimony in that parallel gains are minimized in favor of reversals. The strict consensus tree (Figure 3) reveals those cpDNA goups consistently resolved among all the most parsimonious cladograms. Chloroplast genomes found in Castilleja crista-galli appear in three places on this tree. CACR3 and CACR4, representing genomes identified in only four individuals, form a derived clade whose sister group comprises genomes of all other Castilleja species and the ten other accessions of

Table 3. Chloroplast DNA restriction site mutations used in the cladistic analyses and genetic distance estimations. Number 15 is not phylogenetically informative and was deleted from cladistic analyses.
Numbers designating DNAs refer to their placement in table 1.

Enzyme	Probe Region	Mutation (kb)	Derived DNAs
1. Bam HI	S8	6.4 -> 3.8 + 2.6	2,7,12
2. Bam HI	P3	3.8 + 0.8 -> 4.6	35,16-19
3. Bgl II	P3	2.0 -> 1.6 + 0.4	All Castilleja
4. Bgl II	P8-P10	7.3 + 5.2 -> 12.5	All Castilleja
5. Bgl II	P8-P10	3.6 + 1.0 -> 4.6	All but 47,48,16-19,35
6. Bst BI	S8	3.5 -> 1.9 + 1.6	All but 16-19
7. Dra I	P10	1.4 + 1.5 -> 1.8 + 1.1	All but 35,16-19
8. Eco RI	P8-P10	7.2 -> 4.4 + 2.8	All but 16-19,40,49
9. Eco RV	P14-P1	3.8 + 0.9 -> 4.7	All Castilleja
10. Hind III	P19-18-S8	8.4 -> 6.6 + 1.8	All Castilleja
11. Pvu II	P6-P8	2.5 + 0.9 -> 3.4	All Castilleja
12. Sst I	P3-P6	7.1 + 1.1 -> 5.5 + 2.7	2,7,16-19,47,48
13. Sty I	S8	7.2 + 3.4 -> 10.6	All Castilleja
14. Xho I	S6-P3	10.0 + 10.0 -> 20	33,34
15. Bam HI	P3	10.1 -> 7.1 + 3.0	35
16. Bst BI	S8	2.3 -> 1.5 + 0.8	1,4,9,10,13, 14-16,20,21,22, 24,26,29-34

C. crista-galli.

Significant intraspecific variation was detected by Bst BI, Eco RI, Sty I and Xba I. Both Eco RI and Sty I detect extreme variability in the region spanned by Petunia Pst I probes 4 and 1 (location of Petunia clones in Sytsma and Gottlieb, 1986). This region includes the small single copy region and the ends of the inverted repeat. Eco RI detects the same degree of variability in the Petunia Pst I probe 6

portion of the large single copy region. Mutational "hotspots" in the small single copy region near inverted repeat borders have been noted by other workers (Palmer et al., 1985; Coates and Cullis, 1987), but they differ from the pattern observed here in that they are observed only in small fragments which vary around a mean size. In contrast, Eco RI and Sty I here produced patterns in which fragments of one lane represent a subset of those in a neighboring lane, with the larger fragment sets summing to a total far exceeding that known for the sequence, a pattern which could be explained by either methylation or partial digestion. Eco RI is known to be methylation-sensitive (Zimmer et al., 1988), but a visual evaluation rules out partial digestion. The fragment patterns could also signify heteroplasmy. A very similar pattern was observed in Eco RI digests of Pelargonium (cultivated geraniums) DNA and was shown to result from biparental plastid inheritance (Metzlaff et al., 1981). Because other enzymes do not detect this same variation, methylation might be the most likely alternative, however, clearly defined plastids have been observed in the generative cell of one species of Castilleja (Jensen et al., 1974), suggesting that heteroplasmy could occur. The homogeneity of chloroplast genomes across the sampled populations and across species boundaries is consistent with a paternal or partially paternal mode of plastid inheritance, assuming that pollen is more widely dispersed

than seed. If inheritance were strictly maternal, we would expect any geographical variation noted to be concordant with boundaries of seed dispersal.

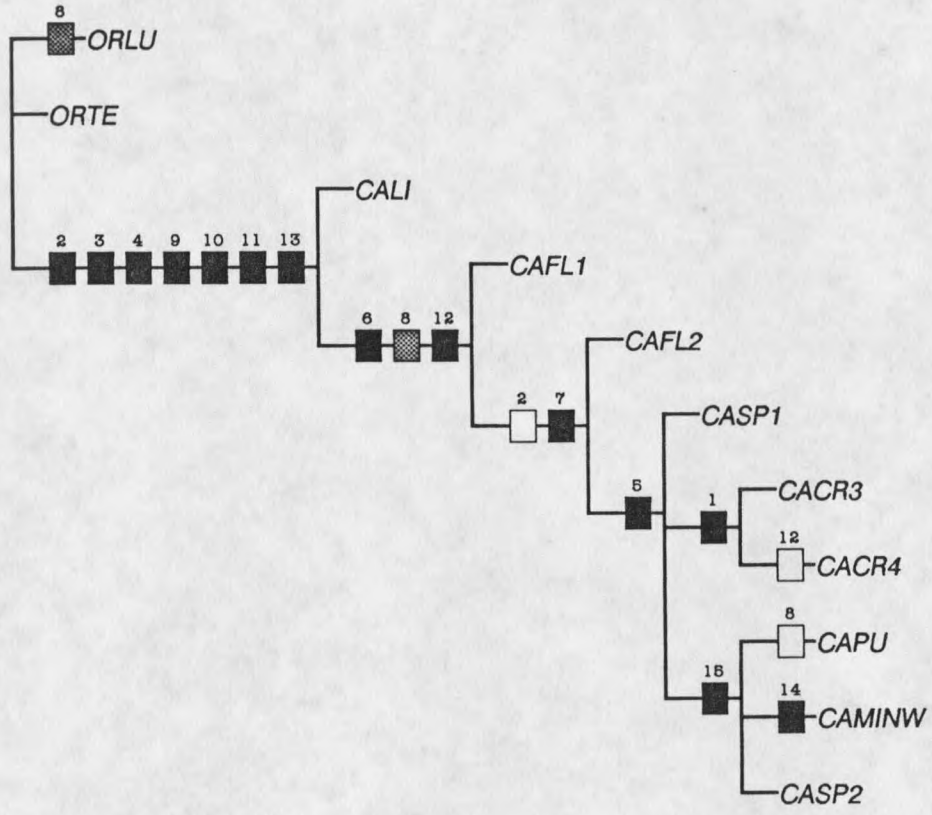

Fig. 2. A most parsimonious cladogram for chloroplast genomes of species of Castilleja and Orthocarpus. Length = 19; CI = 0.789. Numbers denote mutations listed in table 3. Acronyms are defined on pp. 17 and 18. Open boxes represent reversals, hatched boxes represent parallel gains, and filled boxes represent non-convergent gains.

