ABSTRACT

Title of Dissertation: PATTERNS OF MATING SYSTEM EVOLUTION IN

CRYPTANTHA SECTION OREOCARYA (BORAGINACEAE) –

A PHYLOGENETIC APPROACH

Tanya Marie Marushak, Doctor of Philosophy, 2003

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Molecular phylogenetic methods provide new and powerful ways to test

hypotheses concerning the evolution of breeding systems. These methods allow one to

infer the number of evolutionary transitions for specific reproductive characters, thereby

providing a framework for existing models of breeding system evolution to be tested

using exemplar systems. Character mapping has provided insight into the evolution of

plant breeding systems by suggesting the number and direction of changes in many key

reproductive characters, including those that define specific syndromes, e.g., dioecy,

androdioecy, self-incompatibility and heterostyly (Weller and Sakai, 1999).

Heterostyly involves the reciprocal placement of anthers and stigmas among two

(distyly) or three (tristyly) flower morphs, and is usually accompanied by a

sporophytically controlled diallelic incompatibility mechanism that prevents self- and

section Oreocarya of the genus *Cryptantha* (Boraginaceae) contains species that exhibit breeding system characters intermediate to those classically found in heterostylous systems. The goal of this study was to determine the order of events that lead to heterostyly for *Cryptantha* thereby testing the current models for the evolution of this syndrome. Measurements of the stigma position, anther position, and corolla length indicate that stigma height and anther height do not always change between pins and thrums of the same species. Additionally, the size of the corolla is related to the degree of spatial separation between the floral organs. All of the dimorphic species are at least partially self-compatible, while some monomorphic species exhibit incompatibility. Genetic variation among the Oreocarya is limited, and plant species distribution for the section is correlated with geology. The phylogenetic analyses indicated that the heterostylous state may be ancestral in Oreocarya, and it appears that the breeding system is in the process of breaking down, making it difficult to test the models for the evolution of the syndrome.

PATTERNS OF MATING SYSTEM EVOLUTION IN *CRYPTANTHA* SECTION
OREOCARYA (BORAGINACEAE) – A PHYLOGENETIC APPROACH

by

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BEES

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ix

Mating system evolution in *Cryptantha* Section Oreocarya (Boraginaceae) –

A phylogenetic approach

A vast majority of angiosperms have hermaphroditic flowers. In the absence of specific genetic or morphological adaptations, this condition allows for self-pollination to occur. In some plant systems, this may lead to inbreeding depression, i.e., lower fitness in self-crossed seed compared to out-crossed seed. To assert control over 'who mates with whom' within a species, plants have developed an array of complex breeding systems that effectively prevent certain types of matings (Barrett 1992, Richards 1997, Holsinger 2000).

Historically, studies of breeding system evolution were theoretical or descriptive, and at the level of the population. Advances in molecular biology and systematics now allow the study of complex breeding systems in light of a phylogenetic framework. Thus it is possible to test explicit hypotheses regarding the significance of changes in morphological and reproductive characters as intermediate steps in the process of mating system evolution (Schoen *et al.* 1997, Bena *et al.* 1998, Weller and Sakai 1999). Heterostyly is an example of a complex breeding system where two (distyly) or three (tristyly) mating types form discrete groups within a population with respect to the reciprocal placement of their stigmata and anthers (Figure 1, Barrett 1990). The term heterostyly includes many variations on a central theme where the long-styled (pin) and the short-styled (thrum) (Dulberger 1992) morphologies are accompanied by diallelic incompatibility (Barrett 1992), and a suite of ancillary morphological polymorphisms (Lloyd and Webb 1992). Examples of genera that exhibit 'classical' characteristics of

1

Figure 1. The heterostylous polymorphisms distyly and tristyly. Heterostyly is a floral polymorphism with reciprocal placement of the stigma and anthers, coupled with a sporophytically controlled self-incompatibility mechanism. Distyly is controlled by a single locus with 2 alleles, and tristyly has 2 diallelic loci (S and M), with S epistatic to M.

DISTYLY

PIN
SS

THRUM
Ss

TRISTYLY

ssmm   ssM_   Ss_ _
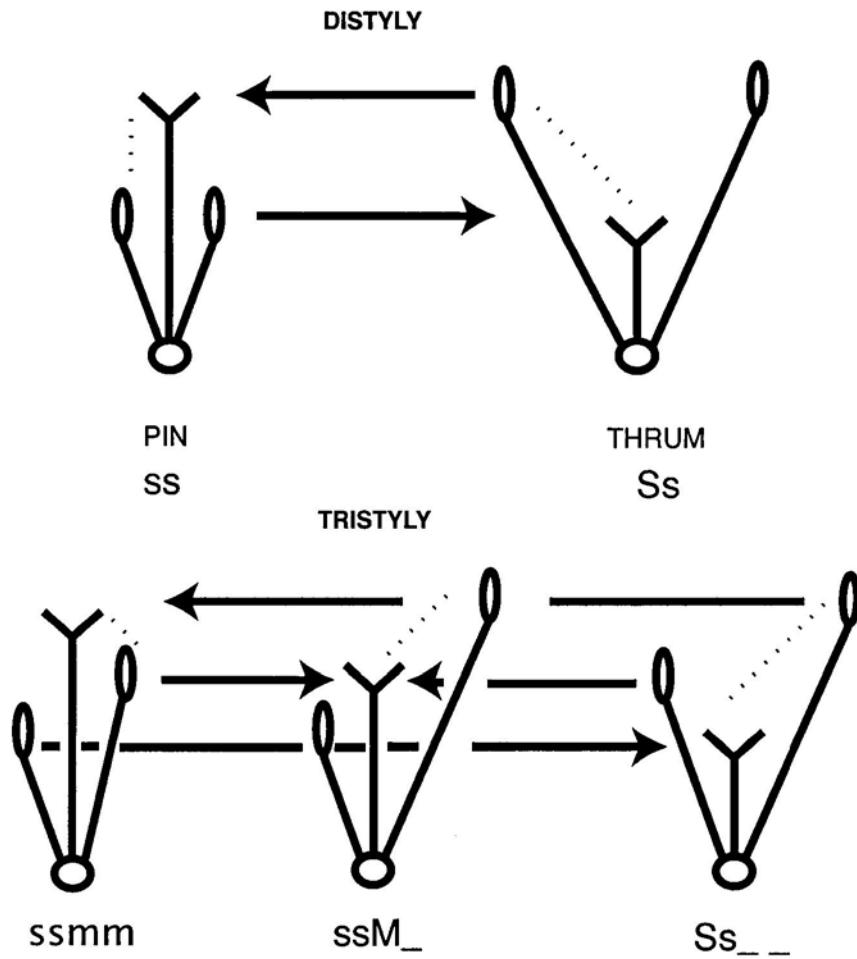
solid lines indicate legitimate pollinations
dashed lines indicate illegitimate pollinations

3

heterostyly include *Fagopyrum, Jepsonia, Connarus, Erythroxylum, Primula, Forsythia, Lithospermum, Houstonia, Psychotria, Eichornia*, and many others. Several examples are known that deviate from the classic form of heterostyly - di- and tri-morphic species that lack self-incompatibility (*Melochia, Amsinkia, Cryptantha, Hedyotis, Oxalis, Biophytum, Quinchamalium, Guettarda, Nivenia*), dimorphic flowers with multi-allelic incompatibility (*Narcissus tarzetta, Anchusa hybirda*, and *A. officianalis*), and stylar polymorphisms unaccompanied by reciprocal changes in anther position (*Narcissus, Anchusa, Lithodora, Linum, Primula, Epacris, Kalmiopsis, Chlorogalum*, and *Anigozanthos*). These types of heterostylous breeding systems (both classic and aberrant) are hypothesized to evolve as mechanisms to promote outbreeding by preventing self-fertilization (Charlesworth and Charlesworth 1979, Barrett 1992, Lloyd and Webb 1992, Richards 1997, Barrett *et al.* 2000).

Since the properties of heterostyly were first described (Darwin 1862, 1877), the breeding system has been well-studied, and much is known about its genetics, outbreeding mechanisms, taxonomic distribution, and morphology (Vuilleumier 1967, Barrett 1992, Dulberger 1992, Lewis and Jones 1992). Despite this concentration of effort, much about the evolutionary origins of heterostyly remains obscure.

Nature and Distribution of Heterostyly

History

A morphological difference in the height of anthers and styles among individuals of the same species was first documented as early as the 16th century in *Primula* (Ornduff 1992). Little attention was given to the syndrome until Darwin (1862) and Hildebrand (1863) speculated on the adaptive significance of dimorphic flowers (Barrett 1992).

4

Hildebrand (1867) coined the term heterostyly, and used it to describe plant species that had two or three different ratios of style length to stamen length within their flowers. Barrett (1992) views Hildebrand's use of the term as a reference to floral morphology only, while Vuilleumier (1965) writes that Hildebrand alluded to heterostylous plants as self-incompatible, though that term was not used specifically. Darwin (1877) proposed that the term heterostyly be reserved for those species that exhibit reciprocal placement of styles and stamens between two or three morphs along with self-incompatibility, since he completed breeding experiments that demonstrated reduced seed set in selfed individuals.

The majority of plants described as heterostylous do possess reciprocal herkogamy (spatial separation of the style and stamen within a flower), sporophytic diallelic incompatibility, and a suite of ancillary floral polymorphisms. However, over the last century, many plant species that exhibit stylar polymorphisms coupled with varying degrees of self-compatibility have been described. Examples include: self-compatibility (both within an individual and within a morph); multi-allelic incompatibility, monomorphic stamen heights, and the absence of ancillary polymorphisms (Barrett 1992); variable stigma/anther separation within a morph (Pailler *et al.* 1998, Thompson & Dommee 2000); and the loss of reciprocal herkogamy due to post-anthesis allometric growth of styles and stamens (Riveros *et al.* 1987). These polymorphic, self-compatible examples have contributed to the confusion associated with an exact description of heterostyly. Nevertheless, the importance of intermediate "non-heterostylous" characters in otherwise heterostylous systems can not be overstated. These traits may be keys to our understanding of the evolution of heterostyly, because

5

they may represent stages in the development of heterostyly, or conversely, they may be stages in the breakdown of the syndrome.

Occurrence

Heterostyly has been documented in 28 angiosperm families (Vuilleumier 1967, Ganders 1979, Barrett 1992, Barrett *et al.* 2000), and may have evolved independently more than 28 times, if one allows for multiple origins within a family. Much of the current information regarding heterostylous plants is based on detailed studies of a relatively small number of taxa (*Primula, Lythrum, Linum, Oxalis*). As more exceptions to early generalizations are described, it becomes apparent that postulates about the evolutionary precedence of certain aspects of the breeding system must be re-evaluated. There are many instances where a breeding system described as heterostylous lacks one of the classically recognized aspects –e.g. reciprocal herkogamy (*Narcissus, Anchusa, Lithodora, Linum, Primula, Epacris, Kalmiopsis, Chlorogalum,* and *Anigozanthos*) or self-incompatibility (*Melochia, Amsinkia, Cryptantha, Hedyotis, Oxalis, Biophytum, Quinchamalium, Guettarda, Nivenia*) (Vuilleumier 1967, Ganders 1979, Lloyd and Webb 1992, Barrett *et al.* 2000). Even if one allows that this variety results from a breakdown of heterostylous systems, the diversity and repeated evolution of the heterostylous syndrome implies that more than one evolutionary pathway to heterostyly may exist (Lloyd and Webb 1992). New taxa are added almost yearly to the list of heterostylous species. When heterostyly is mapped on to a cladogram of the angiosperms, no clear pattern emerges (Figure 2). There are pairs of closely related families that exhibit the breeding system, such as Commelinaceae - Pontederiaceae, and Plumbaginaceae -

6

Figure 2. The distribution of heterostyly among angiosperms. This cladogram is redrawn from analyses of Savolainen *et al*. 2000, Bremer 2000 and Chase *et al*. 1993. Families with heterostyly are labeled in bold. Orders are listed in regular face type to the right of the bracket. Note that there is no distinctive pattern for the distribution of the breeding system in flowering plants.

Solanales
Boraginaceae

Lamiaceae
Oleaceae
Rubiaceae
Loganaceae

Lamiales

Gentianales
Garryales
Apiales
Menyanthaceae
Asterales

Dipsacales
Aquifoliales

Epacridaceae

Polemoniaceae

Ericales

Primulaceae

Cornales
Santalaceae
Santalales
Sapindales
Malvales
Brassicaceae
Brassicales

Myrtales
Lythraceae
Geraniales
Celastraceae
Oxalidaceae
Oxalidales
Erythroxylaceae

Clusiaceae
Turneraceae
Linaceae

Malpighiales

Zygophyllaceae
Fagales
Cucurbitales
Rosales
Fabaceae
Fabales
Lac Vitis
Saxifragaceae
Saxifragales

Aextoxicon Berberidopsis

Caryophyllales

Plumbaginaceae
Polygonaceae
Gunnera Myrothamnus
Trochodendraceae
Buxus Didymeles
Sabia
Proteales
Ranunculales
Liliales
Camassiaceae

Amaryllidaceae

Asparagales

Iridaceae

Pandanales
Commelinaceae
Pontederiaceae
Commelinales

Poales
Dioscoreales
Acorales
Magnoliales
Piperales
Laurales
Nymphaceae
Ceratophyllum
Gymnosperms

8

Polygonaceae. It is possible that there was a single gain of the system in the most recent common ancestor to these groups, and not independent evolution after their divergence.

Factors associated with the distribution of heterostyly

Though the distribution of heterostylous species across angiosperms is diverse, there are some characteristics that are common among most heterostylous groups. The evolution of heterostyly appears to have proceeded with some general constraints (Richards and Barrett 1992). The flowers of heterostylous species tend to be short-lived, with anthesis lasting 1-2 days, and homogamous, with male and female functions acting from the start of anthesis. Heterostyly is usually found in perennials, though there are a few examples of heterostylous annuals (Dulberger 1992). Heterostyly is predominantly found in entomophilous flowers, with a few ornithophilous examples (Feinsinger and Busby 1987). The breeding system has yet to be described in wind-pollinated or unisexual flowers. The typical heterostylous flower is tubular, with a syncarpous ovary, a limited number of stamens and a moderate size. There are only a few examples of heterostylous zygomorphic (bilaterally symmetric) flowers (East 1940, Richards and Barrett 1987, Barrett *et al.* 2000). This may be due to the difficulty in achieving reciprocal herkogamy given that the anthers are frequently positioned at different levels (in multiple whorls) on opposing sides of zygomorphic flowers (Dulberger 1992).

Stamen filaments are typically not adnate to the corolla in heterostylous flowers. When this does occur, the corolla tube must elongate in order to achieve stamen elongation in the thrum morph. Dimorphic flower sizes have been noted in heterostylous species of the Boraginaceae, where filaments are generally adnate to the corolla (Ganders 1979). This condition is found in most heterostylous species in *Cryptantha* (T. Marushak

9

Chapter 2). There are examples of reciprocal herkogamy in the Rubiaceae, where various degrees of adnation of the stamens occur, and dimorphic flower size has not been reported (Dulberger 1992)

Heterostyly is generally absent from primitive flowering plant families (i.e., Magnoliaceae, Piperaceae, Lauraceae and Nymphaceae). This absence may be a function of several aspects of these flowers. Elongated receptacles, apocarpous gynoecia, spirally-arranged, numerous, and indefinite numbers of carpels and stamens would render reciprocal herkogamy functionally difficult to achieve (Dulberger 1992). An increased number of stamens prolongs the time between the start and end of stamen initiation and increases the complexity of positional effects in development (Hufford 1988). Beetle-pollination is common in these families (Dulberger 1992). Heterostyly is typically associated with segregated pollen deposition on different pollinator parts (Barrett *et al.* 2000), and beetles tend to be somewhat imprecise pollinators (Richards 1997). It would be difficult for selection favoring a complex breeding system to proceed in floral types that possess traits associated with indiscriminate forms of pollen dispersal (Lloyd and Webb 1992).

