UNIVERSITY OF CALIFORNIA
RIVERSIDE

AND

SAN DIEGO STATE UNIVERSITY

# The Study of Sexual Systems and Consequences in the Genus *Cylindropuntia*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolutionary Biology

by

Niveditha Ramadoss

September 2024

Dissertation Committee:

Dr. Lluvia Flores-Renteria, Co-Chairperson
Dr. Amy Litt, Co-Chairperson
Dr. Jeet Sukumaran
Dr. Erin Rankin Wilson
Dr. Jon Rebman

The Dissertation of Niveditha Ramadoss is approved:

---

---

---

---

Committee Co-Chairperson

---

Committee Co-Chairperson

University of California, Riverside

San Diego State University

## Acknowledgments

I am grateful to my advisor Dr. Flores-Renteria, without whose help, I would not have been here. Gratitude is extended to my co-advisor, Dr. Litt, for her unwavering support and invaluable guidance throughout this journey. Sincere thanks are also due to all committee members for their support and contributions. In acknowledgment of prior publications, this dissertation incorporates sections from Ramadoss et al., 2022, and Ramadoss et al., 2023. Chapter 5 contains material submitted for review to Molecular Ecology by Ramadoss et al., 2024. The research forming the foundation of this dissertation was directed and supervised by Dr. Lluvia Flores-Rentería, a co-author listed in the aforementioned publications. Additionally, Dr. Jon Rebman co-supervised Chapter 1. Contributions to collections and sample processing were provided by Scarlet Steele, Amy Orduno, and Carlos Portillo. This work is supported by the Hispanic-Serving Institutions Education Grants (HSI) Program [grant nos. 2018–38422-28614 and 2023-70440-40156] from the USDA National Institute of Food and Agriculture. This study was partially funded by Irwin M. Newell Graduate Research Fund (UCR), 2021 Emergency Spring Funding for Student Assistance with Research, Scholarship and Creative Activities (RSCA) SDSU and Paul Jorgensen Research Grant (Anza Borrego Foundation). I wish to express my sincere appreciation to the University Graduate Fellowship (SDSU), AWIS-SD Scholarship, Joshua Tree National Park Graduate Research Grant and Completion of Research and Creative Activity (CORE) Fellowship (SDSU) for their generous support, which was instrumental in the completion of some chapters. I extend my gratitude to Michelle Cloud-Hughes and Marc Baker for their valuable guidance and insights during the course of our research. I thank all the past and

present members of Dr.Flores-Renteria lab who kindly helped me in my field work; San Diego Gas and Electric Company (SDGE), Joshua Tree National Park (JTNP), Bureau of Land Management (BLM) in Imperial County, and Sam Schultz, the owner of the desert view tower, for providing access to the field sites.

This dissertation is dedicated to my supportive parents, Ramadoss and Sivakama Sundari, who have nurtured my academic interests and unwaveringly believed in me. To my little sister, Akshitha, whose boundless energy has been a constant motivation. To my loving husband, Krishna Ramamoorthy, whose support has helped me find balance between work and life, including invaluable assistance during fieldwork. To my caring sister-in-law, Divya Saratha, for her constant encouragement.To my ever inspiring mom-in-law, Amsamani and dad-in-law, Ramamoorthy; And to my emotional support pet, baby Eevee. Lastly, heartfelt appreciation goes to my mentor, Dr. Flores-Renteria, whose dedication and support have been a driving force behind the hard work reflected in this dissertation.

# ABSTRACT OF THE DISSERTATION

The Study of Sexual Systems and Consequences in the Genus *Cylindropuntia*

by

Niveditha Ramadoss

Doctor of Philosophy, Graduate Program in Evolutionary Biology
University of California, Riverside and San Diego State University, September 2024
Dr. Lluvia Flores-Renteria and Dr. Amy Litt, Co-Chairpersons

Flowering plants have a remarkable diversity in sexual systems. These sexual systems have ecological and evolutionary implications, yet comprehensive studies are hindered by the scarcity of species-level sexual system data. In this dissertation, we will focus on the genus *Cylindropuntia* (family Cactaceae), which although has a variety of sexual systems, has been poorly studied. My first goal is to accurately determine the sexual system of *C. wolfii* reported anecdotally as gynodioecious. To achieve this, we carried out experimental crosses and histological analysis of putative female and bisexual flowers. We found that *C. wolfii* is functionally dioecious where the putative bisexual flowers were actually functionally male. Dioecious species often exhibit sexual dimorphism, which can have significant ecological effects, particularly in terms of plant-pollinator interactions. My second goal is to identify whether *C. wolfii* has sexual dimorphism and if that influences pollinator attraction. We conducted a comprehensive analysis of quantitative and qualitative traits in *C. wolfii* flowers and compared them statistically. Our findings indicate that male flowers of *C. wolfii* are larger and more brightly colored, leading to a higher attraction of poten-

tial pollinators compared to female flowers. In this study, we utilized fluorescent dyes as pollen analogues, which are available in various colors. However, previous research has not investigated whether the color of these dyes influences pollinator attraction. Therefore, my third goal was to assess whether there is any bias in pollinator attraction due to the color of the dye. We conducted fluorescent dye assays on: *Oscularia deltoides*, which exhibits consistent flower coloration, and *C. wolfii*, which displays flower color polymorphism. We discovered that the presence of green dye influences the attraction of bees. Dioecy is hypothesized to lead to low genetic diversity and so my fourth goal was to determine whether the dioecious *Cylindropuntia* species have lower genetic diversity than hermaphrodites. We found that dioecious *Cylindropuntia* has comparable diversity to that of hermaphrodites. But, the overall genetic diversity of all *Cylindropuntia* were found to be low signaling the need for protection measures. Our study sheds light on sexual separation in plants and its influence on ecological and genetic factors.

# Contents

# List of Figures

# List of Tables

# Chapter 1

# Introduction

The diversity of sexual systems in angiosperms is vast, and understanding the causes and consequences of this diversity has been a significant area of inquiry since Darwin's time (Darwin, 1851). While hermaphroditism, where both stamens and pistils coexist in the same flower, is the most common sexual system in angiosperms, various other configurations have evolved. For instance, gynodioecious systems consist of individuals bearing hermaphroditic flowers alongside those carrying only female flowers. This system is prevalent in approximately 7% of angiosperm species (Richards, 1997) and is widespread across phylogenetic groups (Jacobs and Wade, 2003), encompassing about 50 families. Additionally, some plants have evolved complete sexual separation under the dioecious system, where female and male flowers are borne on different individuals. Dioecy is found in around 6% of angiosperms, occurring in roughly 40% of angiosperm families, and has independently evolved at least 871 times across various lineages (Renner 2014).

The sexual system of a species carries ecological and evolutionary consequences. For example, Janzen (1971) demonstrated through field studies that dioecious species experience reduced seed predation due to spatial segregation of sexes, resulting in fewer seed-producing females. Muyle et al. (2018) utilized population genetics approaches to reveal that dioecious species in the *Silene* genus exhibit higher genetic diversity and adaptation rates compared to their non-dioecious counterparts. Therefore, sexual systems play a crucial role in determining genetic variation, inbreeding rates, adaptive potential, and long-term evolutionary outcomes such as speciation and extinction. This information can be valuable for designing effective conservation strategies (Janzen 1971; Vamosi and Vamosi, 2005; Tree of Sex Consortium, 2014; Anderson et al., 2015; Tsuji and Fukami, 2018; Muyle et al., 2018).

Plant sexual systems data at the species level are limited, hindering comparative evolutionary studies involving sexual systems and correlated traits (Glick et al., 2016). Accurately identifying the sexual system is crucial in plant studies. In certain cases, non-functional sexual organs may persist as vestigial, potentially leading to the oversight of unisexuality and incorrect descriptions of sexual systems. Ambiguity surrounding the sexual system can impede our comprehension of the evolutionary and ecological significance of species with that particular sexual system (Penagos et al., 2021). Therefore, detailed morphological and developmental analyses or experimental crosses are imperative (Hoffman, 1992; Wang et al., 2017). For instance, in species like *Spachea membranacea* (Malpighiaceae) and *Withania aristata* (Solanaceae), individuals were observed to bear either hermaphroditic or female flowers. However, crosses and histological experiments

revealed that the hermaphroditic individuals functioned as males, leading to the characterization of their sexual system as functionally dioecious (Steiner, 1985; Anderson et al., 2006). Similarly, within the cactus family, descriptions of sexual systems have often been ambiguous and imprecise, primarily relying on anecdotal observations rather than thorough morphological analysis or experimental validation of the system (Hoffman, 1992). Hence, accurately determining the sexual system of plants through developmental studies or experimental crosses is crucial to avoid misinterpretations of evolutionary patterns in sexual systems (Lloyd, 1980; Steiner, 1985). Chapter 1 is focused on accurately determining the sexual system of *Cylindropuntia wolfii* (Cactaceae) previously reported anecdotally as gynodioecious but we expect it to be functionally dioecious.

The *Cylindropuntia* genus consists of both completely cosexual (hermaphroditic) and separate sexual (gynodioecious) systems constituting a suitable model to explore plant sexual systems and its consequences. *Cylindropuntia* forms one of the major groups of cacti that occupy the warm deserts of North America and includes 39 species (Bobich et al. 2014, Majure et al. 2019). In our study, we included six species from this genus, each characterized by distinct sexual systems: three species featuring hermaphroditic sexual systems (*C. echinocarpa, C. ganderi, and C. ramosissima*), one gynodioecy (*C. chuckwallensis*), one dioecy (*C. wolfii*), and one hermaphroditic species with a clonal reproductive strategy (*C. bigelovii*).The coincidence of unisexual flowers, polyploidy, and geographic proximity among *C. chuckwallensis*, and *C. wolfii* is remarkable. Both these species share similar flower color morphs ranging from bright green to yellow to red, and filament color (Baker and Hughes, 2014). Baker and Hughes (2014) hypothesize that these species originated

through allopolyploidy and share a common parent. But there is no genetic data to support or refute this. Several species of this genus are endemic to the deserts of Southwestern US and Mexico (Goettsch et al. 2015). As desert ecosystems are predicted to be more susceptible to global climate change (Hantson et al. 2021), these species are under threat. Consequently, obtaining genetic data related to the *Cylindropuntia* species diversity and population structure is crucial for their conservation.

*Cylindropuntia wolfii* serves as an exceptional model for studying sexual dimorphism and dichromatism, as well as their impact on pollinator attraction. This is due to the presence of six distinct color morphs within the same geographical area and its sexual system (Ramadoss et al., 2022). Throughout the five years of our field study, we did not observe any changes in color morphs within an individual over time. Pollinator attraction is a crucial factor in determining successful reproduction in species with separate sexes. Furthermore, pollinator-mediated selection favors traits that improve pollination success in males and fertilization success in females, leading to sexual dimorphism in many dioecious species (Queller, 1987; Waelti et al., 2009). This phenomenon often results in males being more attractive than females. Male attractiveness can be enhanced through various factors. Generally, many dioecious species develop larger male flowers than females, which tend to attract more pollinators (Stephenson and Bertin, 1983; Delph, 1996; Costich and Meagher, 2001). In certain cases, such as *Silene latifolia*, although male flowers may be smaller, the male plants produce a higher number of flowers, often preferred by pollinators (Meagher, 1992). Additionally, male flowers may emit more floral scent for pollination compared to females (Waelti et al., 2009). In some populations of *S. latifolia*, male flowers produce

higher sugar concentrations, providing higher-quality rewards (Shykoff and Bucheli, 1995). Moreover, in this species, females may evolve to be less attractive, as floral signals can attract both pollinators and florivores (Bopp et al., 2004), potentially impacting sexual reproduction when pollinator abundance is low (Vamosi and Otto, 2002). To counteract this, females may mimic the signals of males (eg. similar scent) to attract pollinators equally, ensuring mating success and maximizing the fitness of both sexes (Renner, 2006). For instance, Hossaert-McKey et al. (2016) demonstrated the stability of dioecy in figs through female chemical mimicry of male floral scents using gas chromatography-mass spectrometry. Chapter 2 is focused on identifying sexually dimorphic traits in the flowers of *C. wolfii* (Cactaceae) and its potential influence on pollinator attraction. Here we report for the first time sexual dichromatism and fluorescent dichromatism in plants.

Pollen transfer or dispersal is a crucial parameter that offers valuable insights into the evolution of plant traits and sexual systems (Pyke, 1981; Perkins, 1977; Williams and Mazer, 2016), and the effectiveness of pollinators (Schmitt, 1980; Diller et al., 2022). A commonly used and successful method involves coating the anthers with fluorescent dye powder (Stockhouse, 1976; Van Geert et al., 2010; Huais et al., 2022). Different colors of dyes have been utilized in studies without assessing their potential differential attractiveness to pollinators. For instance, in a study by Adler and Irwin (2006), green and orange dyes were used to examine the pollination effectiveness of various bee visitors on the distylous plant *Gelsemium sempervirens*. The researchers found differences in pollen-carrying capacities among bee genera, but the impact of dye color attraction on increased pollen load remains uncertain, as bees are known to be attracted to specific flower colors. In our

Chapter 3, we aimed to address the following question: Do different colored fluorescent dye powders affect pollinator attraction, and if so, which colors exhibit a bias?

There are two competing hypotheses that explain sexual separation in plants. The first one states that gynodioecious and dioecious systems have evolved as an adaptation to mitigate inbreeding (in the absence of self-incompatibility) and the associated negative effects of inbreeding depression (Charlesworth and Charlesworth, 1978). In some gynodioecious systems, females are obligatory outcrossers, while hermaphroditic individuals can self-pollinate, with selfing rates being highly variable between populations. Consequently, offspring of females tend to be more outcrossed than those of hermaphrodites, resulting in higher seed quality and quantity in females (Darwin, 1877; Shykoff et al., 2003; Dufay and Billard, 2011). The frequency of females in gynodioecy is linked to the selfing rate of hermaphrodites, relative seed production, and inbreeding depression (Darwin, 1877; Lloyd, 1982; Charlesworth and Charlesworth, 1978; Maki, 1992) In general, the frequency of females is positively correlated to selfing rate of hermaphrodites to avoid inbreeding depression and also positively correlated to seed production (Lloyd, 1982; Charlesworth and Charlesworth, 1978; Maki, 1992). In contrast to gynodioecy, dioecy promotes maximum outcrossing since there are no hermaphrodites within the population (Lloyd, 1982; Charlesworth and Charlesworth, 1978), thereby reducing the risk of inbreeding depression (Thomson and Barrett, 1990; Charlesworth, 1999).

An alternative hypothesis proposes that dioecious clades exhibit lower species richness compared to their non-dioecious sister clades (which included both self-compatible and self-incompatible species) due to heightened extinction rates (Heilbuth, 2000). Heilbuth et

al. (2001) elucidated this hypothesis by highlighting two evolutionary disadvantages of dioecy. Firstly, only half of the population, i.e., females, contribute to seed production and dispersal, potentially leading to fewer seeds and reduced dispersal (resulting in smaller geographic distributions) and increased competition for local resources (resulting in fewer individuals per species) compared to hermaphroditic species (Heilbuth et al., 2001). Secondly, in dioecious species with biotic pollination, intrasexual competition in males drives them to become more attractive to pollinators than females. Consequently, population size fluctuates with pollinator density, impacting genetic diversity (Vamosi and Otto, 2002). Renner (2014) challenges the dead-end hypothesis, suggesting that 43% of dioecious species within species-rich clades remain understudied. Additionally, Muyle et al. (2018) tested one of the predictions of the dead-end hypothesis, which posits that dioecy increases extinction rates, by comparing the genetic diversity and adaptive potential of dioecious species with their close non-dioecious relatives. Contrary to expectations, they found that both dioecious and gynodioecious species exhibited increased genetic diversity compared to their close hermaphroditic relatives (Muyle et al., 2018). However, a contrasting study by Maki (1992) in the *Chionographis* genus showed that gynodioecious species had low genetic diversity and higher inbreeding depression compared to their hermaphroditic relatives. These conflicting studies are the only two assessing the effects of different sexual systems on genetic diversity, highlighting the need for additional research to elucidate which hypothesis applies to angiosperms more broadly. So our chapter 4 is focused on validating these two hypotheses using species of *Cylindropuntia* genus. The sexually separated species, *C. chuckwallensis* and *C. wolfii*, exhibit a close and narrow distribution within California. The convergence

of traits such as unisexuality, hexaploidy, and a spectrum of flower color morphs ranging from bright green to red, as well as filament color, is noteworthy. Baker and Hughes (2014) proposed a hypothesis suggesting shared ancestry between these two species. However, no genetic data currently support this claim. Hence, our chapter 4 also focuses on determining the population structure of six *Cylindropuntia* species to determine if we can discern the shared ancestry of the polyploid species *C. wolfii* and *C. chuckwallensis*. Notably, we are the first to report any genetic estimates on this genus.

# Chapter 2

# Accurate Characterization of Sexual System

## 2.1 ABSTRACT

In certain unisexual flowers, non-functional sexual organs remain vestigial and unisexuality can be overlooked leading to the ambiguous description of the sexual systems. Therefore, to accurately describe the sexual system, detailed morphological and developmental analyses along with experimental crosses must be performed. *Cylindropuntia wolfii* is a rare cactus endemic to the Sonoran Desert in southern California and northern Baja California that was described as gynodioecious by morphological analysis. The aims of our project include accurately identifying the sexual system of *C. wolfii* using histological

The materials presented in Chapter 2 have been published in BMC Plant Biology 2022 as "Unraveling the development behind unisexual flowers in *Cylindropuntia wolfii* (Cactaceae)"

and functional studies and characterizing the developmental mechanisms that underlie its floral development. Histological analyses were carried out on different stages of *C. wolfii* flowers and controlled crosses were performed in the field.Our results identified *C. wolfii* to be functionally dioecious. The staminate flowers were not able to produce fruits and had aborted ovules confirming that they are functionally males and the pistillate flowers produced no pollen but mature fruits confirming that they are functionally females. The ovule and anther development differed between staminate and pistillate flowers. In vivo pollen germination tests showed that the pollen of staminate flowers were viable and the stigma and style of both staminate and pistillate flowers were receptive. This suggests that there are no genetic or developmental barriers in the earlier stages of pollen recognition and pollen germination. Despite being functionally dioecious, we observed that functionally pistillate individuals produced fruits with a large number of aborted seeds. This implies that not only does this species have low reproductive success, but its small population sizes may lead to low genetic diversity.

## 2.2 INTRODUCTION

The breeding or sexual systems of organisms is a critical aspect of natural biology that affects genetic diversity and genome evolution (Charlesworth, 2006). Dioecy is a sexual system in which populations are made of distinct male individuals and female individuals (Freeman et al., 1979). About 6% of angiosperm species have evolved to separate their sexes in a dioecious system and dioecy has evolved at least 871 times independently in 175 families (Charlesworth and Guttman, 1999; Renner, 2014). Dioecy is reported to have

evolved from hermaphroditic flowers by the accumulation of mutations that drive male sterility in pistillate flowers and female sterility in staminate flowers (Charlesworth and Guttman, 1999). Despite heavily depending on pollinators for a successful fertilization (Westergaard, 1958) and having fewer individuals that can produce seeds compared to the hermaphroditic populations, dioecious angiosperms are present in species rich entirely dioecious clades disputing its negative consequences (Renner, 2014). Studies on unisexual flower development have shown that the developmental processes are not consistent among species and unisexual flower development is not well studied in rare species.

In dioecy, two types of developmental regulations are involved in producing unisexual flowers (Mitchell and Diggle, 2005). Type I involves the arrest of one sexual organ at a very early stage which leads to unisexual flowers by inception (Mitchell and Diggle, 2005). Type II involves carpel/stamen abortion after all of the organs have been specified by the flower, but one sexual organ remains functional (Mitchell and Diggle, 2005). This leads to unisexual flowers that carry vestigial organs of the other sex (Sobral et al., 2016). Programmed Cell Death (PCD) has been described as a principal force for driving the development of unisexuality in angiosperms through Type II (Irish and Nelson, 1989; Lebel-Hardenack and Grant, 1997; Wu and Cheung, 2000). However, the abortion of the male and female organs can occur in different stages and tissues (Caporali et al. 2003; Coimbra et al. 2004; Flores-Renteria et al. 2013; Caporali et al. 2019; Hernandez-Cruz et al. 2019) and it is species dependent.

As abortion of one of the sexual whorls occurs late during the development of Type II flowers, non-functional sexual organs remain vestigial and unisexuality can be over-

looked leading to the ambiguous description of the sexual systems. Thus, to describe the breeding system precisely, detailed morphological and developmental analyses or experimental crosses are a necessity (Hoffman, 1992; Wang et al., 2017). For example, some species have been originally considered as gynodioecious or androdioecious, but a closer look at their hermaphroditic flowers has shown they are functionally dioecious. For example, *Spachea membranacea* Cuatrec. (Malpighiaceae) and *Withania aristata* (Aiton) Pauquy (Solanaceae) were observed to have individuals that have either hermaphroditic flowers or pistillate flowers, but crossing and anatomical experiments revealed that the hermaphroditic individuals were functioning as males thus describing their sexual system as functionally dioecious (Steiner, 1985; Anderson et al. 2006). A member of the Caryophyllaceae, *Honckenya peploides* (l.) Ehrh. var. major (Hook.) Abrams, was initially identified as androdioecious based on morphological studies and then proved to be functionally dioecious based on manual outcrosses performed over a consistent two year study period (Tsukui and Sugawara, 1992). Thus, it is of importance to determine the sexual system of plants based on functional studies as their reproductive systems are often not accurately represented by superficial observations of the floral morphology (Lloyd, 1980; Steiner, 1985).

The family Cactaceae has about 2000 species (Anderson and Brown, 2001), most of them are hermaphroditic; although 23 species have Type II unisexual flowers in either dioecious, gynodioecious or trioecious sexual systems (Sánchez and Vázquez-Santana, 2018 and references therein). Within the cactus family, the description of the sexual systems has been ambiguous and imprecise attributed mostly to anecdotal observation rather than detailed morphological analysis or experimental confirmation of the system (Hoffman, 1992).

Recent studies in the Cactaceae have advanced our understanding of the cellular mechanisms by which flowers develop unisexuality as well as their spatial and temporal patterns (Flores-Renteria et al., 2013; Hernandez-Cruz et al., 2018). In most cactus examples, pistillate flowers underwent anther degradation before the completion of meiosis. For example, in the genus *Consolea* Strittmatter et al. (2006) showed that the pistillate flowers underwent anther abortion early at the onset of meiosis leading to sterile anthers bearing no pollen grains. In *Opuntia stenopetala* Englem.,the pistillate flowers undergo various cellular changes (vacuolization, DNA degradation, cytoplasm collapse) in the microsporangium and all cell layers prior to completion of meiosis (Flores-Renteria et al., 2013). Similarly, in four species of *Echinocereus* with pistillate flowers, the microspore mother cell development was arrested prior to meiosis with other cell layers collapsing later (Hernandez-Cruz et al., 2018).

In Cactaceae, female sterility varies considerably between taxa (Hernandez-Cruz et al., 2019). In the functionally dioecious *O. stenopetala*, the ovule abortion in staminate flowers was initiated in early primordial stages (Orozco-Arroyo et al., 2012), whereas in *Consolea* the ovule degeneration was induced prior to anthesis, after the development of embryo sac (Strittmatter et al., 2008). In *Echinocereus*, the seed abortion takes place after the zygote is formed (post-fertilization) (Hernandez-Cruz et al., 2018). The role of PCD in female sterility of Cactaceae is not clearly determined. One recent study in *Opuntia robusta* H.L. Wendl. ex Pfeiff. showed that placental arrest and ovule abortion in staminate flowers were regulated by PCD (Hernandez et al., 2019).

Plants that are dioecious can deviate from the 1:1 sex ratios, especially those restricted to small populations, thereby decreasing their viability (Field et al., 2013; Cuevas et al., 2017). This is because biased sex ratios allow for few mating opportunites which impacts the effective population and thereby the genetic diversity (Field et al., 2013; Cuevas et al., 2017). Among them, male biased ratios are at least twice as frequent as the female biased ones (Field et al., 2013). These biases are attributed to the differences in reproductive costs (i.e sex that spends more on reproduction rather than growth will have a higher mortality), pollen or seed dispersal (For instance, in abiotically pollinated species, males face higher reproductive costs than females to produce rich pollen and hence are more vulnerable whereas in biotic seed dispersal females spend more on reproduction to produce fleshy fruits), sex chromosomes (for example degeneration of Y chromosome influences female bias) and/or differential rate of mortality (Harris and Pannell, 2008; Field et al., 2013). In Cactaceae, subdioecious populations (consisting of males, females and hermaphrodites) of *Consolea spinosissima* (Mill.) Lem. (Strittmatter et al., 2002) and *Pachycereus pringlei* (S. Watson) Britton & Rose (Fleming et al., 1998) as well as functionally dioecious populations of *O. stenopetala* have been reported to have a male biased sex ratio.

Interestingly, several species of *Cylindropuntia* have been described as gynodioecious, with some individuals having perfect flowers and others having functionally female flowers (Rebman, 1998; Rebman and Pinkava, 2001). These species are *C. calmalliana* (J.M. Coult.) F.M. Knuth, *C. chuckwallensis* M.A. Baker and M.A. Cloud-Hughes, C. molesta (Brandegee) F.M. Knuth, *C. sanfelipensis* (Rebman) Rebman, and *C. wolfii* (L. D. Benson) M.A. Baker.

In our study, we focus on *Cylindropuntia wolfii*, which occurs within a very restricted range in extreme southern California, USA and extreme northern Baja California, Mexico (Figs. 1A and 1B). This species has been described as gynodioecious based on field and herbarium specimen observations (Rebman, 1998), but little is known about its reproductive biology. The putative female individuals were assumed because the anthers looked shriveled and no pollen was present. Type II flowers are easily misidentified by superficial observations, therefore, histological studies and experiments (e.g. manual crosses) are needed in order to accurately describe the sexual system in *C. wolfii*. Our goals are to identify the sexual system of *C. wolfii*, to describe the developmental processes contributing to the formation of unisexual flowers using histological observations, experimental crosses, and pollen viability tests, and to estimate the sex ratios in some populations.