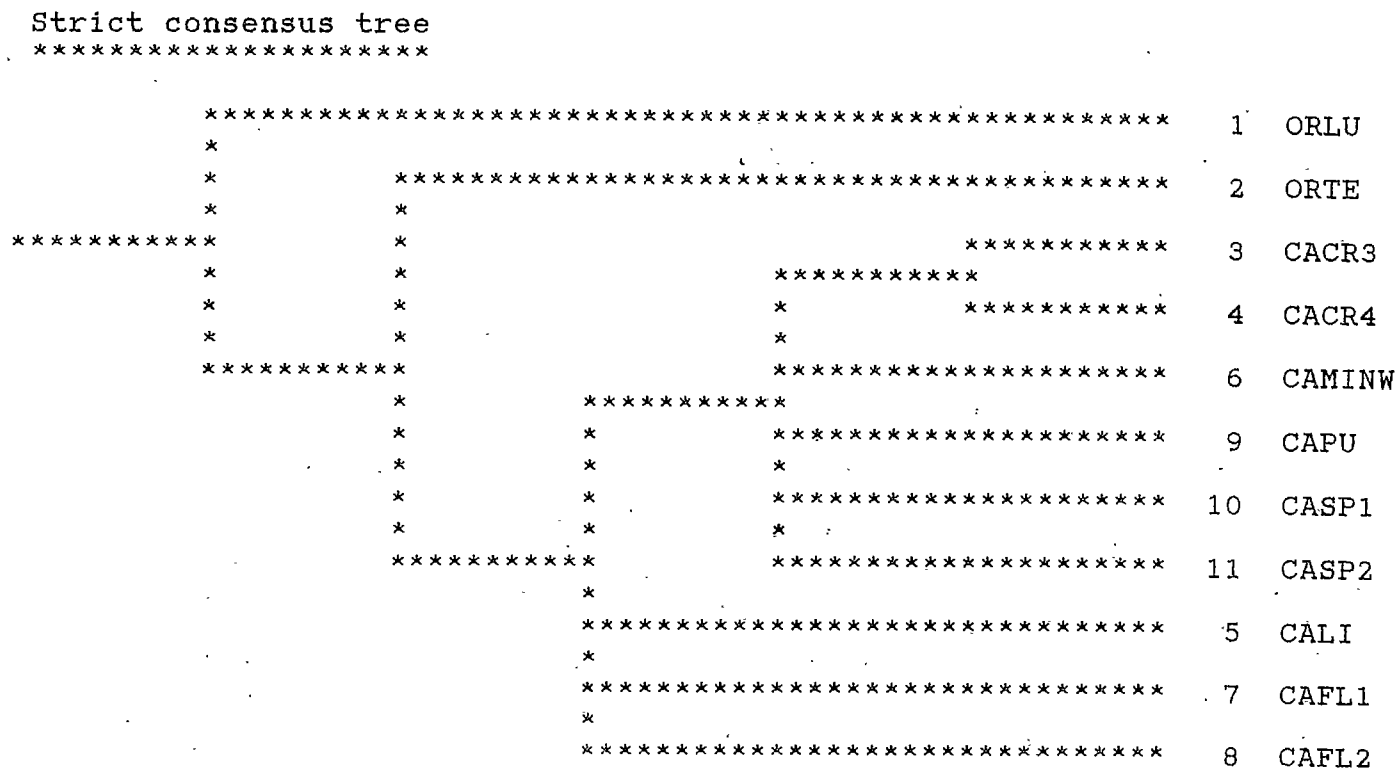

Fig. 3. Strict consensus tree revealing cpDNA groups consistently resolved among all most parsimonious cladograms. Acronyms are defined on pp. 17 and 18.

Sequence Divergence Values

Genetic distances were estimated using the restriction site mutations listed in Table 3, resulting the matrices depicted in Tables 4 and 5. Interspecific divergence values varied from 0.0000 to 0.0163 nucleotide substitutions per site, a range comparable to values reported for other congeneric species, for example, 0.0000-0.0070 for Lycopersicon (Palmer and Zamir, 1982), 0.0017-0.0156 for Clarkia sect. Peripetasma (Sytmsa and Gottlieb, 1986), and 0.0000-0.0260 for Pisum (Palmer et al., 1985). Intergeneric sequence divergence values of the taxa studied ranged from 0.0068-0.0117 nucleotide substitutions per site, a range comparable to the values noted among Castilleja species. In fact, the value of 0.0163 nucleotide substitutions per site between Castilleja crista-galli and C. flava is higher than the value of 0.0117 nucleotide substitutions per site between C. pulchella and Orthocarpus luteus. Distance estimates from restriction site data provide only an index of similarity and these examples may simply indicate that divergence within a lineage may be greater than divergence between lineages. Analysis of discrete characters implies that Orthocarpus is distinct from Castilleja. Levels of intraspecific divergence are high relative to those reviewed in Soltis et al. (in press). The highest intraspecific distance noted, 0.0033 nucleotide

substitutions per site between accessions of C. linariifolia is above the highest they report for Heuchera grossularifolia (0.0030 nucleotide substitutions per site). However, intraspecific divergence in Castilleja may appear greater if introgressed chloroplasts are being compared with those derived from a recent common ancestor. In addition, high levels of divergence within populations were observed, with the value of 0.0031 nucleotide substitutions per site between two individuals of a C. crista-galli population, which is nearly equal to the highest intraspecific value noted. Divergence was observed in two other populations

Table 4. Genetic distances (nucleotide substitutions per site x 100; Nei and Li, 1979) estimated from restriction site data for all pairwise comparisons of the different chloroplast genomes identified in the survey of 60 DNAs.