Structure and Development

Developmental studies

The structural diversity of style-stamen polymorphisms and the broad taxonomic distribution of heterostyly suggest that there may be multiple developmental pathways involved in the independent evolution of the breeding system (Lloyd and Webb 1992). Unfortunately, there have been few detailed structural or developmental studies performed, and data supporting predictions about the number and diversity of

10

developmental pathways associated with heterostyly is lacking (Richards and Barrett 1992).

Tristyly

A few simple models have been proposed for the developmental relationships that lead to the differences in stamen and style length in tristylous species. These include components that differ in the size of stamen and gynoecium primordia, differences in relative growth rates, and size differences between stamens and styles at the time of initiation. There are few data to support any of the proposed models, and none of the models have been rigorously tested (Richards and Barrett 1992). Investigations in the Pontederiaceae show that style differences among morphs arise from differences in the timing of the cessation of growth (Richards and Barrett 1984, 1987). In contrast, heterostylous species in the Lythraceae and Oxalidaceae show differences in the rate of growth of their styles (Stirling 1933, 1936, Richards and Barrett 1992).

Distyly

As with tristylous systems, developmental pathways of distyly need further investigation. Those studies that have been conducted showed that differences in growth rates, times of origin, size at origin, or timing of growth cessation may all contribute to the reciprocal placement of styles and stamens in distylous species (Richards and Barrett 1992). Stirling (1932, 1936) working with species within the Primulaceae and Menyanthaceae showed that stamen and pistil growth rates differed between morphs. Stamen and style cell number in the Primulaceae (Stirling 1932), and cell length in the Plumbaginaceae and Primulaceae (Dulberger 1975, Heslop-Harrison *et al.* 1981) were found to differ between pins and thrums. Faivre (2000) showed that modifications in

11

growth rates of the stamens relative to the corollas in members of the Rubiaceae led to differences in anther heights between pins and thrums. Her measurements also showed that style height differences originated in the bud for some genera, while others showed differences in their relative growth rates between floral morphs (Faivre 2000).

Floral Polymorphisms

There are several ancillary features of the stigmata and pollen grains that typically differ among morphs in heterostylous species. These include differences in: pollen size; exine sculpting of the pollen grains; color of the pollen grains; size of the anthers; the amount of pollen produced; size, shape, and color of the stigmata; size and shape of the stigmatic papillae; and dimorphic structure of the papilla wall (Vuilleumier 1967, Dulberger 1974, Ganders 1979, Bir Bahadur *et al.* 1984, Barrett 1988, Barrett and Richards 1989). Despite the documentation of these differences, there has been little work on the adaptive significance of these characters. Dulberger (1975 a,b, 1992) proposed that these features function in the promotion of legitimate (pin/thrum) pollinations and reduce illegitimate or within morph and self-pollinations.

Genetics of Heterostyly

There are two main forms of incompatibility: gametophytic, found in the Solanaceae, Rosaceae, Papaveraceae, and Scrophulariaceae, and sporophytic, found in the Brassicaceae. The main difference is that incompatibility in gametophytic systems is determined by the genotype of the pollen, and in sporophytic systems, incompatibility is determined by the genotype of the parent plant (Heslop-Harrison 1975). Sporophytic incompatibility is found in heterostylous plants (Lewis 1949) and the discussion here will focus on sporophytic systems, though it is important to note that it is thought that

12

heteromorphic and homomorphic incompatibility are not equivalent (Gibbs 1986, Lloyd and Webb 1992). The gene responsible for the pollen component of the incompatibility recognition process has been recently described.

Genetic studies have been performed in only 11 families on 23 species, and assumptions about the universal nature of genetic systems associated with heterostyly need to be made with caution. Pellew (1928) first proposed the concept of a 'supergene'- a group of loci that are tightly linked and so function as a single gene- as a model for the genetic control of heteromorphic breeding systems. His hypothesis was supported by a series of studies on *Primula* (Mather and De Winton 1941, Mather 1950, Ernst 1936, 1957, Lewis 1954, Dowrick 1956). The supergene model proposed for *Primula* includes three genes with 2 alleles each, where one locus controls each of the following traits: the female gynoecium (*G/g*)- style length and associated features together with incompatibility recognition (female side), pollen size (*P/p*) – along with incompatibility recognition (male side), and the male androecium (*A/a*) - anther position (Ernst 1925, 1936, Lewis and Jones 1992, Wedderburn and Richards 1992). The possibility exists for the involvement of other genes in the complex, especially in light of evidence for a gene that controls incompatibility in *Primula* (Kurian and Richards 1997). Given the array of ancillary characters that exist in heterostylous systems, it seems reasonable that there may be additional genes to control these traits. No heteromorphic chromosomes have been found in heterostylous taxa, even though the system bears similarity to a sexually dimorphic system (Lewis and Jones, 1992).

13

Genetics of pollen behavior in heterostyly

In the sporophytic incompatibility system typically associated with heterostylous plants, pollen behavior is dictated by the genotype of the diploid parent (Barrett 1988). Heteromorphic systems are controlled by an S-gene complex that also governs the structural appearance of the mating type, i.e. pin (ss) vs. thrum (Ss), and many other ancillary characters (Ganders 1979) discussed below. Heterostyly is clearly a sporophytic phenomenon, since characters associated with the stamens and styles are involved in the incompatibility syndrome. Distylous sporophytic systems are of interest in this study, and are characterized by one locus with two alleles (Barrett 1988). Dominance of the short-styled morph has been demonstrated for *Primula* (Bateson and Gregory 1905), *Linum* (Laibach 1923), *Fagopyrum* (Dahlgreen 1922, Garber and Quisenberry 1927), *Amsinkia* (Ray and Chisaki 1957), *Forsythia* (Sampson 1971), *Gelsemium* (Ornduff 1980), *Uragoga (*Baker 1958*)*, *Erythroxylum* (Ganders 1979), *Nivenia* (Ganders 1979), and *Pulmonaria* (Darwin 1877), and was originally thought to be universal. Exceptions have been found including (*Armeria maritima* (Plumbaginaceae), *Hypericum aegypticum* (Clusiaceae), and *Oxalis articulata* (Oxalidaceae)) (von Ubisch 1926, Fyfe 1956, Baker 1966, Ornduff 1979).

The absence of dominance for the thrum in *Oxalis articulata* exemplifies the importance of testing for universal dominance relationships in a family before generalizations are made. The difference in this case is within a genus, which implies that the aberration arose after the basic genetic system had been fully developed. The occurrence of a dominant pin in *Oxalis* complicates the understanding of dominance relationships of the suite of characters found in heterostyly. Assuming that the

14

dominance of all of the characters was determined at the origin of heterostyly for the genus, a subsequent change later in evolution for one species within a group would require a separate dominant operator gene that controls the whole cluster of genes. This change in dominance for the fully developed system would necessitate a crossover, transposition (via transposons), or mutation (Lewis and Jones 1992). There is evidence for the breakdown of incompatibility independently in several heterostylous taxa, where a shift from outcrossing to various degrees of selfing has been reported (Barrett, 1992). These shifts in breeding behavior represent a largely untapped source for studying the ecological and genetic basis of mating system evolution in plants.

The incompatibility system in *Anchusa officinalis* (Schou and Phillip 1984) and *A. hybrida* (Dulberger 1970) is multiallelic and is independent of the heteromorphic characters. The recognition site for incompatibility in these species is also unusual in that it occurs in the ovary and not the stigma or style (Lewis and Jones 1992).

There appear to be two recognition mechanisms that commonly occur in heterostylous species. In the Primulaceae, Clusiaceae, Oxalidaceae, Lythraceae, and Oleaceae the incompatible pollen tubes are inhibited in the style, after they have grown some distance. Style length differences alone are not essential for the incompatibility response. Rather, style length coupled with differential pollen-tube growth rate does appear to dictate the incompatibility response (Baker 1966). The incompatible pollen is inhibited on the stigma in the Linaceae, Plumbaginaceae, and the Polygonaceae, a feature well marked in the sporophytic systems of homomorphic species (Dulberger 1975b, Dulberger 1992).

15

There has been no general mechanism proposed to explain the incompatibility behavior in heterostylous plants. It seems that there have been no recent, rigorous molecular studies of these systems (Raghavan 1997).

Molecular biology of self-incompatibility

The inheritance of the incompatibility components for non-heterostylous plants too remained a mystery until recently, when evidence (Schopfer *et al.* 1999) that separate genes are responsible for the pollen and pistil incompatibility recognition process was provided. The self-incompatibility locus of *Brassica* was the first studied at the molecular level. A gene encoding a stigmatic glycoprotein, *SLG*, was cloned and sequenced. This gene differed between incompatibility types, and showed increased expression at the stage of stigma-development when the incompatibility reaction develops. Discovery of a closely linked gene, *SRK*, followed. *SRK* was named for its stigma-specific *S* locus receptor kinase product. *SRK* is also expressed at the appropriate stage of stigma development, at lower levels. The *SLG-SRK* locus has been studied in several species of *Brassica*, and many different haplotypes have been found (Charlesworth 2000). In some cases, *SLG* is absent, suggesting that *SRK* may be the essential stigma component of the self-incompatibility reaction (Okazaki *et al.* 1997, Cabrillac *et al.* 1999).

Physical mapping of the *Brassica campestris S*-gene region yielded a gene satisfying the criteria for the candidate incompatibility locus including: linkage to the *S* loci previously identified (*SLG* and *SRK*), polymorphism between different alleles, and expression in anthers or pollen. *SGR*, the pollen recognition gene, encodes a cysteine-rich protein similar to a family of pollen coat proteins that were previously identified

16

(Suzuki *et al.* 1999). A paradox revealed by the characterization of this gene is that it is expressed gametophytically, despite the genetic prediction that it should be sporophytically determined. One explanation is that the proteins determined by the two *S* alleles might be secreted during pollen development, and subsequently incorporated into the pollen coat as a mixture, thereby providing the sporophytic phenotype (Charlesworth 2000).

The current model for how the components of the *S* locus interact to form the incompatibility response involves interaction of *SCR* with *SRK* within the stigmatic papilla cells to activate a signal transduction pathway. This pathway leads to the arrest of incompatible pollen early in pollination when the *SCR* carried on the pollen coat is allelic to the recipient stigma. Recognition of *SCR* results in activation of a *SRK* protein kinase domain, that leads to an intracellular signaling cascade in the papilla cell. It has been suggested that the signaling pathway may moderate the availability of water in the stigmatic papillae for incompatible pollen (Franklin-Tong and Franklin 2000).

The Maintenance and Breakdown of heterostyly

Theory predicts a 1:1 equilibrium morph ratio for self-incompatible, distylous populations due to the mode of inheritance and disassortative mating. Frequency dependent selection maintains the mating types in self-incompatible populations. Patterns of inheritance are more complicated for tristylous populations, and the approach to equilibrium is slower (Charlesworth and Charlesworth 1979b, Barrett 1992); but a 1:1:1 ratio of floral types will prevail in a large tristylous population at equilibrium (Heuch 1979). Morph frequencies of dimorphic self-compatible species frequently

deviate from a 1:1 ratio, probably due to founder effects or differences in selfing rates between the morphs (Barrett 1992).

The most common cause of the breakdown of distyly is inefficient insect pollination (Baker 1966, Charlesworth and Charlesworth 1979b). Self-fertile homostyled species of secondary origin from heterostyled ancestors are usually found in marginal habitats for the group, such as extreme northern latitudes, where typical insect vectors are likely absent (Charlesworth and Charlesworth 1979b). When insect pollination was prevented artificially in *Amsinkia spectabilis*, homostyled individuals set more seed than heterostyled plants. Additionally, the geographic range of homostyled individuals in this species corresponds well with the hypothesis that the homostyles have an advantage due to decreased insect pollination at higher latitudes (Ganders 1975). Charlesworth and Charlesworth (1979) mathematically show that when inbreeding depression is reduced below 1/2, the frequency with which self-fertile homostyles can invade increases. The other two classes of recombinant phenotypes they considered would not benefit from the increased benefits of selfing, either because they are self-sterile (self-incompatible homostyles) or because there is a separation of anthers and stigmas in space (self-compatible pins and thrums) that lowers the rate of selfing. The breakdown of heterostyly should occur as a result of the fixation of the self-fertile homostyle that carries the dominant allele controlling pollen type (Charlesworth and Charlesworth 1979b).

Often heterostylous plant genera contain members that are homostylous, small-flowered, highly self-fertile, devoid of isozyme variation, and colonizing species. These observations are suggestive of a repeated, rapid shift to self-compatibility correlated with colonization of temporary habitats (Barrett 1989).

18

There is evidence that tristyly can also breakdown to give rise to self-fertilizing populations (Charlesworth 1979, Barrett 1988). The breakdown typically results in a self-compatible, semi-homostylous (one set of anthers adjacent to the stigma) population. The evolutionary breakdown of tristyly is gradual, and usually develops in stages of increased self-incompatibility (Barrett 1988).

Models for the Evolution of Heterostyly

There are conflicting interpretations of how heterostylous breeding systems originated. Darwin proposed that the breeding system arose due to selective pressures that favored the evolution of approach herkogamy, the condition where the stigma is presented at a height above the anthers, to increase the accuracy of pollen transfer from anthers to stigmas. The self-incompatibility mechanisms and ancillary features evolved subsequently (Darwin 1877). This view has since been supplemented by alternative scenarios: Mather and de Winton (1941) postulate that self-incompatibility arose simultaneously with reciprocal herkogamy; while Baker (1966) and Charlesworth and Charlesworth (1979a) contend that self-incompatibility was the initial feature of the breeding system. Baker (1966) proposed that the first stage in the evolution of distyly was the evolution of diallelic self-incompatibility, followed by observable dimorphisms that were superimposed stage by stage until a strict heterostylous condition was achieved. He based his conclusions on data from the Plumbaginaceae. There has been general acceptance of the latter of the two alternate explanations for the order of events in the evolution of heterostyly (Baker 1966, Lloyd and Webb 1992), and Charlesworth and Charlesworth (1979a) have described a quantitative model that adds credence to this view.

19

Charlesworths' model

In the model of Charlesworth and Charlesworth (1979a) (Figure 3a.), the ancestral flower is monomorphic, with stigmas and anthers at the same height, and diallelic self-incompatibility arises first as a mechanism to decrease selfing, thereby reducing inbreeding depression. They propose that evolution favored the spread of a functionally female mutant, with pollen that was self-incompatible and incompatible with the stigma of the original floral type, due to its increased female fitness (i.e. the wild type pollen was compatible with the stigma of the mutant). They postulate the evolution of a second mutant with a stigma mutation. This linked stigma mutation would then be compatible with the mutant pollen, and not with the original pollen type. This scenario would allow for the development of diallelic incompatibility; and, eventually reciprocal herkogamy would follow as a mechanism to promote efficient cross-fertilization. They found that a polymorphism for stigma height was not easily maintained, and thereby concluded that a change in stigma position was unlikely to lead to the evolution of heterostyly in a self-compatible species. Their model considers how changes in stigma height affect pollen transfer and female fertility (Charlesworth and Charlesworth 1979a).