## 2.3 METHODS

### 2.3.1 Plant Material

*Cylindropuntia wolfii* is listed on the Inventory of Rare and Endangered Plants by the California Native Plant Society, rare plant program (2021) at CA Rare Plant Rank 4.3 due to its limited distribution, but the populations are not very threatened. It is narrowly distributed in the Sonoran Desert of southern California and Baja California (Benson and Baker, 2003) Fig.2.1. It is a hexaploid succulent shrub that is densely branched with stout cylindrical stems having terminal branchlets that are not easily detached. The stems have dense spination and oblong to obovate tubercles bearing the spines (Pinkava et al. 1992). The flowers have a variable tepal color on different individuals ranging from yellow-bronze

to red with a pink to red style and stamens with yellow to red filaments. The fruits are dry at maturity, densely spined to bur-like, and most often do not contain seeds.

Samples from *C. wolfii* were collected from around the Desert View Tower (Imperial County, CA) (32.6592 N, 116.0999 W) and Mountain Springs (Imperial County) (32.674509 N, 116.098834 W), California between March 2017 and July 2019. We tagged 47 individuals, 27 putative hermaphrodites (flowers with developed anthers which released pollen at anthesis and a normal pistil, Fig. 2.2A, 2.2C) and 20 which superficially looked female (with reduced anthers and a lack of pollen at anthesis, Fig. 2.2B, 2.2D). The flowers were collected at different development stages, ranging from early pre-anthesis to late post-anthesis based on the presumed sex of the parent plants via observed flower morphology.

### 2.3.2 Histological methods

To analyze the morphological development, flowers and buds from *C. wolfii* were collected at different stages for separating and sectioning the anthers and ovules. The samples were fixed in 4 % paraformaldehyde in 1× phosphate-buffered saline (PBS). This was followed by dehydration using ethanol in increasing concentrations. Fixed samples were embedded through LR White Resin medium grade (Electron Microscopy Sciences, Fort Washington, PA, USA). LR-White-embedded samples were sectioned at 1-3 µm using an ultramicrotome (Reichert- Jung Ultracut E) and stained with toluidine blue solution (1% toluidine blue and 1% sodium borate in distilled water) using Hoffmann et al. (1983) procedure in Polychrome Stains for High Resolution Light Microscopy. Stained samples were mounted with Kleermount® solution. The mounted samples were viewed under a

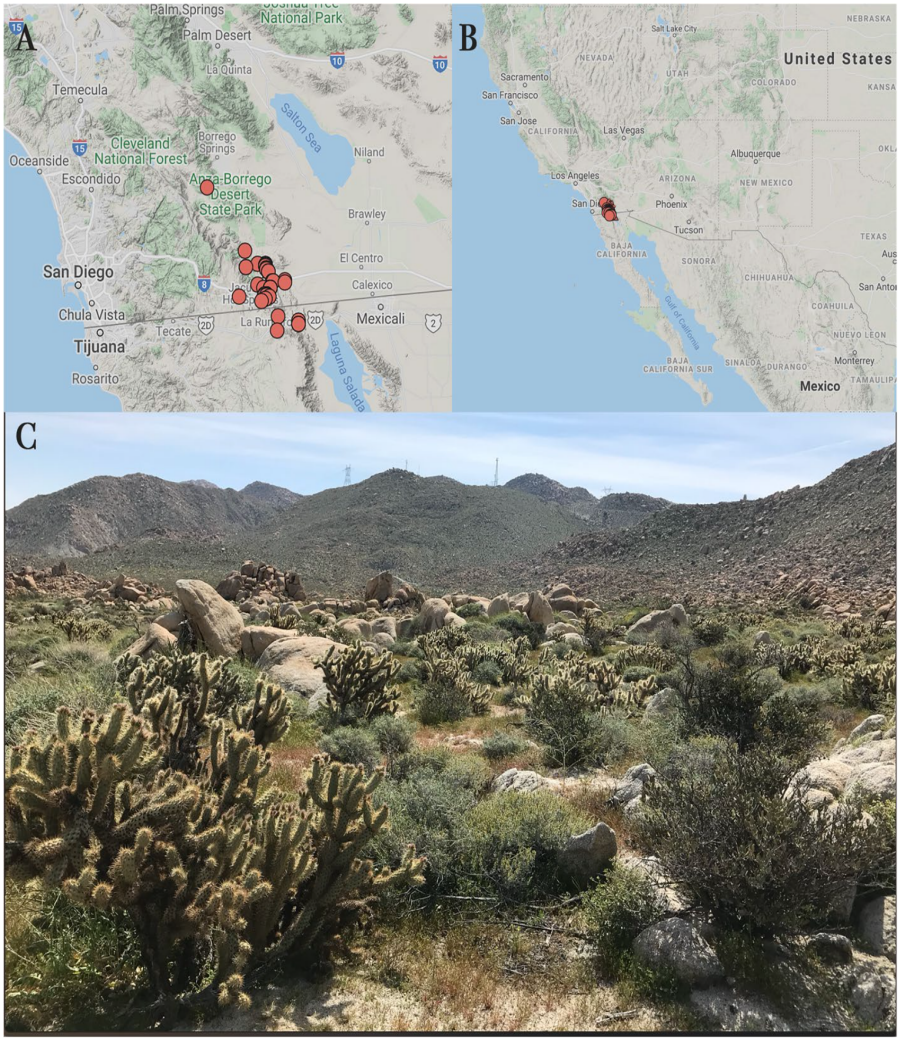


Figure 2.1: Distribution of *Cylindropuntia wolfii* (A). This species has a narrow distribution in both California and Baja California, Mexico (B). Red points show the current distribution of *C. wolfii*. Occurrence data that included only precise points were extracted from SEINet Portal (www.swbiodiversity.org), misidentifications were excluded and the map was generated using Google imagery. (C) Natural occurrence of *C. wolfii* in Mountain Springs, Imperial County, California. Figures A and B were obtained from SEINet Portal and were collated with figure C in Adobe Photoshop

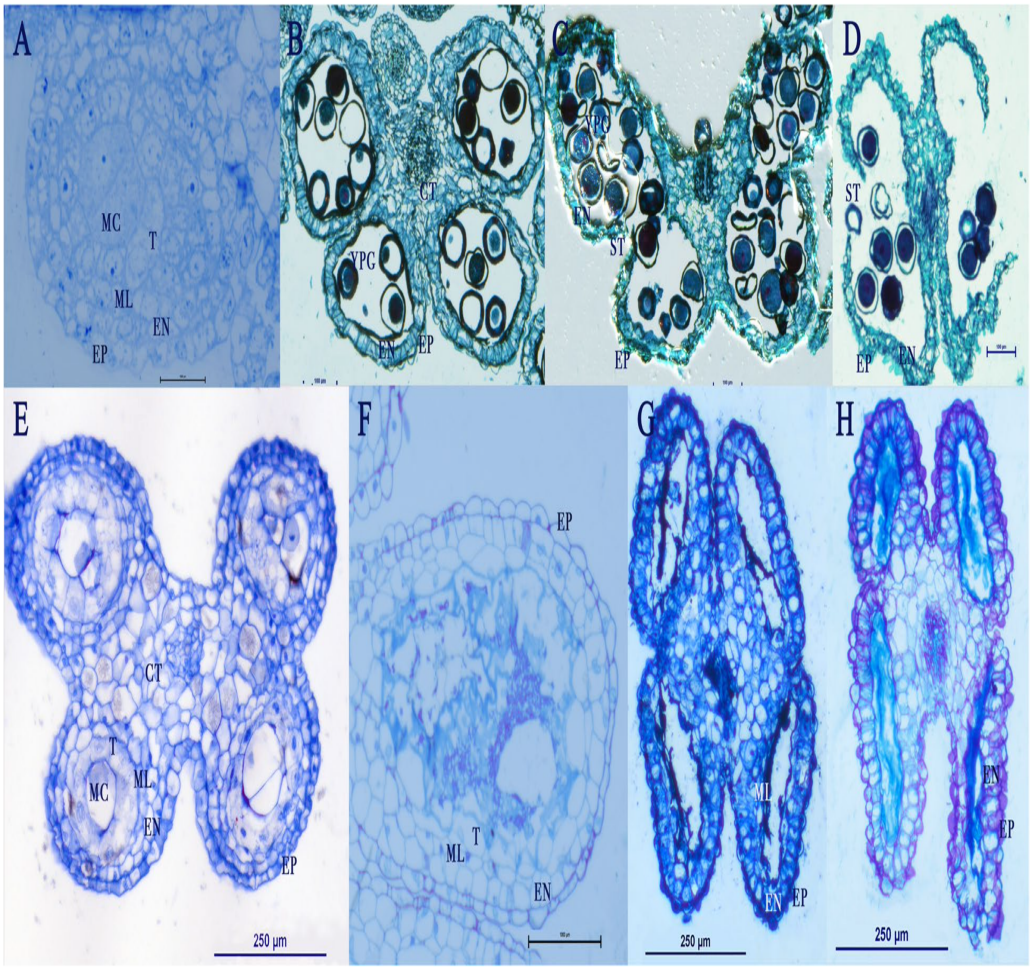


Figure 2.2: Anther development of flowers of *Cylindropuntia wolfii*. A) Anther of staminate flowers shows four layers at pre-meiosis of the microspore mother cells (MC). (B) Tetralocular male anther in pre-anthesis with young pollen grain (YPG) developing with tapetum fully disintegrated but endothecium (EN), epidermis (EP) and connective tissues (CT) intact. (C) Male anther in late pre-anthesis with stomium (ST) initiation indicating that the anther is about to dehisce. (D) Male anther at anthesis releasing fully mature pollen grains. (E) Female flower anther in pre-anthesis with MiMC (MC) developing. All cells in the anther wall such as tapetum (T), middle layer (ML), endothecium (EN) as well as the MiMC start showing hyper-vacuolization. (F) Microspore mother cells and tapetum show complete disintegration by this stage and only few tapetal cells are visible. (G) Female anther in late pre-anthesis showing the disintegration of the MiMC, nuclei are visible in cells of the anther wall. (H) Aborted anther in female flower at anthesis lacking nuclei in most remaining cells

compound microscope (Nikon Microphot-FX) at different objectives.

### 2.3.3 In-vivo pollen germination test

In order to test for pollen viability and stigma receptivity, a pollen germination test was performed. Manual crossing was done between different sexes at the time when the flowers were fully open. The flowers were collected approximately 48hrs after the manual crossing and the pistils were removed. The pistils were prepared for analysis by following the procedure mentioned by Livia et al. (2015) with a few modifications. The pistils were fixated in 3:1 ethanol: acetic acid solution for 24 hrs. After fixation, the samples were cleared by immersion in 1M sodium sulphite for 25 mins. This was followed by immersion in 1% aniline blue overnight. The pistils were then sectioned longitudinally and flattened on a clean glass slide. Glycerol at 50% concentration was used as the mounting medium. The samples were viewed with a compound microscope (Nikon Microphot-FX) at 10X objective under UV light.

### 2.3.4 Crosses

In order to further test for ovule and pollen viability, controlled crosses were conducted between the putative hermaphroditic and female individuals in the field and in the growth chamber (Percival E-36L2). Plant segments having buds were cut and planted in a soil mix containing 70% potting mix, 15% sand and 15% perlite. The planted pots were placed in the growth chamber with 12 hours of daylight and an optimal temperature of 25°C in the day and 15°C in the night. A total of 130 crosses were performed including both the plants in the field and the segments in the growth chamber. Plants in the field

were surveyed in early March 2018 and 2019 for floral buds, and in April 2018 and 2019 pollination tests were performed. Crosses were divided into 4 types: 1) natural controls – bee pollinated flowers, 2) outcross – pollen from hermaphroditic flowers was manually deposited on stigmata of pistillate flowers and anthers from pistillate flowers were removed and rubbed on the stigmas of hermaphroditic flowers as pistillate flowers lack pollen, 3) selfing - manually pollinated self-crosses for hermaphroditic and pistillate flowers and 4) negative control – cross pollination was avoided by bagging the buds with pollination bags. Each cross was noted with a colored tag and protected with fabric bags (ULINE, 2018). A monthly survey was conducted and dry fruits were collected from August-September 2018 and August-September 2019. Ovule counts, developed seed counts, and terminated ovule counts were done using a Nikon SMZ25 stereoscopic microscope. The seed set was calculated as the ratio of developed seeds with respect to the total number of ovules. A value of 0 indicates that no seeds were formed or no ovules were fertilized and a value of 1 indicates that all the ovules were fertilized to form seeds. A Welch two sample t-test was performed in the R program (R Core Team, 2017) to check if the seed set mean of outcrosses vs natural crosses differed significantly between the pistillate flowers. The fruit set was calculated as the ratio of fruits with seeds with respect to the total number of flowers. A value of 0 indicates that no fruits were formed and a value of 1 indicates that all the flowers developed to fruits.

### 2.3.5 Sex Ratio

We surveyed the plants present in a 600 meter transect in Mountain Springs, Imperial County, California. The sexes were identified through the presence/absence of de-

hiscent anthers releasing mature pollen at anthesis and in some flowers through the presence of ovules in flowers and confirmed by performing viability tests and cross sections in the lab as described above. The observed sex ratio was compared to the expected 1:1 sex ratio through chi square test in MS Excel using CHISQ.TEST function.

## 2.4 RESULTS

Based on histological examinations and cross-pollination experiments, we discovered that *C. wolfii* is functionally dioecious with individuals that have bisexual flowers that abort to one sex during the development process. Thus, the previously identified hermaphrodites are hereafter referred to as males or staminate flowers.

### 2.4.1 Androecium development

#### Staminate flowers

Staminate flowers develop a typical tetralocular anther with an anther wall composed of 4 single cell layers surrounding the Microspore Mother Cells (MiMCs). The wall is made of epidermis (Ep), endothecium (En), middle layer (Mi) and tapetum (Ta). Meiosis of the MiMCs results in four microspores, at this point the middle layer disappears and the tapetum disintegrates which contributes to the pollen nutrition and wall ornamentation of the intermediate pollen grain (IPG) (Fig. 2.3A). The epidermal cell begins to change into a conical shape. In late pre-anthesis, the anthers become bilocular with the disintegration of the connective tissues that form the septum of the microsporangia, and a stomium forms (Fig. 2.3B). During anthesis, the last layer consists of endothecium and epidermis in conical

shape. The wall dehydrates, and eventually dehisces (Fig. 2.3C) to reveal visible pollen (Fig. 2.2A and 2.2C).

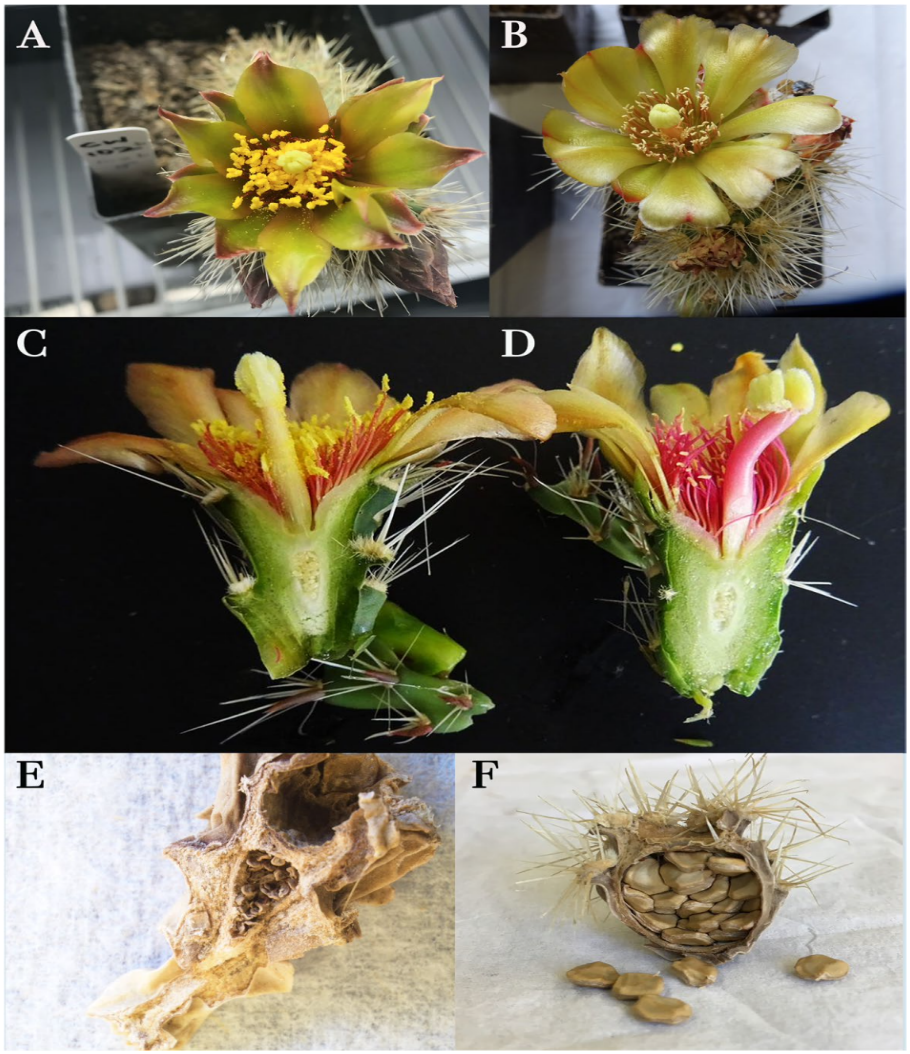


Figure 2.3: (A) *Cylindropuntia wolfii* staminate flower (left) and pistillate flower (right). (B) Lateral cross-section of staminate flower (left) and pistillate flower (right). Fruits collected from a staminate plant (C) and pistillate plant (D). Fruits collected from male (E) and female plants (F)

**Pistillate flowers**

Anther development of pistillate flowers is interrupted after the formation of the MiMCs and does not undergo meiosis. At this stage, the MiMCs and the cells of the anther wall are highly vacuolated (Fig. 2.3D). The MiMCs and the tapetum completely disintegrate, while the middle layer is atypically retained (Fig. 2.3E). This is followed by the hypervacualization of the epidermis and the endothecium cells and the disintegration of their nuclei (Fig. 2.3F). In pistillate flowers, the anthers at anthesis do not dehisce. The septum that separates the two microsporangia in each thecae remains visible (Fig. 2.3E), whereas it disintegrates in the male counterpart (Fig. 2.3B). Here, the anther development halts and no pollen is formed and no dehiscence occurs; therefore, no pollen is readily visible on the anthers of pistillate flowers (Fig. 2.2B and 2.2D).

### 2.4.2 Gynoecium development

**Staminate flowers**

In staminate flowers, differentiation of the ovule primordia is similar to that of the pistillate flower until the formation of the megagametophyte. The ovule primordia develops the integuments (It), the funicle, and Megaspore Mother Cells (MeMC) (Fig. 2.4A). During the megagametogenesis, the young circinotropous ovule degenerates. The degeneration starts on the megagametophyte and extends to the nucelle (Fig. 2.4B). The funicle exhibited some signs of disruption but it is not known if that is a direct disintegration or an artifact due the lack of internal support of the ovule during the sectioning. At anthesis, the ovule is completely disintegrated and cellular debris can be observed along with tannins

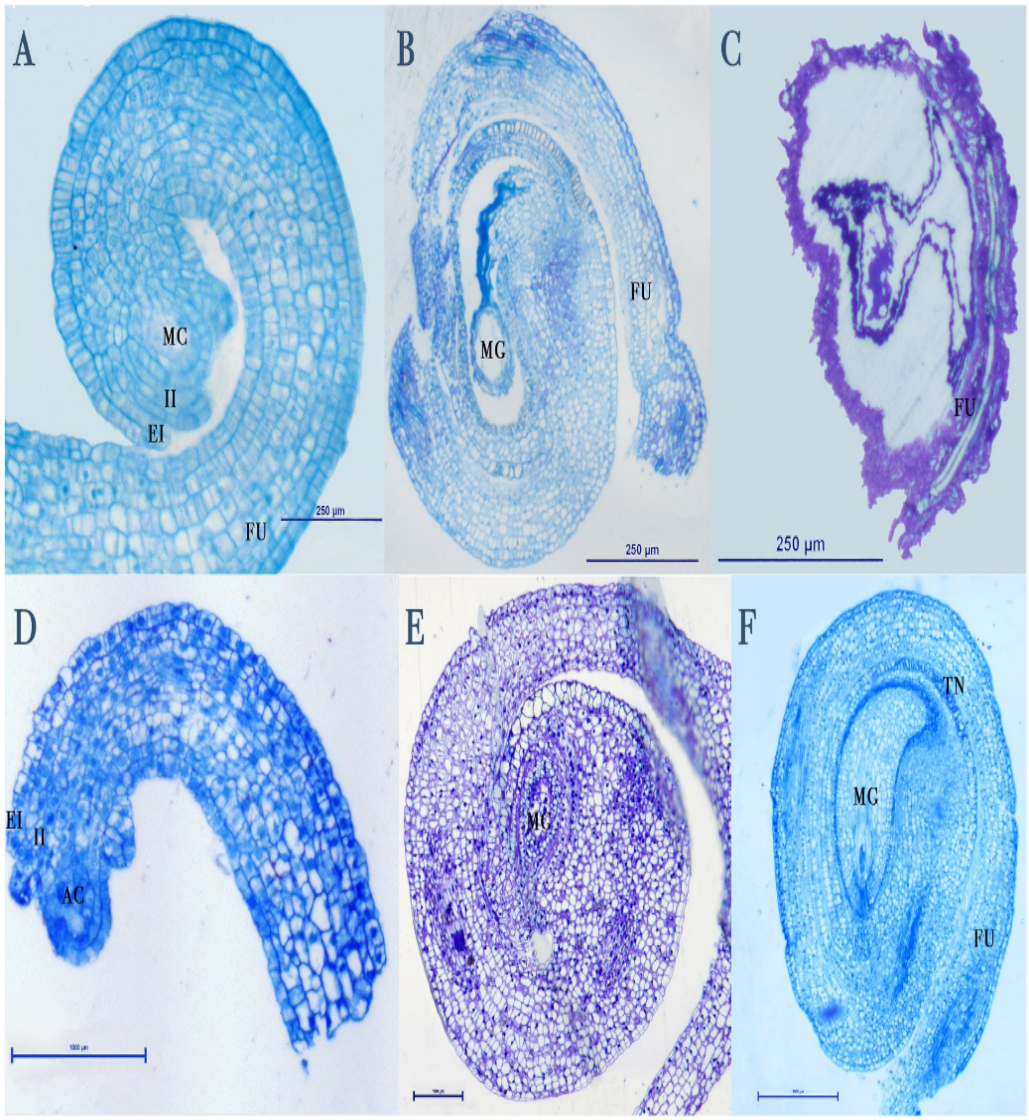


Figure 2.4: Ovule development of flowers of *Cylindropuntia wolfii*. (A) Ovule in male flower at pre-anthesis, with long funicle (FU) and integuments (external (EI) and internal (II)) developing with a Megaspore Mother Cell (MC). (B) Ovule of male flower in late pre-anthesis showing the collapse of the megagametophyte (MG). (C) Aborted ovule in male flower completely collapses in male flowers, at maturity only cellular debris are observed. (D) Ovule of female flower in early pre-anthesis, with a long funicle (FU) and integuments (external (EI) and internal (II)) developing with an archesporial cell (AC). (E) Ovule of female flower in early pre-anthesis showing the formation of the megagametophyte (MG). (F) Fully mature ovule of female flower in early pre-anthesis showing well developed megagametophyte and tannin (TN) deposits

in the integuments and vascular system in the debris of the funicle (Fig. 2.4C). Although staminate flowers' ovaries carry ovules early in development, (Fig. 2.2C) they are considered nonfunctional.

### 2.4.3 Pistillate flowers

In pistillate flowers, the primordial ovule developed starting with wrapping itself around with the long funicle, forming the integuments and the MeMCs (Fig. 2.4D). The pre-anthesis ovule has a curved nucelle, the MeMCs divides into 7 cells to form the megagametophyte. At this stage the development of the integuments is completed with the internal integument defining the micropyle (Fig. 2.4E). Tannin deposits in the integument are visible during megagametogenesis (Fig. 2.4E). At anthesis, the fully mature ovule is campylo-circinotropous. The megagametophyte is well developed and surrounded by the nucelle. The chalazal region is formed by the conjunction of the funicle and the integuments which have tannin deposits (Fig. 2.4F). The embryonic sac develops seven cells, three antipodal cells are ephemeral and only four cells are found at maturity including the central cell, the synergids and the egg at the micropylar end (Fig. 2.5).