01=Castilleja crista-galli *Mathews s.n.*; 02=C. crista-galli *Mathews 280, 036*; 03=C. crista-galli *Mathews 280; Lavin 6245*; 04=C. crista-galli *Mathews s.n., 148, 035, 010, 140, 184, 03, 036; Lavin 6263* and C. miniata *Mathews s.n., 274, 240, 141; Lavin 6265*; 05=C. crista-galli *Mathews 035, 184, 192, 295* and C. miniata *Mathews 281, s.n., 151, 193, 047, 294, 056; Lavin 6246* and C. linariifolia *Mathews 290, 291, 070*; 13=C. linariifolia *Mathews 292, 293; Lavin 6268, 6270*; 24=C. miniata *Mathews 298; Siebert s.n.*; 25=C. flava *Mathews 272*; 26=C. flava *Mathews 272*; 27=C. flava *Lavin 6264* and C. pallescens *Mathews 300; Siebert s.n.*

	01	02	03	04	05	13	24	25	26	27
01	0.00									
02	0.37	0.00								
03	0.44	0.08	0.00							
04	0.31	0.06	0.13	0.00						
05	0.25	0.11	0.19	0.06	0.00					
13	0.59	0.33	0.25	0.27	0.33	0.00				
24	0.31	0.17	0.25	0.11	0.06	0.39	0.00			
25	1.63	1.36	1.45	1.30	1.32	1.22	1.39	0.00		
26	1.47	1.21	1.30	1.15	1.17	2.40	1.24	0.15	0.00	
27	1.41	1.47	1.23	1.08	1.04	1.30	1.17	0.22	0.07	0.00

of C. crista-galli (values of 0.0006 and 0.0014 nucleotide substitutions per site), in one population of C. flava (0.0016 nucleotide substitutions per site) and in one population of C. rhexifolia (0.0006 nucleotide substitutions per site). Dendrograms resulting from cluster analysis of the distance matrices are depicted in Figures 4 and 5.

Table 5. Genetic distances (nucleotide substitutions per site x 100; Nei and Li, 1979) estimated from restriction site data for all pairwise comparisons of the different chloroplast genomes identified in the survey of 30 DNAs.
29=C. pulchella *Mathews 288*; 30= C. rhexifolia *Mathews 266*; 31=C. rhexifolia *Mathews 266*; 33=C. rhexifolia *Mathews 080, 051; Lavin 6266* and C. gracillima *Mathews 275, 040* and C. miniata *Mathews 151* and C. pallescens *Mathews 300*; 40=Orthocarpus luteus *Mathews 296; Rumely and Lavin s.n.*; 41=C. crista-galli *Mathews 148* and C. linariifolia *Mathews 070*; 45=Orthocarpus tenuifolius *Mathews s.n.*

	29	30	31	33	40	41	45
29	0.00						
30	0.48	0.00					
31	0.51	0.03	0.00				
33	0.45	0.08	0.10	0.00			
40	1.17	0.76	0.79	0.68	0.00		
41	0.48	0.10	0.08	0.03	0.70	0.00	
45	1.05	0.65	0.68	0.57	0.10	0.60	0.00

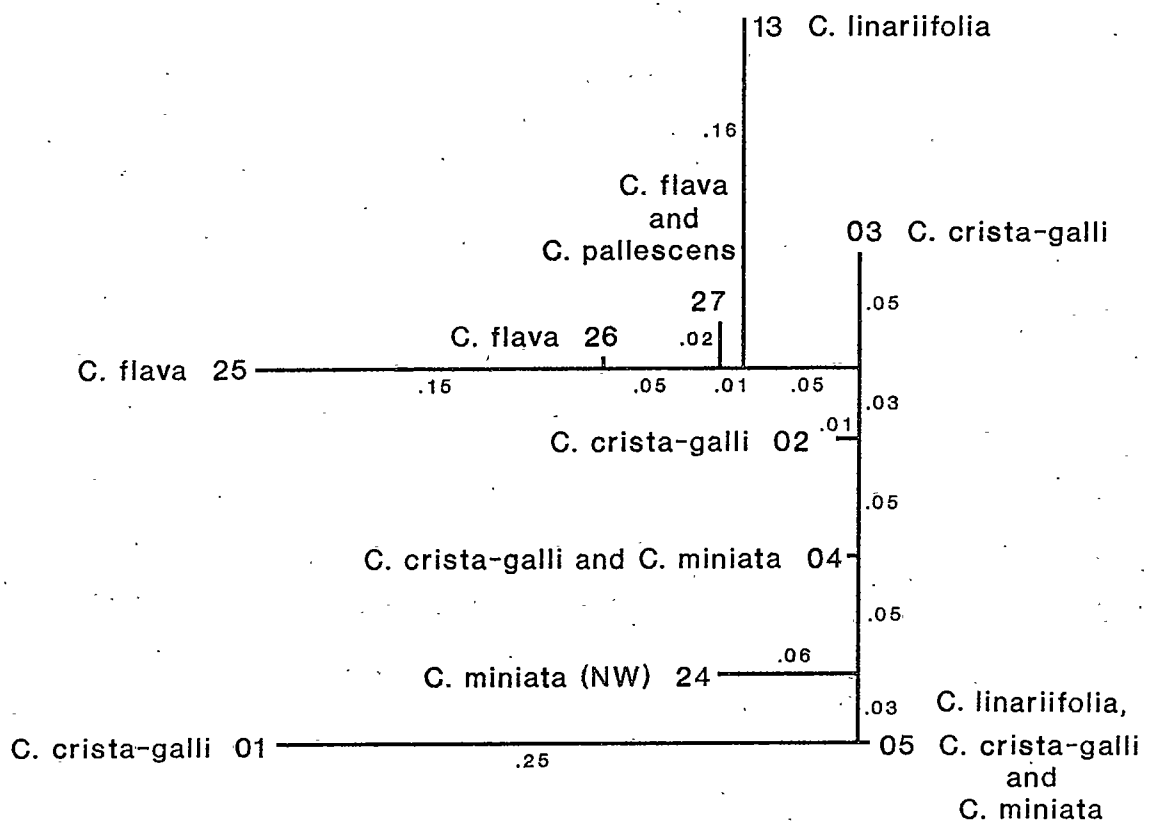

Fig. 4. Dendrogram of genetic distances (nucleotide substitutions per site x 100) estimated from restriction site data for all pairwise comparisons of the chloroplast genomes identified in the survey of 60 DNAs. Populations represented by each taxon are listed in table 4.