Some support for this theory comes from evidence that the prevention of self-fertilization appears to be achieved more effectively by self-incompatibility mechanisms alone than by reciprocal herkogamy alone (Lloyd and Webb 1992). Description of the supergene that controls distyly in Primula (Ernst 1955, Dowrick 1956), led to the tacit assumption that comparable stigma and anther height was the ancestral condition for heterostylous taxa (since recombination of the supergene leads to a homostylous condition). This assumption resulted in additional support for the concept that the

20

Figure 3. Models for the Evolution of Heterostyly. Charlesworth and Charlesworth (1979) (a) focus on the effects of inbreeding depression as a selective force in their selfing avoidance model. Lloyd and Webb (1992) propose a pollen transfer model to explain the evolution of heterostyly (b).

a) Selfing Avoidance Model: Charlesworth and Charlesworth

Inbreeding Depression

functionally gynodioeecious stage

Homostyly

original homostyle

mutant with new pollen type

Diallelic Incompatibility

mutant with new stigma type compatible with new pollen

mutant with new pollen type

Efficient Cross Pollination

Distyly
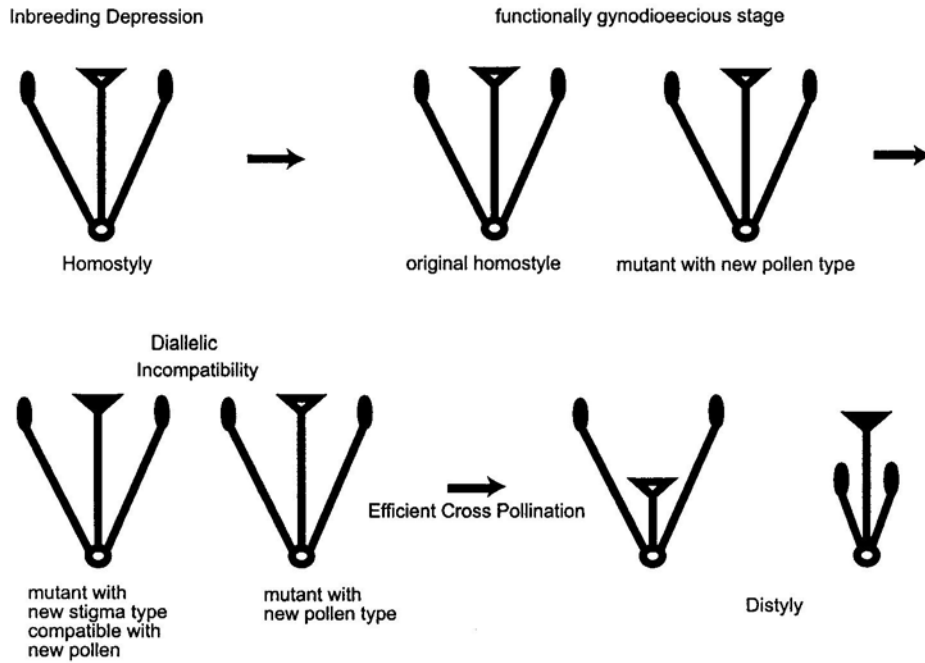
b) Pollen Transfer Model: Lloyd and Webb

Approach Herkogamy

Efficient Cross Pollination

Reciprocal Herkogamy

Distyly

incompatibility evolved as a pleiotropic by-product of selection for increased pollen competitive ability on the style type to which pollen most frequently transferred

22
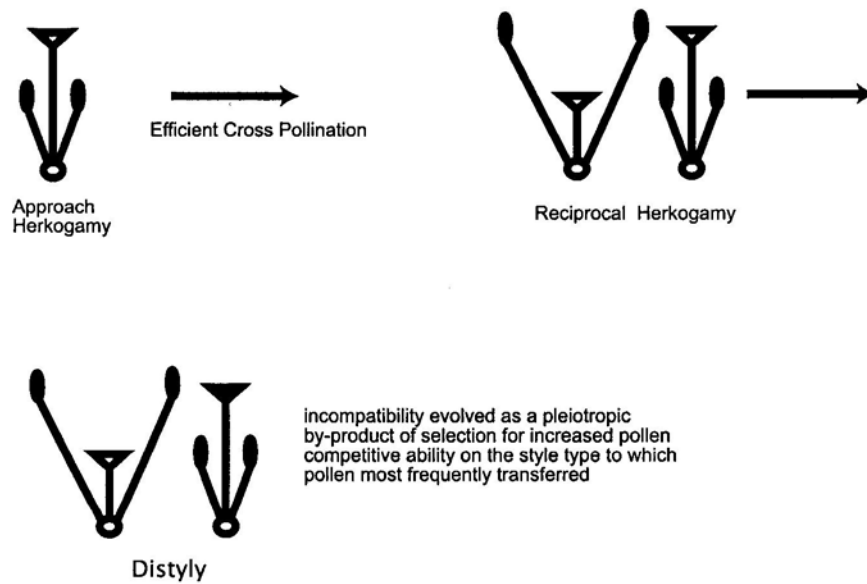
selective advantage of heterostyly lies in the ability to prevent selfing, and that self-incompatibility evolved before the polymorphism in stigma and anther height (Lloyd and Webb 1992). Ganders (1979a) disagreed with the proposed homostylous ancestral condition for this model, but agreed with the overall sequence of events. Lloyd and Webb (1992) also believe that an ancestral homostylous condition was unlikely, especially since the homostylous state is the result of a breakdown in heterostyly. They consider it improbable that the product of reshuffling a specialized set of coadapted characters (i.e. the supergene) would resemble the ancestors of heterostylous species which presumably lacked most of the advanced features of the breeding system (Lloyd and Webb 1992).

Lloyd and Webb's model

Lloyd and Webb (1992) (Figure 3b) follow Darwin (1877) and propose that evolution of reciprocal herkogamy arose first from an ancestral approach herkogamous individual. Self-incompatibility is proposed to have evolved secondarily as a pleiotropic by-product of selection for increased pollen competitive ability on the style type to which pollen is most frequently transferred. This scenario reduces the cost of lost ability to fertilize plants of the same incompatibility type, which is high in the first model. Lloyd and Webb (1992) focus on how changes in stigma height affect pollen donation and male fertility. In their model, the spread of the reverse herkogamous (thrum) mutant is favored due to its increased male fitness relative to the average approach herkogamous plant. The increased male fitness is attributed to the decreased interference by the female organs in the reverse herkogamous individuals. A number of stigma-height polymorphisms have been described in genera with heterostylous, self-compatible members, which could represent the plesiomorphic state (Lloyd and Webb 1992).

23

Support for this model derives from the pattern of inheritance in distyly. An advantageous mutant can be favored by selection immediately if it is caused by a dominant allele, and is consequently expressed in every individual that carries it (Fisher 1922, Haldane 1927). Assuming that this factor has influenced the establishment of dominant stylar mutants found in heterostylous systems, the observed pattern of dominant short-styled (Ss) mutants would arise only if the form with reverse herkogamy (short stigma and long anthers- the thrum) is the derived condition. That is, the plesiomorphic state would be approach herkogamy (ss).

Interpretations

It is important to note that the two models differ in their interpretation of the character state for the intermediate stages of heterostyly. The Charlesworth and Charlesworth model (1979a) assumes that the self-compatible heterostylous condition is apomorphic. They predict that a crossover during meiosis in the region of the supergene controlling heterostyly accounts for the loss of self-incompatibility (Charlesworth and Charlesworth, 1979b). The Lloyd and Webb (1992) model predicts the occurrence of self-compatible heterostylous individuals as the pleisiomorphic condition, since they propose that reciprocal placement of the anthers and the stigmas among the two forms arose before self-incompatibility. The two models emphasize different selective forces; Charlesworth and Charlesworth (1979) invoke antiselfing, while Lloyd and Webb (1992) propose cross-pollination as the mechanism responsible for the assembly of the heterostylous syndrome (Barrett *et al.* 1996).

Empirical tests of the models

Stone and Thompson (1994) looked at pollen transfer in artificial flowers to determine if empirical pollen-transfer probabilities support the conditions necessary for the evolution of distyly. They found support for establishment of a stigma-height polymorphism, the first step proposed by the Lloyd and Webb model. The conditions for the second step in the model, an anther-height polymorphism, were not met by pollen transfer probabilities alone. However, the authors derive support for the model from these data, since inbreeding depression was not measured, and inbreeding levels are an important component of the model. Kohn and Barrett (1992), assuming the thrum was the ancestral state, showed support for the idea that the selective basis for the establishment of heterostyly could have been increased pollen transfer and not higher outcrossing levels. Faivre (2000) used a developmental approach to show how the morphological transitions predicted by Lloyd and Webb (1992) may be achieved. She compared the floral development of two approach herkogamous individuals to that of two heterostylous individuals in the Rubiaceae. Differences in the relative rates of style elongation suggested the addition of a step where stylar growth in the ancestral approach herkogamous morph is adjusted to that typical of the long-styled individual (pin). Scribailo (1987) and Barrett (1990) found that there was no uniform rejection response in tristylous *Pontederia* (Pontederiaceae) in pins and thrums. They found that incompatibility in this system operates through failure of pollen tubes to grow in particular stylar environments, rather than from recognition of shared pollen and style specificities. These data support the concepts put forth in Lloyd and Webb's model.

25

Graham and Barrett (1995) and Kohn *et al.* (1996) also found support for Lloyd and Webb's model in the Pontederiaceae. These studies used partial sequences from *rbcL* and ndhF and chloroplast restriction site data to reconstruct the phylogeny among approximately two-thirds of the family, and mapped the reproductive characters (tristyly, self-incompatibility, and enantiostyly) onto the phylogenetic tree. Tristyly was found to evolve either once or twice, and the evolution of a reciprocal polymorphism in stigma height preceded the evolution of self-incompatibility according to their data (Kohn *et al.* 1996). Schoen *et al.* (1997) surveyed restriction site variation in the chloroplast of *Amsinkia* (Boraginaceae) to analyze the evolutionary history of the mating system. When distyly was treated as ancestral in the group, or when the loss of distyly was considered common relative to its gain, their results support the hypothesis that self-fertilizing taxa are of recent origin from outcrossing relatives. The authors did not interpret these results as support for either model. These studies are among the first to use a molecular approach to determine the order of evolution for reproductive characters in breeding systems.

Significance

A major problem in determining the path for the evolution of heterostyly has been the inability to identify patterns in floral variation which represent the intermediate steps in the assembly of the polymorphism (Barrett 1992). Very little is known about the vast majority of heterostylous groups, which makes it difficult to identify the floral characteristics of heterostylous lineages (Barrett 2000). Few heterostylous families show a clear development of the polymorphism, and there is disagreement regarding the order

26

of establishment of the physiological and morphological aspects of the breeding system (Charlesworth and Charlesworth 1979, Lloyd and Webb 1992, Barrett 2000).

In the genus *Cryptantha* (Boraginaceae), a variety of mating systems are present, some of which appear to represent intermediate stages in the evolution of distyly (Figure 4). At present, the ancestral character state for heterostyly is unknown, and phylogenetic studies of heterostylous groups are required to determine the characteristics found in the ancestors of heterostylous taxa. In the following chapters, floral characters associated with heterostyly in *Cryptantha* will be evaluated and mapped on to a phylogeny for the group to determine the relative order of the evolution of self-incompatibility and dimorphism.

Figure 4. Variation in floral morphology in *Cryptantha*. All combinations of self-incompatibility and mono/dimorphism have been observed in *Cryptantha*. The monomorphic condition is always reverse herkogamous, with the anthers positioned above the stigma.

Breeding system variation in *Cryptantha*
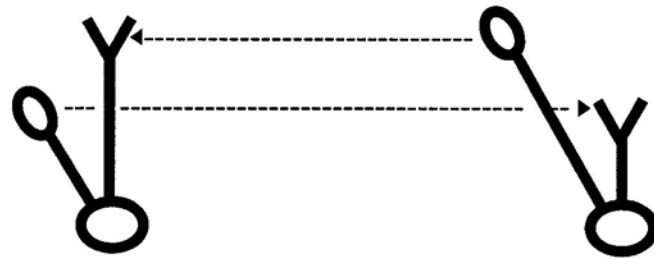
Dimorphic and self-incompatible

Dimorphic and self-compatible
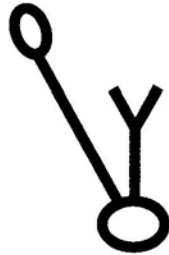
Monomorphic and self-incompatible

Monomorphic and self-compatible

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Evaluation of self-compatibility in homostylous and heterostylous members of *Cryptantha* Section Oreocarya

Introduction

Plants display an array of mating systems, ranging from obligate outcrossing to obligate selfing (Schemske and Lande 1985, Lloyd and Schoen 1992). Distinguishing patterns of self- and cross-fertilization in a phylogenetic framework is key to developing an understanding of mating system evolution (Weller and Sakai 1999), especially due to the debate regarding the ancestral condition in angiosperms. The historical view is that self-incompatibility is ancestral and was a major factor in the success and radiation of the angiosperms (Whitehouse 1950, Crowe 1964). However, preliminary phylogenetic data support the idea that the earliest angiosperms were self-compatible, though this result varies with different weighting schemes (Weller et al. 1995).

Self-incompatibility in angiosperms collectively represents a complex assortment of breeding systems (DeNettancourt 1977) that results in rejection of self-pollen or pollen of the same incompatibility type (Charlesworth 1985, Weller et al. 1995). The different forms of self-incompatibility are characterized by distinct genetic systems and physiologies (Richards 1997). Homomorphic incompatibility is found in 60-90 angiosperm families (Charlesworth 1985). In this type of incompatibility the mating types are morphologically indistinguishable, but self or closely related matings do not produce viable seed. The reactions that lead to incompatibility between sperm and egg can be classified as sporophytic or gametophytic, depending on the genotype expressed by the pollen donor (DeNattencourt 1977). Homomorphic incompatibility systems are

37

usually multi-allelic, i.e., incompatibility recognition factors are controlled by many alleles at the same locus (Richards 1997).

In heteromorphic incompatibility systems, the incompatibility types are morphologically distinct, with reciprocal placement of stigmata and stamens in each morph (Barrett 1990). Distylous systems have two morphs, the long-styled 'pin', with a genotype of (ss), and the short-styled 'thrum', with a genotype of (Ss). Tristylous systems have three morphs: short-styled (Ssmm), mid-styled, (ssMm) and long-styled (ssmm) (Lewis and Jones 1992). The mating system chromosomes (S and s) act in an allelic fashion, so that the control of the mating system is effectively diallelic (Richards 1997). The two mating types may differ from one another in a suite of characters (Dulberger 1992). Some of these traits have been shown to recombine in crossing experiments (Lewis and Jones 1992), demonstrating that S and s represent a coadapted linkage group often referred to as a 'supergene' (Richards 1997). Heteromorphic incompatibility is sporophytically controlled, and found in approximately 25-27 angiosperm families (Weller *et al.* 1995).

Detection of self-incompatibility can be difficult (Charlesworth 1985). Studies range from the most thorough involving a complete genetic analysis for the determination of gametophytic versus sporophytic self-incompatibility, to those with crossing programs involving selfs and outcrosses. Thorough studies of self-incompatibility systems are rare, especially in woody plants with long generation times. Mating systems are more commonly evaluated by crossing experiments (Weller *et al* 1995).