### 2.4.4 Pollen tube visualization

The aniline blue dye used to stain the callose in pollen tubes, enabled clear visualization of the germinated pollen in different crosses (Fig. 2.6). The pollen tubes that germinated were observed in male $\times$ male selfed crosses (Fig. 2.6A) and female $\times$ male crosses (Fig. 2.6B). This germination of pollen in stigmas of pistillate and staminate flow-

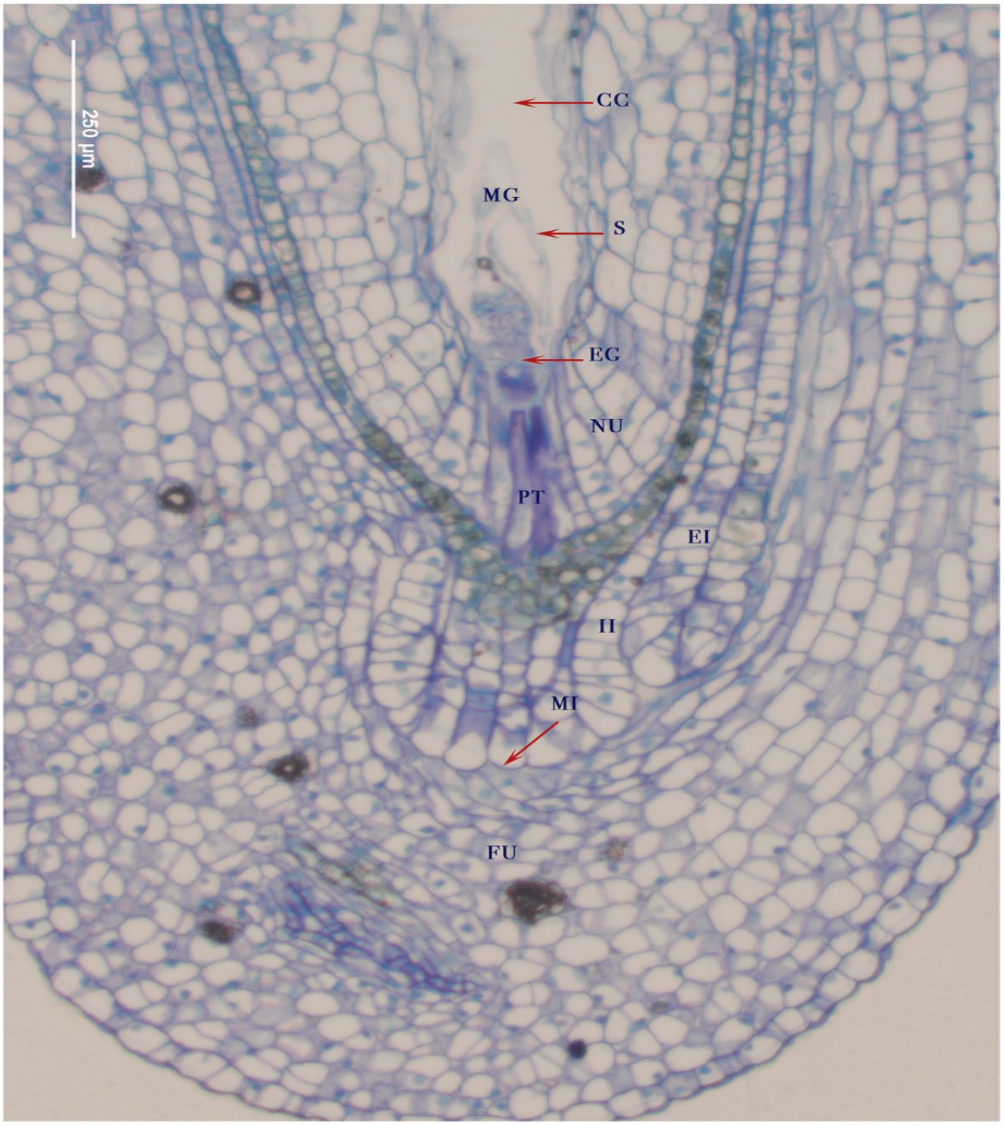


Figure 2.5: Fertilization of the egg (EG) by pollen tube (PT) in megagametophyte (MG) of ovules of female flowers. The pollen tube enters through the micropyle (MI). The megagametophyte is surrounded by nucelle (NU), internal integument (II) and external integument (EI), which in turn is surrounded by the funicle (FU). Four cells are found at maturity including the central cell (CC), two synergids (S, only one visible) and the egg (EG)

ers indicates that the stigma of pistillate and staminate flowers are functionally viable. As pistillate flowers did not contain any pollen, there were no pollen tubes observed between male x female crosses and female x female selfed crosses.

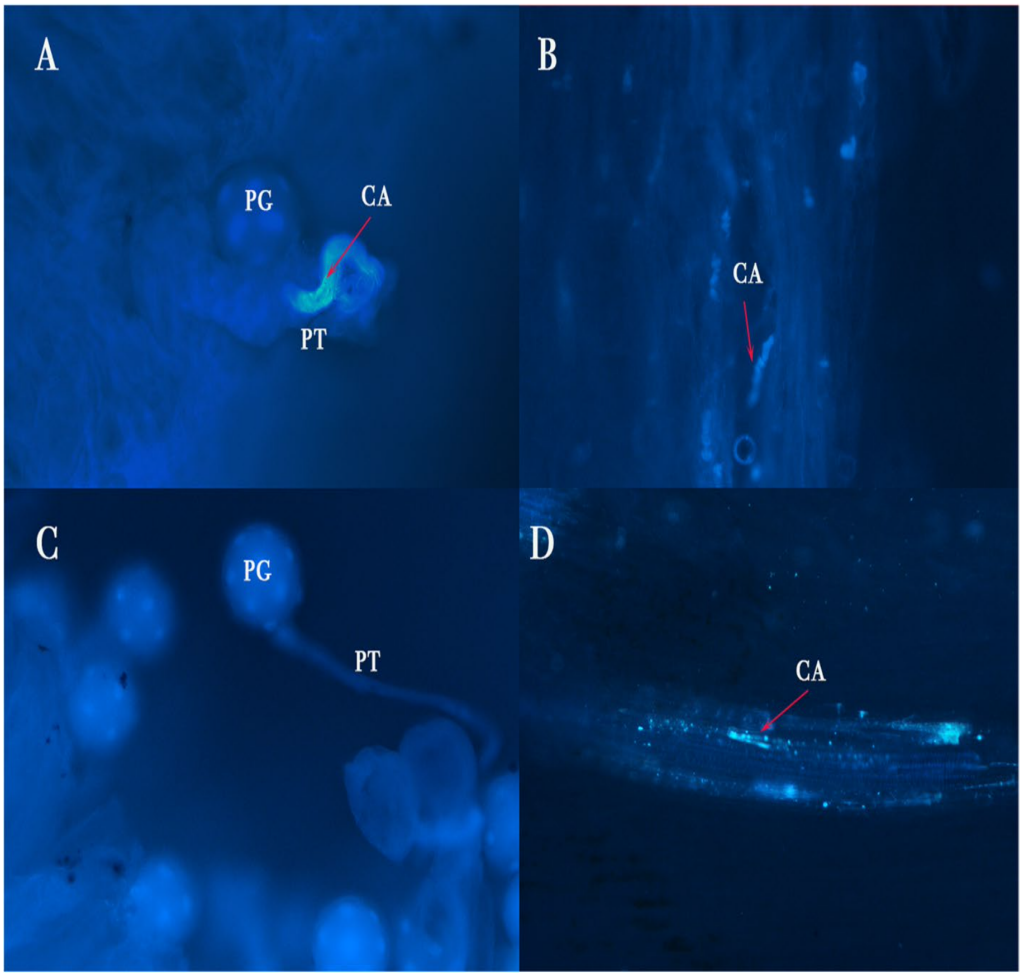


Figure 2.6: *Cylindropuntia wolfii* pollen tube (PT) development from pollen grain (PG) on stigma and style of flowers used for the crosses (A, B) male x male (selfing) male x female (C, D). The callose (CA) in the pollen tube fluoresces as a result of staining by aniline blue. Other crosses were not possible as females do not form pollen. Magnification:10X

### 2.4.5 Fruit development and Seed Set from Natural and Artificial Crosses

The fruits of *C. wolfii* become dry at maturity. Functionally female individuals were the only ones that formed mature fruits. Functionally male plants aborted the ovules, but ovaries were retained and dried (Fig. 2.2C). Thus, the fruits of female individuals are larger than male aborted fruits (Fig. 2.7A). However, a large number of fruits also aborted in functionally female plants, and some produced a few mature seeds with most ovules aborted, flattened, and located against the wall of the ovary (Fig. 2.7B). In contrast, no mature seeds were produced in functionally male individuals, (Fig. 2.7C). Some mature seeds of functionally female individuals lacked embryos (Fig. 2.7D), but most developed mature embryos (Figs. 2.7E and 2.7F). Since aborted ovules were visible under the microscope in both female and male fruits, we were able to estimate the seed set in both sexes in manual and natural crosses.

Using the controlled crossing experiment, we confirmed the ovule viability by formation of seeds. Fruits were collected when they appeared dry, plump, and brown (Fig. 2.7A). From the collected fruits, we calculated the average seed set for each type of cross (Fig. 2.8A). The seed set values of females acting as pollen receptors were as follows: 1) natural controls had a seed set of 0.05; 2) outcross had a seed set of 0.25; 3) selfing had a seed set of 0 and 4) negative controls had a seed set of 0.01. Male individuals acting as pollen receptors had a seed set of 0 in every cross type. The fruit set of males was 0 in all types of crosses and for females the values are as follows - 1) natural controls had a fruit set of 0.14; 2.2) outcross - 0.61; 3) selfing - 0 and 4) negative controls had 0.05 (Fig. 8B). Our data showed that the staminate flowers produced no fruits or seeds in any type of crosses

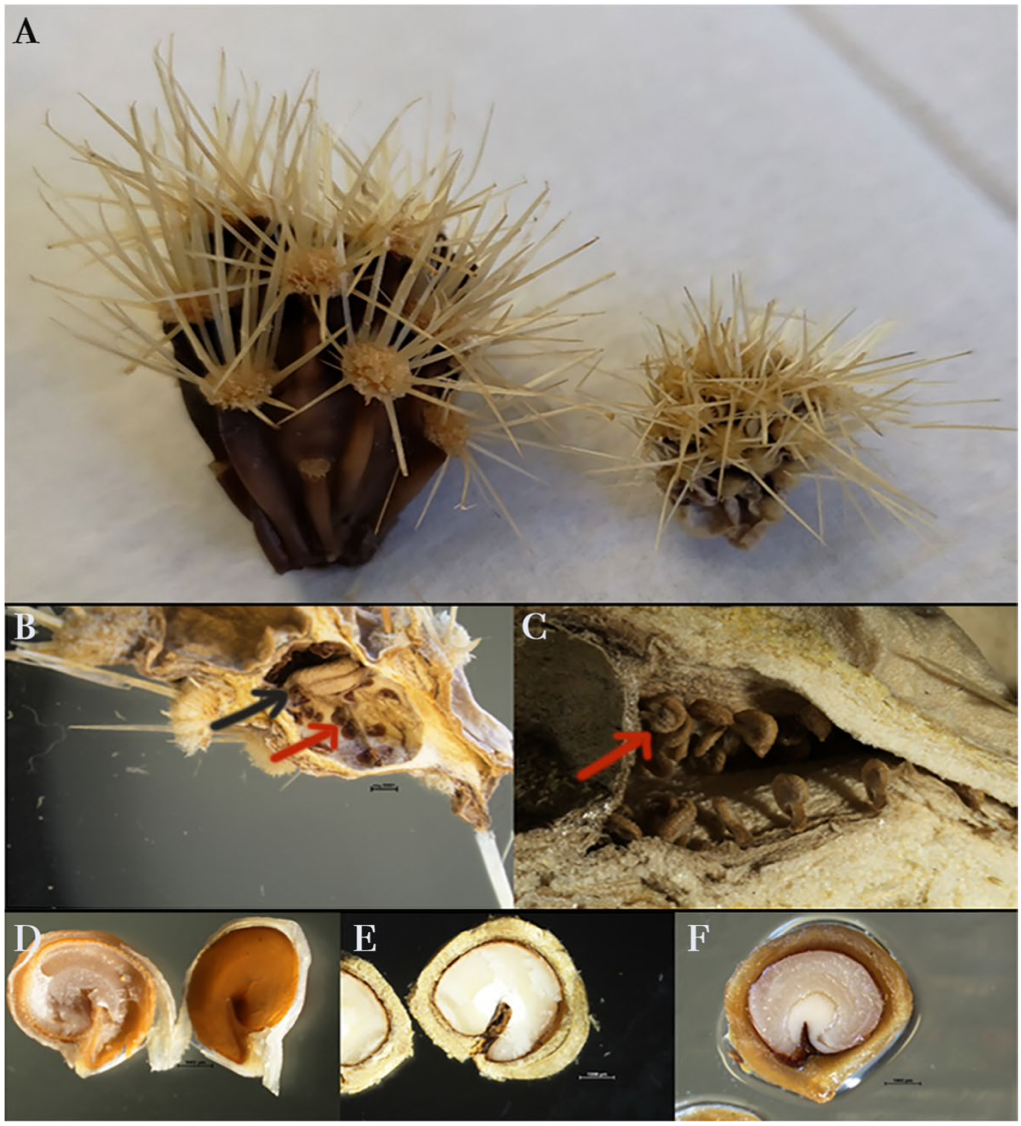


Figure 2.7: *Cylindropuntia wolfii* fruit and seed development. A) Fruits of female (left) and male (right) individuals. Aborted ovules are found in fruits of both female (B) and male (C) individuals (red arrows), however, fruits of female individuals also form some mature seeds (black arrow). D-F) Mature seeds from fruits of female individuals showing the thick funicular coat. D) Seed with abnormal development that lacks the white solid embryo as shown in E-F. The latter shows a mature seed with endosperm consumed by the fully developed embryo and remaining perisperm (arrow).

which denotes that even though they superficially appear to have a functional stigma and style, their ovules were not viable. On the other hand, we observed pistillate flowers to produce fruits and seeds in outcrosses, natural crosses and also in a single negative control individual. Overall, our results suggest that the pistillate flowers have viable ovules. Both sexual morphs subjected to selfing did not produce any seeds. This further supports that the sexual system of *C. wolfii* is functionally dioecious.

Our analysis shows that in general the seed set of *C. wolfii* is low, 0.25 for outcrosses and 0.05 for natural crosses. This difference in seed set between natural and outcrosses are significantly different (t = 3.2568, df = 50, p-value = 0.002027).

### 2.4.6 Sex ratio

A total of 137 plants were surveyed in the Mountain Springs area, 42 were identified as female individuals (31%) and 95 were identified as males (69%). The sex ratio of male: female was calculated to be 42:95, therefore there are approximately 2 males for every female in the sampled population. The male biased sex ratio is significant with p-value of 0.00000595177, chi-square statistic = 20.5036, df=1.

## 2.5 DISCUSSION

Given the recent evolution and wide polymorphism in reproduction, Cactaceae are considered an ideal family to study sexual systems in plants (Hernández-Cruz et al., 2019). Using histological observations, pollen viability tests, and controlled experimental crosses, we identified the sexual system of the rare cactus, *Cylindropuntia wolfii*, as functionally

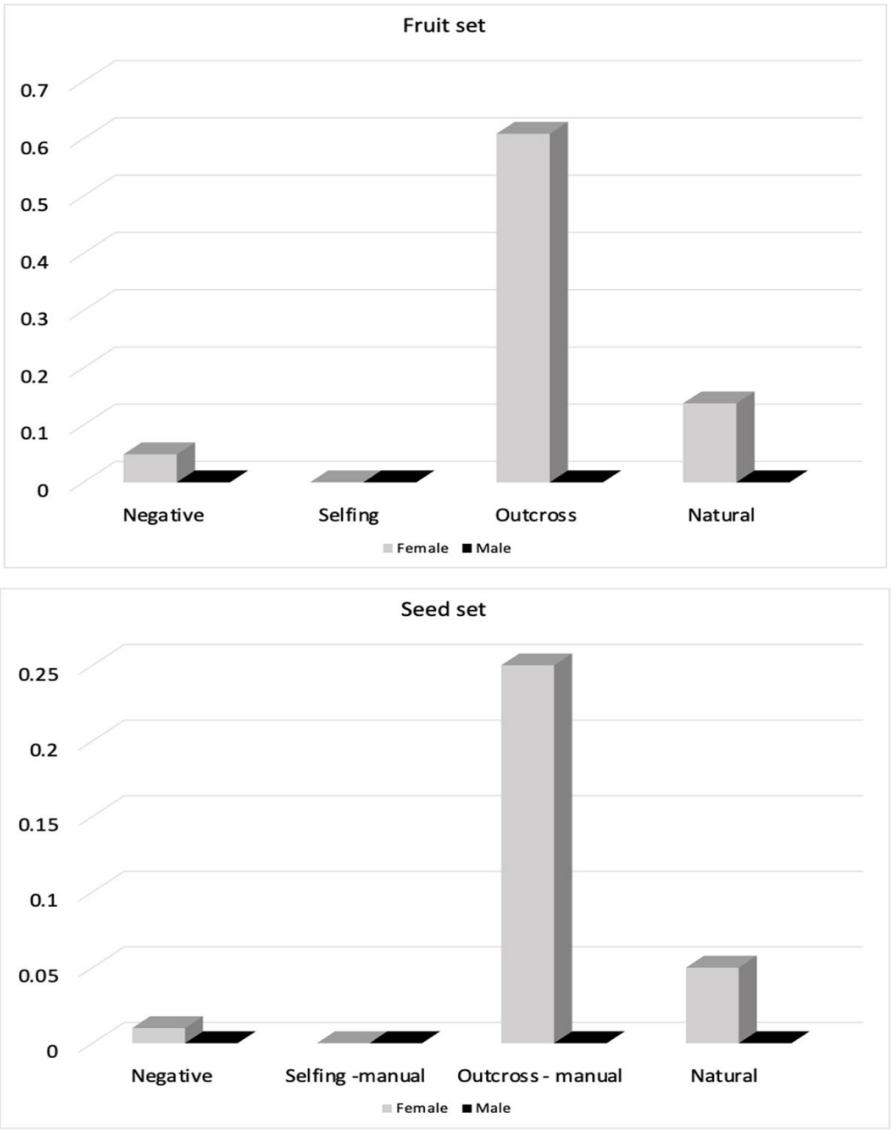


Figure 2.8: Experimental crosses in *Cylindropuntia wolfii*. (A) Bar chart showing the average fruit set (Y axis) obtained from various controlled crosses (X axis) in functionally female (gray bars) and functionally male (black bars) individuals. (B) Bar chart showing the average seed set (Y axis) obtained from various controlled crosses in functionally female (gray bars) and functionally male (black bars) individuals

dioecious and not gynodioecious (individuals with hermaphroditic flowers and individuals with pistillate flowers) as previously described (Rebman, 1998). This species has a Type II flower, with functionally female individuals retaining aborted anthers and functionally males retaining aborted ovules. In the staminate flowers of *C. wolfii* the differentiation of the ovule primordia is similar to that of the pistillate flower until the formation of the megagametophyte. During megagametogenesis the young circinotropous ovule degenerates starting on the megagametophyte and extending to the nucelle. At anthesis, the ovule is completely aborted, but it is retained. The retention of the ovules on staminate flowers during anthesis, makes it difficult to identify whether they are aborted, reinforcing the idea that histological analyses are needed to accurately characterize the sexual system. The female sterility pattern exhibited in *C. wolfii* is similar to the one presented in *Consolea spinosissima* in which the ovules abort just before anthesis and start with the disintegration of nucelle proceeding to a complete break down of the ovule (Strittmatter et al., 2002). In comparison, in both *O. robusta* and *O. stenopetala* female sterility happens much earlier suppressing the ovule in its primordial stage (Orozco-Arroyo et al., 2012; Hernandez-Cruz et al., 2019). On the other hand, in *Echinocereus* the ovule abortion takes place only after the zygote is formed (post-fertlization) (Hernandez-Cruz et al., 2018), requiring careful observation to determine the sexual system. These observations imply that the cellular mechanisms or pathways behind ovule abortion in *C. wolfii* might be different from that of other species in the Cactaceae.

From our histological data, it was revealed that the androecium development in pistillate flowers was interrupted in late pre-anthesis by the degradation of the tapetum

and MiMCs. The early degradation of the tapetum is known to affect the nutrient supply to developing microspores by causing variations in callose metabolism and other tapetal enzymes critical for normal development (Dong et al., 2005; Xu et al., 2014; Zhang et al., 2014). This pattern of tapetal disintegration is common in dioecious species of Cactaceae studied thus far although the temporal patterns might differ (Hernández-Cruz et al., 2019). The mechanism behind tapetal disintegration of most of the cacti species - *Opuntia robusta* (Hernández-Cruz et al., 2019), *O. stenopetala* (Flores-Rentería et al., 2013), and in the dioecious species of *Echinocereus* (Hernández-Cruz et al., 2018) is reported to be PCD. Moreover, tapetal disintegration by itself is a hallmark of PCD because mutations in male developmental genes lead to premature PCD or retarded PCD ultimately damaging the tapetum (Balk and Leaver, 2001). The ovule abortion in staminate flowers occurs after the anther abortion in pistillate flowers. This suggests that the unisexuality expression is asynchronous, a phenomenon reported in *Opuntia robusta* (Hernández-Cruz et al., 2019) and *O. stenopetala* (Flores-Rentería et al., 2013). In conclusion, tapetal disintegration to attain male sterility might be a conserved trait of the Cactaceae, but as the timings vary, the regulating pathways might be evolving separately.

Similar to *C. wolfii, O. robusta* and six species of *Consolea* have staminate flowers that appear to be hermaphroditic having a functional looking stigma and style. Our pollen germination tests showed that the stigma of staminate flowers in *C. wolfii* is functional which is also true for *O. robusta* and *Consolea* (Negrón-Ortiz and Strittmatter, 2004, Strittmatter et al., 2008; Hernandez et al., 2019). This suggests that there are no genetic or developmental barriers in the earlier stages of pollen recognition or pollen germination in

*C. wolfii*. In contrast, the staminate flowers of *O. stenopetala* are devoid of stigmatic tissues suggesting a strict self-incompatibility mechanism (Orozco-Arroyo et al., 2012). Thus, the degree of degradation in stigma and style differs among cactus species. The ovule abortion is the main cause for female sterility in most species of Cactaceae although the timing and degree of abortion differs. Although in *O. robusta*, female sterility was regulated by PCD (Hernandez et al., 2019), it is unclear if this mechanism is conserved throughout the Cactaceae.

The controlled experimental crosses showed that functional males were not capable of producing seeds through any crosses, further confirming that their ovules are non-functional. The pistillate flowers produced seeds in outcrosses, natural crosses, and also in one functional female used as a negative control. The seeds found in the female negative control could be an artifact or a sign of apomixis (i.e. adventive embryony) - which is the formation of seeds without pollination and this kind of reproduction has been reported previously in a variety of *Opuntia* species (Griffith, 2001) and in *C. fulgida* (Engelm.) F.M.Knuth (Baker and Pinkava, 1987). Overall, our results suggest that the pistillate flowers have viable ovules. Based on these results we predict that the abortion of one sex in unisexual flowers of *C. wolfii* is happening later in the developmental stages - after anthesis. This confirms that the sexual system of *C. wolfii* is functionally dioecious.

The seed set data from controlled crosses showed that, in general, the seed set of *C. wolfii* is low, and has been reported to produce generally abortive seeds (Mayer et al., 2011), but very little is known about its reproductive biology. A similarly low value of seed set (avg: 37 seeds/ 158 ovules = 0.23 seed set) has been observed in *O. microdasys* (Lehm.)

Pfeiff. where sexual recruitment by seeds is rare. Moreover, the seed set of *C. wolfii* was significantly lower in natural crosses as compared to manual crosses. This suggests that, in 2019, natural pollination may not be as effective as manual pollination in *C. wolfii*, possibly because of a low number of pollinators. Ecological studies are needed to ascertain the factors causing the reduced seed set in our system. About 40% of our outcrossed fruits failed to produce seeds. Some species of Cactaceae are narrowly distributed and dioecy could evolve to avoid the negative effects of inbreeding depression (Austerlitz et al., 2012). If dioecy prevents inbreeding depression through avoidance of selfing then why is the seed set of *C. wolfii* low? The low seed set suggests *C. wolfii* is struggling for sexual reproduction and the lack of easily detached segments suggests it does not use clonal propagation as its main way of reproduction. These combined might explain its restricted distribution. This, in turn, might lead to low levels of genetic diversity and high levels of inbreeding (Silvertown, 2008, Hamadeh et al., 2018) reducing the adaptive potential, and population genetic studies are necessary to address this concern and develop conservation strategies.

The sex ratio of the *C. wolfii* population in Mountain Springs (Imperial County) was significantly male-biased. Among dioecious species, a male-biased sex ratio is twice as common as the female-biased one (Field et al., 2013). A high female reproductive investment towards production of fruits may be the cause of the male-biased sex ratio (Charnov, 1982). In order to precisely identify the causes of a male-biased ratio in *C. wolfii*, we need to evaluate the stage of lifecycle in which the bias is established and factors that contribute to sex ratio differences such as life history traits (survival, growth, flowering, clonality, etc.) and phylogenetic relationships (Field et al., 2013). One of the consequences of a male-biased

sex ratio is a lower effective population size that can lead to bottleneck effects (Sinclair et al., 2012). As bottlenecks can cause inbreeding, it is of interest to resolve if dioecy with male biases is really adaptive.

The evolution of sexual separation and its association to polyploidy has been known for a long time (Stebbins, 1950), but it is uncertain whether it is a cause, consequence or both. This relation is also seen in many species of Cactaceae. For example, *Pachycereus pringlei*, *O. robusta, Consolea* species and five species of *Echinocereus* have a sexual system with unisexual flowers (e.g. trioecious, gynodioecious or dioecious) and have a level of polyploidy either as tetraploid, hexaploid or octaploid (Ferguson, 1989; Rebman, 2003; Baker, 2006; Segura et al., 2007; Majure et al., 2012). This pattern is consistent in the genus *Cylindropuntia* in which polyploid species are either gynodioecious, e.g. *C. chuckwallensis, C. sanfelipensis, C. calmalliana,* and *C. molesta* (see Baker and Cloud-Hughes, 2014) or functionally dioecious such as in *C. wolfii.* Exceptions to this rule include *O. stenopetala* and *O. grandis* Pfeiff. which are subdioecious and diploid (Pinkava et al., 1977; Baker and Cloud-Hughes, 2014), *C. arbuscula* (Griffiths) F.M.Knuth. which is triploid, but does not have a dioecious system although it seems to propagate mostly by clonal means (Cloud-Hughes, 2014, Pinkava and McLeod, 1971), hermaphroditic species such as *C. bigelovii* (Engelmann) F.M.Knuth. which is mostly triploid and rarely diploid, *C. prolifera* (Engelmann) F.M.Knuth. which is triploid and *C. cholla* (F.A.C.Weber) F.M.Knuth. which is mostly diploid and rarely tetraploid (Baker et al. 2009). However, *C. wolfii* was previously described as gynodioecious. but in this study we have shown it has a functional dioecious system. Therefore, a detailed study is warranted for the other polyploid species

of *Cylindropuntia* whose breeding systems are described based on superficial observations (e.g., *C. arbuscula, C. calmalliana, C. chuckwallensis, C. molesta, C. sanfelipensis*).