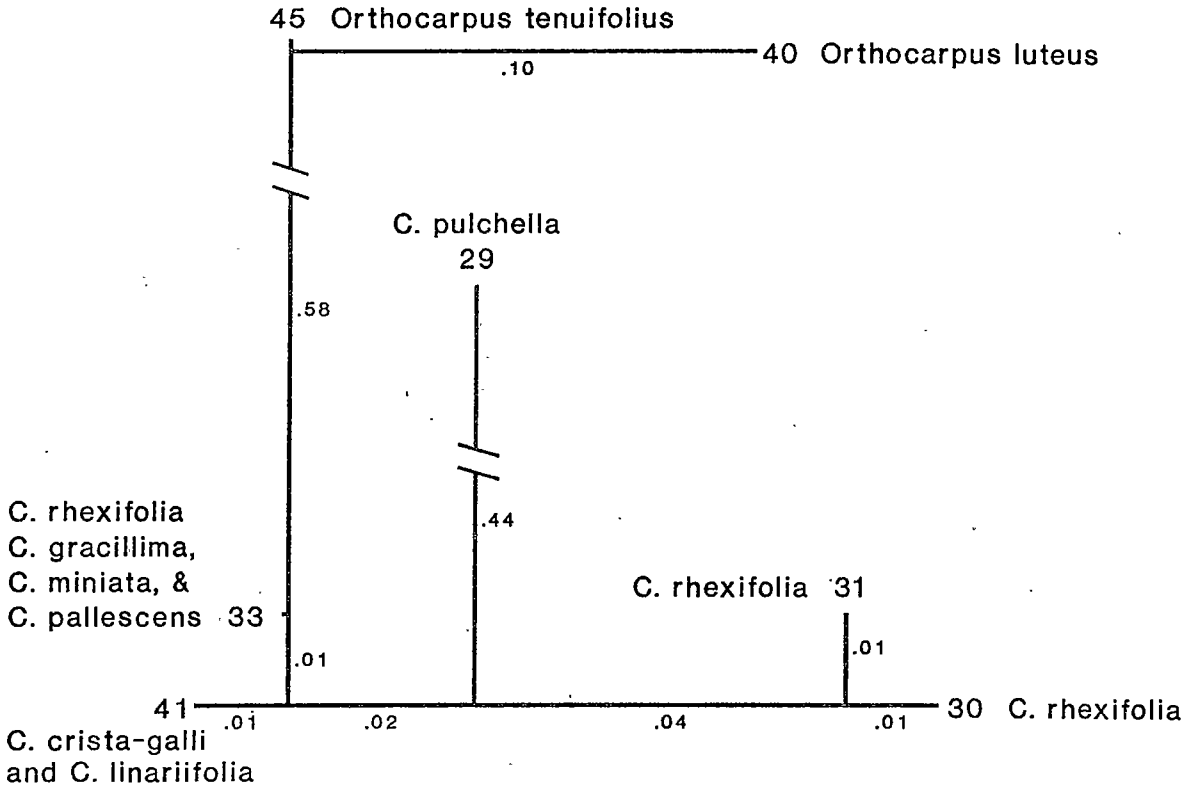

Fig. 5. Dendrogram of genetic distances (nucleotide substitutions per site x 100) estimated from restriction site data for all pairwise comparisons of the chloroplast genomes identified in the survey of 30 DNAs. Populations represented by each taxon are listed in table 5.

Ribosomal DNA

Genes encoding rRNAs exist in tandemly repeated units in the genomes of all higher organisms (Appels and Honeycutt, 1986). Each unit consists of regions coding for the 18s, 5.8s and 26s rRNAs. The 5.8s region is separated from the other two coding regions by small internally transcribed spacer sequences, and a length variable intergenic spacer (IGS) lies between the 5' end of the 26s gene and the 3' end of the 18s gene. Restriction enzymes which cut a repeat unit in a single place produce a single band on a Southern blot, allowing size analysis of one unit. In Castilleja and Orthocarpus, the sizes of the rDNA repeats are 9.9 and 11.3 kb. This is similar to sizes reported for repeat units of other higher plants (7.8-18.5 kb; Appels and Honeycutt, 1986). Monomorphic fragment patterns for all accessions were produced by ten of the thirteen enzymes employed. Bam HI, Bst BI and Eco RV detected site mutations among rDNA repeat units. Restriction site polymorphism is apportioned both among individuals and among repeat units within single individuals. For example, Sst I detected two rDNA repeat units in all individuals of the survey (Table 6). Sequential probing with pGmr1, pGB28s and pFH84 (clones containing a whole repeat and two separate portions totaling the whole, respectively) allowed identification of contiguous fragments. The number of variable characters

resulting from the rDNA analysis proved too few and too inconsistent in taxonomic distribution for a meaningful phylogenetic analysis, although taxa are more homogenous for rDNA restriction site mutations than for cpDNA mutations. Qualitative groupings of accessions are presented in Table 6. In contrast to these findings, other workers have found nuclear rDNA to be species specific when cpDNA variation was not. Zimmer et al. (1988) used repeat mutations to group species of Zea and Tripsacum, corroborating morphological, cytological and biochemical data, and Whittemore and Schaal (in press) found that species of oak lacking distinctive cpDNA genomes were well marked by rDNA mutations. The model of molecular drive applied to repeated nuclear sequences predicts that unequal crossing-over and/or gene conversion will cause variant repeats to spread to all gene loci first within individuals (homogenization) and thereafter throughout a population (fixation; Dover and Tautz, 1986). A discussion of Castilleja data relative to other findings and to predicted models is found in Chapter Four.

Table 6. Taxa grouped by rDNA repeat units.