The genus *Cryptantha* (Boraginaceae) is a group of about 150 species, mostly found in dry, rocky, or montane habitats. Members of *Cryptantha* in North America are

38

subdivided into four sections: Krynitzkia, Oreocarya, Eremocarya, and Piptocalyx (Higgins 1971). This study will focus on members of section Oreocarya, which has both monomorphic and dimorphic members (Cronquist 1984). Many of the heteromorphic species lack the self-incompatible condition typical of the breeding system (Casper 1985). Those species in the group that are monomorphic are reverse herkogamous, with their stamens presented above their stigma (Chapter 3). In general, the plants included in Oreocarya are herbaceous with a highly branched, woody caudex that bears a rosette of leaves and gives rise to a flowering stem, and a variably branched taproot. The members of this group are perennials that occupy mostly xerophytic habitats at middle elevations (Higgins 1971).

This study was designed to examine patterns of self-incompatibility in species of *Cryptantha* Section Oreocarya. The goal was to determine the strength of the incompatibility reactions in the group and to compare plants that have been described as heterostylous with those that are monomorphic.

Self-compatibility and self-incompatibility are not absolute classifications and are often distinguished quantitatively rather than qualitatively (Fryxell 1957, Becerra and Lloyd 1992, Lloyd and Schoen 1992). Frequently, criteria are developed that place arbitrary boundaries that categorize a taxon as self-compatible or self-incompatible in comparison to the seed set of outcrossed individuals. For example, a plant may be considered self-incompatible if selfing yields less than 20% of the seed set obtained by outcrossing (Bawa 1974, Zapata and Arroyo 1978). Vogler and Kalisz (2001) recommend the treatment of mating systems as a continuous variable, rather than a discrete character, due to the range of intermediate outcrossing rates for animal-pollinated

39

species. Accordingly, a self-compatibility index was calculated after Becerra and Lloyd (1992) for this study.

Methods

Breeding system data were gathered in the spring of 1999 and 2000. Populations with at least 10 plants were located for the species listed in Table 1, primarily in Utah, but with a few in Arizona. Plants with flowers that were in late bud stage were located and flowers were marked with a permanent marker, using different colors for self and outcross treatments. Flowering stalks were covered with a fine mesh bag for 2-4 days. Pollination was accomplished by brushing an anther sac on the stigma of the treated flower. The anther was repeatedly brushed on the stigma until a coating of pollen was evident through a 10X hand lens. Flowers were either selfed by using an anther from an open flower on the same plant, or outcrossed by using an anther from an open flower of another plant. Thrums and homostylous species (all homostylous species in this study exhibit the thrum morphology) were emasculated in bud the day the plant was bagged. Anther sacs were removed with the aid of a pair of fine tweezers, and care was taken not to displace the stigma during application of the pollen. Tweezers were cleaned between pollinations by dipping them in 95% Ethanol. When the anther sacs were located deep within the corolla, the corolla was sliced lengthwise with tweezers to gain access. Hand-pollinations were done between 2 and 4 days after the plant was bagged. Time and sample size limitations did not allow a separate treatment to investigate cryptic incompatibility. All flowers in the appropriate stage were used in pollination treatments. In some cases (*Cryptantha grahamii, C. osterhoutii, and C. longiflora*), the populations were smaller than 10 plants, or 10 plants were bagged, but the bags were displaced by

40

Table 1. Location data for species used in this study

Species	Location	
*C. abata**	N 37° 42.031'	W 112° 16.715'
*C. bakeri**	N 39° 04.092'	W 109° 41.941'
*C. breviflora**	N 40° 35.716'	W 109° 26.194'
C. capitata	N 37° 05.212'	W 111° 59.067'
C. cinerea var.	N 36° 51.157'	W 111° 59.565'
*C. cinerea var. cinerea**	100 m S of Red Canyon Campground, Garfield county Utah	
C. confertiflora	N 37° 05.250'	W 111° 59.052'
C. flava	N 40° 35.716'	W 109° 26.194'
C. fulvocanescens	N 38° 55.949'	W 110° 36.107'
*C. grahamii**	N 39° 50.832'	W 109° 37.147'
*C. humilis**	behind Univ. of Utah hospital center	
C. johnstonii	N 39° 10.620'	W 110° 29.901'
C. jonesiana	N 38° 53.032'	W 110° 39.389'
C. longiflora	N 38° 37.818'	W 109° 47.971'
*C. osterhoutii**	N 38° 49.531'	W 109° 17.058'
C. paradoxa	N 38° 53.139'	W 110° 39.469'
*C. rollinsii**	N 39° 45.630'	W 109° 36.641'
*C. tenuis**	N 38° 43.678'	W 109° 21.016'
*C. virginenesis**	N 37° 15.335'	W 113° 37.656'
*C. wetherillii**	N 39° 17.496'	W 110° 52.401'
*C. fendleri**	N 37° 15.335'	W 113° 37.656'
*C. racemosa**	N 37° 15.335'	W 113° 37.656'
*C. torreyana**	N 36° 51.456'	W 112° 43.905'

GPS data were not available for *C. cinerea var. cinerea* or *C. humilis*.
All monomorphic species (based on Chapter 3 results) are marked with an *.

wind or animals when revisited for pollination treatments (or collection of seed). In these cases, treatments were performed on the remaining plants.

The bags were collected approximately two months after the pollination date by breaking the bagged flowering stalk at the base, and placing the covered stalk within a paper bag. The differences in the marks made on each calyx remained apparent after the calyx turned brown, but to minimize error, a dissecting scope was used to identify separate treatments. Count data representing the number of nutlets produced for each type of cross are shown in Table 2. Nutlets were categorized either as undeveloped or fully developed. The percent of the total nutlets that developed was calculated for both types of crosses. For two additional species, *Cryptanatha humilis*, and *C. flava*, count data were from Casper (1984, 1985). *C. humilis* is monomorphic and self-incompatible (Casper 1984), while *C. flava* is dimorphic but self-compatible (Casper 1985).

There are a maximum of 4 nutlets per fruit possible, though it is common in this genus for fewer than 4 to mature per flower (Welsh et al. 1987, Casper1981). When a nutlet falls out of the calyx, a clear scar is left behind (Brenda Casper pers. comm.). In a few cases there was no apparent scar, so the nutlet was not scored.

The self-compatibility index was calculated as follows for each taxon: the fraction of fully developed nutlets from selfed flowers was divided by the fraction of fully developed nutlets from outcrossed flowers. As the index approaches unity, the species is more self-compatible.

Seed set data were analyzed with a Wilcoxon Signed Rank test. The percent developed nutlets was compared with the percent undeveloped nutlets for monomophic

42

Table 2. **Seed set data for hand pollinations in** *Cryptantha.* The percent of developed seed for selfed and outcrossed pollinations are presented. Sample size for both the number of plants and the number of flowers are shown., with the number of outcrossed and selfed treatments in parentheses. The plant sample sizes below 10 are due to loss of pollination bags by abiotic and biotic factors, except for *C. osterhoutii*, where only small populations were found. Monomorphic species are located in the upper half of the table, and heteromorphic species are in the lower half. Two geographically separate populations of *C. cinerea* were sampled, one from central Utah and one from Northern Arizona.

Species	# of plants	# of flowers (selfed, outcrossed)	Selfed developed	undeveloped	% developed	Outcrossed developed	undeveloped	% developed	Self-compatibility index
C. abata	11	38 (12, 21)	15	53	22%	33	51	39%	0.6
C. bakeri	10	36 (17, 19)	31	35	47%	37	39	49%	1.0
C. breviflora	20	70 (31, 39)	4	120	3%	10	146	6%	0.5
C. cinerea var. cinerea	17	34 (17, 17)	36	72	33%	62	46	57%	0.6
C. cinerea var. cinerea	16	43 (19, 47)	13	63	17%	36	58	38%	0.4
C. grahamii	6	13 (8, 5)	3	27	10%	15	5	75%	0.1
C. humilis var. *nana*	10	94 (51, 43)	6	45	12%	35	8	81%	0.2
C. osterhoutii	4	22 (11,11)	5	39	11%	22	22	50%	0.2
C. virginenesis	16	56 (29, 27)	16	100	14%	55	53	51%	0.3
C. capitata	12	68 (36, 33)	17	124	12%	50	81	38%	0.2
C. confertiflora	16	78 (40, 38)	35	125	22%	66	86	43%	0.5
C. flava	28	324 (213, 111)	159	54	75%	73	38	66%	1.1
C. fulvocanescens	10	40 (22, 18)	35	53	40%	34	36	49%	0.8
C. johnstonii	10	62 (32, 30)	28	100	22%	32	88	27%	0.8
C. jonesiana	15	64 (34, 30)	66	70	49%	70	48	59%	0.8
C. longiflora	5	32 (16, 16)	9	55	14%	19	42	31%	0.5
C. paradoxa	16	45 (21, 24)	28	56	33%	48	48	50%	0.7
C. rollinsii	13	77 (43, 34)	46	126	27%	58	78	43%	0.6
C. tenuis	11	37 (17, 20)	15	53	22%	28	52	35%	0.6
C. wetherillii	10	35 (18, 17)	43	29	60%	39	29	57%	1.0

44

and heteromorphic species, respectively (Table 2). A Mann-Whitney U test was used to compare the self-incompatibility indices between homomorphic and heteromorphic taxa.

Results

   Cryptantha breviflora appears to be easily damaged during hand-pollination; it was common for the stigma to begin to brown as soon as one day after emasculation. Seed set in this species was very low for both selfed and outcrossed flowers. *Cryptantha grahamii* appeared to be frost damaged during the unusually cold spring of 2000, resulting in very small sample sizes for this species. The small sample sizes for *C. grahamii* and *C. osterhoutii* make conclusions about their degree of self-compatibility tenuous, despite the statistically significant differences between selfed and outcrossed flowers.

   There was a significant difference between selfed and outcrossed seed set for both the heteromorphic (p= .0355) and monomorphic (p≤ .0001) taxa. The self-compatibility index for the dimorphic species ranged from 0.2 to 1.1 (Table 2). The self-compatibility index was derived from published data (Casper 1984, 1985) for *Cryptantha humilis* and *C. flava*. The heteromorphic taxa surveyed were more self-compatible than the monomorphic taxa (p=.0180, Table 2); that is, the monomorphic species had significantly lower mean seed sets for selfed compared to outcrossed flowers.

Discussion

   Outcrossed seed set ranged from 6-75%, with the lowest value in *Cryptantha breviflora* (6%). An overall low seed set, with only one or two of the four nutlets developing per flower, was previously demonstrated in *Cryptantha* (Casper 1985). The

45

low values for outcrossed seed set for *C. breviflora* and *C. grahami* may be a result of extrinsic factors (i.e. sensitivity to treatments, frost damage - see Methods) and may not actually reflect typical results (especially given the small sample size for *C. grahamii*).

The self-compatibility index helped to correct for random error associated with fertilization; it diluted the effect of unexplained failure of pollination thus providing a more objective measure of self-compatibility (Becerra and Lloyd 1992). Nevertheless, the increased levels of self-incompatibility in the monomorphic species compared to the heteromorphic species are contrary to what one might expect. The heteromorphic species have been described as heterostylous (Higgins 1971, Cronquist 1984), and diallelic incompatibility is one of the hallmarks for this breeding system (Barrett 1990). The higher levels of self-incompatibility in monomorphic species may result from the close proximity of the styles and stamens (Chapter 3). Limited separation of the stigmata and stamens may be expected to have increased rates of self-fertilization (Lloyd and Webb 1986). If the resultant progeny were of low fitness (due to inbreeding depression, etc), barriers to self-fertilization (self-incompatibility) would be advantageous, and selection favoring its maintenance may have been strong. Additionally, self-incompatibility appears to be ancestral in Oreocarya (Chapter 4). This means that self-compatibility in the heteromorphic species is a derived condition, implying the presence of an ancestral heteromorphic, self-incompatible ancestor in *Cryptantha*.

Results from crossing experiments comparing seed set from selfed and outcrossed pollination treatments can be confounded by inbreeding depression (Weller *et al.* 1985). Inbreeding depression cannot be ruled out as a factor contributing to the observed low seed set after self-pollination for the monomorphic species of *Cryptantha*. To determine

46

the influence of inbreeding on the observed differences in seed set between selfed and outcrossed treatments, one would first have to screen a subpopulation for each species to determine the magnitude of inbreeding depression, which can be estimated by measures of heterozygosity (Futuyma 1998). This could be done with a codominant molecular marker, such as microsatellites (Whitehouse and Harley 2001). Once a population had been surveyed, selfed and outcrossed treatments could be performed on the individuals with known inbreeding coefficients, and these coefficients could be compared with the self-compatibility index to determine if a correlation was present. If higher levels of inbreeding consistently resulted in a low self-compatibility index, one could argue that the low index was a result of inbreeding, and thus not a robust estimate of self-compatibility.

The data presented here constitute a broad survey of self-compatibility in Oreocarya. It is possible that the data characterize a species as self-compatible, while the actual frequency of self-pollination is extremely low, due to herkogamy, or other factors that promote cross-pollination. The most direct way to provide the advantages of both self- and cross- pollination is by delayed selfing, which gives outcrossing a temporal preference (Lloyd 1992). Recently, self-incompatibility has been described as a quantitative, phenotypically-plastic trait, rather than a qualitative trait of the breeding system. Many species exhibit pronounced phenotypic variation in the expression of self-incompatibility that is often related to environmental conditions (Stephenson *et al.* 2000). The expression of self-incompatibility has been shown to vary 1) with the action of weak versus strong *S*-alleles, 2) with the expression of modifier genes, 3) with the relative quantities of self-pollen versus mixtures of self and cross pollen, 4) with environmental

47

conditions (e.g. temperature), and 5) with stylar conditions (e.g. age of flower, time of year, presence of developing fruit (Ascher and Peloquin 1966, de Nattencourt 1977, Stephenson and Bertin 1983, Mulcahy 1984, Lloyd and Schoen 1992, Levin 1996, Vogler *et al.* 1998).

When both self- and cross-pollen are deposited on the same stigma, their relative competitive abilities are likely to differ (Lloyd and Schoen 1992). Darwin (1876) coined the term prepotency to describe the phenomenon where cross pollen outcompetes self-pollen, thereby limiting the frequency of self-fertilization in self-compatible species. More recently, the term cryptic-incompatibility has been used to describe the advantage of outcross-pollen relative to self-pollen in mixed pollinations of self-compatible plants (Bateman 1956, Casper et al. 1988,Schoen and Lloyd 1992, Eckert and Allen 1997). Additional names have been adopted to describe variations in the expression of self-incompatibility including incomplete, weak, leaky, partial, pseudo-incompatibility, etc. Evolutionary biologists have only recently begun to consider the potential roles of plasticity in the expression of self-incompatibility (Stephenson *et al.* 2000). The levels of self-compatibility observed here are first estimates of self-compatibility for *Cryptantha*. The presence of cryptic incompatibility was not tested in this study; however, Casper (1985) demonstrated that cryptic incompatibility was absent in *C. flava.* Incompatible cross-pollinations (i.e. any cryptic incompatibility) will yield an inflated self-compatibility value. More extensive study of compatibility in *Cryptantha* is needed to determine if there is variation in self-compatibility between individual populations within a species, and to verify the potential for cryptic incompatibility within the group.