## 2.6 CONCLUSION

Our study aimed to identify the sexual system and present the developmental process of unisexual flowers of a rare and endemic cactus of the Sonoran Desert - *Cylindropuntia wolfii*. Through controlled cross experiments, histological analysis, and pollen viability tests, it was confirmed that the sexual system of *C. wolfii* is functionally dioecious rather than gynodioecious as previously described. The ovule abortion in staminate flowers and pollen abortion in pistillate flowers occurred during and before anthesis respectively, presenting asynchrony in formation of unisexuality. Pistillate flowers underwent anther disintegration to attain male sterility and staminate flowers underwent ovule abortion for female sterility. In species of cacti with unisexual flowers, the developmental mechanisms underlying sterility are similar, although the timing of abortion differs. By characterizing the developmental events of unisexuality, our study complements the existing literature on the emergence of different sexual systems. Furthermore, this is the first study describing the development of unisexuality in the genus *Cylindropuntia*. Evidence from the seed set of *C. wolfii* suggests that this species might be struggling to reproduce sexually and occupies a very restricted distribution which could lead to inbreeding depression. Further field and genetic studies will offer opportunities for gaining more insights into the consequences of dioecy in this rare native species.

# Chapter 3

# Ecological Ramifications of Sexual Separation

## 3.1 ABSTRACT

Sexual separation in plants is associated with sexual dimorphism and identifying dimorphic traits is critical in advancing our knowledge on plant–pollinator interactions. For example, dimorphism in floral colors, or sexual dichromatism, is a crucial mediator of pollinator choice on foraging decisions. We studied *Cylindropuntia wolfii*, a model system, with diverse flower colors and a functionally dioecious sexual system. However, evidence suggests that sexual reproduction is limited in this species as it has a low seed set especially in naturally pollinated fruits. Thus, it is critical to this native species' conservation to

The materials presented in Chapter 3 have been published in Oecologia 2023 as "Influence of sexual dimorphism and dichromatism on reproductive success in a rare native cactus"

investigate its relationship with pollinators. Our goals were to: (a) investigate the sexual dimorphism including the sexual dichromatism in the flowers of the cactus, and (b) determine whether sexually dimorphic traits affect the pollinator attraction of both the sexes. We measured several quantitative and qualitative traits and compared them between male and female flowers. Then we recorded the pollinator visitation rate in nature for both sexes and tracked pollinator color preference using fluorescent dyes as pollen analogues. Our study showed that male flowers of *C. wolfii* are bigger and brighter, and they attract more potential pollinators than females, supporting the hypothesis that sexual dimorphism influences pollinator visitation preference. Fluorescence dichromatism, in which female flowers' anthers fluoresce more than male flower anthers suggest this could be female flowers' strategy to compensate for their dark colors and small size. The results from this study showed that *C. wolfii* exhibits sexual dichromatism and fluorescence dichromatism, which is a novel finding in plant research.

## 3.2 INTRODUCTION

Dioecy, a sexual system where female and male flowers are separated in individuals, is present in 6% of angiosperms and its occurrence is observed in about 40% of the angiosperm families (Renner 2014). Dioecy is reported to have evolved independently in many lineages at least 871 times (Renner 2014). One common factor that sexual separation is accompanied by is sexual dimorphism (Correns 1928; Lloyd and Webb 1977; Barrett and Hough 2013). Sexual dimorphism is defined as the morphological differences between males and females in secondary characters, including traits other than reproductive organs.

In dioecious plants, sexual dimorphic traits include floral size (Delph et al. 1996), flower longevity (Primack 1985), number of flowers per individual (Delph et al. 2005), scent (Ashman 2009), nectar production (Bawa and Opler 1975). However, sexual dimorphism in terms of color, also known as sexual dichromatism, has not been reported in plants to the best of our knowledge. In contrast, it has been widely reported in animals such as birds (Badyaev and Hill 2003), fishes (Kodric-Brown 1998) and reptiles (Olsson et al. 2013). Studies on sexual dimorphism in plants have identified few species that exhibit intersexual mimicry to overcome pollinator visit discrimination. This mimicry is especially prevalent in plants where the females do not offer any rewards such as pollen or nectar (Delph et al. 1996; Ashman 2009). While this mimicry is not always successful, theory predicts that it should not effectively affect reproduction as long as there is no pollinator limitation (Yang et al. 2022).

The evolution of sexual dimorphism has been explained by sexual selection, especially in animals (Darwin 1851; Bateman 1948). Darwin (1851) suggested that two factors contribute to sexual selection of traits: male–male competition and female choice. In the past decades, this principle has also been increasingly applied to plants (Delph and Ashman 2006). Male–male competition in plants results in male flowers becoming more attractive to pollinators by evolving a brighter display, more potent scent, and other relatable traits (Stanton 1994). However, there can be other architectural traits that are sexually selected but do not have an effect on biotic pollinator attraction, in the wind pollinated species (Tonnabel et al. 2019). Even within the insect pollinated species, forces other than pollinator-mediated selection like fertility selection in females, can select for traits that im-

prove the reproductive success (Barbot et al. 2022). From the female's perspective, studies have observed selection for fertility traits such as ovule number and flower number (Delph and Herlihy 2012; Barbot et al 2023), although flower number can also be a result of pollinator mediated selection. Studies have shown that the mating success (finding mates) increases with reproductive success (siring offspring) in males while they are not correlated in females and hence the difference in selection acting on different floral traits (Tonnabel et al. 2021; Kwok and Dorken 2022).

On the other hand, cryptic female choice is predicted to occur in plants, but there is little evidence as it usually occurs a long time after fertilization through pre-selected abortion of ovules during the embryogenesis (Moore and Panell 2011). Recently, Tonnabel et al. 2022 reported female choice in wind pollinated species which involved traits such as pollen tube growth, size of stigma and expression of pollen proteins that facilitate competition for ovules. This suggests that sexual selection continues to occur even after the pollen dispersal phase of plant reproduction.

Studies on plant sexual dimorphism will advance our knowledge on plant–pollinator interactions and the influence of pollinators' preference on plant sexual separation. The key factors that contribute to the study of plant–insect pollination and their conservation in the face of climate change and loss of biodiversity are floral strategies and floral resource availability (Vanbergen and Insect Pollinators Initiative 2013). One of the crucial mediators of plant and pollinator interaction is flower color polymorphism (Weiss 1991). While many factors contribute towards pollinator choice for a particular flower, a large number of studies have suggested that flower color widely influences their choice on foraging decisions (Dafni

et al. 1990; Ômura and Honda 2005; Dötterl et al. 2014; Reverte et al. 2016). Diurnal insect pollinators have trichromatic vision which covers a broader range of spectrum than humans. Their vision includes visible light in the UV, fluorescent, blue, and green spectra (Chittka 1992; Mori et al. 2018). Based on phylogenetic analysis, it has been shown that pollinators were pre-adapted to trichromatic vision long before the widespread radiation of angiosperms. This evidence leads to the hypothesis that flowers have evolved their beautiful colors to visually attract the pollinators (Chittka 1997; Reverte et al. 2016). Among floral color traits, UV patterns play a major role in pollinator preference since UV-absorbance and reflection guide the pollinators to foraging parts (Lunau et al. 2017). Several studies have demonstrated the importance of UV patterns in bees through artificial manipulation of UV patterns in flowers (Welsford and Johnson 2012; Rae and Vamosi 2013; Brock et al. 2016; Klomberg et al. 2019). Recently, a study by Rao and Ostroverkhova (2015) demonstrated the strong attraction of a wide variety of wild bees to fluorescence under UV excitation. Therefore, studies in plants displaying dimorphism in floral traits and the effect of floral color polymorphism, including UV and fluorescence patterns, on pollinator attraction is needed. Sexual dichromatism in fluorescence has only been recently reported in blue winged parrotlets (Barreira et al. 2012), highlighting the need to further study the role of fluorescence patterns on sexual dimorphism. Moreover, the relationship between sexual sterility in plants and the specific autofluorescent properties of anthers remains an area of scientific investigation that has not yet been explored fully (Araki et al. 2020; Nakashima et al. 1984).

*Cylindropuntia wolfii* (Cactaceae) is an exceptional model to study sexual dimorphism and dichromatism, and its influence on pollinator attraction owing to its six distinct color morphs within the same geographical location (Fig. 3.1) and its functionally dioecious sexual system (Ramadoss et al. 2022). The sexual system is called functionally dioecious as their flowers carry vestigial organs that are non-functional. Both the male and female flowers have anthers but males have pollen on them and females lack pollen (Ramadoss et al. 2022). This species is rare and native to California and Baja California. *Cylindropuntia wolfii* is ranked 4.3 on the Inventory of Rare and Endangered Plants by the California Native Plant Society (1968 onwards) implying it has limited distribution but the populations are not threatened. Moreover, our previous study on *C. wolfii* showed that it had a lower seed set compared to its close relatives (Ramadoss et al. 2022). This seed set was significantly lower in naturally pollinated fruits compared to manually pollinated ones. Thus it would be beneficial to this native species' conservation to explore its relationship with pollinators. Our goal here is to investigate the sexual dimorphism of the cactus *C. wolfii* and determine its effect on pollinator attraction.

## 3.3 METHODS

### 3.3.1 Study site and species

The present field work was carried out in Mountain Springs (Imperial County, CA) from April 2021–July 2022. *Cylindropuntia wolfii* is a rare endemic shrub which is narrowly distributed only in the Sonoran Desert of southern California and Baja California. This is the only known population of this species. It is densely branched with stout cylindrical

stems and sheaths on their spines. The flowers have six tepal colors ranging from green to red with green to dark pink filaments on their stamens (Fig. 3.1).

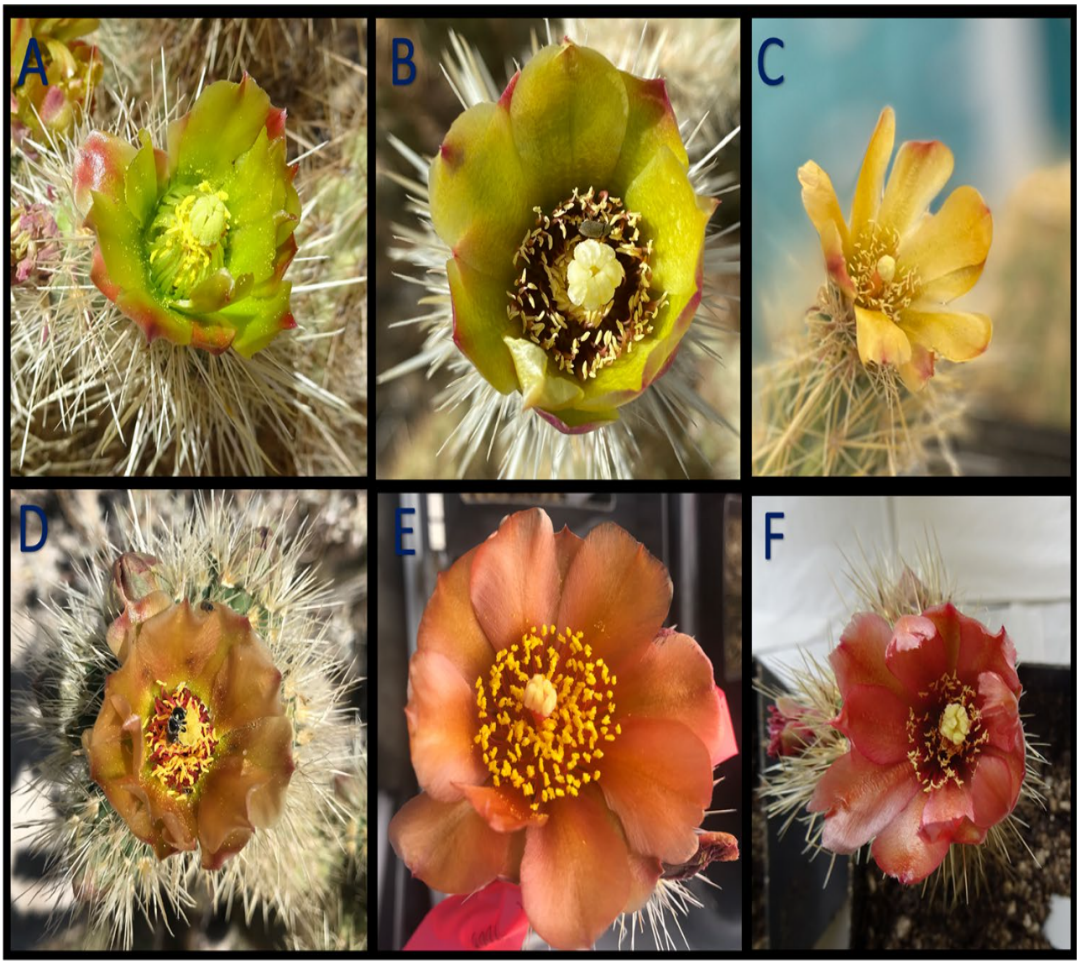


Figure 3.1: Six color morphs are found in C. wolfii flowers— Green (A), Greenish yellow (B), Yellow (C), Yellowish orange (D), Orange (E) and Red (F). In addition, three different filament color morphs are present, for example (A) green filaments (C) pink filaments, and (D) red filaments

### 3.3.2 Flower dimorphism measurements

To determine sexual dimorphism, we measured and compared several quantitative and qualitative characters between the male and female flowers. The qualitative floral traits refer to flower color and filament color, and quantitative factors refer to flower display diameter, tepal length, tepal width, and number of flowers open at a time per plant. For the qualitative factors, we recorded the flower color and sex of only 53 plants along a 600 m transect in Mountain Springs (Imperial County, California). The flower colors were attributed based on a self-built color map with the help of herbarium color reference chart (McCamy et al. 1976). The six flower color morphs were further distinguished into two groups as brighter (green, greenish yellow and yellow) and darker (yellowish orange, orange and red) colors. During our pollinator survey, we visually and statistically observed a pattern where brighter morphs attracted more than the darker morphs so we split the six color morphs into two groups based on their color gradient, grouping 3 morphs per group. The low sample size we have here is due to asynchronous blooming and reduced number of plants. The filament color was recorded from 94 plants we collected from the field. A Fisher's independence test was performed to check if the qualitative factors are related to sex.

Then, quantitative factors which are display diameter, tepal length and tepal width were measured and compared between the two sexes. To control for the variability in tepal factors, we randomly chose 2 tepals per flower and took the average measure of them. The survey was done in 2022 with a sample size of 94 plants with at least seven plants per sex and color combination. A general linear model of ANOVA was used to assess the significant

differences in quantitative traits between sexes after testing for normality using Shapiro's test and homoscedasticity using Bartlett's test. We then explored the correlation between color morph and flower size to assess if brighter colored flowers, regardless of sex, are bigger using a two-way ANOVA.

### 3.3.3 Ultraviolet (UV) and green fluorescence (GF) measurement

To determine sexual dimorphism in terms of UV and GF, we measured their emission intensity in male and female flowers. Plant fragments with buds were collected. At least seven fragments per color and sex combination (i.e male reds, female reds, male greens and so on) were obtained and tracked until blooming to assess dimorphic differences. The fragments were potted in 70% soil, 15% perlite, and 15% sand mixture and grown in a growth chamber. The photoperiod was set to 12 h with day temperature of 25 °C and night 15 °C. The bloomed flowers were collected and mounted under a NIKON stereomicroscope (SMZ-25/18) at 0.5X magnification. Photographs were taken using RBG (Red Green Blue) filter for human eye view and Blue, green, and UV filter for bee's eye view (Fig. 3.2) based on the false color photography protocol by Verhoeven et al. (2018). In addition, GFP (Green Fluorescent Protein) filter cube photographs were also taken to capture the green autofluorescence of anthers in male and female flowers. Photographs were analyzed using Nikon BRanalysis software to measure the emission intensity of UV and GF per unit area, from the flowers. The emission intensities were compared by ANOVA and post hoc tests in R and R studio.

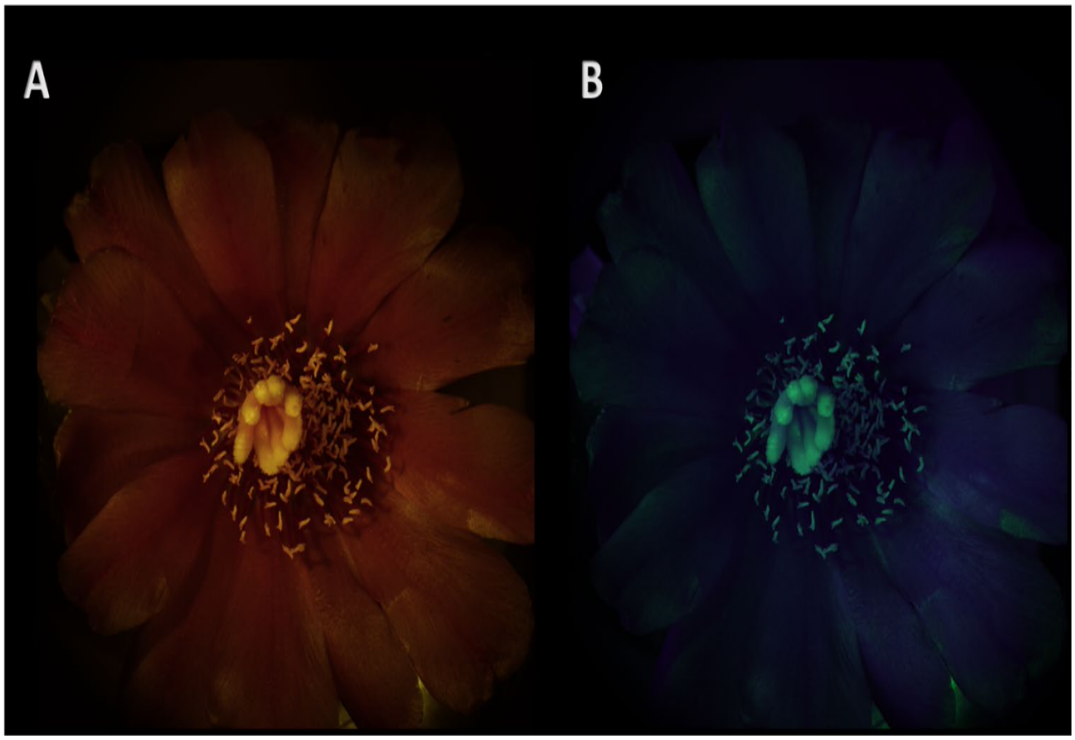


Figure 3.2: Human eye view (A) vs Bee's eye view (B) of a red *C. wolfii* flower

To further investigate the autofluorescence in female flower anthers, buds from *C. wolfii* females were collected from the pre-anthesis stage as that is when the premature degradation of tapetum occurs (Ramadoss et al. 2022). The anthers were placed in glass vials containing 70% ethanol for preservation. The anthers were frozen in the cryostat's freeze medium and then cut into sections with a thickness of 10 µm using a Leica CM1950 cryostat. The cryo-sectioned samples were placed on SuperFrost Plus microscope slides (Fisher Scientific, USA) and observed under Nikon stereo microscope under regular vs GFP light for observing the location of autofluorescence.

### 3.3.4 Pollinator visitation rate

To determine the pollinator visitation rate between the sexes, we followed three steps. First, we determined whether *C. wolfii* flowers attract potential pollinators by manually surveying the diurnal floral visitors during the peak flowering season, April–May of 2021. We surveyed 24 plants including both sexes and all colors except for male red as they are hard to find, for 10 min each during the daytime (between 8 am and 2 pm when the pollinators were present) in the Mountain Springs field site on a clear sunny day. The number of visits and types of visitors (i.e., bees, moths, beetles) were noted down. The percentage of visits from each type of visitor was calculated. Two samples from each pollinator species were collected and identified.

After confirming the presence of potential pollinators in the first step, we quantified the pollinator visitation rate per flower for the two sexes and tested whether one sex or color attracts pollinators more than the other in the secind step. Here we manually surveyed the plants and noted the number of pollinators' visits for each flower. We surveyed 75 plants

for all six colors with at least 11 plants per color and 32 males and 43 females that had one branch with one flower in the field and observed them for 10 min each. This survey was conducted for diurnal floral pollinators during the peak flowering season, April–May of 2021. We used a two-way ANOVA to test if pollinator visits were influenced by sex, color or interaction of both. We log transformed the visits data to achieve a normal distribution. The visits data then satisfied the assumptions of normality and homogeneity of variance. We controlled for the time of day by using time as a random factor in ANOVA. We also assessed the influence of flower color on pollinator visitation, categorizing it into brighter hues encompassing green, greenish yellow, and yellow, and darker shades, which included yellowish orange, orange, and red flower colors.

Finally, in order to corroborate that the potential pollinators exhibiting biased visits to these flowers were indeed responsible for pollen transportation, we employed the fluorescent dye powder. These fluorescent dyes act as pollen analogues, and this method is a good indicator of pollen dispersal (Fenster et al. 1996). We used three colors of dye: green, violet, and orange due to restricted availability. Subsequently, we organized the six color morphs into pairs based on their proximity along the color gradient, resulting in the creation of three primary flower color categories, grouping them as follows: green=green/greenish yellow; yellow=yellow/yellowish-orange; red=orange/red. It was ensured that significant differences in pollinator visits were not observed within these pairs. We selected male plants that are of these three colors in four separate locations along a 188 m transect. Since females do not have any pollen we did not include them in this experiment. The selected plants were marked with flags and GPS points. We then applied the color dye using a sterile

brush on the anthers. The following colors were green, violet, and orange corresponding to green, yellow, and red flowers respectively. To avoid cross contamination, three individuals performed the dye deposition separately. The deposition was carried out on a clear sunny day early morning to avoid dye carryover by abiotic factors. We visited the study site that same day after sunset to observe the flowers in the area under UV light. We surveyed only the plants with flowers present within a 950 m2 quadrant around the dyed flowers. We manually noted the presence or absence of fluorescent dye in 55 flowers of the neighboring plants around the four locations. Then we calculated the percentage of flowers that had the different colored dyes on them from the flower counts data.

## 3.4 RESULTS

### 3.4.1 Males flowers are bigger and brighter

Our study on sexual dimorphism in floral traits revealed three quantitative traits were significantly different between sexes with males always having a higher value for each: tepal length ($P < 0.01$), tepal width ($P < 0.01$), and display diameter ($P < 0.01$) (Fig. 3.3). In addition the color of the flowers also influenced the size traits such as diameter ($P < 0.01$), tepal length ($P = 0.01$), tepal width ($P < 0.01$) but we did not see a consistent pattern that explains brighter color flowers regardless of sex being bigger. For display diameter, yellow flowers regardless of sex are bigger than green ($P < 0.01$). For tepal length, yellowish orange flowers are bigger than green ($P = 0.01$). For tepal width, yellowish orange flowers are bigger than green ($P = 0.01$) and orange flowers ($P < 0.01$). The other colors were not significantly


Figure 3.3: Box plots showing *Cylindropuntia wolfii* males have a significantly larger display diameter (A), tepal width (B) and tepal length (C) than females

different from the others.

We found that flower color and sex are related (X2=18.63; P=0.03). For example, male flowers are usually associated with green and yellow shades while the females are mainly associated with orange and red shades (Table 3.1). We acknowledge that the number of females is low. Unfortunately, this is due to the small sample size and likely also influenced by the male-biased sex ratio, as we found few females. In addition, the filament color (Fig. 3.4) differed between the six color morphs and had a correlation with brighter colors generally displaying brighter filament colors (green and pink) ( $P<0.01$) (Fig. 3.4). There was no significant difference between the number of flowers in males and females. Both sexes had a mean of about 26 flowers per plant.