Enzyme	Fragment Molecular Weight,kb	& Taxa	Fragment Molecular Weight,kb	& Taxa
Bam HI	11.3 and 4.9 4.2 0.9	C. miniata C. linariifolia C. pulchella O. luteus	7.1 4.2 and 4.2 4.1 2.1 0.9	C. crista-galli C. flava C. gracillima C. pallescens C. rhexifolia
Bst BI	11.3	C. crista-galli C. linariifolia C. miniata (*Mathews s.n., 056, 280*)	6.8 4.5	C. miniata
Eco RV	11.3 and 5.0 4.9	C. miniata C. gracillima	11.3	C. crista-galli C. pulchella C. rhexifolia C. flava (*Lavin 6264*)
	11.3 and 7.3 4.0	C. linariifolia C. flava (*Mathews 272*) C. pallescens (*Siebert s.n.*)		C. pallescens (*Mathews 300*) O. luteus

Cytological Analyses

The basic haploid number of 12 has previously been established for Castilleja (Gillett, 1954; Heckard, 1958), and polyploidy has been established for more than half the taxa in the genus, with levels ranging from 2x through 12x (Heckard, 1968; Heckard and Chuang, 1977). Chromosome counts obtained in this study for C. miniata, C. linariifolia, C. rhexifolia, C. pallescens and C. gracillima are in agreement with those in the literature (Heckard 1968;

Heckard and Chuang 1977). Additional counts to those already established are here reported for C. crista-galli, C. flava and C. gracillima. Tetraploid and diploid counts are reported for C. crista-galli, previously known only as an octoploid, and diploid counts are established for C. flava and C. gracillima, previously known only as tetraploids. Two uneven counts, n=9 for C. miniata and n=18 for C. linariifolia (Table 1) are added to the report of n=32 (Heckard and Chuang, 1977) for C. crista-galli. While C. miniata is known to have polyploid races ranging up to n=72, only diploid counts are noted in this survey. This data combined with the lower counts for C. crista-galli and C. flava are in agreement with the trend observed by Heckard and Chuang (1977) that diploid populations occupy more northerly portions of species' ranges. Lower counts for these two taxa also indicate that hypotheses of allopolyploid origin based partially on chromosome number (Heckard and Chuang, 1977; Holmgren, 1984) are in need of reexamination. In three instances (Table 1), different chromosome numbers were found within single populations. These differences were not correlated with morphological or habitat variability, as has been noted for populations of C. miniata and C. rhexifolia (Heckard and Chuang, 1977).

Morphological Analyses

Forty-five most parsimonious cladograms were generated by the branch-and-bound search option of the Penny algorithm of PHYLIP, each tree having a length of 18 and a consistency index of 0.667. A representative tree is found in Figure 6. The strict consensus tree of Figure 7 reveals those groups consistently resolved among the most parsimonious cladograms. Castilleja crista-galli occurs in an unresolved polytomy with C. miniata, C. linariifolia and C. hispida on 100% of these. Thus, a relationship is indicated between C. crista-galli and both putative parents, but also with C. hispida. This group in turn is consistently placed in a polytomy with all other Castilleja species. Closer examination of Figure 6 also demonstrates how tentative groups based on this data set can be, for example, the clade comprising CACO, CAHI, CACR, CAMI and CALI, is resolved solely on inflorescence color which is gained in parallel in Orthocarpus tenuifolius, and which is a character likely to undergo convergent evolution. If hummingbird pollination acts as a selective force in Castilleja (Grant and Grant, 1968; Brown and Kodric-Brown, 1979), there are most likely other characters which are convergent, including the long galeas and further degree of lip reduction which resolve the clade containing C. crista-galli. Results of the cladistic analyses required reversal of polarity in characters 1,

3,and 6 (duration, bract margin, and calyx clefts, respectively) in order for their most parsimonious distribution on the cladogram. Such a distribution does not alter the hypothesis of phylogeny presented, but may change our assessment of the direction of character evolution.

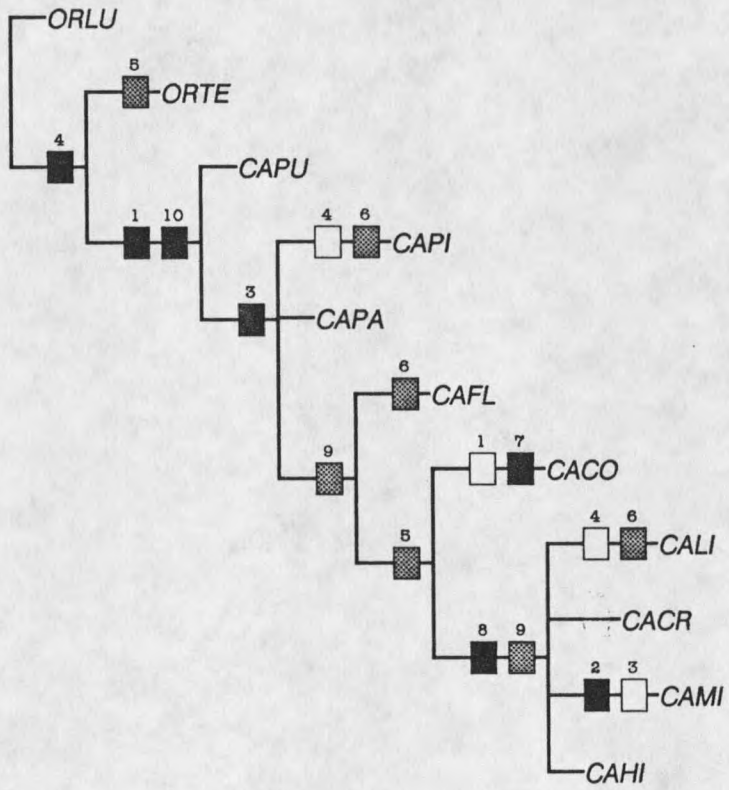

Fig. 6. A most parsimonious cladogram from morphological analysis of C. crista-galli, Orthocarpus, and 8 sections of Castilleja. Numbers denote characters defined in table 2. Length = 18; CI = 0.667. Open boxes represent reversals, hatched boxes represent parallel gains, and filled boxes represent non-convergent gains. Acronyms are as follows: ORLU = O. luteus, ORTE = O. tenuifolius, CAPU = C. pulchella, CAPI = C. pilosa, CAPA = C. pallescens, CAFL = C. flava, CACO = C. coccinea, CALI = C. linariifolia, CACR = C. crista-galli, CAMI = C. miniata, CAHI = C. hispida.