48

Casper et al. (1988) found that cryptic incompatibility (or prepotency) was acting in *Amsinkia douglasiana*. A very small percentage of seeds developed from self-pollen when a mixture of self and outcross pollen was deposited on the same stigma. Partial incompatibility would allow self-fertilization to enhance seed set when outcrossing levels are low. The 'best-of-both-worlds' hypothesis as described by Becerra and Lloyd (1992) explains the maintenance of intermediate levels of selfing, and seems to be acting in *Cryptantha*. They propose that the advantages of both outcrossing and selfing are best accomplished when there is some level of 'prepotency'(cryptic incompatibility) that allows outcrossing to prevail when feasible.

Charlesworth and Charlesworth (1979) used mathematical models to demonstrate that heterostyly can break down as the result of fixation of a modifier that renders the incompatibility reactions ineffective. The modifier is unconnected to the supergene associated with the control of heterostyly, and can spread in a population and become fixed when inbreeding depression is less than 1/2 (Charlesworth and Charlesworth 1979). Empirical evidence supporting the evolution of modifier loci responsible for phenotypic changes in floral architecture that result in increased self-fertilization has been demonstrated in *Eichornia paniculata* (Fenster and Barrett 1993). There are no studies to date that indicate similar patterns in distylous species, but the potential exists that similar patterns occur in the breakdown of distyly (Charlesworth and Charlesworth 1979).

Lloyd (1992) reviews a series of genetic and functional factors that favor the maintenance of intermediate levels of self-fertilization. These include local adaptations enjoyed by self-fertilized progeny when they are dispersed within close range to the parent plant, compared to the hybrid advantage of outcrossed progeny in more distant

49

environments (Holsinger 1986), and the advantage of being able to self to compensate for a deficit in seed set.

These factors could influence the maintenance of a mixed mating system in *Cryptantha*. Observations in both plants and animals demonstrate an increased incidence of developmental instability under stress conditions (Lerner 1954, Beardmore 1960, Griffing and Langridge 1963, Huether 1968, Levin 1970). Barrett and Harder (1992) found that unfavorable growing conditions promote the production of self-compatible flowers in genotypes displaying floral instability in both natural and greenhouse populations of *Eichornia paniculata*. The unique geological substrates of Utah provide different soil types over short distances. It seems reasonable that both selfed and outcrossed progeny would experience frequent dispersal events to soil types different from that of the parental generation. If their ability to survive in a new environment varies, it seems reasonable that both cross- and self-fertilization could be favored under different selection regimes.

The high elevation desert southwest experiences extreme variations in annual weather patterns (Cayan et al. 1998). It is not uncommon to have a cold or dry spring. Naturally, these fluctuations affect the population densities of the local flora and fauna, including insect pollen vectors (Carroll et al. 2001). If there were variation in the number of pollinators from one year to the next, selection would favor the ability to set seed via self-fertilization in the absence of the vectors that facilitate cross-pollination (Lloyd 1992). Limited pollen flow could be exacerbated if *Cryptantha* population densities also decrease due to climatic variation thus limiting potential mates (Cruzan *et al.* 1994). Stephenson *et al.* (2000) showed that the outcrossing rate of individual plants of

50

Campanula rapunculoides could vary with pollinator availability (which varies by year and location), and conclude that plasticity in the self-incompatibility system translates to plasticity in the breeding system of *C. rapunculoides*. Self-compatible dimorphic species are found throughout the Boraginaceae (Ganders 1979, Barrett 1992). The presence of variability in the levels of self-compatibility in these species related to external factors (environment, etc.) may reveal selective regimes that lead to steps in the breakdown of the heterostylous syndrome.

Herkogamy may be enough to increase the frequency of cross-pollination in *Cryptantha*, thus limiting the amount of pollen available for self-fertilization. Spatial separation of anthers and stigmas in self-compatible species, or in species that exhibit some degree of self-compatibility, likely contributes to the avoidance of self-fertilization, as well as reducing the interference between pollen receipt and pollen dispersal. Minimizing pollen-stigma interference may be accomplished by the spatial separation of pollen dispatch and pollen receipt within a hermaphroditic flower (Webb and Lloyd, 1986).

Additional pollination treatments to test for success of outcross vs. self pollen when deposited on the same stigma would address the presence of cryptic incompatibility in this genus. In order to directly test the cause for the maintenance of self-compatibility in *Cryptantha*, more tests needs to be done, across populations of the same species, to obtain rigorous data. These data are especially remarkable due to the heterostylous nature of the more 'self-compatible' species in this group, and may represent species in the process of becoming heterostylous, or of a breakdown in heterostyly. The

51

phylogenetic relationship between the heteromorphic and monomorphic species within

this genus should shed light about the stage of breeding system evolution in the group.

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54

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Variation associated with style and stamen position in heteromorphic and monomorphic species of *Cryptantha* (Boraginaceae)

Introduction

Angiosperms show considerable variation in floral traits that influence mating patterns (Riveros *et al*. 1987, Barrett *et al*. 1996, Mal and Lovett-Doust 1997, Hermann *et al*. 1999, Thompson and Dommee 2000, Faivre and McDade 2001). If variation in floral morphology affects reproductive success, then we may expect natural selection to act on variation among conspecific populations and closely related congeneric species. For example, the prevalence of certain pollinators may select for changes in floral size, reward, or color to increase the effectiveness of pollination (Nilsson 1988, Cresswell and Galen 1991, Galen 1996, Johnson and Steiner 2000). Self compatibility (selfing) may be favored in new habitats or at the edge of a species range, and this may lead to changes in floral traits that would increase the effectiveness of selfing. In the *Mimulus* complex, selfing and out-crossing species have dramatic differences in floral morphology (Ritland and Ritland 1989).

Herkogamy, the spatial separation of stigmas and anthers, has been interpreted as a mechanism to limit interference of male and female function in hermaphroditic flowers (Webb and Lloyd 1986, Lloyd and Webb 1992). Herkogamy is divided into classes delimited by the number and type of variation within a species, including a single floral type (homomorphic herkogamy), two floral types - one with long stamens and short

57

styles, and another with short stamens and long styles (reciprocal herkogamy), and interfloral forms, including a class with variation in either style or stamen length, but not both (Kerner 1902, Webb and Lloyd 1986, Barrett *et al.* 1996). Homomorphic herkogamy can be further subdivided into unordered, where anthers and stigmas are separated in a fashion that allows a pollinator to contact both in various sequences, and ordered, where the pollinator usually contacts the anther and stigma in the same order once per visit. Approach herkogamy, where the stigma is positioned above the anthers, and reverse herkogamy, where the anthers are positioned above the stigma, are examples of ordered herkogamy (Webb and Lloyd 1986).

Heterostyly is a breeding system with reciprocal herkogamy, and is believed to promote outcrossing by limiting self-fertilization (Charlesworth and Charlesworth 1979, Lloyd and Webb 1992). Heterostyly includes distylous systems, which have a long-styled (LS) and short-styled (SS) morph, coupled with short and long stamens, respectively. Heterostyly may also include tristylous systems, which have a LS, mid-styled, and SS morph (Darwin 1877, Vuilleumier 1965). The stylar polymorphism is often accompanied by a sporophytically controlled, diallelic self-incompatibility system that prevents self and within morph fertilization (Charlesworth and Charlesworth 1979, Shore and Barrett 1985, Barrett 1990, Barrett 1992).

The genus *Cryptantha* (Boraginaceae) section *Oreocarya*, includes species that have either monomorphic-reverse herkogamous- or dimorphic flowers, as well as species that are either partially or completely self-incompatible (Higgins 1971, Cronquist 1984, Casper 1985). The presence of variation in both floral morphology, and self-incompatibility in *Cryptantha* implies that the breeding system of this group may be in a

58

state of flux. The taxonomic literature makes qualitative descriptions for members of the *Oreocarya* as heterostylous or homostylous, but most taxa have not been described quantitatively (Higgins 1971, Cronquist 1984). To evaluate evolutionary status of heterostyly within the group requires an objective, quantitative analysis of floral morphology. Accordingly, measurements presented here examine the patterns of sex-organ variation in 28 perennial species of *Cryptantha* (Boraginaceae).

There exist two theoretical models describing the evolutionary pathway to heterostyly. The two models differ in the predictions for the evolutionary order of self-compatibility and reciprocal herkogamy, and in the presumed ancestral condition (homostylous- with stigmata and anthers at equal positions within the flower-, and approach herkogamous) (Charlesworth and Charlesworth 1979, Lloyd and Webb 1992).

The objective of this study was to determine the between species variability for stigma position, anther position, and corolla size for both heteromorphic and monomorphic species of *Cryptantha*. These data will identify patterns associated with herkogamy in species of *Cryptantha*, which can be compared to the predictions of the mathematical models describing the evolutionary process for heterostyly. Furthermore, these data will help to clarify the classification of *Cryptantha* species previously described as 'partially' or 'incompletely' heterostylous, and to identify the role these species assume in the evolution of the breeding system (Higgins 1971, Cronquist 1984).

Methods

Flowers were collected for measurement from natural populations and from herbarium sheets for 28 species of *Cryptantha* during May and June of 1999, 2000, and 2001. Vouchers for all populations that were sampled are stored at the Norton-Brown

59

Herbarium (MARY) at the University of Maryland, College Park campus. Herbarium sheets from the Brigham Young herbarium (BYU) and from the Smithsonian herbarium (US) were used as a source for flowers, to increase sample size, or to obtain samples from species that were otherwise inaccessible. Flowers from herbarium sheets were rehydrated with a 10% soap solution prior to morphological measurements.

Anther height, corolla length, and stigma height were measured as illustrated in Figure 5. Since members of the Boraginaceae possess a gynobasic style – one that is attached to an elongation of the receptacle- (Welsh *et al.* 1993), the point of attachment of the corolla to the receptacle was used as a baseline for measurements. To minimize pseudo-replication, no more than three flowers per plant (fewer from herbarium sheets) were sampled. Flowers that were collected in the field were placed in 70% ethanol and were transferred to a 3:1 ethanol:glacial acetic acid solution for storage. To expose the anthers and styles for measurement, the corolla was sliced lengthwise with a pair of fine tweezers. Measurements were taken at 1.5x magnification under a dissecting microscope using a conventional Quilix probe (Hu-Friedy, Chicago, Illinois) calibrated with mm increments ranging from 1-15 mm. Observations were rounded to the nearest 0.5 mm. Micrographs were taken of exemplar pins, thrums, homostyles, and approach herkogamous monomorphic species (Appendix I).

All statistical analyses were conducted in Statistica, 1999 edition (StatSoft, Inc. Tulsa OK). To quantify variation in floral morphology between individuals within a species, a k-means cluster analysis was used on the data for anther height, stigma height, and corolla length. The k-means cluster analysis groups observations into a specified number of clusters. These data were analyzed with the parameter k=2, choosing the

Figure 5. Diagrammatic representation of the measurement variables used to quantify floral morphology in various *Cryptantha* species. Measurements were taken with a standard Quilix probe calibrated with mm increments at 1.5 X magnification using a dissecting microscope.

62
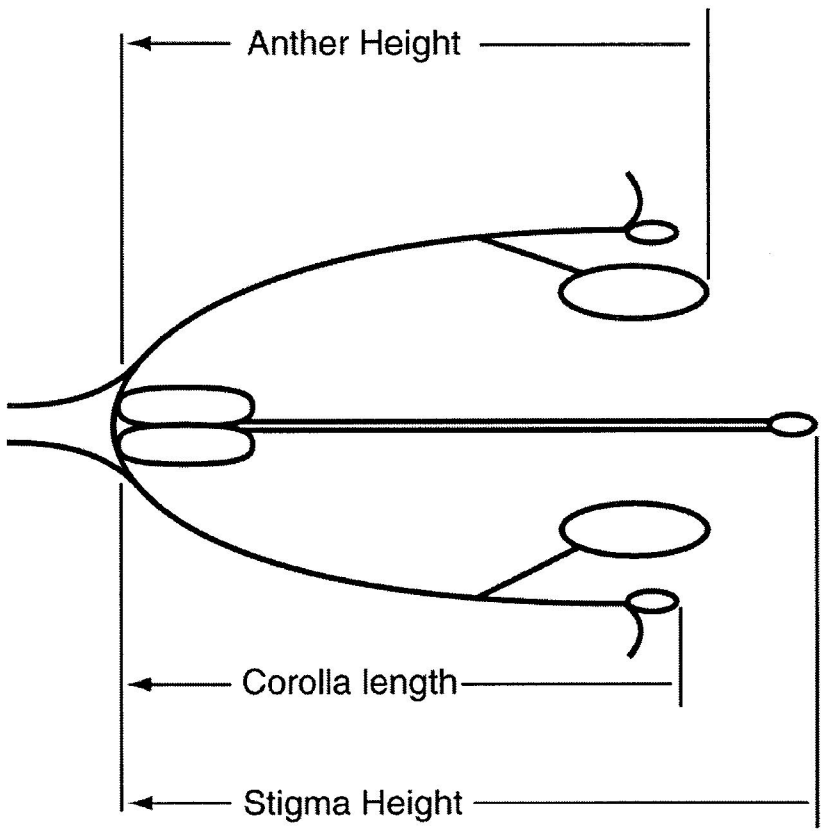
option where the program finds the first 2 significantly different data clusters, at $p \leq 0.05$. The value of 2 was chosen because distyly is the heteromorphic condition described for *Cryptantha*. An initial screening of the data for 3 clusters did not have any positive results. Cluster analysis for 2 groups resulted in the following outcomes: no significant clustering in the data; significant clustering due to a change in one parameter only; or, significant clustering due to changes in two or more of the parameters. After determining whether or not clusters existed in the data, an ANOVA was performed to test for significant differences between mean stigma height, mean anther height, and mean corolla length in each cluster.

Results

Higgins (1971) grouped *Cryptantha* species into three groups, monomorphic, partially heteromorphic, and heteromorphic (Table 3). The mean corolla size measured for the heteromorphic species was greater than that of the partially heteromorphic species, which was greater than that of the monomorphic species (Table 3, Figure 6). Sorting species by corolla length indicated two breaks in the data, one between monomorphic and partially heteromorphic species, and the second between partially heteromorphic and heteromorphic species (Figure 6).

The K-means cluster analysis placed morphs into the first two clusters that could be found in the data, regardless of the parameter that caused the clustering (Table 4). In some cases, no clusters were found, e.g., *C. breviflora, C. celosoides, C. grahami*, and *C. sericea* (Table 4). In species classified by Higgins (1971) as monomorphic (Figure 7), clusters were found due to differences in corolla length only, e.g., *C. cana* and *C. cinerea*

63

Table 3. Means ± standard deviations for stigma, anther, and corolla heights of clusters determined from k-means cluster analysis. Data from herbarium sheets and field-collected specimens were pooled and analyzed with a k-means cluster test, using the first two clusters found in the data. Data within each cluster were then used to calculate means and standard deviations. Highlighted values represent cases where stigma and anther height reversed positions relative to each other, indicative of heterostyly. The table is organized into three sections based upon the classification given by Higgins (1971) as monomorphic, partially heterostylous, and heterostylous, respectively. Sample sizes for cluster 1 and cluster 2 are provided after the species name.