Table 3.1: Counts of sexes found in different tepal colors. Males are gradually decreasing and females are gradually increasing with darker shades of colors

| Tepal color | No. of males | No. of females |
|---|---|---|
| Green | 9 | 1 |
| Greenish yellow | 10 | 2 |
| Yellow | 5 | 2 |
| Yellowish orange | 8 | 2 |
| Orange | 3 | 4 |
| Red | 2 | 5 |

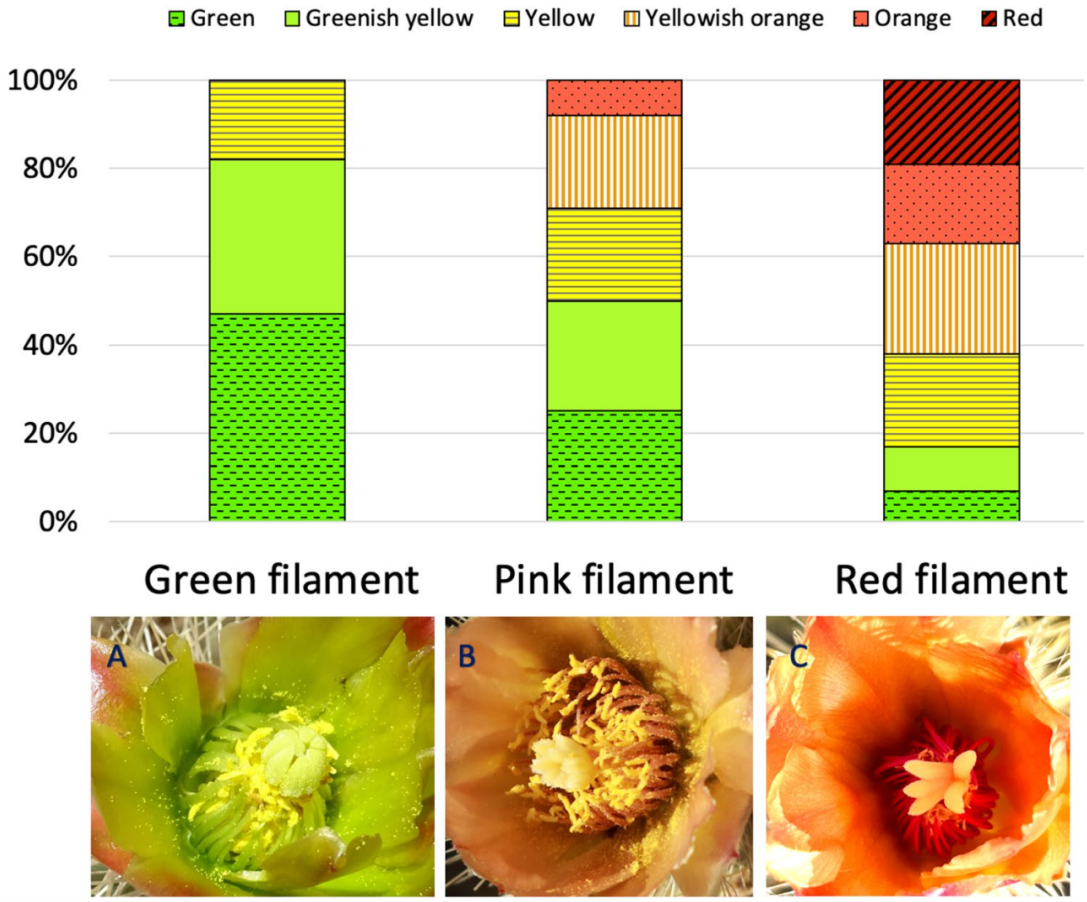


Figure 3.4: (Top) Bar plots showing the frequency of the three fila- ment colors (green, pink, and red) across the six flower color morphs (green, greenish yellow, yellowish orange, orange, and red). Green filament color is not seen in darker shades of flower color (e.g., yellowish orange, orange and red). (Bottom) *Cylindropuntia wolfii* has flow- ers with three different filament color morphs—Green (A), Pink (B) and Red (C)

### 3.4.2 Female flowers fluoresce more than males

A total of 113 flowers were imaged under RGB, UV, and GFP filter cubes. We sampled at least seven flowers per sex and color. The mean UV emission intensity per-unit area and mean GF emission intensity per-unit area were measured using the Nikon BR elements application. The intensity values were compared between the two sexes and among the six colors using a linear model in ANOVA and Tukey's post hoc test. The data fit the normality and homoscedasticity assumptions of ANOVA. Sex and color were fixed factors and individuals were random factors. We checked if the emission intensity of the individuals changed as a factor of sex or color or interaction between sex and color. The ANOVA analysis showed that there were no significant differences in the UV emission intensity between the sexes ($P=0.09$) or color ($P=0.11$). However, the GF emission intensity in the anthers differed significantly between the sexes. Anthers of females were shown to have a higher emission of GF compared to that of males (Fig. 3.5; $P<0.01$). This could be due to the obstruction of fluorescence by pollen in male anthers. Further, the GF emission intensity of tepals did not vary significantly as a factor of sex or flower color. When the cryo-sectioned female anther sections were viewed under the GFP filter light, autofluorescence was observed in the areas of degraded tapetum (Fig. 3.6).

### 3.4.3 Males attract more potential pollinators

We observed that *C. wolfii* flowers attracted diverse insects such as beetles (5%), moths (5%), and potential pollinators like bees (90%). Two species that we collected were identified as *Apis mellifera* (European honey bee) and *Diadasia spp.* (native cactus bee).

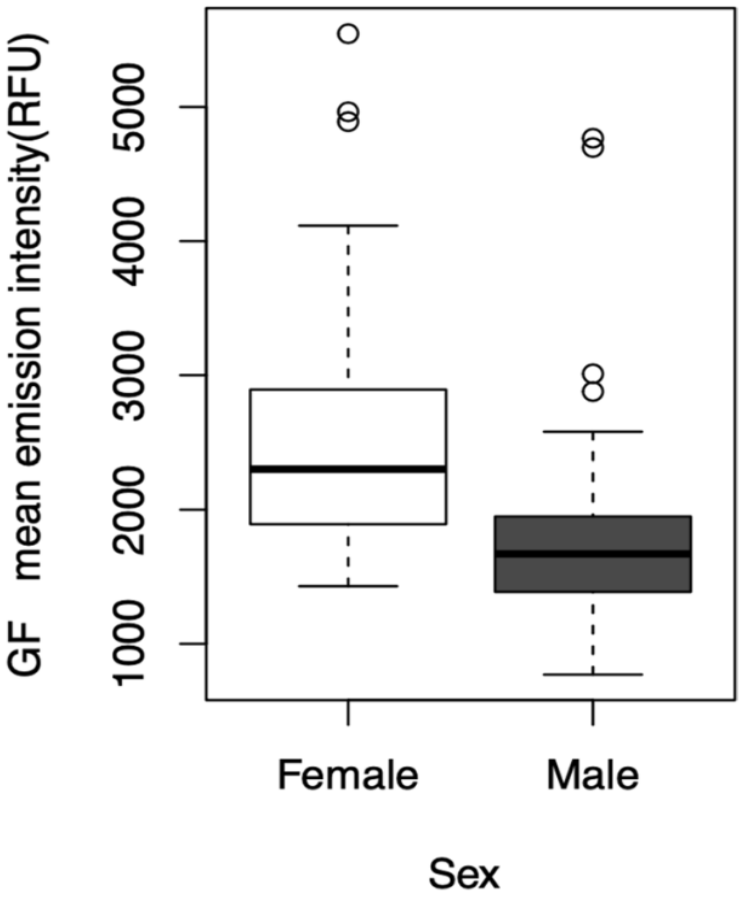


Figure 3.5: Boxplot showing GF emission intensity of anthers in females being higher (i.e. more fluorescent) than males

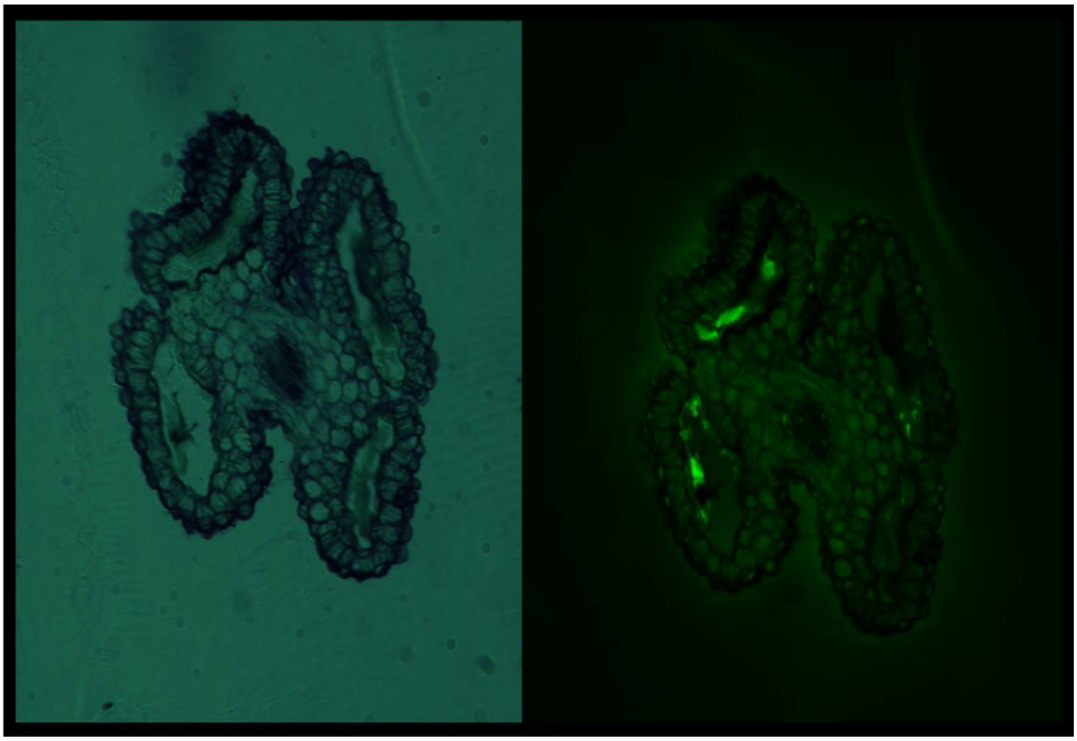

Figure 3.6: Cross sections of female anthers at pre-anthesis under regular light (left) vs GFP light (right)

We identified the beetles as belonging to the Nitops genus and the moth as a member of the hummingbird moth taxon (*Hemaris spp.*).

Our ANOVA results on the potential pollinator visitation rate showed that sex and tepal color influenced the pollinator visitation rate. Males attract more potential pollinators than females (P=0.02) (Fig. 3.7A). In terms of colors, we observed that brighter flower colors attracted more pollinators than darker flower colors (P=0.02) (Fig. 3.7B) but there was no significant interaction between flower color and sex in influencing pollinator visits (P=0.12). The time of the day did not have a significant effect (P=1).

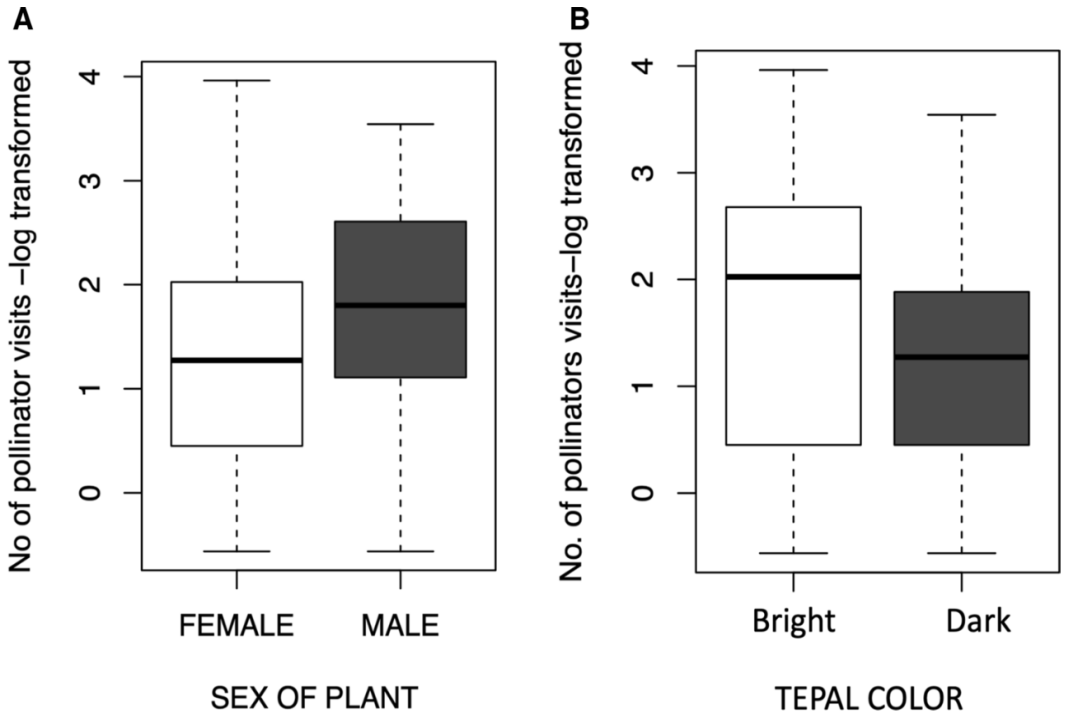


Figure 3.7: Box plots showing pollinator visitation rate with respect to sex (A) and flower color (B). In (A), males attract significantly more potential pollinators than females. In (B) Bright green-yellow flower colors (related to male sex) attracted significantly more potential pollinators than dark (related to female sex) flowers

Our experiment using dye pigments to track pollen movement and validate pollinator preference showed that out of the total 55 flowers, the majority of them (39/55) did not have any dye. About 20% (11/55) had green dye on them, which corresponds to the green/greenish yellow male flowers. None of the flowers (0/55) had the violet dye which was applied to yellow/yellowish orange flowers. Five flowers (5/55) had the orange dye that corresponds to orange/red flowers.

## 3.5 DISCUSSION

Our study focused on measuring the dimorphic floral traits of a functionally dioecious native cactus, *C. wolfii*, which is a valuable model system to study because of the sexual dimorphism/dichromatism relation and the effects of varying floral traits on pollinator floral preferences within a single population. The flowers of *C. wolfii* carry vestigial organs i.e., non-functional anthers in female flowers and non-functional pistil in the male flowers (Ramadoss et al. 2022). This is considered as a 'deceit strategy' where the plants try to deceit the pollinators into visiting their non-rewarding flowers equally as rewarding flowers to ensure successful sexual reproduction (Dafni 1984). This strategy of developing both sexes whorls in unisexual flowers and later arresting the development of the opposite sex called as cryptic dioecy is observed in at least 78 species from 18 families (Mayer and Charlesworth 1991), commonly in Anacardiaceae (Zohary 1952) and Rosaceae (Cronk and Muller 2020). Despite this strategy, this rare species is limited in its sexual reproduction (Ramadoss et al. 2022) and studying its floral ecology might provide us with some valuable insights.

Our results on sexual dimorphism showed that male flowers were larger and brighter than female flowers. Large flower size is a trait that is often selected by pollinator-mediated selection as they attract more pollinators and facilitate more pollination (Parachnowitch and Kessler 2010; Glaettli et al. 2008, Delph et al. 1996). In *C. wolfii*, we observed that the quantitative traits such as tepal length, tepal width, and tepal display diameter were significantly higher in male flowers than female flowers. Intersexual mimicry theory suggests that in rewardless females (i.e. females that lack pollen or nectar) qualitative traits such as scent composition do not differ significantly between the sexes to ensure unbiased pollination and successful reproduction (Ashman 2009). However, *C. wolfii* did not completely support this theory. With respect to the qualitative trait—color, females are usually found to be in orange-red shades while males tend to be mostly in brighter colors of green-yellow shades. However, there is no strict dimorphism cutoff in the flower color trait. For instance, males are generally more brightly colored, but they can also exhibit darker flower colors. We predict that males are biased towards brighter colors because it is potentially advantageous as pollinators, especially bees, tend to be attracted to brighter colors, such as yellow flowers, and the male reproductive success depends on pollen dispersal (Acharya et al. 2022). On the other hand, we predict that females are biased towards darker shades as brighter colors might be more apparent and attract florivores and herbivores which might affect the fruit production (McCall and Irwin 2006). But we do not have collected data to support this. Some birds can see fluorescence, but we are unsure about the specific identities of the florivores in this species (Martins et al. 2018). Additionally, florivores have a longer visual range than bees, and petals serve as long-distance signals, while anthers are used for

short-distance decisions, making petals more attractive to herbivores or florivores (Martins et al. 2018). Additionally, the color of the flower also seems to be correlated with the size of the flower. In general, the green flowers appear to exhibit reduced size dimensions in comparison to their yellow or yellowish orange counterparts.

We found an interesting relation between flower color and filament color in which lighter colored flowers usually have lighter colored filaments. A similar association was observed in *Bixa orellana* flowers where deeper colors had darker filaments and it was suggested that this color combination could act as visual cues to pollinators because with the background colors blending the anthers could stand out with better contrast (Joseph and Siril 2013). In fact, a study on bumble bees has shown that the contrast of flowers against their background is more critical for identifying their targets than color patterns within the flower (Whitney et al. 2013). Alternatively, within-flower contrast helps in guiding pollinators towards the nectar (Goldblatt et al. 1998; Hempel de Ibarra and Vorobyev 2009; Sletvold et al. 2016). Additionally, factors such as scent and nectar composition also play a major role in female attractivity (Bawa and Opler 1975; Ashman 2009). Previous studies in *Mammillaria magnimamma* (Callejas-Chavero et al. 2021) have shown that females have smaller nectar chambers and that in *Opuntia quimilo* (Diaz and Cocucci 2003) have lower nectar supply than hermaphrodites. Future studies on dioecious *Cylindropuntia* could focus on measuring these differences between the sexes and how they may influence pollinator attraction.

Moreover, it is critical to consider patterns that are visible by insects only, such as UV and fluorescence. Floral fluorescence is proposed to be a visual signal for insect

pollinators (Gandia-Herrero et al. 2005). Several plant species have been reported to have auto blue or green fluorescence in their petals, stigma, ovaries and nectaries (Iriel and Lagorio 2010; Lagorio et al. 2015; Mori et al. 2018). Therefore, we examined the insect perspective by measuring emission intensity in the UV and GF spectra. Our results suggested that there was no significant difference in UV emission intensity between sexes and/or colors. However, we observed that GF emission intensity of anthers differed as a factor of sex. The anthers of females had a higher emission intensity than the male anthers. Though fluorescence signals are proposed to be involved in communication of plant–pollinator systems, there are few studies that performed behavioral experiments to understand the perceiving capabilities of bees for fluorescence (Gandia-Herrero et al. 2005; Garcia-Plazaola et al. 2015). A recent study by Mori et al. (2018) showed that honeybees in the field were attracted to blue fluorescence of anthers and pollen. Thus, brighter anther fluorescence patterns found in females could be attractive to bees that possess green light sensitivity and attractivity to brighter targets. This could be one way the female flowers are compensating for their dull flower colors.

We propose that the mechanism by which anthers of female flowers have brighter fluorescence patterns is associated with the sterility cellular mechanism. Autofluorescence in pollen and anthers has been reported in some plants (Castro et al. 2010). Persistent autofluorescence in anthers of female flowers only has been reported in mutant soybeans *Glycine max*. It was suggested that due to early degeneration of tapetum in male sterile flowers, the autofluorescence pigment is not transferred to the microspores and ends up piling in the tapetum, which leads to persistent autofluorescence in male-sterile flowers only

(Nakashima et al. 1984). Premature disintegration of the tapetum is a hallmark of male sterility in *C. wolfii* (Ramadoss et al. 2022). In the pre-anthesis stage of female anthers of *C. wolfii*, autofluorescence was detected inside the anther where the microspores deteriorated suggesting that *C. wolfii* follows a similar pattern as that of the mutant female soybeans which highlights the potential for fluorescence sexual dichromatism in other functionally dioecious species. Additionally, premature tapetal disintegration is not uncommon in dioecious Cactaceae *Opuntia* (Orozco-Arroyo et al. 2012; Flores-Rentería et al. 2013), *Consolea* (Strittmatter et al. 2002, 2006, 2008) and *Echinocereus* (Hernández-Cruz et al. 2018). Particularly, in *O. stenopetala* autofluorescence has been reported in male sterile flowers at later stages of development due to abnormal callose retention in the anthers (Flores-Renteria et al. 2013). These observations underscore the potential for fluorescent dichromatism in those species as well. Sexual dichromatism in fluorescent signals has only been recently observed in birds (Barreira et al. 2012) and studies about its role on animal evolution are still limited (Ancillotto et al. 2022). We report for the first time fluorescence sexual dichromatism in a plant species which has important implications for future research on the evolution of sex in plants.

Our results on pollinator visitation showed that male flowers attract more pollinators than females. However, discerning the relative influence of color or size on pollinator visitation rates is not possible with our data. Our results showed that males had larger flowers than females. This could be evolved by growth-sexual investment tradeoff where females generally allocate more resources towards production of seeds thereby affecting their growth in terms of flower size, while males directly allocate their resources towards growth

(Stearns 1992; Rabska et al. 2022). Several studies have found flower size to positively influence pollinator visitation (Bell 1985; Eckhart 1991; Dudash et al. 2011; Lazaro et al. 2013; Barbot et al. 2023) as larger flowers are expected to be more attractive. Alternatively, the pollinator visitation bias observed could be due to sexual dichromatism in our species.

Our investigation revealed a statistically significant preference for brighter tepal colors in attracting a higher number of pollinators compared to darker hues. This observation aligns with the notion that bees have limited capacity to perceive the color red, but exhibit heightened sensitivity to the color green, as substantiated by prior studies (Kevan et al. 1996; Chittka and Waser 1997; Acharya et al. 2022). This was in agreement with our observations that about 67% of the flowers that received dye moved by pollinators was green suggesting that the green/greenish yellow flowers were visited more frequently than the other colors. Based on our empirical data, the enhanced pollen dispersal from green/greenish yellow male flowers explains the higher prevalence of brighter coloration in male flowers as opposed to darker variants. This suggests that male flowers evolved to become more attractive through intrasexual competition that occurs as a consequence of sexual selection (Stearns 1992, Paterno et. al 2020; Barbot et al 2023).

Our experiment with dyes to track pollen movement found low pollinator visitation. About 71% of the flowers we surveyed did not have any dye on them which might be an indication of low pollinator density or low pollinator visits or inefficient movement of pollen or the dyes we used to the *C. wolfii* flowers in general. Concordantly, we have previously observed that natural pollinations of *C. wolfii* resulted in a reduced average seed set (0.05) compared to that of hand pollination (0.25) (Ramadoss et al. 2022). Studies

have theorized and empirically shown that while male attractiveness is optimal during high pollinator visits, this strategy could lead to inefficient sexual reproduction when the pollinator density is low due to the biased visit to the most attractive sex, which is usually the males (Vamosi and Otto 2002; Moquet et al. 2022). Experimental studies have shown that plants can abort fruits that have reduced seed number due to low pollen deposition to save their resources (Stephenson 1981). We acknowledge the presence of multiple variables beyond pollination that may exert influence on reduced seed production such as resource availability, predation, and environmental conditions, which have not been subjected to empirical investigation in our study.

## 3.6 CONCLUSION

Studying sexually dimorphic traits is essential for understanding plant–pollinator interactions, especially on species with restricted distributions like *C. wolfii*, which is limited with low seed production. Our results suggest that *C. wolfii* exhibits sexual dimorphism with male flowers being bigger than females. Additionally, they exhibited sexual dichromatism with males usually having brighter tepals and females having darker tepals. We also observed an unprecedented phenomenon of fluorescent sexual dichromatism, which had not been previously observed in plants. The mechanism for autofluorescence of sterile anthers might be prevalent in other functionally dioecious species and more studies need to be undertaken. Our pollinator survey showed that males attracted more pollinators than females which could be a consequence of the observed sexual dimorphism. This observation could potentially account for the reduced seed production observed in this endemic species, as

when pollinator density is low female plants have less opportunity for ovules to be fertilized and therefore produce mature fruits.

# Chapter 4

# Unveiling Bias with Fluorescent Dyes in Pollination Studies

## 4.1 ABSTRACT

Assessing pollen transfer in angiosperms is crucial for comprehending plant-pollinator interactions. One effective method to study this is through the use of fluorescent dye powder. However, studies have utilized various dye colors without comparing their potential variations in attractiveness to pollinators. Our research addresses this gap by examining the dispersal patterns of different dye colors on both uniform and polymorphic flowers. We conducted fluorescent dye assays on two plant species—one with consistent flower color *(Oscularia deltoides)* and the other displaying flower color polymorphism *(Cylindropuntia wolfii)*. We recorded the number of flowers with each colored dispersed dye to assess differences and compared them to determine if one color was more dispersed than another. Our

findings revealed a discrepancy in pollen transfer, with the green dye showing the highest transfer frequency in both *O. deltoides* and *C. wolfii*. We hypothesize that this is due to the common pollinator in both systems, *Apis mellifera*, being attracted to green color, and consequently, to the green dye used. This chapter highlights that fluorescent dye color significantly influences pollinator attraction, particularly the green dye, thereby introducing a potential bias in pollination experiments.

## 4.2 INTRODUCTION

In angiosperms, pollen transfer/dispersal serves as a critical metric to study various aspects of plant biology, including population structure (Schaal, 1975; Motlep et al., 2021), mating patterns (Harder et al., 1996; Wessinger, 2021), evolution of plant traits and sexual systems (Pyke, 1981; Perkins, 1977; Williams and Mazer, 2016), and pollinator effectiveness (Schmitt, 1980; Diller et al., 2022). However, the limited capacity to track the movement of pollen grains has rendered pollen dispersal one of the less explored aspects of plant reproductive biology (Minnaar et al., 2019).

Previous studies have attempted to estimate pollen transfer by recording the distances traveled by pollinators (Schmitt, 1980; Waser and Price, 1982). However, this method presents significant challenges, as pollen taken from one flower may not be entirely deposited onto the next flower, and monitoring pollinators in natural environments can be complex (Waser and Price, 1982). To address these challenges, more specific methods have been developed to track pollen grains, such as the use of radioisotopes (Colwell, 1951; Schlising and Turpin, 1971; Reincke and Bloom, 1979) or heavy metals (Gaudreau and Hardin, 1974)

to mark pollen grains. But they are expensive and time-consuming (Waser and Price, 1982; Minnaar and Anderson, 2019). Alternatively, pollen polymorphisms, such as differences in color or size, have occasionally been employed for pollen tracking (Thomson and Plowright, 1980). However, the applicability of these methods is limited to species exhibiting such polymorphisms (Minnaar and Anderson, 2019). Recent advancements in molecular tools have enabled scientists to sequence microsatellite markers from pollen and identify the pollen donor (Matsuki et al., 2007). Nevertheless, this method requires substantial labor and financial resources due to the meticulous process of isolating individual pollen, extracting DNA, and sequencing them (Minnaar and Anderson, 2019).

An often-utilized and successful approach is the use of fluorescent dye powder to coat the anthers (Stockhouse, 1976; Van Geert et al., 2010; Huais et al., 2022). These powders, characterized by their minute particle sizes and emission of fluorescence in near-UV wavelengths, are available in a variety of colors. Employing fluorescent dyes as substitutes for pollen has notable advantages, like simplicity and cost-effectiveness (Waser and Price, 1982; Van Geert et al., 2010). Furthermore, formaldehyde-free dyes, when administered to insects, have been observed to have minimal or no adverse effects on survival and mobility (Rojas-Araya et al., 2020).