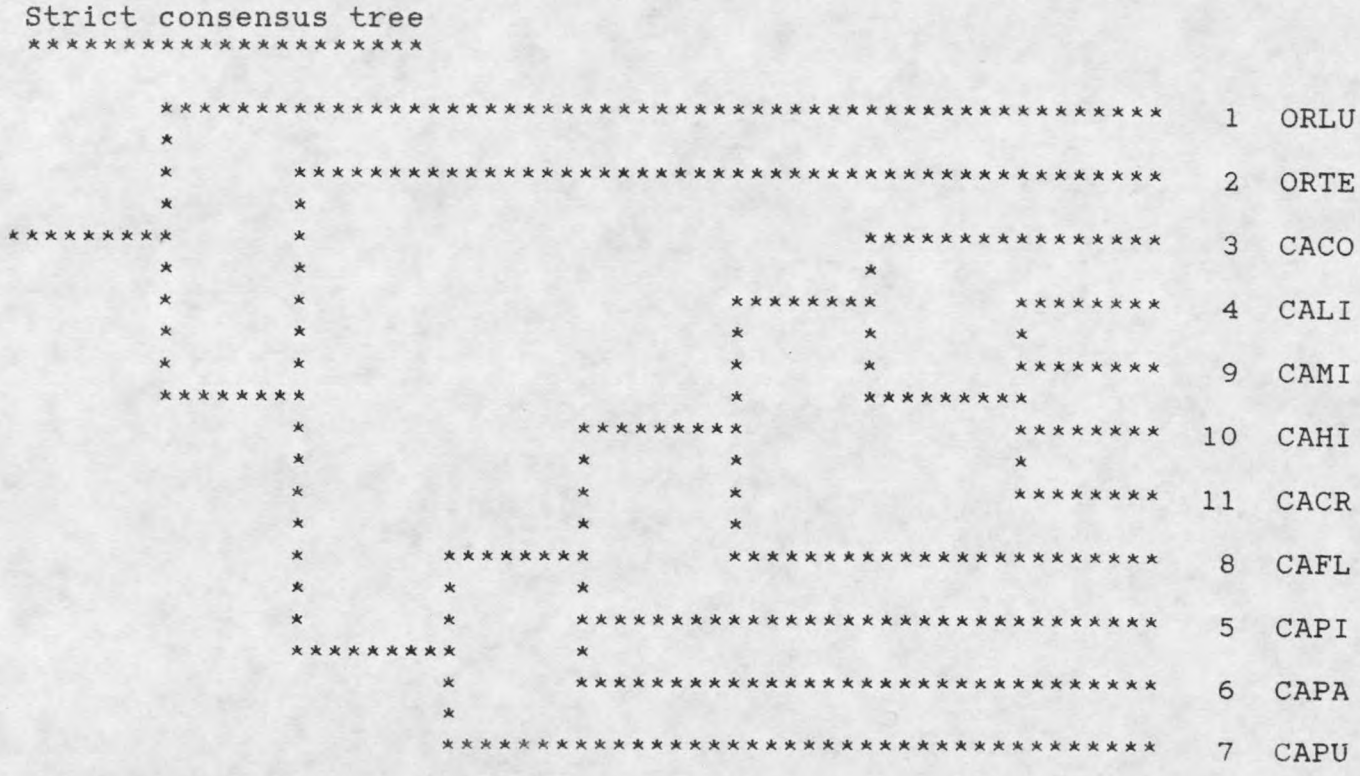

Fig. 7. Strict consensus tree revealing those groups consistently resolved in the morphological analyses. Acronyms are defined in figure 6.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

Data presented herein support a model of reticulate evolution for Castilleja, but a simple allopolyploid origin for Castilleja crista-galli is not supported. The data suggest that Castilleja species within a region form a large system of interbreeding individuals similar to that usually found within populations of a single species. Such a system is indicated in particular by the wide taxonomic distribution of chloroplast genotypes. Species representing six different sections within the genus share identical chloroplast genomes. In addition, two species, C. linariifolia and C. miniata, have populations at the edge of outside the sampled range which are marked by unique cpDNA mutations, whereas each variant genome found within the the range is shared by several or all species. This pattern is suggestive of that found in species of oak (Whittemore and Schaal, in press), in which cpDNA types are concordant with geographical locations rather than species boundaries. Yet the data for Castilleja suggest a more complex pattern of relationships than that found in oaks, where length variants of the nuclear ribosomal repeat are species specific.

In fact, in the many examples of organellar genomes crossing species boundaries (Avise, 1986; Soltis et al., in press), a concurrent observation was made of the constancy of nuclear markers to species boundaries. This is not the case observed so far in Castilleja, in which each individual restriction site marker in the ribosomal repeat places C. crista-galli in combination with a different set of species. Although only one type of nuclear marker is assessed in this study, it is one that is expected to be highly homogenous within lineages (Dover and Tautz, 1986). It is uncertain how the model of molecular drive would apply to combinations of more than one genome within a polyploid individual introducing more than one variant repeat type. Several workers have noted nonrandom distribution of repeat length variants among chromosomes (summarized in Dowling et al., 1990), suggesting less efficient homogenization among chromosomes than within them. If the model is correct for Castilleja, it may signify that recombination of genomes is recent and that sorting out is not complete.

Heckard (1968) has suggested that widespread, morphologically and cytologically complex species in Castilleja may have arisen via diverse combinations of genomes from up to four species, with introgression occurring among the polyploid derivatives. Such events are corroborated by the molecular data here presented, with C. miniata implicated in the formation of at least two

complexes. Such a scenario might be considered for C. crista-galli. While it is more restricted in distribution than the complexes referred to, it shows morphological variation toward C. miniata, C. linariifolia and C. hispida, and possibly a fourth for color variation. Conversely, characters typical of C. crista-galli occasionally appear in more variable populations of C. miniata in the region. Indeed, Heckard and Chuang (1977) interpreted a variable population from Yellowstone National Park to be the result of hybridization between the two. The chloroplast genomes of each are equally polymorphic for the same set of mutations, whereas cpDNAs of the other species studied showed less polymorphism and most often grouped with just one of the types found in C. miniata and C. crista-galli. However, this could result from sampling bias toward these two. High ploidy levels (eg. n=60,72) were integral parts of Heckard's species complexes (Heckard, 1968). It is unclear from data presented here, and from further observations by Heckard and Chuang (1977), whether difference in chromosome number provides much of a sterility barrier. Chromosome numbers of variable ploidy levels (including odd-ploids and aneuploids) within presumably interbreeding populations were noted in this study and by Heckard and Chuang (1977). In addition, Heckard and Chuang (1977) made successful artificial crosses between different species of different ploidy levels. Also integral to

Heckard's hypotheses of allopolyploid origin was an observed lack of diploid populations. This study adds diploid counts for C. crista-galli and C. flava, both putatively of allopolyploid origin. Yet taken together with data provided by crossability studies and observations in natural populations, diploid counts do not seem a strong basis for rejecting hypotheses of hybrid origin.