Species	Cluster 1			Cluster 2		
Monomorphic	Stigma	Anther	Corolla	Stigma	Anther	Corolla
C. abata, n= 28,13	2.00±0.00	2.20±0.28	3.02±0.09	1.46±0.14	1.96±0.14	2.81±0.38
C. bakeri, n= 9,33	3.11±0.33	3.83±0.25	4.39±0.42	2.71±0.33	3.30±0.25	3.94±0.21
C. breviflora, n= 66	2.45±0.50	3.17±0.38	4.06±0.38	----------	----------	----------
C. cana, n= 21,12	3.00±0.27	3.79±0.41	4.05±0.35	2.88±0.31	3.50±0.37	3.00±0.00
C. celosoides, n= 26	2.50±0.40	3.02±0.17	3.60±0.28	----------	----------	----------
C. cinerea, n= 14,26	2.11±0.21	2.75±0.33	3.00±0.00	2.06±0.29	2.88±0.21	3.71±0.25
C. compacta, n= 10,27	1.60±0.21	2.85±0.24	3.60±0.21	1.56±0.21	2.13±0.26	2.89±0.25
C. elata, n= 17,23	1.76±0.36	3.32±0.25	4.18±0.28	2.96±0.30	3.52±0.46	4.33±0.42
C. grahami, n= 32	2.41±0.51	3.39±0.40	4.16±0.48	-----------	-----------	-----------
C. mensana, n= 4,35	1.63±0.25	2.38±0.25	3.13±0.25	3.13±0.31	4.1±0.36	4.49±0.41
C. osterhoutii, n= 8,10	2.06±0.18	3.06±0.18	3.00±0.00	2.30±0.26	3.40±0.21	3.40±0.21
C. sericea, n= 10	1.65±0.34	1.95±0.16	2.70±0.35	----------	----------	----------
C. virginensis, n= 13,17	2.85±0.24	3.96±0.14	4.62±0.22	2.79±0.31	3.24±0.26	3.97±0.12
Partially heterostylous						
C. barnebyi, n= 25,34	5.88±0.93	6.16±0.92	6.68±0.80	6.84±0.53	7.29±0.46	7.60±0.40
C. rollinsii, n= 41,29	6.78±1.21	7.28±0.92	7.76±1.15	5.13±1.19	5.90±1.09	6.38±0.65
C. tenuis, n= 41,26	4.30±0.68	5.99±0.33	6.04±0.32	5.67±0.62	7.00±0.45	7.35±0.56
C. wetherillii, n= 24,77	3.21±0.62	5.94±0.93	7.74±1.00	8.81±1.55	7.00±0.79	9.77±1.13
Heterostylous						
C. capitata, n= 20,20	5.35±0.40	10.75±0.77	11.83±0.83	10.40±0.95	8.03±0.41	12.23±0.73
C. confertiflora, n= 15,19	6.90±0.95	11.83±1.33	12.73±1.43	10.39±0.61	7.87±0.57	11.95±1.08
C. creuzfeldtii, n= 25,24	12.06±1.19	9.72±1.37	13.24±1.14	6.27±0.64	10.29±0.81	11.83±0.75
C. flava, n= 19,9	9.74±0.89	6.66±0.58	11.03±0.72	5.17±0.25	9.28±0.75	10.5±0.79
C. flavoculata, n= 16,16	9.22±0.82	7.34±0.75	9.63±0.65	5.31±1.06	8.97±0.97	10.22±1.05
C. fulvocanescens, n= 51,27	5.54±0.73	9.02±1.01	10.64±1.01	9.31±0.91	7.70±1.01	10.70±0.76
C. jonesiana, n= 36,31	5.38±1.10	9.76±1.42	11.58±1.21	11.53±0.89	9.24±0.96	12.39±1.17
C. johnstonii, n= 16,15	5.66±0.54	12.19±0.54	13.44±0.60	9.83±1.20	6.93±0.59	12.03±0.61
C. longiflora, n= 16,18	6.34±1.41	11.84±0.93	13.28±1.11	12.13±1.99	8.67±1.66	13.53±1.87
C. paradoxa, n= 33,21	9.73±1.22	6.38±0.57	10.68±0.65	4.55±0.89	9.57±1.20	10.69±0.90
C. semiglabra, n= 11,16	11.05±0.35	7.41±0.49	11.73±0.61	6.78±0.68	12.44±0.97	13.28±0.97

64

Figure 6. Comparison of corolla length for each cluster in a k-means cluster analysis of 28 different *Cryptantha* species. From left to right, monomorphic species*, partially heterostylous species**, and heterostylous species, from Higgins (1972). Clusters are defined arbitrarily, so that pins and thrums may be cluster one or two for the heterostylous species. Monomorphic species have smaller corollas than both heterostylous and partially heterostylous species.
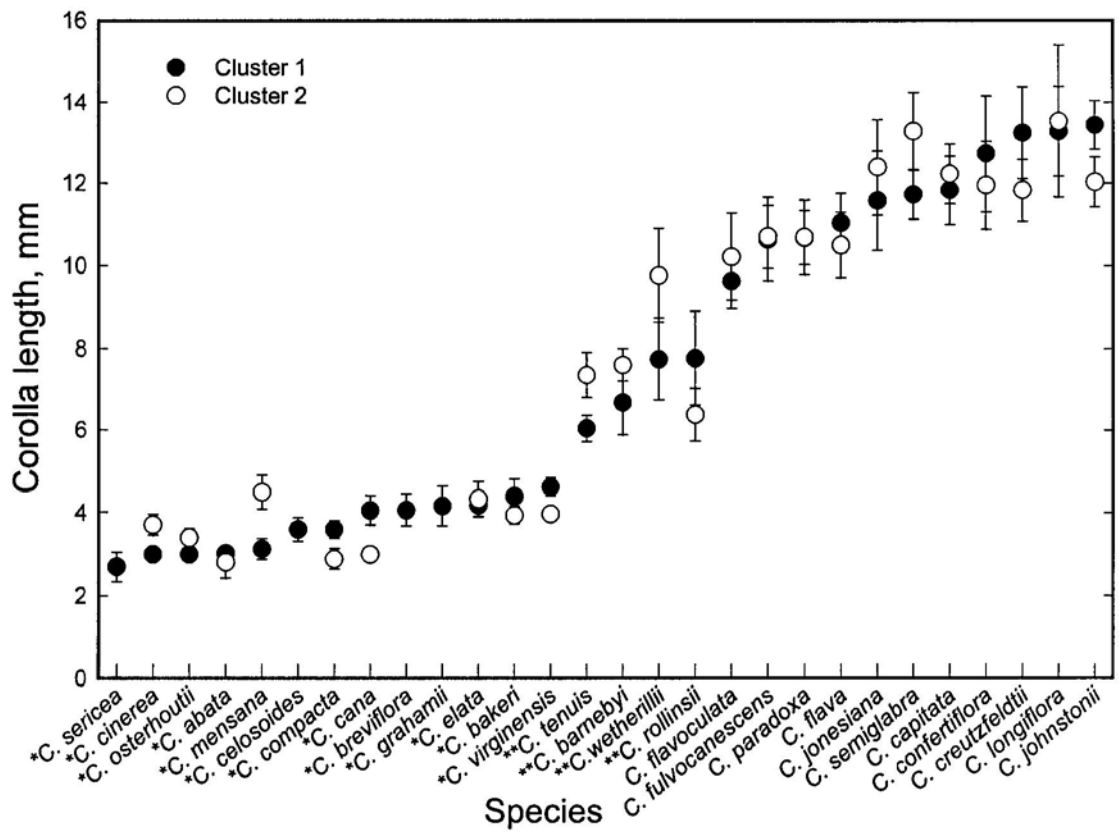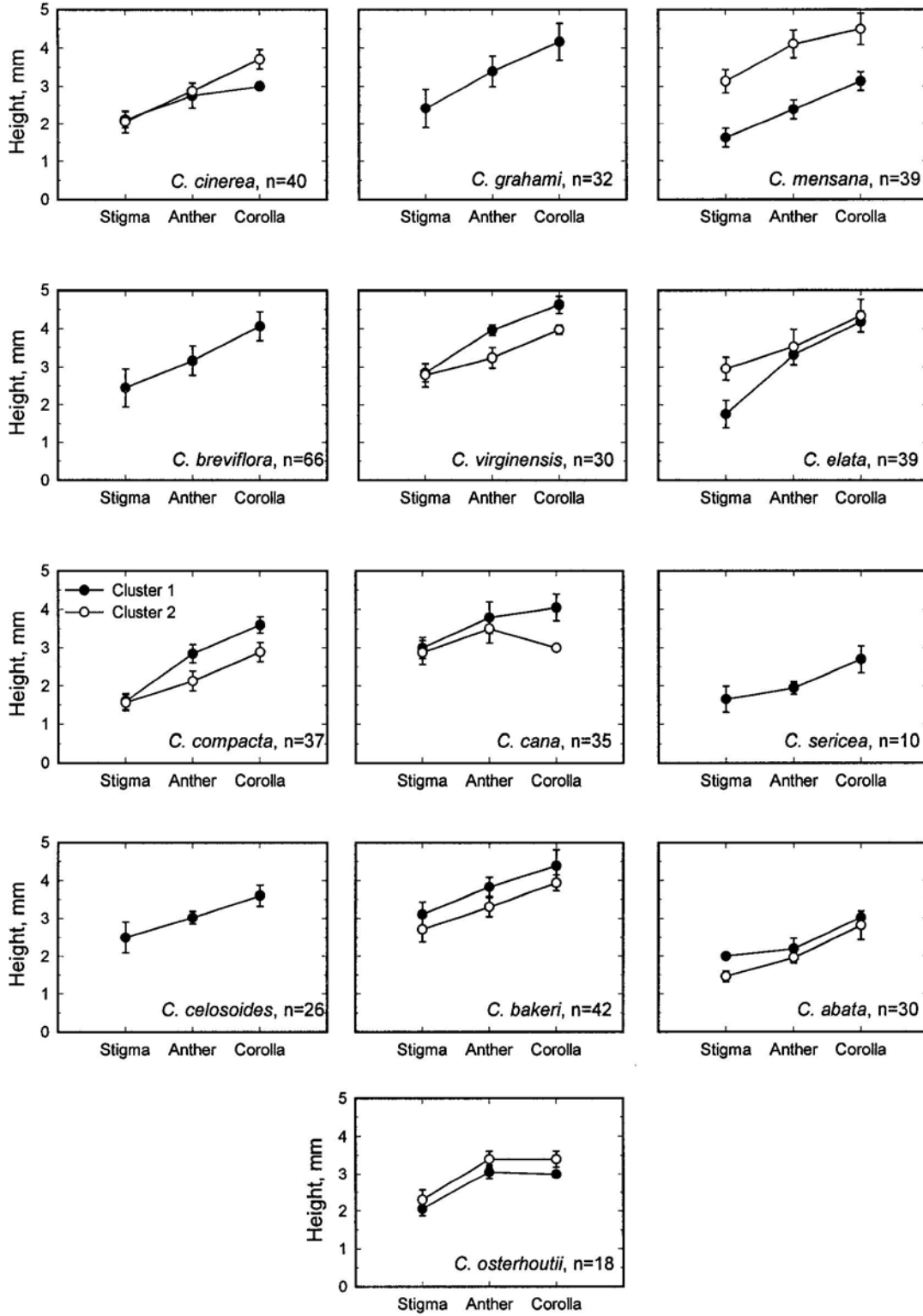
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Table 4. ANOVA results for three floral measurements based on clusters resulting from a k-means analysis. The k-means cluster analysis used the first 2 clusters that could be identified in the pooled data for each species. The ANOVA then tested for significant differences in stigma, anther, and corolla height between these two groups. Sample sizes for each species are provided after the species name and represent the number of flowers measured. Significant values are highlighted.

Species	Stigma F-value	Stigma p-value	Anther F-value	Anther p-value	Corolla F-value	Corolla p-value
C. abata, n= 41	435.02	0.00	7.96	0.01	7.61	0.01
C. bakeri, n= 42	10.22	0.00	32.21	0.00	20.65	0.00
C. breviflora, n= 66	--------	--------	--------	--------	--------	--------
C. cana, n= 33	1.44	0.24	4.04	0.05	105.94	0.00
C. celosoides, n= 26	-------	--------	--------	--------	--------	--------
C. cinerea, n= 40	0.31	0.58	2.48	0.12	110.35	0.00
C. compacta, n= 37	0.32	0.57	57.10	0.00	62.49	0.00
C. elata, n= 40	131.40	0.00	1.68	0.20	1.00	0.32
C. grahami, n= 32	--------	--------	--------	--------	--------	--------
C. mensana, n= 39	89.39	0.00	86.15	0.00	41.47	0.00
C. osterhoutii, n= 18	4.90	0.04	13.09	0.00	28.44	0.00
C. sericea, n= 10	--------	--------	-------	--------	--------	--------
C. virginensis, n= 30	0.25	0.62	84.36	0.00	105.59	0.00
C. barnebyi, n= 59	25.13	0.00	38.52	0.00	33.58	0.00
C. rollinsii, n= 68	31.81	0.00	32.91	0.00	34.00	0.00
C. tenuis, n= 67	69.36	0.00	114.62	0.00	146.82	0.00
C. wetherillii, n= 101	668.78	0.00	25.37	0.00	70.60	0.00
C. capitata, n= 40	476.21	0.00	194.77	0.00	2.60	0.12
C. confertiflora, n= 34	169.96	0.00	137.11	0.00	3.36	0.08
C. creuzfeldtii, n= 49	441.88	0.00	3.13	0.08	25.94	0.00
C. flava, n= 28	225.85	0.00	102.99	0.00	3.09	0.09
C. flavoculata, n= 32	136.03	0.00	28.05	0.00	3.72	0.06
C. fulvocanescens, n= 78	394.39	0.00	29.74	0.00	0.090	0.77
C. jonesiana, n= 67	620.46	0.00	3.00	0.09	7.59	0.01
C. johnstonii, n= 31	158.64	0.00	661.42	0.00	41.48	0.00
C. longiflora, n= 34	93.59	0.00	45.71	0.00	0.211	0.65
C. paradoxa, n= 54	281.96	0.00	173.71	0.00	0.00	0.97
C. semiglabra, n= 27	360.86	0.00	308.73	0.00	22.28	0.00

67

Figure 7. Means for stigma height, anther height, and corolla length of the K-means clusters for monomorphic species. Only one cluster was found for *C. grahami*, *C. breviflora*, *C. sericea*, and *C. celosoides*

C. cinerea, n=40 · C. grahami, n=32 · C. mensana, n=39 · C. breviflora, n=66 · C. virginensis, n=30 · C. elata, n=39 · C. compacta, n=37 · C. cana, n=35 · C. sericea, n=10 · C. celosoides, n=26 · C. bakeri, n=42 · C. abata, n=30 · C. osterhoutii, n=18

69

(Table 4), differences in stigma height only, e.g., *C. elata*, differences in both anther height and corolla length, e.g., *C. compacta* and *C. virginensis,* and differences in all three measures, e.g., *C. abata, C. bakeri, C. mensana,* and *C. osterhoutii.* For species classified by Higgins (1971) as partially or fully heterostylous (Figure 8), significant differences between clusters were found for all three measures in *C. barnebyi, C. rollinsii, C. tenuis, C. wetherillii, C. johnstonii,* and *C. semiglabra* (Table 4). Significant cluster differences for both the stigma and anther height, but not for corolla length were found for *C. capitata, C. confertiflora, C. flava, C. flavoculata, C. fulvocanescens, C. longiflora,* and *C. paradoxa.* Data for *C. creutzfeldtii* and *C. jonesiana* had significant differences in stigma height and corolla length, but not anther height.