Various colors of dyes have been employed in studies without comparing their potential differential attractiveness to pollinators. For instance, in a study by Adler and Irwin (2006), green and orange dyes were used to evaluate the effectiveness of different bee visitors in pollinating the distylous plant *Gelsemium sempervirens*. The researchers noted variations in pollen-carrying capacities among different genera of bees. However, the

potential influence of dye color attraction on the increased pollen-carrying load remains uncertain. In another study, Diniz et al. (2019) utilized three distinct fluorescent dye colors as surrogate pollen to explore the contribution of bats to pollen movement between three isolated urban populations of *Crescentia cujete* (Bignoniaceae). The researchers observed short-distance dispersal of the dye among these populations, a phenomenon inconsistent with the anticipated dispersal capacity and foraging range of bats. However, the underlying cause of this discrepancy, particularly whether it arises from the attractive nature of the dye colors remains unclear.

In a parallel study, Ramadoss et al. (2023) investigated the influence of various flower color morphs on pollinator visitation rates, utilizing fluorescent dyes as pollen analogs. Focusing on the species of interest, *C. wolfii*, which exhibits six distinct flower color morphs, the authors employed three different colored dyes (Ramadoss et al., 2023). The researchers found that green flowers experienced a higher visitation rate, corresponding to the increased dispersal of the green dye applied to them, a result consistent with their direct observations of pollinator behavior. However, a crucial question that remains unanswered is whether applying green dye to different flower colors would yield a similar increase in dispersal, potentially indicating a bias toward the color of the dye in pollinator attraction.

In this chapter, we aimed to address the following questions:

1) Do different colored fluorescent dye powders affect pollinator attraction?

2) Does the application of green dye enhance pollen dispersal compared to other dye colors in *C. wolfii* species?

To achieve these objectives, our study compares the dispersal patterns of dyes

on two plant species—one with uniform flower color and the other exhibiting flower color polymorphism.

## 4.3 METHODS

### 4.3.1 Species of interest

To compare dye dispersal patterns in systems with uniform and dimorphic flowers, we selected two species for our investigation. For the uniform flower color system, we chose *Oscularia deltoides* (family Aizoaceae) that produces pink colored flowers with yellow colored pollen. This ornamental succulent is grown in the Mediterranean garden of San Diego State University and has been observed to attract *Apis mellifera*, the European honeybee. For the system with flower color polymorphism, we studied *Cylindropuntia wolfii* (family Cactaceae). The flowers of *C. wolfii* exhibit six different color morphs, including green, greenish yellow, yellow, yellowish orange, and red (Ramadoss et al., 2023) with yellow pollen. Previous research has suggested that *C. wolfii* faces limitations in seed production (Ramadoss et al., 2022). They are known to attract *Apis mellifera* and *Diadasia sp.*, the native cactus bee in the field (Ramadoss et al. 2023).

### 4.3.2 Fluorescent Dye Tracking Assay

To control for flower trait variables and isolate the potential influence of dye color on pollinator attraction, we conducted the fluorescent dye assay on the uniform-looking pink flowers of *Oscularia deltoides*. Six different colored dyes (ultra violet, horizon blue, signal green, saturn yellow, blaze orange, aurora pink from DayGlo Color Corp) with a

consistent amount of 1/2 teaspoon were randomly assigned to different flowers, with four flower replicates for each dye color (sample size = 24 flowers with one flower per plant on 24 different individuals). To avoid edge effects, we selected plants located within a core area, ensuring they were surrounded by neighboring plants within a 10-meter radius. Dye application took place in the morning, and subsequent observations were conducted in the evening. The presence or absence of dye on nearby flowers (within a 10m radius) was manually recorded by illuminating them with UV light. We then counted the number of flowers with each colored dispersed dye to identify any differences.

We conducted a fluorescent dye assay on the polymorphic flowers of *C. wolfii*, employing a different approach from Ramadoss et al. (2023). Specifically, we applied green dye to yellow flowers. To have a systemic and reproducible way of assigning dyes, each of the six different color morphs was paired with a fluorescent dye based on ascending wavelengths: green flowers with violet dye, greenish yellow flowers with blue dye, yellow flowers with green dye, yellowish orange flowers with yellow dye, orange flowers with orange dye, and red flowers with pink dye. In this approach, the flower color and dye color were easily distinguishable by the observer. We employed four flower replicates for each dye color (sample size = 24 flowers with one flower per plant on 24 different individuals). To avoid edge effects, we selected plants located within a core area, ensuring they were surrounded by neighboring plants within a 20-meter radius. We applied dyes to the anthers of flowers as they began to bloom. A consistent amount of 1/2 teaspoon of dye was added to all flowers. In the evening, we manually inspected neighboring flowers within a 20m radius to determine dye dispersal. Due to the larger area of *C. wolfii* plants in the field as opposed to *O. deltoides*

in the SDSU garden, we employed a greater radius. This assessment was conducted using a black light, serving as an indirect measure of pollen dispersal. We recorded the presence or absence of dye on these neighboring flowers. This methodology variation was introduced to explore whether the preference observed for green and greenish-yellow flowers in the previous study (Ramadoss et al. 2023) remains when green dye is applied to yellow flowers.

## 4.4 RESULTS

To uncover biases attributed to dye color, we conducted a fluorescence dye assay on 24 uniform-looking pink flowers of *O. deltoides*. Most of the neighboring flowers that were probed did not have any dye on them (114/213 =54%). The dye counts (i.e flowers with at least one grain of dye powder were counted as "yes") are as follows: Signal Green - 72; Horizon Blue - 9; Blaze Orange - 8; Aurora Pink - 5; Saturn Yellow - 4; Ultra Violet - 2. The observed pattern indicates a higher propensity for dispersion with the Signal Green dye compared to other colors (Fig. 4.1).

To evaluate potential biases towards green dye color preference, we performed a fluorescence dye assay using six different dyes, each corresponding to the six flower color morphs in *C. wolfii*, where 24 flowers were marked with the dyes. A majority of the flowers (103 out of 227, or 46%) showed no dye dispersion. Notably, the green dye (36 flowers) and yellow dye (33 flowers) exhibited higher dispersion rates than other dyes. These dyes were associated with yellow and yellowish-orange flowers, respectively. The remaining dye counts are as follows: Aurora Pink - 17 flowers; Horizon Blue - 16 flowers; Blaze Orange - 13 flowers; and Ultra Violet - 9 flowers.

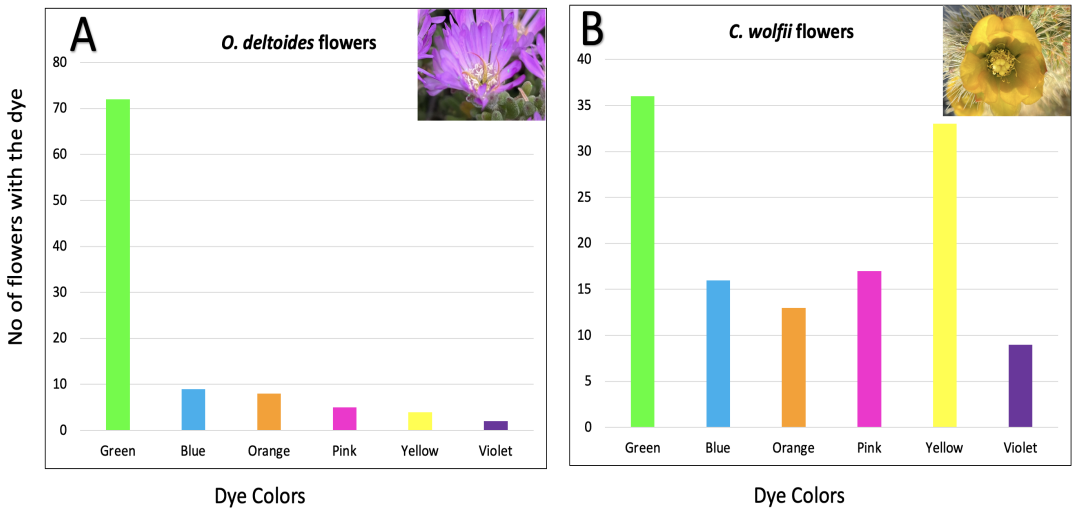


Figure 4.1: Bar plot showing the counts of A) *O. deltoides* and B) *C. wolfii* flowers having the dispersed dye. The x-axis labels indicate the dye colors.

## 4.5 DISCUSSION

Several studies have utilized various colored dyes to assess the impact of different floral traits on pollinator visitation rates (Van Geert et al., 2010; Diniz et al. 2019). But an intriguing gap remains: few have examined the direct effect of dye color itself on pollinator visitation. Our current study takes the initial step toward addressing this gap by investigating the dispersal patterns of various dye colors on uniform versus polymorphic flowers.

The uniform flowers *O. deltoides's* dye dispersal indicated that the majority of flowers with dispersed dye had green dye (72%). This observed pattern suggests that dye color may indeed influence pollinator visitation, with green dye being the most deposited on different flowers. These findings align with previous research suggesting that some bees have heightened sensitivity to green (Kevan et al. 1996; Acharya et al. 2022). Moreover

the complementary color to pink is green. Therefore, we can hypothesize that the green dye may stand out more against the pink background, making it appear more attractive to pollinators.

The fluorescent dye assay conducted in *Cylindropuntia* demonstrated that the majority (approximately 67%) of flowers with dye exhibited green or yellow dye powder, indicating that yellow or yellowish-orange flowers received more frequent visits from pollinators compared to other colors. This finding was consistent with their direct observations of pollinator visitation (Ramadoss et al., 2023). However we acknowledge that our data does not quantify the degree of contrast between the dyes and petal colors. Therefore, as a next step to confirm pollinator color preference towards green dye our future study would test each of the color morphs with all (or the same subset) of dyes. This would control for background matching (or contrast) relative to the dyes.

Most bees can perceive colors within the 300 to 600 nm visual spectrum (Menzel et al. 1991). While specific information on the vision of many solitary bees like *Diadasia* is still under study, *Apis mellifera* bees are known to have trichromatic color vision with highly sensitive photoreceptors at 344 nm (ultraviolet), 436 nm (blue), and 544 nm (green) (Kevan et al. 1996; Acharya et al. 2022). In both species - *O. deltoides* and *C. wolfii*, there is a notable dispersal of green dye (which could be confounded by flower color). Our hypothesis is that the frequent visitation by *Apis mellifera* bees to both plant species might be contributing to the increased spread of the green dye. Moreover, the heightened dispersal of yellow dye in *C. wolfii* may be influenced by native *Diadasia* bees (not observed in *O.deltoides*), potentially contributing to the observed attraction bias (Ramadoss et al.,

2023) This is because *Diadasia*-visited flowers were more frequently in the bee-green (human yellow) color (Messinger, 2013). However, it is not yet clear whether *Diadasia* bees have an innate preference for this color. This highlights the intricacy of ecological interactions and emphasizes the importance of interpreting results cautiously within specific habitats. Although we lack the spectral information for the dyes, it would be interesting to investigate whether the spectrum falls within the sensitivity range of the bee's vision and if they can distinguish between the green and yellow dyes. If they are unable to distinguish between the green and yellow dye colors, it might suggest that they are attracted by the yellow pollen that is common in both the plant species and the dyes as pollen analogues could increase the respective flowers's attractivity. Another step for future research is to test whether the degree of color difference between the flower color and the dye color affects visitation. This would help determine how bees perceive the dyes relative to the flowers.

Similarly to dye color, the color of bee pan traps is also known to influence bee attraction (Leong and Thorp, 1999). Leong and Thorp (1999) proposed that specialist bees, such as *Diadasia sp.*, are attracted to traps resembling the color of their host flowers. However, data from Wilson et al. (2008) refutes this hypothesis. Interestingly, they found that two Diadasia species (*Diadasia australis* and *D. diminuta*) show a preference for fluorescent blue pan traps. These bee species are specialists on Cactaceae (pink, yellow and orange flowers) and Malvaceae (orange flowers) whose flowers are not blue. This is indicative of the fact that in the absence of a preferred color, bees visit other colors.

Another pantrap study by Acharya et al. (2022) investigated how different light wavelengths and reflectivity of vane traps affect bee capture rates. Vanes in various col-

ors—dark blue, bright blue, dark yellow, bright yellow, purple, and red—were assessed for their light reflectance properties and attractiveness to different species bees including *Apis mellifera*. Similar to Wilson et al. (2008), the study found that bright blue vanes had the highest light reflectance, while bright yellow traps had the second highest capture rates. The authors suggest that reflectance intensity from different colored vanes in traps influences the number of bees attracted to the trap (Vrdoljak et al. 2012; Acharya et al. 2022). Therefore, it is possible that the green and yellow fluorescent dye powders have higher reflectance than other colors, potentially attracting *Apis mellifera* and *Diadasia* bees.

Overall our findings suggest that the dye powders used as pollen analogs may have an influence on bee attraction potentially introducing bias in pollinator studies. We acknowledge that our methodology would be more convincing if we reversed the dye colors on the flower colors by using descending wavelengths of dye on ascending wavelengths of flower color and observed the same pattern. However, the dye remains in the field for several months after application, and the flowers only bloom once a year. Consequently, while this study cannot be immediately replicated, it can be repeated in future blooming seasons to better supplement our data. While our data suggests that dye color influences pollinator attraction, we acknowledge the possibility of other underlying flower characteristics (such as scent or nectar production) that may have also influenced attraction, but our data cannot elucidate it.

## 4.6 CONCLUSION

The main outcome of our investigation highlights the potential influence of fluorescent dye color on pollinator attraction, potentially biasing pollination experiments. Specifically, we observed that green dye is the most dispersed in systems visited by *Apis mellifera*. However we acknowledge that our data does not quantify the degree of contrast between the dyes and petal colors. Therefore, as a next step to confirm pollinator color preference towards green dye our future study would test each of the color morphs with all (or the same subset) of dyes. Given the growing imperative for food sustainability, the utilization of green dye may potentially aid in attracting honeybees, which are crucial for the pollination of food crops. We recommend that researchers using fluorescent dye powders carefully examine the potential impact of dye color on pollinator behavior, especially when employing multiple colored dyes in systems with polymorphic flowers.

# Chapter 5

# Genetic Ramifications of Sexual Separation

## 5.1 ABSTRACT

Dioecy, the separation of sexes, is found in 6% of flowering plants. One hypothesis suggests that it is an evolutionary tactic to mitigate inbreeding risks. Another hypothesis suggests that dioecy might be an evolutionary dead end due to only half of the population (females) contributing to seed production and dispersal. Despite phylogenetic studies challenging the second hypothesis, the scarcity of population genetics studies restricts conclusive testing. Only two such studies have compared genetic diversity among species with different sexual systems, yielding conflicting results. Our first goal is to test the two evolutionary

The materials presented in Chapter 5 have been submitted to Molecular Ecology for review as "Genetic Diversity Patterns in Cacti: Assessing Inbreeding Avoidance and the Dead End Hypothesis"

hypotheses in the genus *Cylindropuntia* (Cactaceae) using population genetics. This genus encompasses native endemic species with diverse sexual systems, with the separation of sexes observed solely in polyploid species. Notably, polyploid species with separate sexes (*C. wolfii* and *C. chuckwallensis*) share similar ploidy, flower colors, geographic proximity and narrow distribution raising speculation about their shared ancestry. To address this gap, our second goal is to investigate the shared ancestry of the sexually separated species *C. wolfii* and *C. chuckwallensis* by investigating their genetic structure. One of the species, *C. wolfii* has been reported to struggle to sexually reproduce based on previous evidence and so, our third objective is to investigate whether *C. wolfii* is dominated by clonal reproduction.We collected six species with different sexual systems and sequenced them for SNPs (single nucleotide polymorphisms) which were used to estimate the genetic diversity parameters and population structure. The clonality of *C. wolfii* was assessed using a combination of field survey and genetic analysis. The comparisons of genetic diversity between species with different sexual systems did not support the dead end hypothesis. The field survey of *C. wolfii* revealed no seed recruitment but the genetic analysis on the current adult plants showed low signs of clonality suggesting that this species has recently shifted to clonal reproduction. Results showed that overall this genus had low genetic diversity and high differentiation implying that it is vulnerable to environmental threats.

## 5.2 INTRODUCTION

The sexual systems found in angiosperms exhibit a wide range of diversity. Roughly 90% of flowering plants have a hermaphroditic system, containing both male and female

reproductive structures within the same flower of an individual. Some plant species have developed floral unisexuality through spatial separation of their flowers (Yampolsky and Yampolsky, 1922; Renner, 2014). These plants may exhibit male and female reproductive structures on different flowers of the same plant which is known as the monoecious sexual system or, have male and female flowers on separate male and female plants respectively, known as dioecious sexual system (Darwin, 1877; Barrett, 2002). While the evolution of dioecy has been a source of fascination for plant biologists, it is infrequent, constituting approximately 6% of the total species in flowering plants (Renner, 2014). Gynodioecy sexual system has been suggested to be a pathway for shifting from hermaphroditism to dioecy in angiosperms, thus gynodioecious populations are composed of female individuals and hermaphroditic individuals (Darwin, 1877; Renner, 2014). Consequently, gynodioecy is often viewed as a less stable sexual system, characterized by its potential for reversibility (Charlesworth and Charlesworth, 1978; Spigler and Ashman, 2012).

Angiosperm species with individuals bearing hermaphroditic flowers typically exhibit a mixed mating system, leading to seed production through both self-pollination and cross-pollination. There are two hypotheses that explain the evolution to sexual separation in dioecious systems. The first hypothesis is widely accepted and explains the separation of sexes in gynodioecious and dioecious systems is believed to have evolved as an adaptive strategy to minimize the risks associated with inbreeding and the subsequent detrimental effects of inbreeding depression (Charlesworth and Charlesworth, 1978; Olito and Connallon, 2019). In gynodioecious systems, female individuals are obligatory outcrossers, while hermaphrodites may have the capacity for self-pollination. These dis-

tinctions result in the offspring of females having an enhanced seed quality and quantity compared to that of hermaphrodites, as a result of greater degree of outcrossing (Darwin, 1877; Shykoff et al., 2003; Dufay and Billard, 2011). In gynodioecy, the presence of self-compatible hermaphroditic individuals is associated with the high selfing rate which can lead to inbreeding depression (Darwin, 1877; Lloyd, 1982; Charlesworth and Charlesworth, 1978; Maki, 1992). In contrast, dioecy achieves maximal outcrossing due to the absence of hermaphrodites in the population (Lloyd, 1982; Charlesworth and Charlesworth, 1978), thereby reducing the chances of inbreeding depression (Thomson and Barrett, 1990; Charlesworth, 1999).

Alternatively, the 'dead-end' hypothesis as proposed by Heilbuth (2000) posits that dioecious lineages exhibit reduced species diversity in contrast to their non-dioecious counterparts, possibly due to elevated rates of extinction. Heilbuth et al. (2001) elucidated this hypothesis by highlighting two evolutionary drawbacks associated with dioecy. First, only half the population, i.e females, contribute to seed production and dispersal, causing less dispersal (smaller geographic distributions) and more competition for local resources (fewer individuals per species) compared to hermaphroditic species (Heilbuth et al., 2001). Second, in dioecious species with biotic pollination, the competition among males within the species drives them to develop traits that make them more appealing to pollinators than the females. As a consequence, the population size fluctuates with pollinator density, which in turn will affect the genetic diversity (Vamosi and Otto, 2002).

Renner (2014) challenges the dead-end hypothesis by proposing that 43% of dioecious species within species-rich clades have not been included in the study by Heilbuth

(2000). If the pollinator and seed-dispersal drawbacks of dioecy exist, they should result in dioecious species having smaller effective population sizes (Ne) compared to nondioecious species. Thus this hypothesis can be tested by using genetic diversity as a proxy to assess the long-term Ne effects in both dioecious and nondioecious species (Muyle et al. 2018). Muyle et al. (2018) tested the dead-end hypothesis by comparing the genetic diversity and adaptive potential of dioecious *Silene* species to their close non-dioecious relatives and thus evaluate whether dioecy increases the extinction rates of species. In contrast to what one might expect based on the dead-end hypothesis, Muyle et al. (2018) discovered that both dioecious and gynodioecious species exhibited higher genetic diversity when compared to their nearby hermaphroditic counterparts. On the other hand, a similar study by Maki (1992) in the *Chionographis* genus showed that gynodioecious species have lower genetic diversity compared to their hermaphroditic close relatives. To the best of our knowledge, these contrasting studies are the only two comparing the genetic diversity between species with different sexual systems in the same genus. Hence, further investigations are necessary to determine the validity of the hypotheses related to sexual separation.

In this chapter, we focus on the genus *Cylindropuntia*, which belongs to the Cactaceae family and Opuntioideae subfamily, because it has hermaphroditic, gynodioecious and dioecious systems allowing us to evaluate both hypotheses of evolution to sexual separation. The dioecious and gynodioecious species exhibit characteristics suggestive of evolutionary handicaps, including reliance on insect pollination and the absence of fleshy fruits, which enables testing of the dead-end hypothesis (Baker and Hughes, 2014; Muyle et al., 2018).

Although the *Cylindropuntia* genus has diverse sexual systems, the dioecy or gynodioecy is only observed in polyploid species *C. chuckwallensis, C. calmalliana, C. sanfelipensis, C. wolfii* and *C. molesta* (Baker and Hughes, 2014; Ramadoss et al., 2022). Moreover, the coincidence of separate sexes, hexaploidy, diversity with up to six different colors, geographic proximity and narrow distributions among *C. chuckwallensis*, and *C. wolfii* is remarkable (Baker and Hughes, 2014). Baker and Hughes (2014) hypothesize that these two species may be of hybrid origin (allopolyploidy), and share ancestry but there is no genetic data to support this, and provide clear species delimitation for both. In response to the gap in knowledge regarding the shared ancestry of these two species, our study aimed to investigate this by population structure analysis.

The family Cactaceae belongs to the taxonomic groups facing the highest threat levels analyzed to date with about 31% of the species classified in danger of extinction (Goettsch et al., 2015). The reported low seed set in *C. wolfii* even with manual pollination (Ramadoss et al., 2022) has raised conservation concerns, prompting an understanding of the population's genetic variation. The low seed set is suggesting challenges in successful sexual reproduction (Ramadoss et al., 2022) of *C. wolfii*. This emphasizes the importance of investigating whether the *C. wolfii* population is predominantly reproducing through clonal ways. There is a lack of studies on other species of *Cylindropuntia* to assess if the seed set is similarly affected.

The sexual system of the subfamily Opuntioideae (which *Cylindropuntia* belongs to) has been investigated at the ecological, morphological and cellular level (Ramadoss et al., 2022; 2023; Flores-Renteria et al., 2013; Strittmatter et al., 2008), however, it stands out for

their limited exploration in genetic studies at the population level, marking a notable paucity in research (Nassar et al., 2002; Helsen et al., 2011; Guerrero et al., 2019). This genus encompasses about 39 species occurring throughout the major deserts of North America (Majure et al., 2019). Our study is the first to report genetic parameters for species of this genus. By exploring the genetic makeup of these species, we not only gain insights about sexual separation but also provide valuable data for conservation efforts. We included six species from this genus, each characterized by distinct sexual systems: three species featuring hermaphroditic sexual systems (*C. echinocarpa, C. ganderi,* and *C. ramosissima*), one gynodioecy (*C. chuckwallensis*), one dioecy (*C. wolfii*), and one hermaphroditic species with a clonal reproductive strategy (*C. bigelovii*).

Our first goal was to test the two hypotheses related to sexual separation using the species of *Cylindropuntia*. We expect that if the dead end hypothesis holds true, dioecious species would exhibit lower genetic diversity compared to the hermaphrodites. We expect that if the adaptive strategy hypothesis holds true, the sexually separated systems (dioecious and gynodioecious) species would exhibit higher genetic diversity compared to the hermaphrodites. Our second goal is to investigate whether there is shared ancestry between the two species *C. wolfii* and *C. chuckwallensis* that share similar morphological traits, including unisexual flowers, geographic proximity and narrow distribution using clustering analysis. We expect that if the two species have shared ancestry they will cluster closely or together. Our third objective is to investigate whether *C. wolfii* is dominated by clonal reproduction. We hypothesize that *C. wolfii* is dominated by clonal groups as they are known to have reduced seed production (Ramadoss et al. 2022). This study lays a

foundation for future research on dioecy evolution and conservation approaches concerning *Cylindropuntia*.

## 5.3 METHODS

### 5.3.1 Sampling

To validate the two sexual separation hypotheses in the genus *Cylindropuntia*, we examined the variations in genetic diversity among three different sexual systems that are hermaphroditism, gynodioecy, and dioecy within genus *Cylindropuntia*. For hermaphroditic sexual systems we collected fragments of at least 15 individuals from 3 species - *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR). For the dioecious sexual system we collected 124 fragments of *C. wolfii* (CW) and for the gynodioecious system we collected 30 fragments of *C. chuckwallensis* (CC).(Table 5.1). All the hermaphroditic species we collected except *C. bigelovii* (triploid) are diploid whereas the gynodioecious and dioecious are hexaploid. There are no gynodioecious nor dioecious diploid species in this genus. To mitigate bias introduced by high ploidy levels within the system i.e., higher diversity observed due to the hexaploid nature of the gynodioecious and dioecious species, we specifically collected specimens of a triploid species with a hermaphroditic reproductive system, known as *Cylindropuntia bigelovii* (CB). If the triploid species exhibits significantly higher genetic diversity than the diploids, we can infer that the observed diversity in hexaploid species may be influenced by ploidy level, thereby preventing direct comparisons. All the collected fragments were potted in the greenhouse. We ensured to collect only diploid individuals of *C. ramosissima* by sampling from Joshua Tree National Park, CA, USA as they are

also found to have a tetraploid population in Arizona (Baker et al., 2009). We adopted a population genomics strategy, leveraging DArt-seq technology, which has a complexity reduction step that effectively diploidizes polyploid data generating thousands of SNPs from the same homeologs, useful for comparison (Sansaloni et al., 2020). The SNP fragments are derived from a single homeolog by utilizing variations in sequence and methylation status among the homeologs. After sequencing the restriction fragments, DArTseq clusters all similar sequences and then parse these clusters into SNP loci using specialized bioinformatic algorithms. During the parsing process, it efficiently removes paralogs from the data. Subsequently, the obtained SNPs were subjected to analysis for the assessment of genetic diversity and population structure in each species.