Results from cladistic analyses of morphological characters indicate that these may be of limited use in discerning evolutionary relationships among Castilleja species, most likely due to reticulate evolution that has occurred in the genus. Nevertheless, unique combinations of the characters in question allow us to identify separate entities in the landscape. Castilleja crista-galli shares perianth morphology with C. miniata (eg., the calyx is subequally cleft with acute secondary segments from 3-9 mm), but is distinguished by its linear lower leaves and deeply 3- to 5-lobed bracts and upper leaves. In turn, C. hispida is similar to both, but is distinguished by a calyx with shorter secondary segments (1-7 mm) which are sometimes obtuse rather than acute. Its lower leaves are lanceolate like those of C. miniata, but its upper leaves and bracts are deeply lobed, with wider, less divergent lobes than the digitate ones of C. crista-galli. Castilleja linariifolia is most like C. crista-galli, but differs in having upper leaves and bracts with only 3 divergent lobes. In addition,

the calyx of C. linariifolia exceeds the bracts, while in C. crista-galli, it is equal to or shorter than the bracts. Each member of this pair has a corolla which is exserted well forward from the calyx in anthesis, with that of C. linariifolia exserted through a more deeply cleft front. Entities thus recognized are further separated spatially and temporally to varying degrees. Castilleja crista-galli blooms earlier than C. miniata and occupies talus slopes or dry sites in forest openings, while C. miniata is found in mesic meadows and along streambanks. Where the ranges of C. miniata and C. hispida overlap, a similar separation is noted, with C. hispida being the early bloomer and an inhabitant of grassy slopes and forest openings. Castilleja hispida occurs mainly to the north and west of C. crista-galli, reaching Vancouver Island to the northwest and extending south along the coast to Benton Co., Oregon, and extending west from Montana as far as Payette Co., Idaho and Grant Co., Oregon. Only the range of C. miniata overlaps that of C. linariifolia, where the latter grows in sagebrush grasslands and dry woodlands. Castilleja miniata blooms from middle to late summer, C. linariifolia from late summer to fall. Each of these four is hummingbird-pollinated, although there is separation among other species of Castilleja for pollinators, which include bees and moths. Only one species is known to be self-pollinated.

That separations possible based on morphology and

ecology are not corroborated by either chloroplast or nuclear data present an added challenge to identifying lineages within Castilleja. The data suggest that introgression is frequent and that only limited, if any, molecular divergence occurs between hybridization events among species in a region. It seems likely that diploid progenitors have themselves undergone hybridization. What nuclear markers could prove informative in this situation? A technique developed to understand genomic evolution in the wheat genus (Triticum), identifies repetitive sequences specific to diploid genomes of interest and uses them as probes to assess relationships between polyploid species and diploid progenitors (Talbert et al., in press). This technique and enzyme electrophoresis rely on existence of progenitors with fixed, species-specific alleles to be informative. If genomes among species of Castilleja are as homogenous as they appear to be, it is possible that techniques which assess variation at the population level in other groups would be informative at the species level in Castilleja. Fingerprinting probes which detect hypervariable sequences in nuclear DNA of animals have recently been used to identify cultivars in rice (Dallas, 1988) and in the apple subfamily (Nybom et al., 1990), as well as population subdivision in Rubus (Nybom and Schaal, 1990). While chloroplast genomes are homogenous among species of a region, cpDNA data show that divergent lineages

(eg. Castilleja and Orthocarpus) are marked by chloroplast mutations. Mainland Mexico is the putative center of dispersal of Castilleja (Holmgren, 1976), and Holmgren (1976) describes the mainland Mexican species as distinct, with few close relatives in the United States. He also states that the Costa Rican species show greater affinity with Andean South American species than with other Central American and North American species (Holmgren, 1978). Additionally found in Mexico are two monotypic genera, Gentrya and Ophiocephalus, which are interfertile with Castilleja and Orthocarpus (Chuang and Heckard, 1986). These more divergent lineages likely have distinct chloroplast genomes, and might, when better understood, provide a reference point for species-level studies. Comparison of cpDNAs from closely related species may be informative in other ways, however. For example, sampling a species assemblage from additional geographical regions within the range of C. miniata and C. linariifolia would allow discernment of whether the cpDNAs of those species are most similar to those of C. miniata and C. linariifolia elsewhere in their ranges, or to the set of species they co-occur with in any geographical region. Concurrent with this, identifying modes of plastid transmission is possible. Seed is available from two populations of C. linariifolia which differ for cpDNA mutations, such that crosses could be made and progeny tested.

While identification of lineages might prove possible, the question of how morphological integrity is maintained among closely related species without apparent molecular divergence remains open. Multivariate analyses of morphological traits to assess overall similarity, intermediacy, and divergence among populations of C. cristagalli and closely related and sympatric species is suggested as a necessary corollary to further molecular studies. The priority for molecular studies should be the assessment of nuclear rDNA data in light of enzyme analyses.

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APPENDIX

CASTILLEJA CRISTA-GALLI

SPECIMENS EXAMINED

Specimens examined. U.S.A. Montana: Gallatin Co., Bridger Mountains, 17 June 1897, *Rydberg and Bessey 4950* (type: NY!).