Figure 8 shows clearly that not all of the species classified by Higgins (1971) as partially or fully heterostylous demonstrate a cluster pattern indicative of heterostyly; that is, the stigma and anther heights reverse positions relative to each other. The lines connecting cluster means for *C. barnebyi, C. rollinsii,* and *C. tenuis* never cross, but rather run parallel (Figure 8). These data support a classification of the floral morphology for these species as monomorphic (Table 3), especially since the patterns are similar to those found in other monomorphic species of *Cryptantha* (Figure 7). Species such as *C. jonesiana, C. creutzfeldtii,* and *C. elata* show a third cluster pattern (Figures 7, 8). Here, there are significant differences in stigma height, but not in anther height (Table 4). These species may more appropriately be classified as stylar morphs (Table 5). *Cryptantha wetherillii* shows a unique pattern, where the lines connecting the cluster means do not cross, but are not parallel. The pattern for this species is most similar to

70

Figure 8. Means for stigma height, anther height, and corolla length of the K-means clusters for heteromorphic species of *Cryptantha*. The lines connecting stigma height and anther height for each cluster should intersect to form approximately right angles if the floral organs change in a reciprocal fashion. Note that this is not the case for *C. barnebyi, C. rollinsii, C. tenuis,* and *C. wetherillii.*
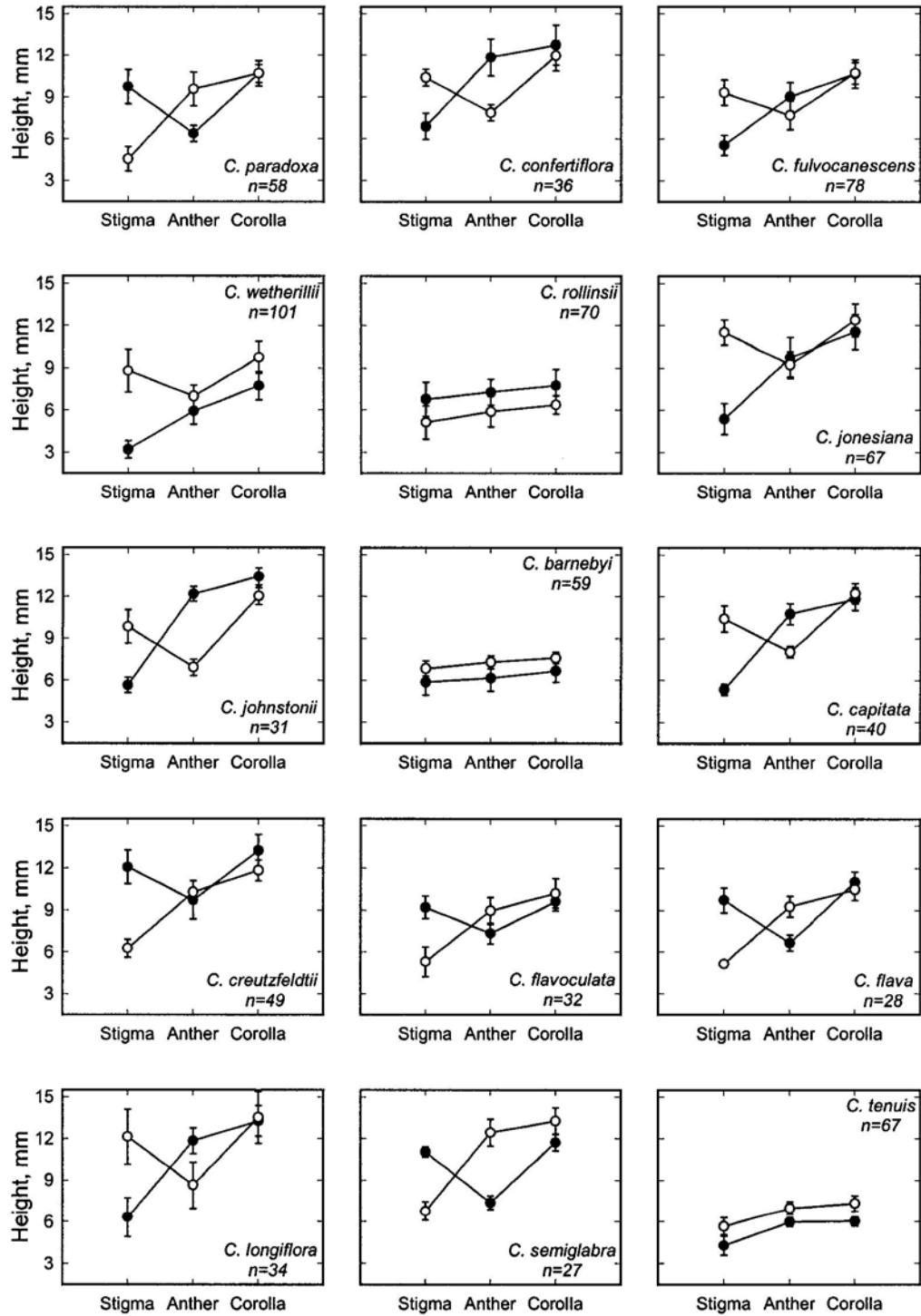
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Table 5. Classification of the floral morphology for species in *Cryptantha* section Oreocarya based upon the results of k-means cluster analysis and ANOVA. The classical description for each species is listed followed by the classification based upon the data presented here. Species where the anther height did not change between clusters are highlighted.

Species	Higgins/Cronquist classification	ANOVA/K-means analysis classification
C. abata, n= 41	Monomorphic	Monomorphic
C. bakeri, n= 42	Monomorphic	Monomorphic
C. breviflora, n= 66	Monomorphic	Monomorphic
C. cana, n= 33	Monomorphic	Monomorphic
C. celosoides, n= 26	Monomorphic	Monomorphic
C. cinerea, n= 40	Monomorphic	Monomorphic
C. compacta, n= 37	Monomorphic	Monomorphic
C. elata, n= 40	Monomorphic	Style morph
C. grahami, n= 32	Monomorphic	Monomorphic
C. mensana, n= 39	Monomorphic	Monomorphic
C. osterhoutii, n= 18	Monomorphic	Monomorphic
C. sericea, n= 10	Monomorphic	Monomorphic
C. virginensis, n= 30	Monomorphic	Monomorphic
C. barnebyi, n= 59	Partially heteromorphic	Monomorphic
C. rollinsii, n= 68	Partially heteromorphic	Monomorphic
C. tenuis, n= 67	Partially heteromorphic	Monomorphic
C. wetherillii, n= 101	Partially heteromorphic	Monomorphic
C. capitata, n= 40	Heteromorphic	Heteromorphic
C. confertiflora, n= 34	Heteromorphic	Heteromorphic
C. creuzfeldtii, n= 49	Heteromorphic	Style morph
C. flava, n= 28	Heteromorphic	Heteromorphic
C. flavoculata, n= 32	Heteromorphic	Heteromorphic
C. fulvocanescens, n= 78	Heteromorphic	Heteromorphic
C. jonesiana, n= 67	Heteromorphic	Style morph
C. johnstonii, n= 31	Heteromorphic	Heteromorphic
C. longiflora, n= 34	Heteromorphic	Heteromorphic
C. paradoxa, n= 54	Heteromorphic	Heteromorphic
C. semiglabra, n= 27	Heteromorphic	Heteromorphic

73

that found in the stylar morphs (Figure 7), but given the significant difference in anther height between clusters (Table 4), the species was classified as monomorphic (Table 5).

Bubble plots (scatter plots where the diameter of the point represents the frequency of a particular measurement) of stigma height versus anther height also serve to demonstrate the patterns of differentiation in floral morphology among species (Figures 9, 10). For monomorphic species, the data clusters around one central mean, e.g., *C. bakeri* (Figure 9). In species classified as stylar morphs (Table 5), there are two foci, with significant scatter along the x-axis, but little scatter along the y-axis, e.g., *C. creuzfeldtii, C. jonesiana* (Figure 10). In species with distyly, there are two foci of data, with separation along both x and y axes, e.g., *C. johnstonii* (Figure 10).

Discussion

Some of the monomorphic, and all heteromorphic taxa in this survey, showed differences among clusters that designated them statistically as individual groups. However, when clusters were found in the monomorphic species, it was typically due to differences in corolla size (Figure 7), hence these clusters were not indicative of different floral morphologies. Differences in floral size may have arisen due to population differences and/or phenological differences, but as long as the cluster lines were roughly parallel, they do not represent changes in any herkogamous characters. With this reasoning, the partially heterostylous species *Cryptantha barnebyi, C. rollinsii*, and *C. tenuis* are best described as monomorphic, and not partially heteromorphic.

Cryptantha barnebyi, C. rollinsii, C. wetherillii, and *C. tenuis* are described as partially or incompletely heterostylous by Higgins (1971) and Cronquist (1982). These species have corolla lengths intermediate between those of the heteromorphic species and

74

Figure 9. Stigma height versus anther height for monomorphic *Cryptantha* species. Species names and sample sizes are identified in the lower right hand corner of each graph. For these monomorphic species, the data clusters around one central mean.

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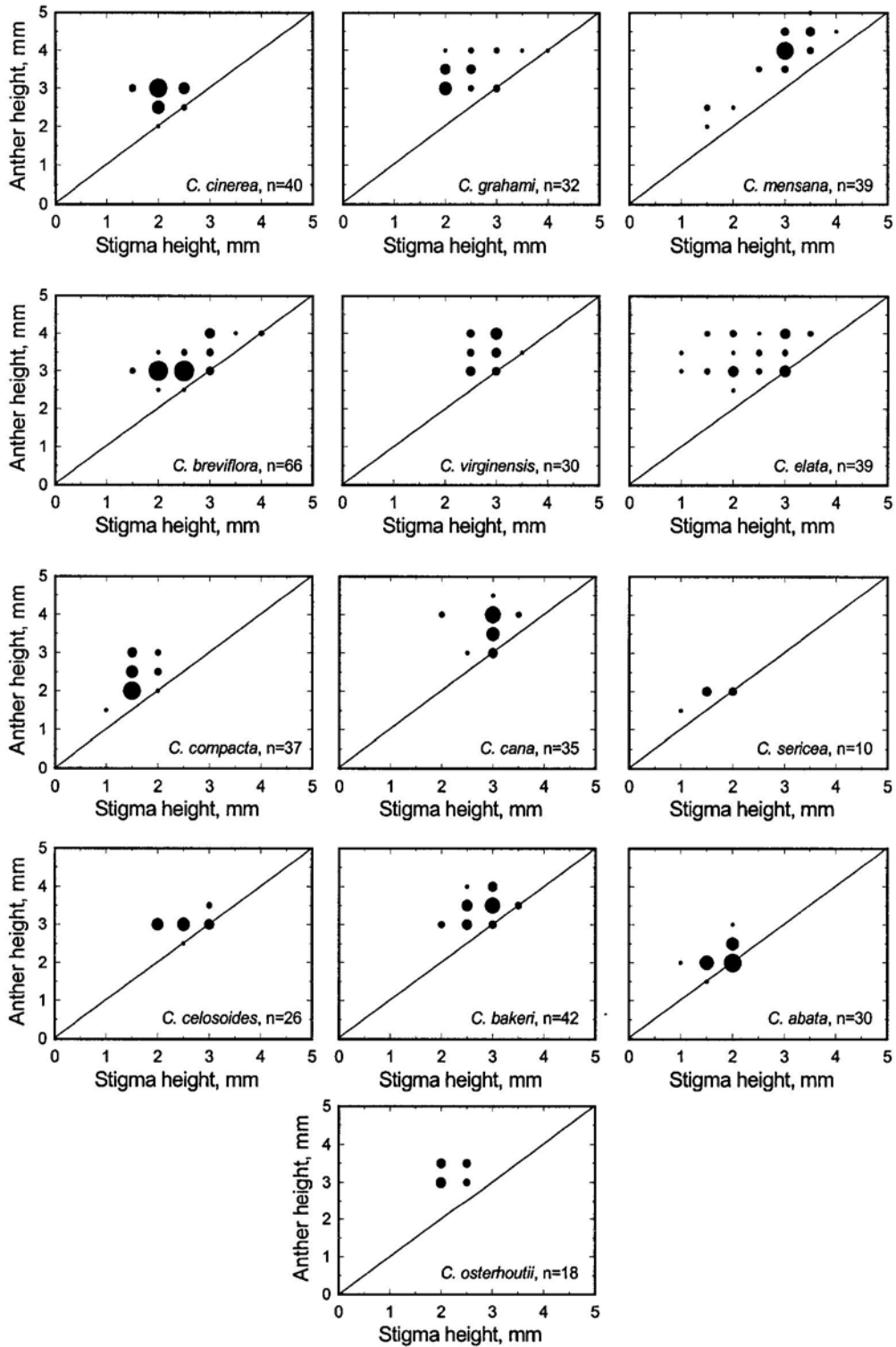
Figure 10. Stigma height versus anther height for previously described heteromorphic *Cryptantha* species. Species names and sample sizes are in the lower right hand corner. Distylous species of *Cryptantha* show two foci of points separated along both x and y axes, stylar morphs show either two foci, separated only along the x-axis, or a scattering of points spread along the x-axis, and monomorphic species show a single foci of points.

78
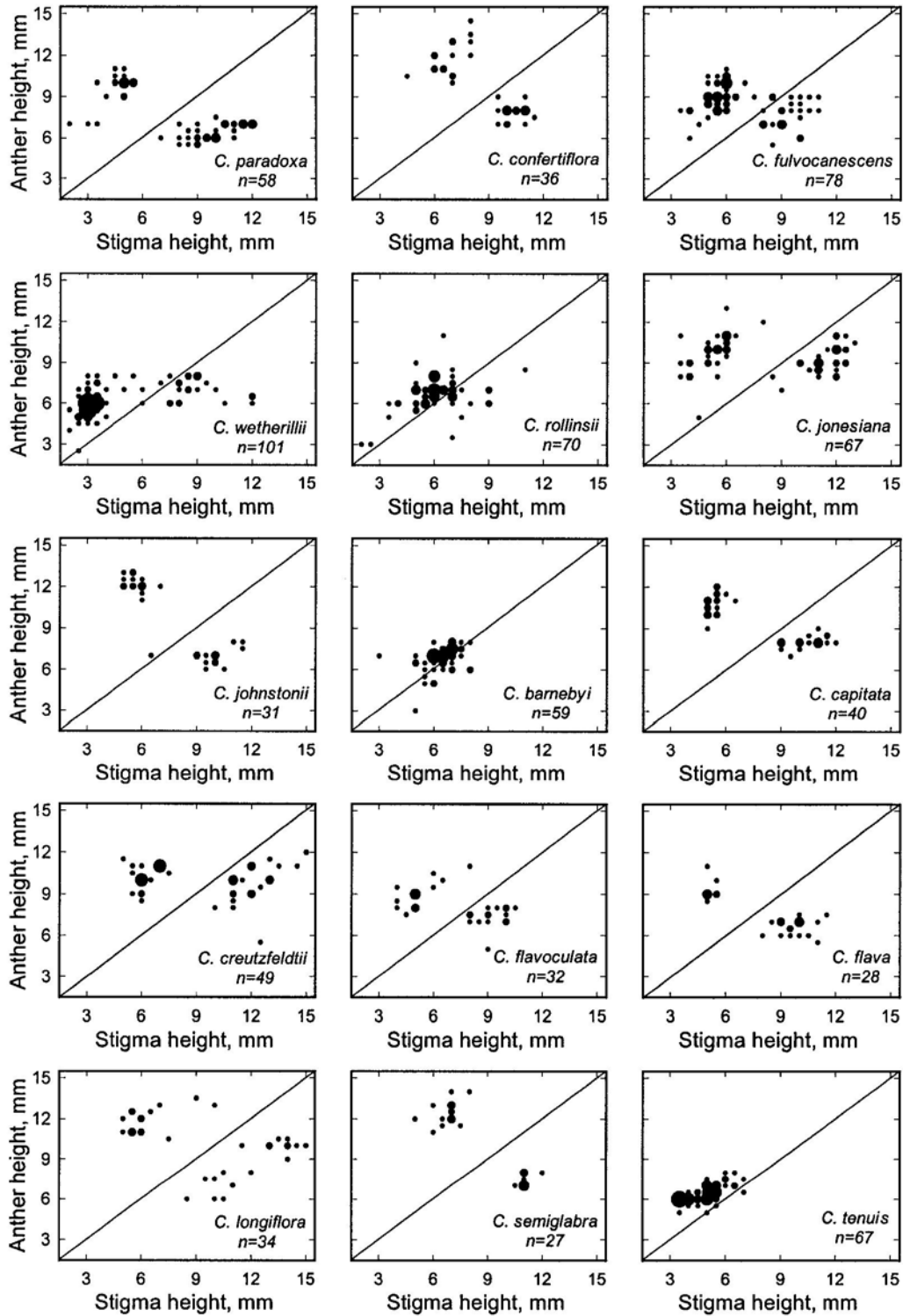
the monomorphic species. These species also possess a funnelform corolla, while the heteromorphic species exhibit a salverform corolla. The shape of the funnelform corolla may limit the spatial separation of the anthers and stigmata in these taxa, or conversely, their smaller floral size may constrain spatial separation of floral organs. *Cryptantha wetherillii* showed a stylar morph type of floral variation, with little scatter in anther placement, but large scatter for the stigma height (Table 4, Figure 8); however, anther height was significantly different between the clusters, so this species was treated as monomorphic. One of the clusters in the *C. wetherillii* data approaches the size of the smallest flowered heteromorphic species (Figure 6), supporting the role of overall flower size in promoting the separation of stigma and anthers.