Table 5.1: Sampling locations with coordinates and number of individuals sampled per population.

| **Species** | **Lat** | **Long** | **Location** | **Individuals** |
|---|---|---|---|---|
| *C wolfi* | 32.672 | -116.097 | Mountain Springs, Imperial county, CA | 124 |
| *C ganderi* | 32.677 | -116.104 | Mountain Springs, Imperial county, CA | 15 |
| *C chuckwallensis* | 33.733 | -115.812 | Joshua Tree National Park, CA | 28 |
| *C ramosissima* | 33.733 | -115.812 | Joshua Tree National Park, CA | 20 |
| *C echinocarpa* | 33.792 | -115.792 | Joshua Tree National Park, CA | 20 |
| *C bigelovii* | 33.925 | -115.928 | Joshua Tree National Park, CA | 20 |

### 5.3.2 DNA Extraction and Sequencing

A small piece of tissue (about 100mg) from the cholla fragments was collected and placed in silica beads for about 2 weeks. The tissue was carefully cut to remove just the epidermis and collenchyma part to avoid excess moisture. The nuclear DNA was extracted using DNAeasy Plant Mini kit by Qiagen. The DNA quality was assessed by running a 1%

agarose gel electrophoresis. After confirming the presence of high molecular weight bands from gel electrophoresis, the samples were shipped to DArT for sequencing following Buck et al. (2020, 2023). This technology uses a fusion of complexity reduction techniques and next-generation sequencing (NGS) platforms based on Illumina to genotype thousands of SNPs, as outlined by Jaccoud et al. (2001). Genome reduction is achieved by employing specific endonucleases that target regions of low-copy DNA (Wenzl et al., 2004). DArTseq technique has been known to be effective in genetic diversity studies of polyploids as well as non-model organisms (Wenzl et al., 2004; Sohail et al., 2015; Shams et al., 2019). Moreover, this technology delivers the capacity to analyze and compare genetic measures across ploidy levels as the SNPs are derived from just one homeolog based on Bioinformatic algorithms that can filter them based on sequence similarity and methylation status that potentially vary between homeologs (Sansaloni et al. 2020).

A total of 227 samples were sequenced and a high density assay provided 47,857 SNPs. We further filtered the data for loci having less than 100% reproducibility, call rates of ranges 100% (no missing data), 90%, 80%, 70% and 60%, monomorphs, departures from Hardy-Weinberg equilibrium, and loci that have more than one locus per sequence tag. The filtered data underwent conversion into suitable file formats for subsequent genetic analyses through the utilization of the R package called dartR (Gruber et al., 2018).

### 5.3.3 Genetic Diversity

Different measures of genetic diversity and inbreeding were calculated using GenoDive (Meirmans and Van Tienderen, 2004). The total number of alleles (A), expected heterozygosity (He), observed heterozygosity (Ho), and inbreeding (Fis) were obtained for

all the 227 individuals from the six species - *C. wolfii, C. chuckwallensis, C. echinocarpa, C. ganderi, C. ramosissima* and *C. bigelovii*. The confidence intervals for the statistics were obtained by bootstrapping over loci (10,000 bootstraps). In our study, we conducted a one-sample t-test to compare the estimates of gynodioecious species with those of hermaphrodites, assuming that gynodioecious *C. chuckwallensis* represents the expected mean. We performed a similar analysis to compare the estimates of dioecious *C. wolfii* with those of hermaphrodites.

### 5.3.4 Genetic Differentiation and Population Structure Analysis

To analyze the shared ancestry of *C. wolfii* and *C. chuckwallensis* we estimated genetic differentiation and population structure from 227 individuals from the six species - *C. bigelovii* (CB), *C. wolfii* (CW), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR). The Fst was obtained using the stamppFst command in R package adegenet using the Weir and Cockerham (1984) method . The 95% confidence interval was obtained by 1000 bootstraps. We utilized the 10,000's of SNP loci obtained from different call rates in four distinct complementary genetic clustering methods: This included non-model based approaches such as Principal Coordinates Analysis (PCoA) and Discriminant Analysis of Principal Components (DAPC) and model based analyses like fastSTRUCTURE, and Construct. Principal Coordinates Analysis (PCoA), conducted using dartR (gl.pcoa.plot) (Gruber et al., 2019), used differences in allele frequencies to delineate the populations. Genetically unique clusters were ascertained through Discriminant Analysis of Principal Components (DAPC) in R, employing the adegenet package (Jombart, 2008; Jombart et al., 2010). DAPC uses the Bayesian Information Criterion (BIC) for com-

paring different cluster solutions. The selection of the optimal cluster solution was based on the lowest BIC score. Cross-validation was performed using the xvalDapc command to determine the appropriate number of retained principal components (Jombart and Collins, 2015).

A Bayesian analysis of population clustering was executed using the software fastSTRUCTURE (Raj et al., 2014). Here we employed the logistic prior with five cross-validations on the dataset comprising 10,839 loci without any missing data. Subsequently, we raised the missing data threshold (call rates of 90%, 80%, 70%, and 60%) to incorporate additional SNPs, to observe if this inclusion provides a more detailed population structure.The selection of model complexity (K) was facilitated by the chooseK command in fastSTRUCTURE. Subsequently, the Q mean bar plots derived from the analysis were visualized using Microsoft Excel. A subsequent sub-structuring analysis was conducted in fastSTRUCTURE for population cluster resolution. This involved utilizing the simple prior as we have well structured populations.

We also inferred the genetic structure patterns through an additional spatial analysis known as conStruct (Bradburd et al., 2018). In spatial conStruct analyses, geographic data is utilized, and the assumption is that allele frequencies exhibit a positive covariance linked to geographic locations due to isolation by distance (Bradburd et al., 2018). We evaluated spatial models across K = 3 to K = 10. The assessment of model performance involved a combination of cross-validation and layer contribution scores. The determination of the optimal K value was therefore based on selecting the model that preserves discernible layer contributions and highest predictive accuracy.

### 5.3.5 Clonality Analysis

To test whether *C. wolfii* is dominated by clonal reproduction, we conducted clonality analysis in our SNP dataset. When employing nuclear markers to delineate multilocus genotypes, the potential for misallocation of individuals exists, attributed to scoring errors, PCR artifacts, and somatic mutations (Meirmans and Van Tienderen, 2004). To mitigate this bias, we utilized the GenoDive program (Meirmans and Van Tienderen, 2004), which groups individuals above certain threshold genetic differences (calculated based on an infinite allele model) into a clonal lineage. To establish a threshold for genetic differences among pairs of individuals, while excluding scoring errors or differences arising from somatic mutations, we adopted the approach outlined by Wetzstein (2022). Here, the clonal threshold was determined by doubling the maximum observed error rate in the dataset—specifically, doubling the maximum genetic distance between replicated samples from the same individual.

Complementary to the genomic analysis, we conducted a field survey to identify sexual recruitment ie. seedling of *C. wolfii*. The study was conducted in Mountain Springs, focusing on a prominent area of *C. wolfii*. Four quadrants with a size of 20 x 20 m were selected. Manual scouting was done during daylight hours to thoroughly explore the designated area. We looked for juvenile plants that are not around an adult plant and that were less than 10 cm tall to distinguish them from clonally propagated individuals. Observations were recorded manually for each quadrant, noting its location within the quadrants and any relevant characteristics. The number of observations were manually counted to determine the prevalence of sexual reproduction in *C. wolfii*.

## 5.4 RESULTS

### 5.4.1 Genetic Diversity Analyses

Following the implementation of the DArTseq method, a comprehensive set of SNPs markers were identified. A 100% call rate yielded a total of 10,839 SNPs, followed by 23,563 SNPs for 90% call rate, 33,157 SNPs for 80% call rate, 39,190 SNPS for 70% call rate and 44,589 SNPs for 60% call rate. To validate the two sexual separation hypotheses in the genus *Cylindropuntia*, we compared the genetic diversity between the sexually separated systems (gynodioecy, and dioecy) and the hermaphroditic systems. The genetic diversity measures (Fig. 5.1; Table 5.2) for every sampled population/species were estimated to be lower. The observed heterozygosity (Ho) estimates ranged from 0.032 - 0.14, with the *C. chuckwallensis* having the highest. The expected heterozygosity (He) estimates ranged from 0.034 - 0.093, with the *C. chuckwallensis* again having the highest. In most cases, the observed heterozygotes were more than the expected value. For diversity in terms of numbers of alleles, *C. chuckwallensis* has the highest followed by *C. wolfii* which are both sexually separated species. According to the t-test results, it was found that *C. chuckwallensis* exhibited significantly higher values ($p < 0.05$) than its hermaphroditic counterparts across all genetic estimates except for inbreeding. Conversely, for *C. wolfii*, the t-test indicated significantly higher values only in terms of the number of alleles ($p = 0.02$) when compared to its hermaphroditic counterparts. The estimated inbreeding coefficients for each population were low (Table 5.2; Fig. 5.2), indicating avoidance or low levels of mating among closely related individuals in this genus.

Table 5.2: Genetic diversity estimates for each species. The species abbreviations are as follows : *C. bigelovii* (CB), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR), *C. wolfii* (CW). The asterisk in the gynodioecious and dioecious species indicate the significantly different values from the hermaphroditic counterparts.

| **Species** | **No of samples** | **No of alleles** | **Effective No of alleles** | **Ho** | **He** | **Inbreeding coefficient** | **Sexual system** |
|---|---|---|---|---|---|---|---|
| CC | 28 | 1.321* | 1.159* | 0.14* | 0.093* | -0.497 | Gynodioecious |
| CW | 124 | 1.301* | 1.06 | 0.053 | 0.036 | -0.464 | Dioecious |
| CG | 15 | 1.152 | 1.061 | 0.054 | 0.037 | -0.443 | Hermaphroditic |
| CE | 20 | 1.183 | 1.049 | 0.032 | 0.034 | 0.047 | Hermaphroditic |
| CR | 20 | 1.25 | 1.082 | 0.05 | 0.054 | 0.065 | Hermaphroditic |
| CB | 20 | 1.206 | 1.06 | 0.059 | 0.034 | -0.709 | Hermaphroditic |

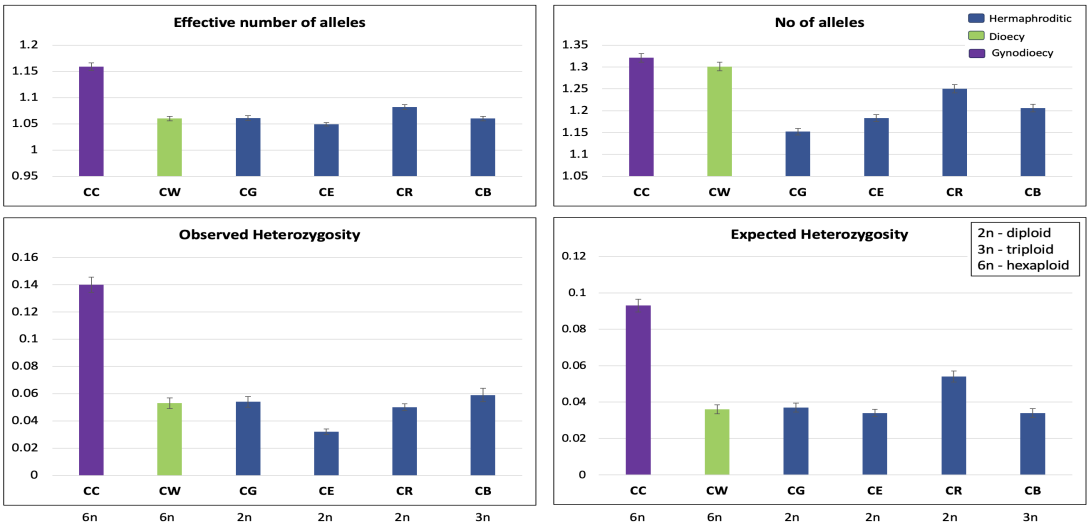


Figure 5.1: Genetic diversity parameters for gynodioecious (purple), dioecious (green) and hermaphroditic (blue) systems. The error bars represent confidence intervals from bootstrapping over loci.

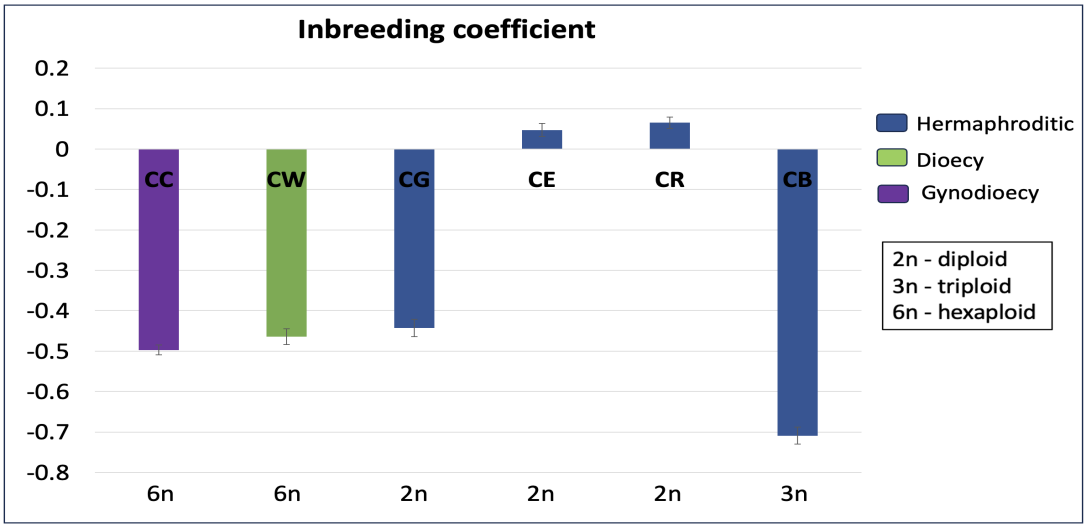


Figure 5.2: Inbreeding coefficient for gynodioecious (purple), dioecious (green) and hermaphroditic (blue) systems. The error bars represent confidence intervals from bootstrapping over loci.

### 5.4.2 Genetic Differentiation and Clustering Analyses

To assess hybrid origins or shared ancestry between CC and CW, we performed population differentiation and clustering analyses. Fst estimates have a value from 0 - 1 with lower numbers indicating lesser differentiation. Our results (Table 5.3) show high genetic differentiation between all species, except CW and CG that are geographically closer. Estimated values range from 0.005 (between CG and CW) to 0.9 (between CE and CB). All the Fst values are significantly different from 0.

Principal Coordinate Analysis (PCoA) indicated that the first two axes accounted for 68.2% of the variation, highlighting the separation of all 227 individuals into four distinct populations (Fig. 5.3a). Notably, with the exception of individuals CG and CW, each group formed its own cluster based on their species group.

Consistent with PCoA, Discriminant Analysis of Principal Components (DAPC)

Table 5.3: Pairwise species F-st estimates between species. The species abbreviations are as follows : *C. bigelovii* (CB), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR), *C. wolfii* (CW).

| **Species** | **CC** | **CW** | **CG** | **CE** | **CR** | **CB** |
|---|---|---|---|---|---|---|
| **CC** | 0 | | | | | |
| **CW** | 0.4 | 0 | | | | |
| **CG** | 0.295 | 0.006 | 0 | | | |
| **CE** | 0.452 | 0.629 | 0.634 | 0 | | |
| **CR** | 0.433 | 0.683 | 0.641 | 0.735 | 0 | |
| **CB** | 0.798 | 0.881 | 0.883 | 0.9 | 0.873 | 0 |

also showed the formation of five genetically distinct clusters corresponding to the species (Fig. 5.3b). Intriguingly, individuals from CG and CW were observed to share the same cluster. Then we further did hierarchical analysis of PCoA and DAPC including just CG, CW, CC and CE and recovered 3 different groups that account for CG-CW cluster, CC and CE respectively. Subsequent sub-DAPC and sub-PCoA analysis focusing just on CG and CW, reaffirmed their joint clustering. In revised analyses of complete and sub groups, where the call rates were adjusted to 90%, 80%, 70%, and 60%, resulting in an increased number of SNPs, the genetic data did not show a clear separation between CG and CW.

FastStructure analysis showed that K=5 to be the optimal number of clusters. Again, CW and CG formed one cluster and the rest of them formed their own cluster based on the species they belong to. No signs of admixture were evident from the plot (Fig. 5.4). Substructuring using just CW, CG, CC and CE with call rates ranging from 100%-60% showed that K=3 is the optimal number of clusters. Here again CW-CG was one cluster and the other two species formed their own cluster. Further substructuring with CW and CG samples only and with 100%, 90%, 80%, 70% and 60% missing data showed that K=1 to be the optimal number of clusters.

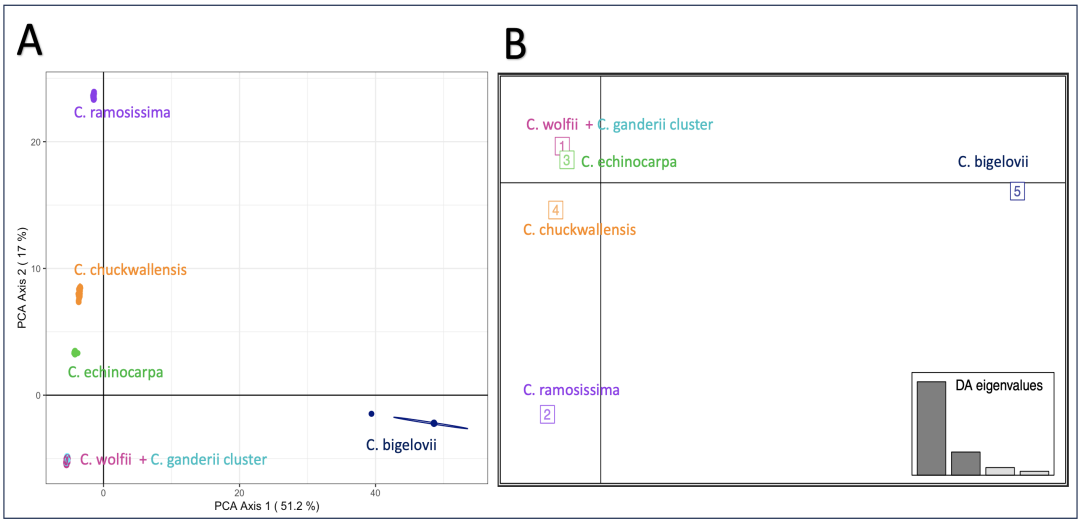


Figure 5.3: (a) Principal Coordinate Analysis (PCoA) of 227 samples using SNP markers. The percentages of total variance explained by each coordinate are provided in parentheses. (b) Discriminant Analysis of Principal Components (DAPC) for the same 227 samples. The axes in this plot represent the first two linear discriminants (LD). Each square within the plot corresponds to a distinct cluster, and the numerical labels denote the different groups identified by DAPC.

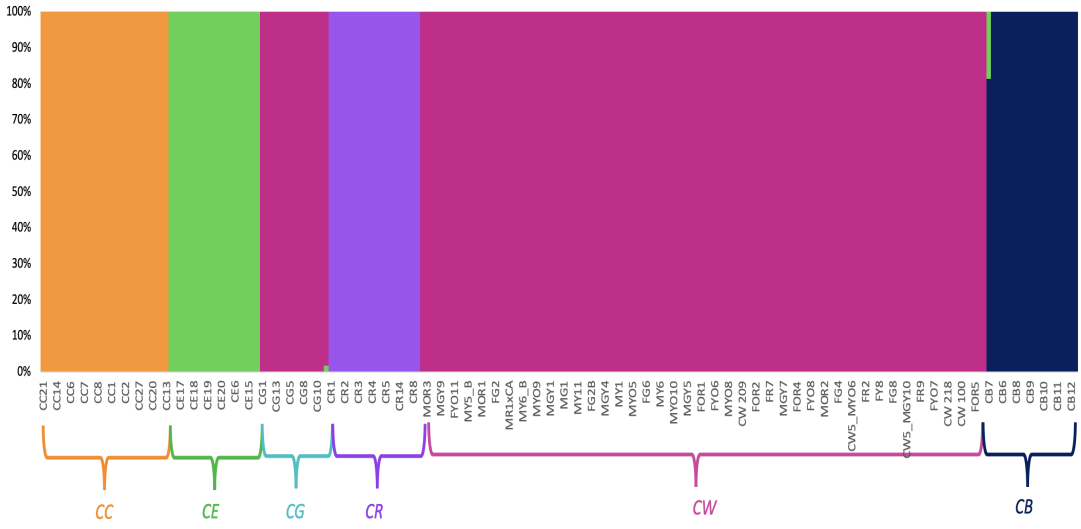


Figure 5.4: fastSTRUCTURE plot showing five genetic clusters (K=5) colored by genetic identity. Each line on the x-axis represents an individual and the proportion of ancestry derived from a certain genetic cluster is represented by the y-axis.The species abbreviations are as follows: *C. bigelovii* (CB), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR), *C. wolfii* (CW).

The CONSTRUCT analysis, based on 0% missing data, indicated that K=4 is the optimal number of clusters, as determined by layer contribution analysis and cross validation analysis (Fig. 5.5). The four clusters were separated as follows: CE, CR, CB and CW-CG and finally CC is observed to have shared ancestry from all the four clusters mentioned earlier with genes derived almost equally from all four distinct clusters. However, in the presence of 10% missing data and an increased number of SNPs, the analysis suggested that K=5 is optimal based on layer contribution and cross validation analysis. Remarkably, this outcome aligns closely with the results obtained from FastStructure analysis. Once again, CW and CG clustered together, forming one distinct group, while the remaining entities constituted a separate cluster based on their respective species. Notably in spatial analysis, indications of admixture were present, particularly in CC, CR, CW, and CG. In CC, there was discernible ancestry derived from CR, CE, CB and CW (Fig. 5.6).

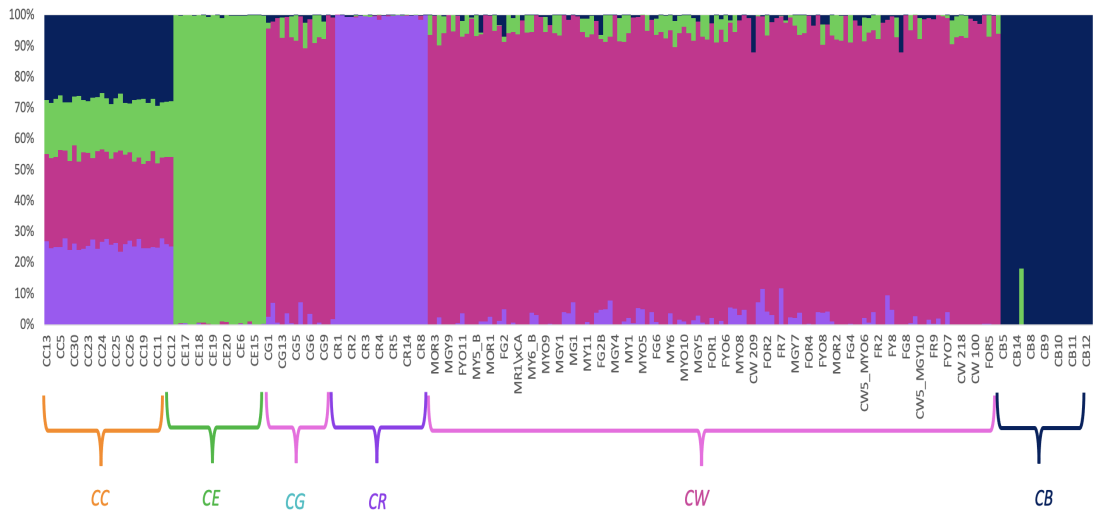


Figure 5.5: CONSTRUCT plot based on 0%missing data, showing four genetic clusters (K=4) colored by genetic identity. The species abbreviations are as follows: *C. bigelovii* (CB), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR), *C. wolfii* (CW).


Figure 5.6: CONSTRUCT plot based on 10% missing data, showing five genetic clusters (K=5) colored by genetic identity.The species abbreviations are as follows: *C. bigelovii* (CB), *C. chuckwallensis* (CC), *C. echinocarpa* (CE), *C. ganderi* (CG) and *C. ramosissima* (CR), *C. wolfii* (CW).

### 5.4.3 Clonality Analysis and Field Survey

Our results showed partial evidence of clonal reproduction in *C. wolfii*. The maximum clonal distance (i.e., pairwise individual genetic distance) between the replicated samples was computed to be 78. Doubling this value results in a threshold of 156, and this was employed in the analysis to identify different genets. Out of the 124 individual *C. wolfii* ramets subjected to genotyping, GenoDive identified a total of 112 distinct clonal lineages plus six clonal groups with two ramets each.

In contrast, no new recruitment of *C. wolfii* seedlings was detected in the field as evidence of sexual reproduction. Out of the four surveyed quadrants, one quadrant revealed the presence of a juvenile cactus (32°40'27"N, 116°05'51.0"W) exhibiting characteristics suggestive of seed germination. However, it is highly probable that this juvenile cactus

belongs to the species *Echinocereus engelmannii*, as it is native to the area. Notably, we did not encounter any seedlings of *Cylindropuntia spp.* during our investigation. Contrastingly, an average of 39 fragments per quadrant was observed in proximity to adult plants within the surveyed area.