U.S.A. Idaho: Blaine Co., alpine slopes at base of Devil's Bedstead, Sawtooth Range, 28 July 1936, *J.W. Thompson 13563* (WS), Custer Co., vicinity of Castle Peak, White Cloud Range, head of stream immediately E of Castle Peak, 8 August 1944, *C.L. Hitchcock and C.V. Muhlick 10922* (WS), Mt. Hyndman, 11 August 1939, *R.J. Davis 1680* (WS), Cherry Cr. divide, 7 July 1939, *R.J. Davis 1267* (WS), sandy bank at head of draw ca 11 mi W of Challis, 3 July 1944, *C.L. Hitchcock and C.V. Muhlick 9520* (WS), Garden Cr. W of Challis, 24 June 1931, *F.W. Pennell 15259* (WS), on upper slopes of Mt. Borah, Chilly, 23 June 1923, *J.H. Christ and W.W. Ward 10408* (WS), 9 mi N of Bonanza on Jordan Cr., 23 July 1946, *J.H. Christ 15650* (WS), Lemhi Co., Panther Cr. 8 mi N of Cabin Cr., 1 July 1949, *C.L. Hitchcock and C.V. Muhlick 14265* (WS), Brazil's, Birch Cr., 30 June 1939, *R.J. Davis 1039* (WS), Fremont Co., About 14 mi N of Macks Inn, below Targhee Peak, 16 July 1949, *J.H. and C.B. Christ 19035* (WS). Montana: Gallatin Co., lower Route 191, Specimen Creek, Yellowstone National Park, 24 July 1938, *F.W. Pennell and R.L. Schaeffer Jr. 23532* (WS), About 24 km S of Gallatin Gateway, avalanche chute near Squaw Creek CG, 19 June 1988, *S. Mathews 010* (MONT), About 10 km S of Bozeman, above Hyalite Creek, Gallatin National Forest, 6 June 1988, *S. Mathews 035* (MONT), about 30 km S of Big Sky, above Gallatin River near abandoned talc mine, 5 July 1989, *S. Mathews 148* (MONT), Gallatin National Forest, 32 km NE of West Yellowstone, along trail above Cabin Creek, 3 August 1989, *S. Mathews 280* (MONT), Mt. Baldy, Bridger Range, 20 June 1989, *Lavin 6245* (MONT), Sacagewea Peak, Bridger Range, 16 July 1989, *Lavin 6263* (MONT), Madison Co., Cedar Mountain, E of Ennis, 26 August 1931, *F.W. Pennell 15952* (WS), vicinity Lazyman Hill, 19 July 1959, *P.F. Stickney PFS-292* (WS), Red Hill, Gravelly Range, 26 July 1947, *C.L. Hitchcock 16920* (WS), Beaverhead National Forest, Mill Creek Recreation Site, 9.6 km E of Sheridan, 3 July 1988, *S. Mathews s.n.* (MONT), Park Co., between dam and sawmill site at falls of the Clarks of the Yellowstone River, about 4 mi E of Cooke City, 28 June and 12 July 1948, *J.G. Witt 1192* (WS), along road to Sheep Creek Basin NW of Cooke City, 28 June 1949, *J.G. Witt 1677* (WS), 4.8 km E of Cooke City, Kersey Lake Trailhead under power line, 10 July 1988, *S. Mathews 295*(MONT), Gallatin National Forest, about 16 km SE of Pray, Mill Creek drainage above Snowbank CG, 14 June 1989, *S. Mathews 081* (MONT), Gallatin National Forest, 16 km SE of Livingston, limestone hogback on divide between Tie and Mill Fork Creeks, 28 June 1989, *S. Mathews 139* (MONT), 16 km SE of Livingston, above confluence of Tie and Mill Fork Creeks, 28 June 1989, *S. Mathews 140* (MONT), Gallatin National Forest, Mill Creek drainage about 18 km SE of Pray, in

clearcut above Wicked Creek, 11 July 1989, *S. Mathews 192* (MONT), Sweet Grass Co., 7 mi E of Box Canyon Ranger Station, along Boulder River, 16 July 1947, *C.L. Hitchcock 16471* (WS). Wyoming: Park Co., Northern Absarokas, along Sunlight Cr. above Lee City on the trail to Hoodoo Basin, ca. 38.5-39.5 air mi. WNW of Cody. 22 July 1985, *B.E. Nelson and R.L. Hartman 12676* (RM), Northern Absarokas, ca. 1.5 air mi. NW of Sugarloaf Mountain above Clarks Fork Canyon on WY 296, ca. 30.5 air mi. NW of Cody, 12 July 1985, *B.E. Nelson 12337* (RM), Northern Absarokas, along Pilot Cr., ca. 2 air mi. S of Pilot Peak, ca. 50 air mi. NW of Cody, 27 July 1985, *B.E. Nelson 12826* (RM), Northern Absarokas, U.S. 212, 3 mi. SE of MT state line, 1 mi. NW of crossing of the Clarks Fork, 19 July 1985, *R.L. Hartman and B.E. Nelson 21110* (RM), Northern Absarokas, ridge NE of Windy Mountain, 1.5-2.5 mi. from summit, 6-7 air mi. N of Sunlight Ranger Station, 17 August 1985, *R.L. Hartman 21916* (RM), Northern Absarokas, rim of natural coral [sic] and ridge above, 17 July 1985, *R.L. Hartman 20910* (RM), Absaroka Mountains, north fork Shoshone River drainage, along river just W of ski area, 17 July 1983, *E.F. Evert 5484* (RM), North fork of Shoshone River drainage, divide between Nutter and Moss creeks, 9 July 1979, *E.F. Evert 1478* (RM), North fork of Shoshone River drainage, Grinnell Cr. trail, 1.5 mi. N of road, 15 July 1979, *E.F. Evert 1496* (RM), North fork of Shoshone River drainage, Clayton Mt. trail, 29 July 1979, *E.F. Evert 1528* (RM), Absaroka Mountains, north fork Shoshone River drainage, ca. 4 mi. N of U.S. 14, 16 & 20 on ridge above and E of Libby Cr., 28 July 1981, *E.F. Evert 3325* (RM), North fork of Shoshone River drainage, Kitty Cr., 5 July 1979, *E.F. Evert 1448* (RM), shale slope below lookout station, 3 mi. W of Bear Tooth Lake, 20 July 1947, *C.L. Hitchcock 16662* (RM, WS), Yellowstone National Park, Sylvan Pass, 4 August 1979, *R.W. Lichvar 2241* (RM), Yellowstone National Park, Glen Cr., 29 June 1899, *A. Nelson and E. Nelson 5562* (RM), Yellowstone National Park, Mt. Washburn, 5 July 1928, *J. Thorp 14* (RM), Yellowstone National Park, Sylvan Pass, 16 August 1979, *R.D. Dorn 3390* (RM), Yellowstone National Park, Mammoth Hot Springs, 13 July 1906, *W.S. Cooper s.n.* (RM), Yellowstone National Park, roadside, Bunsen Peak road, 29 June, 1942, *L. Winiecki 19* (WS), Yellowstone National Park, near beaver ponds off old road to Gardiner, 11 July 1942, *L. Winiecki 112* (WS), July 1906, *I.T. Worthley 117* (RM), 1906, *I.T. Worthley 130* (RM), Rattlesnake Mt. area, Pat O'Hara Quad., 3 August 1979, *R. York 323* (RM), Yellowstone National Park, Bunsen Peak road, 25 June 1988, *S. Mathews 033* (MONT), Yellowstone National Park, above Lava Creek picnic ground, 26 June 1988, *S. Mathews 036*(MONT), Yellowstone National Park between Mammoth Hot Springs and Tower Junction, just W of Phantom Lake, 23 June 1990, *S. Mathews 311* (MONT).