Environmental pressures often affect floral traits. For example, the breeding system of Himalayan *Primula floribunda* varies with altitude. Low altitude populations are distylous, while populations at higher elevations are homostylous, and populations at intermediate elevation show an intermediate breeding system (Richards 1997). Similar environmental data correlated with population level variation in floral morphology in *Cryptantha* could reveal selective pressures favoring the breakdown/evolution of heterostyly, both in terms of morphology and self-compatibility.

Two of the 'incompletely heterostylous' species, *Cryptantha rollinsii* and *C. wetherilli*, are often found as single plants, or scattered in low-density populations. These two species are fully self-compatible (Chapter 2) and are reportedly biennials (Higgins 1971, Cronquist 1984) or short-lived perennials. This habit may force these two species to flower in years when pollinators and resources are scarce due to drought or

79

temperature fluctuations. It is possible that these climatic fluctuations have selected for increased selfing rates (Takebayashi and Morrell 2001), in turn favoring a decrease in the spatial separation of stamens and stigmas within the corolla (Richards 1997). The presence of homostyles in otherwise heteromorphic populations has been documented previously (Ernst 1936, Bahadur 1970, Ganders 1975, Ganders 1979, Charlesworth 1979, Richards and Koptur 1993). Charlesworth and Charlesworth (1979) suggest that the presence of homostyles in a population may indicate that heterostyly is breaking down. Typically, homostylous individuals/populations are found at the edge of a distylous species range and are more scattered in their distribution (Ganders 1979). *Cryptantha rollinsii* and *C. wetherillii* fit this pattern, at least in terms of their distribution (Higgins 1971).

For the majority of the heterostylous taxa in this study, separate LS and SS clusters were apparent (Figure 10). The degree of continuous variation associated with the stigma and anther positions within flowers among individuals of the same species helps to clarify the 'degree of heterostyly' in terms of the stage of the evolution of the breeding system. For example, a species in transition from a monomorphic to heteromorphic state, or vice versa, should show variability across a range of anther and stigma heights (Faivre and McDade 2001), with no apparent LS and SS cluster. There were several species that fit this pattern in *Cryptantha*, including *C. wetherillii*, *C. jonesiana*, *C. creuzfeldtii*, and *C. rollinsii*. The continuum of stigma and anther heights across individuals of the same morph indicates that these plants are not strictly heterostylous. At the population level, heights of these floral organs were not of two distinct classes. More studies that measure individuals from multiple populations within

80

species are needed to demonstrate the frequency with which plants of heterostylous species are in 2 or 3 distinct classes, and whether variation in populations is non-random in relation to the range of the species.

Those species that showed no significant difference for the mean anther position (*C. elata, C. creuzfeldtii, C. jonesiana*) in the K-means cluster analysis demonstrate the presence of a herkogamy without a reciprocal correspondence of the anthers. Thus the differences in variance components that determined separate groupings for each morph were due to variability primarily in the height of the stigma. Barrett *et al.* (2000) developed a new category for stylar polymorphisms, termed stigma-height dimorphism, to encompass those species where reciprocal style morphs exist without a complementary change in anther position. It is worth noting that one of the monomorphic species, *C. elata*, exhibits the same pattern, a significant change in stigma height, without a reciprocal change in anther height (Figure 7). This species was designated as a style morph as well. *Cryptantha wetherillii* and *C. fulvocanescens* graphically appear to be stylar morphs; however statistically they qualify as monomorphic and heteromorphic, respectively. Additional measurements in these taxa may reveal that they are in fact stylar morphs, since the standard deviation for anther height for the clusters overlaps in both species (Figure 8).

Stigma-height dimorphism has been documented in other heterostylous families, i.e. *Anchusa* (Dulberger 1970), *Lithodora* (Barrett *et al.* 2000), and in *Primula* (Al Wadi and Richards 1993). Higgins (1971) reported that the stamens are always at the same position in *Crypttantha oblata*, a species that has been described as heterostylous. Duhlberger (1964) showed that *Narcissus tarzetta* was monomorphic for stamen length,

81

dimorphic for style length, and possessed multiallelic self-incompatibility. Charlesworth and Charlesworth (1979) note that their model favors the evolution of a stigma polymorphism over a change in anther position, and that this polymorphism is difficult to maintain. They predict that when heterostyled individuals exist that lack the full complement of character differences, the difference in anther position should be absent more often than dimorphisms in stigma position (Charlesworth and Charlesworth 1979). There are instances of stigma dimorphisms in *Cryptantha*, and these data support the addition of the stigma-height dimorphism category developed by Barrett *et al.* (1996), and support the Charleworth's prediction that a stigmatic poylmorphism is more stable.

Lloyd and Webb also favor stigma-height dimorphism as the intial step in their model since a number of stigma-height polymorphisms were previously described. They also comment that no instances of anther-height polymorphism without a stigma-height polymorphism are known, and hypothesize that this may be due to the fact that many heterostylous species have anthers that are adnate to the corolla, so that a sudden discrete change in stamen length may be prohibited due to the close association with the corolla (Lloyd and Webb 1992).

The theoretical models for the evolution of heterostyly differ in the assumed ancestral condition, and in the evolutionary order of self-compatibility and reciprocal herkogamy. Lloyd and Webb (1992) postulate the introduction of a herkogamous morph (with a different stigma height) to an ancestral approach herkogamous population. There are no examples of approach herkogamy in *Cryptantha*. They state that reverse herkogamy could be the ancestral condition for some heterostylous species, and emphasize the introduction of a stigma-height polymorphism into the ancestral

82

herkogamous population (be it approach or reverse). In order to apply the Lloyd and Webb (1992) model to *Cryptantha*, one would have to assume the ancestral condition was reverse herkogamy (all of the monomorphic species in *Cryptantha* are reverse herkogamous), and that an approach herkogamous mutant invaded, based upon the absence of any extant approach herkogamous species. The only factor that is influenced by this scenario is the genetics of distyly for the group. Lloyd and Webb point out that an advantageous mutant can be selectively favored as soon as it arises if it is caused by a dominant allele, and use this argument to support the concept that approach herkogamy should be the ancestral condition given the known genetics of the heterostylous morphs. There are very few demonstrated exceptions to the dominance of the thrum morph (Ganders 1979, Lloyd and Webb 1992). The genetics associated with heterostyly in *Cryptantha* are unknown, and so it may be that the dominance relationships are reversed in this group. If that is the case, then the intermediate forms of the breeding system found in *Cryptantha* could represent transitional stages predicted in by Lloyd and Webb. Specifically, the self-compatible heteromorphic species would represent a transition toward heterostyly as interpreted by their model.

Charlesworth and Charlesworth (1979) assumed a homostylous ancestor in their model. Many of the monomorphic species examined here have overlap between stigma and anther positions within the corolla, and so could be classified as homostylous- with no spatial separation between the stigma and anthers within the corolla (Figure 9, Figure 10, Table 3), and so their model can be applied to *Cryptantha* species as well. The self-incompatible monomorphic species would represent the stage prior to the transition to heteromorphy. The incompletely heterostylous species (*Cryptantha barnebyi, C.*

83

rollinsii, C. wetherillii, and *C. tenuis*), and the stylar morphs (*C. elata, C. creuzfeldtii, C. jonesiana*) would represent a secondary loss of heterostyly in light of the Charlesworthian model, since all of these species are partially self-compatible (Chapter 2), and the Charlesworths' model predicts the evolution of self-incompatibility as a precursor to spatial separation of the stigmata and anthers. Under the Lloyd and Webb (1992) model, these species could be in transition to the heterstylous condition, since they propose that herkogamy arises before self-incompatibility. There is no real way to know the order of evolution of the partially heterostylous, stylar morphs, or self-compatible heteromorphic character states without determining the phylogenetic relationships among the species exhibiting these phenotypes. Thus it is difficult to support either proposed model based on these data alone, since there is no way to determine the relative order from one intermediate form of heterostyly to another, though it is apparent that the breeding system in many species of *Cryptantha* appears to be actively undergoing change.

There were several species (*Cryptantha barnebyi, C. confertiflora, C. creuzfeldtii, C. jonesiana, C. johnstonii, C. rollinsii, C. semiglabra, C. tenuis*, and *C. wetherillii*) that showed significant differences between the corolla lengths of the floral morphs. Ganders (1979) noted a few cases where pins and thrums had differences in the size of their corollas, and when size differences were present, the thrum had the larger corolla. Nine out of the fifteen heterostylous (including the partially heterostylous) species of *Cryptantha* investigated here showed significant differences in corolla size between clusters, so the phenomenon is not uncommon for this genus. Of these species, *C. confertiflora* and *C. wetherillii* had larger corollas in the pin morphs, while *C. johnstonii* and *C. semiglabra* had larger corollas in the thrum morph. *Cryptantha barnebyi* and *C.*

84

tenuis are not classified as heterostylous based on these data, so differences in the corolla size between clusters cannot be related to the pin/thrum morphology. Development of the stamens and corolla are often correlated such that male sterile mutants produce smaller corollas and reduced stamens (Ganders 1979). Ganders (1979) posits that the same developmental processes that produce longer stamens in the thrum are responsible for larger corolla sizes. The data presented here do not support this hypothesis, since instances of significantly larger corollas were present in both morphs. There is evidence that corolla size dimorphism is a derived character in *Lithospermum* (Ganders 1979b). He suggested that larger corollas were related to asymmetric pollen flow, such that the larger corolla preferentially attracted pollinators (Ganders 1979b). This hypothesis could be tested in *Cryptantha*, given the species specific differences in corolla size, although additional data on corolla width would be needed to clarify size differences and pollinator preference among species. It is likely that pollination dynamics of *Cryptantha* are affected by differences in corolla size, especially since the anthers are adnate to the corolla.

There have been several studies that present data to question the evolutionary status of heterostyly in different species (Riveros *et al.* 1987, Barrett 1989, Barrett and Richards 1990, Negron-Ortiz 1996, Barrett *et al.* 1996). The data presented here provide a survey of the variability in the floral characters associated with the heterostylous breeding system in *Cryptantha*. Relationships among transitory taxa can provide insight to the importance of various selective regimes that can result in variation in floral morphology, and ultimately changes in mating systems.

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87

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90

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A phylogenetic investigation of *Cryptantha* Section Oreocarya (Boraginaceae) – the influence of geography on speciation patterns

Introduction

Plant species distributions are shaped by biotic and abiotic factors including climate, elevation, soil type, and pollination syndromes (Levin 2001). The unique physiography of the North American Intermountain West provides a series of uncommon substrates over a relatively small geographic area. Basins, ranges, plateaus, deserts, and plains constitute the terrain that is bordered by the Sierra Nevada and Cascades on the West, and the Rocky Mountains on the East (Cronquist *et al*. 1984, Chronic 1990). The plants that occupy this region grow in widely variable ecological conditions from shale ridges and gravel outcrops, to sandy desert soils. Colonization of newly exposed substrates, or unoccupied habitats requires the ability to survive and reproduce in novel, sometimes stressful environments. Plants in the genus *Cryptantha* have been particularly successful in such a radiation. This genus includes many species that are adapted to narrow geographic ranges and specific geologic substrates found in the Intermountain West (Higgins 1971, Cronquist 1984).

The genus *Cryptantha* (Boraginaceae) contains approximately 150 species, divided among five sections; Cryptantha, Eremocarya, Krynitzkia, Oreocarya, and Piptocalyx. The section Cryptantha is located entirely in the Andean region of South America, and is unique in that it has cleistogamous (closed) and chasmogamous (open) flowers. Eremocarya and Piptocalyx are monotypic and along with Krynitzkia comprise the annual species located in southwestern North America. These annual species are generally well defined taxonomically by morphological characters (Cronquist 1984).

91

However, morphological traits are less definitive in the perennial section, the Oreocarya, which also occurs in the Great Basin and Intermountain West of North America. Species boundaries in the Section Oreocarya are not always clear, leading some authors to hypothesize that interspecific hybridization may have occurred (Higgins 1971, Cronquist *et al*. 1984). Nevertheless hybridizations have not yet been demonstrated (Higgins 1971, Cronquist *et al*. 1984). Higgins (1971) presented a phylogenetic hypothesis for Section Oreocarya based on the species descriptions of Johnston (1924, 1925), Payson (1927), and his own observations, but his summary did not make use of cladistic or other formal phylogenetic methods (Figure 11). To date there has been no quantitative evaluation of the group.

The relationships among the species of the Oreocarya are of interest for several reasons. In a purely systematic sense, the molecular phylogeny for this section of *Cryptantha* will clarify questions about species relationships that have been debated by taxonomists since the genus was first described (Johnston 1925, Higgins 1971, Cronquist 1984). In particular, there has been controversy regarding the species status of several entities, among them *Cryptantha abata*, *C. caespitosa*, *C. celosoides*, *C. compacta*, *C. humilis, C. flava*, and *C. confertiflora* (Cronquist 1984). Many of the perennial species of *Cryptantha* are narrow endemics, and understanding the relationships of these taxa to more widespread species within the group has the potential to provide insight to the selective action of the environment as a speciation force (Higgins 1971, Tidwell *et al*., 1972). Furthermore, the branching order of the annuals relative to the perennials is unclear. Higgins (1971) proposed that the annuals were polyphyletic, having arisen