## 5.5 DISCUSSION

The genetic diversity comparisons between species revealed that the gynodioecious species CC exhibited significantly higher genetic diversity compared to all hermaphroditic counterparts. Interestingly, dioecious species CW demonstrated comparable genetic diversity to that of hermaphrodites. These findings provide partial support for the inbreeding avoidance as a potential driving force behind sexual separation and do not support the dead end hypothesis which suggests that dioecious species will have a lower genetic diversity than that of hermaphrodites. Certainly, the higher number of alleles observed within the CC and CW species compared to the remaining species suggests an advantage in terms of genetic variation for sexually separated systems. However, when we compare the effective number of alleles, CC had a higher value than the hermaphrodites and CW had a comparable value. Thus, this observation of the high number of alleles in CW might be influenced by the larger number of individuals compared to other species in our dataset.

The inbreeding coefficients reveal that both the gynodioecious and dioecious systems exhibit outbreeding while some hermaphroditic species (CE, CR) exhibit minor inbreeding. These findings provide further support for inbreeding avoidance, suggesting that sexual separation in *Cylindropuntia* species may indeed have evolved to avoid inbreeding.

The outbreeding in CC and CW can be attributed to the inability of females in the gynodioecious population and that of all individuals in dioecious populations to self-fertilize. CC is morphologically classified as gynodioecious; however, experimental crosses or embryological studies are needed to accurately determine the sexual system of this species (Ramadoss, 2022). Therefore, it is possible that it is functionally dioecious as CW. In such cases, CC would rely entirely on outcrossing, explaining the observed high levels of outbreeding. Furthermore, CC is also hypothesized to be of hybrid origin from *C. acanthocarpa* and *C. multigeniculata* (Baker and Hughes, 2014) based on morphological similarities (tepal and stigma color from *C. acanthocarpa* and stem number per trunk, habit from *C. multigeniculata*) and could have led to high levels of heterozygosity. Although we did not include the parental species hypothesized by Baker and Hughes (2014), our clustering analysis indicates that CC likely originated from hybridization. Contrarily, the hermaphroditic species CE and CR demonstrated low inbreeding, aligning with expectations given their bisexual flowers. Interestingly, the hermaphroditic CG displayed outbreeding despite the presence of bisexual flowers, suggesting the potential presence of self-incompatibility strategies. The CB species is postulated to engage in clonal reproduction, a characteristic attributed to its triploid nature (Fong et al., 2019). However, the elevated out breeding coefficient for CB observed in our study suggests an alternative explanation. This phenomenon can be explained by the Meselson effect, a mechanism known to sustain or potentially enhance heterozygosity over successive generations in asexual species through irreversible accumulation of mutations in homologous chromosomes (Welch and Meselson, 2000).

Overall the genetic diversity values of *Cylindropuntia* were extremely low and comparatively similar to that of the critically imperiled Florida Tree cactus *Pilosocereus robinii* measured from SNP markers, also reported to have lower genetic diversity (Fotinos, 2013). The inbreeding coefficient observed in *Cylindropuntia* is comparatively lower than that reported for *P. robinii* , a species where inbreeding is known to impact genetic diversity (Fotinos, 2013). Hence, this implies that factors besides inbreeding may be influencing the reduced heterozygosity levels in *Cylindropuntia*. s. We can attribute it to genetic drift as it tends to have a more pronounced effect in small populations (Nabutanyi and Wittmann, 2022). Additionally, in dioecious species with insect pollination, competition among males could render them more appealing to pollinators than females, thereby causing the overall population size to be contingent upon variations in pollinator density (Vamosi and Otto, 2002). Consequently, fluctuating population sizes will lead to low levels of genetic diversity (Muyle et al., 2018). A previous study on *C. wolfii* demonstrated a pollinator preference bias towards males over female flowers (Ramadoss et al., 2023), attributed to their larger flower size and more vibrant colors. Thus, this phenomenon might elucidate the observed low genetic diversity in *C. wolfii* in our current study.

Genetic differentiation and clustering analyses not only helps us understand the ancestry of species but also provides parameters for effective biodiversity conservation (Pearse and Crandall, 2004; Ottewell et al., 2016; Buck et al., 2023). According to Nei's (1986) qualitative indices for Fst, the findings in *Cylindropuntia* species suggest high genetic differentiation, with the exception of CG and CW, implying that five out of six are different species. Based on morphological characteristics such as prominent spines on upper stems,

hiding stems and tubercles (Chester, 2007), it can be discerned that CW is more closely related to the CG clade than to the CE or CR clades (Majure et al., 2019). Additionally, our genetic clustering analysis like PCoA, DAPC, ConStruct and FastSTRUCTURE also supplement the data by suggesting that there are 5 genetically distinct clusters grouping according to their species except for CW-CG cluster. The clustering of CW and CG could be attributed to several plausible explanations. Firstly, it is possible that ongoing hybridization events are occurring between CG and CW, as they coexist in sympatry. Furthermore preliminary observations suggest that the CG species collected for the study have physical characteristics indicative of hybrids originated from CG and CW. Secondly, considering CW is hypothesized to have a hybrid origin, it is plausible to speculate that CG may serve as one of its parent species. Additionally, given that Dartseq diploidizes the polyploid data, it is conceivable that the captured SNPs may represent the haplotype of one parent, potentially CG in this context. Alternatively, the genetic markers we sequenced may not have completely sorted into distinct lineages as not enough time has passed (ILS - Incomplete Lineage Sorting). Additional data such as Whole Genome Sequencing or conducting more advanced coalescent-based phylogenetic analyses, may help elucidate the underlying processes contributing to the clustering of CW-CG.

Notably, the next pair with the least genetic differentiation, are the two hexaploids CC and CW, which share similar flower color polymorphism, unisexual flowers, and a predicted shared common ancestor of *C. acanthocarpa* (Baker and Hughes, 2014). In the DAPC and PCoA plots, CC clustered close to the CW and CG cluster suggesting it could be phylogenetically closely related to them. Although no shared ancestry between CC and CW

was evident in the FastSTRUCTURE analysis, we observed admixture between CC and CW individuals in the ConStruct analysis. Specifically, in both the K=4 model, CC cluster consisted of equally shared ancestry with all the other four gene clusters, while the CW individuals exhibited some admixture with the other four gene pools, implying the potential for an allopolyploid origin. In the K=5 model, CC exhibited its own gene pool with admixture with all the other four gene clusters. This implies that the other species may either be the parent or share genetic material with the parents of CC. In the phylogenetic context, CB is recognized as the sister taxon to the remaining members of the *Cylindropuntia* genus included in the study by Majure et al. 2019, thereby accounting for its high genetic differentiation from other species within the group.

The overall low genetic diversity raises significant conservation concerns for the *Cylindropuntia* species (Fahrig, 2002; Reed and Frankham, 2003).The low genetic diversity is directly related to a constrained evolutionary potential (Spielman et al., 2004). Consequently, the six *Cylindropuntia* species in our study might encounter challenges in adapting to forthcoming extreme climatic changes (Zscheischler et al. 2020). Some species have overcome this low genetic diversity with the help of gene flow (Teukeng et. al 2022; Buck et al., 2023). But our data does not show evidence for a lot of gene flow in these species except for CC, potentially because of difference in ploidy or other kinds of barriers that could be temporal, mechanical or behavioral.

The observed clonality from genetic markers does not account for the low genetic diversity within the *C. wolfii* population. Excluding the known replicates, two clonal lineages were identified, each comprising a pair of ramets from different individuals. In one

lineage, the individuals were geographically proximate (50 ft), both being females. However, one had orange-red flowers, while the other exhibited a red flower morph. This shows that even though they are from a single clonal group they can accumulate genetic variations that can influence a slight shift in their flower color morphs. The second clonal pair, despite being 365 feet apart, consisted of both male individuals with green flower color morphs.This suggests limited clonal recruitment in the adult population or current generation that exists in Mountain Springs. However, during our sampling process, we specifically collected individuals that were at least 20 feet apart. Thus it is possible that our sampling method may have led to an underestimation of clonality among the adult plants present in Mountain Springs. The observations from the field survey however suggest a phenomenon consistent with clonal propagation for the next generation, as the fragments we noted are likely a result of asexual reproduction process associated with falling of fragments from mature plants. Thus we can suggest that this species has shifted from sexual reproduction to clonal reproduction and this shift could be influenced by climate change, decrease in pollinators, negative mutation or a combination of all such factors (Barrett, 2009; Barrett, 2015; Hamann et al., 2021).

## 5.6 CONCLUSION

Based on the findings of our study, it is evident that the *Cylindropuntia* system does not support the dead end hypothesis. The dioecious system displayed genetic diversity parameters that were comparable to those of hermaphrodites. Interestingly, the gynodioecious species exhibited higher genetic diversity compared to the hermaphrodite

species. However, it is crucial to acknowledge the ambiguity about the sexual system of *C. chuckwallensis*, as it is only morphologically identified as gynodioecious. Nevertheless, it is imperative to recognize the limitations of our study. The inclusion of only one dioecious and one gynodioecious species restricts the generalizability of our conclusions, as observed patterns may be influenced by factors beyond the sexual system itself. Furthermore, our genetic structure analysis revealed intriguing clustering patterns with *C. wolfii*-*C. ganderi* forming one cluster, suggesting potential hybridization or incomplete lineage sorting with *C. ganderi* possibly serving as one of the parents of *C. wolfii*. Overall, our investigation highlights the relatively low genetic diversity within the *Cylindropuntia* genus, with *C. wolfii* exhibiting a notable shift towards clonal reproduction, as indicated by field surveys. These findings underscore the urgent need for conservation efforts to protect these species and their genetic diversity.

# Chapter 6

# Conclusions

The genus *Cylindropuntia* is a useful model in the study of sexual separation and its consequences. Based on histological and developmental crosses, we have identified the sexual system of *Cylindropuntia wolfii* to be functionally dioecious which was previously noted as gynodioecious. This highlights the necessity of accurately identifying the sexual systems of other species within the genus that are also morphologically described as gynodioecious. Our study is the first in the genus of *Cylindropuntia* to describe the development of unisexual flowers. The male sterility pattern in this species was observed to be similar to other Cactaceae species suggesting a common underlying mechanism in this family. Furthermore, we also identified sexual dimorphism in floral traits of *C. wolfii* where the male flowers are significantly bigger than female flowers. Additionally, we observed sexual dichromatism, with males displaying brighter tepals and females exhibiting darker ones. Moreover the females flowers have significantly higher fluorescence in their anthers compared to the male counterparts. This phenomenon is called fluorescent dichromatism

and it has only been reported in birds so far. Our study introduces, for the first time, fluorescence sexual dichromatism in a plant species, highlighting significant implications for future studies on the dioecious plant-pollinator interactions. The mechanism underlying the autofluorescence of sterile anthers may be widespread among functionally dioecious species, warranting further investigation. Our pollinator survey revealed that male flowers attracted more potential pollinators than females, potentially linked to the observed sexual dimorphism. This observation might explain the decreased seed production observed in the *C. wolfii* species, as lower pollinator densities could result in fewer opportunities for female ovules to be fertilized, leading to reduced fruit maturation. Additionally, we also found that the pollinator attraction can also be influenced by the choice of fluorescent dye used as pollen analogues in ecological surveys. Particularly the green dye introduces a potential bias in bee pollination experiments by having a higher attraction rate. We suggest that researchers utilizing fluorescent dye powders should investigate the potential impact of dye color on pollinator behavior, particularly when utilizing multiple dye colors.

Our population genetic estimates revealed that the *Cylindropuntia* system deviates from the dioecy dead end hypothesis. The dioecious species *C. wolfii* displayed genetic diversity comparable to those of the hermaphrodites. Notably, the gynodioecious species *C. chuckwallensis* exhibited higher genetic diversity than the hermaphrodites. However, it's essential to acknowledge the uncertainty regarding the sexual system of *C. chuckwallensis*, as it is only morphologically identified as gynodioecious suggesting that it could be functionally dioecious like *C. wolfii*. Nevertheless, it is imperative to recognize the limitations of our study. The inclusion of only one dioecious and one gynodioecious species restricts the

generalizability of our conclusions, as observed patterns may be influenced by factors beyond the sexual system itself such as ploidy level, geographic location or population size. Overall, our investigation highlights the relatively low genetic diversity within the *Cylindropuntia* genus, with *C. wolfii* exhibiting a notable shift towards clonal reproduction, as indicated by field surveys. These findings underscore the urgent need for conservation efforts to protect these species and their diversity.

Finally, our genetic structure analysis revealed intriguing clustering patterns with *C. wolfii* and *C. ganderi* forming one cluster, suggesting potential hybridization or incomplete lineage sorting with *C. ganderi* possibly serving as one of the parents of *C. wolfii*. Future studies should prioritize accurate identification of the sexual system of *C. chuckwallensis* through comprehensive histological analysis and leverage the power of Whole Genome Sequencing (WGS) to uncover the elusive parental origins of allopolyploid species within this genus. Additionally, employing coalescent-based approaches can illuminate the evolutionary history behind the observed low genetic diversity across these species, paving the way for a deeper understanding of their genetic dynamics. By advancing the knowledge on sexual systems and its consequences, our study aims to aid in the conservation of cacti, the fifth most threatened group on Earth.

# Bibliography

[1] Acharya, R. S., Burke, J. M., Leslie, T., Loftin, K., and Joshi, N. K. (2022). Wild bees respond differently to sampling traps with vanes of different colors and light reflectivity in a livestock pasture ecosystem. Scientific Reports, 12(1), 1-1.

[2] Adler, L. S., and Irwin, R. E. (2006). Comparison of pollen transfer dynamics by multiple floral visitors: experiments with pollen and fluorescent dye. Annals of Botany, 97(1), 141-150.

[3] Ancillotto, L., Vignoli, L., Martino, J., Paoletti, C., Romano, A., and Bruni, G. (2022). Sexual dichromatism and throat display in spectacled salamanders: a role in visual communication? Journal of Zoology, 318(2), 75-83.

[4] Anderson, E. F., and Brown, R. (2001). The Cactus Family. Timber Press.

[5] Anderson, G. J., Anderson, M. K., and Patel, N. (2015). The ecology, evolution, and biogeography of dioecy in the genus *Solanum*: with paradigms from the strong dioecy in *Solanum polygamum*, to the unsuspected and cryptic dioecy in *Solanum conocarpum*. American Journal of Botany, 102(3), 471-486.

[6] Anderson, G. J., Bernardello, G., Opel, M. R., Santos-Guerra, A., and Anderson, M. (2006). Reproductive biology of the dioecious Canary Islands endemic *Withania aristate* (Solanaceae). American Journal of Botany, 93(9), 1295-1305.

[7] Araki, S., Le, N. T., Koizumi, K., Villar-Briones, A., Nonomura, K. I., Endo, M., ... Komiya, R. (2020). miR2118-dependent U-rich phasiRNA production in rice anther wall development. Nature Communications, 11(1), 3115.

[8] Ashman, T.-L. (2009). Sniffing out patterns of sexual dimorphism in floral scent. Functional Ecology, 23, 852–862.

[9] Austerlitz, F., Gleiser, G., Teixeira, S., and Bernasconi, G. (2012). The effects of inbreeding, genetic dissimilarity and phenotype on male reproductive success in a dioecious plant. Proceedings of the Royal Society B: Biological Sciences, 279(1726), 91-100.

[10] Badyaev, A. V., and Hill, G. E. (2003). Avian sexual dichromatism in relation to phylogeny and ecology. Annual Reviews of Ecology, Evolution and Systematics, 34, 27-49.

[11] Baena-Díaz, F., Fornoni, J., Sosenski, P., Molina-Freaner, F. E., Weller, S. G., Pérez-Ishiwara, R., and Domínguez, C. A. (2012). Changes in reciprocal herkogamy during the tristyly–distyly transition in *Oxalis alpina* increase efficiency in pollen transfer. Journal of Evolutionary Biology, 25(3), 574-583.

[12] Baker, M. (2006). A new florally dimorphic hexaploid, *Echinocereus yavapaiensis sp. nov.* (section Triglochidiati, Cactaceae) from central Arizona. Plant Systematics and

Evolution, 258, 63–83.

[13] Baker, M. A., and Pinkava, D. J. (1987). A cytological and morphometric analysis of a triploid apomict, *Opuntia × kelvinensis* (subgenus Cylindropuntia, Cactaceae). Brittonia, 39(3), 387-401.

[14] Baker, M. A., and Cloud-Hughes, M. (2014). *Cylindropuntia chuckwallensis* (Cactaceae), a new species from Riverside and Imperial counties, California. Madroño, 61, 231–243.

[15] Baker, M. A., and Pinkava, D. J. (2018). Chromosome numbers in some cacti of western North America—IX. Haseltonia, 25, 5–29.

[16] Baker, M. A., Rebman, J. P., Parfitt, B. D., Pinkava, D. J., and Zimmerman, A. D. (2009). Chromosome numbers in some cacti of western North America-VIII. Haseltonia, 2009(15), 117-134.

[17] Balloux, F., Lehmann, L., and de Meeus, T. (2003). The Population Genetics of Clonal and Partially Clonal Diploids. Genetics, 164, 1635–1644.

[18] Barreira, A. S., Lagorio, M. G., Lijtmaer, D. A., Lougheed, S. C., and Tubaro, P. L. (2012). Fluorescent and ultraviolet sexual dichromatism in the blue-winged parrotlet. Journal of Zoology, 288(2), 135-142.

[19] Barrett, S. C., and Hough, J. (2013). Sexual dimorphism in flowering plants. Journal of Experimental Botany, 64(1), 67-82.

[20] Barrett, S. C. (2015). Influences of clonality on plant sexual reproduction. Proceed-

ings of the National Academy of Sciences of the United States of America, 112(29), 8859-8866.

[21] Barrett, S. C. H. (2002). The evolution of plant sexual diversity. Nature Reviews Genetics, 3(4), 274–284.

[22] Barrett, S. C. H., Ness, R. W., and Vallejo-Marín, M. (2009). Evolutionary pathways to self-fertilization in a tristylous plant species. New Phytologist, 183(3), 546–556.

[23] Bawa, K. S., and Opler, P. A. (1975). Dioecism in tropical trees. Evolution, 29, 167–179.

[24] Bell, G. (1985). On the function of flowers. Proceedings of the Royal Society of London. Series B. Biological Sciences, 224(1235), 223-265.

[25] Benson, L. D., and Baker, M. A. (2003). Calflora: Information on California plants for education, research and conservation, with data contributed by public and private institutions and individuals, including the Consortium of California Herbaria 2021. Berkeley: The Calflora Database. Website. https://www.calflora.org [accessed: 27 03 2021].

[26] Bobich, E. G., Wallace, N. L., and Sartori, K. L. (2014). Cholla mortality and extreme drought in the Sonoran Desert. Madroño, 61(1), 126-136.

[27] Bopp, S., and Gottsberger, G. (2004). Importance of *Silene latifolia ssp. alba* and *S. dioica* (Caryophyllaceae) as host plants of the parasitic pollinator *Hadena bicruris* (Lepidoptera, Noctuidae). Oikos, 105(2), 221-228.

[28] Bradburd, G. S., Coop, G. M., and Ralph, P. L. (2018). Inferring continuous and discrete population genetic structure across space. Genetics, 210, 33–52.

[29] Brock, M. T., Lucas, L. K., Anderson, N. A., Rubin, M. J., Markelz, R. J., Covington, M. F., ... Weinig, C. (2016). Genetic architecture, biochemical underpinnings and ecological impact of floral UV patterning. Molecular Ecology, 25, 1122–1140.

[30] Buck, R., Hyasat, S., Hossfeld, A., and Flores-Rentería, L. (2020). Patterns of hybridization and cryptic introgression among one-and four-needled pinyon pines. Annals of Botany, 126, 401–411.

[31] Buck, R., Ortega-Del Vecchyo, D., Gehring, C., Michelson, R., Flores-Rentería, D., Klein, B., ... Flores-Rentería, L. (2023). Sequential hybridization may have facilitated ecological transitions in the Southwestern pinyon pine syngameon. New Phytologist, 237(6), 2435-2449.

[32] Buxton, M. N., Anderson, B. J., and Lord, J. M. (2022). Moths can transfer pollen between flowers under experimental conditions. New Zealand Journal of Ecology, 46(1), 1–5.

[33] California Native Plant Society, Rare Plant Program. (1968 onward continuously updated). Inventory of rare and endangered plants of California (online edition, v8-03 0.39). Website http://www.rareplants.cnps.org [accessed 08 03 2022].

[34] Caporali, E., Spada, A., Marziani, G., Failla, O., and Scienza, A. (2003). The arrest of development of abortive reproductive organs in the unisexual flower of *Vitis vinifera ssp. sylvestris*. Sexual Plant Reproduction, 15, 291–300.

[35] Caporali, E., Testolin, R., Pierce, S., and Spada, A. (2019). Sex change in kiwifruit (*Actinidia chinensis* Planch.): a developmental framework for the bisexual to unisexual floral transition. Plant reproduction, 32(3), 323–330.

[36] Castro, A. J., Rejon, J. D., Fendri, M., Zafra, A., Jimenez Lopez, J. C., Rodriguez Garcia, M. L., and Alche, J. D. (2010). Taxonomical discrimination of pollen grains by using confocal laser scanning microscopy (CLSM) imaging of autofluorescence. In A. Mendez and J. Diaz (Eds.), Microscopy: science, technology, applications and education (pp. 607–613). Formatex, Spain.

[37] Charlesworth, D., and Guttman, D. S. (1999). The evolution of dioecy and plant sex chromosome systems. In Sex determination in plants (pp. 25–49).

[38] Charlesworth, D. (2006). Evolution of plant breeding systems. Current Biology, 16(17), R726–35.

[39] Charlesworth, D. (1999). Theories of the evolution of dioecy. In Gender and sexual dimorphism in flowering plants (pp. 33–60). Springer, Berlin.

[40] Charlesworth, D., and Charlesworth, B. (1978). Population genetics of partial male-sterility and the evolution of monoecy and dioecy. Heredity, 41, 137–153.

[41] Charlesworth, D., and Charlesworth, B. (1987). Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics, 237-268.

[42] Charnov, E. L. (1982). The theory of sex allocation. Princeton: Princeton University Press.

[43] Chester, T. (2007). Plants of Southern California: Opuntia echinocarpa, O. ganderi, O. parryi, and O. wolfii: Pictorial Identification Guide. The Plants Analysis Database. Retrieved from https://tchester.org/plants/analysis/cholla/ganderietal.html

[44] Chittka, L. (1992). The colour hexagon: a chromaticity diagram based on photoreceptor excitations as a generalized representation of colour opponency. Journal of Comparative Physiology A, 170, 533–543.

[45] Chittka, L. (1997). Bee color vision is optimal for coding flower color, but flower colors are not optimal for being coded – why? Israel Journal of Plant Sciences, 45, 115–127.

[46] Coimbra, S., Torrao, L., and Abreu, I. (2004). Programmed cell death induces male sterility in *Actinidia deliciosa* female flowers. Plant Physiology and Biochemistry, 42, 537–541.

[47] Colwell, R. N. (1951). The use of radioactive isotopes in determining spore distribution patterns. American Journal of Botany, 38, 511–523.

[48] R Core Team. (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

[49] Cornet, C., Noret, N., and Van Rossum, F. (2022). Pollinator sharing between reproductively isolated genetic lineages of *Silene nutans*. Frontiers in Plant Science, 13, 927498.

[50] Correns, C. (1928). Bestimmung, Vererbung und Verteilung des Geschlechtes bei den höheren Pflanzen. In: Baur E Hartmann M (Eds.), Handbuch der Vererbungsseissenschaft (pp. 1–128).

[51] Costich, D. E., and Meagher, T. R. (2001). Impacts of floral gender and whole-plant gender on floral evolution in *Ecballium elaterium* (Cucurbitaceae). Biological Journal of the Linnean Society, 74(4), 475–487.

[52] Cuevas, E., Pérez, M. A., and Sevillano, L. (2017). Population size, sex-ratio and sexual dimorphism in *Fuchsia parviflora* (Onagraceae) an endemic dioecious shrub. Botanical Sciences, 95(3), 401–408.

[53] da Cunha, N. L., Xue, H., Wright, S. I., and Barrett, S. C. (2022). Genetic variation and clonal diversity in floating aquatic plants: Comparative genomic analysis of water hyacinth species in their native range. Molecular Ecology, 31(20), 5307-5325.

[54] Dafni, A., Bernhardt, P., Shmida, A., Ivri, Y., Greenbaum, S., O'Toole, C., and Losito, L. (1990). Red bowl-shaped flowers: convergence for beetle pollination in the Mediterranean region. Israel Journal of Plant Sciences, 39(1-2), 81-92.

[55] Darwin, C. (1851). A Monograph on the Sub-class Cirripedia: The Lepadidæ; or, pedunculated cirripedes (Vol. 1). Ray society.

[56] Darwin, C. (1897). The different forms of flowers on plants of the same species. D. Appleton. Chapter 7.

[57] Darwin, C. R. (1877). The Different Forms of Flowers on Plants of the Same Species. Murray, London, UK.

[58] Dawson, J., Sözen, E., Vizir, I., Van Waeyenberge, S., Wilson, Z. A., and Mulligan, B. J. (1999). Characterization and genetic mapping of a mutation (ms35) which prevents anther dehiscence in *Arabidopsis thaliana* by affecting secondary wall thickening in the endothecium. The New Phytologist, 144(2), 213-222.

[59] Delph, L. F., and Ashman, T. L. (2006). Trait selection in flowering plants: how does sexual selection contribute? Integrative and Comparative Biology, 46, 465–472.

[60] Delph, L. F., Gehring, J. L., Arntz, A. M., Levri, M., and Frey, F. M. (2005). Genetic correlations with floral display lead to sexual dimorphism in the cost of reproduction. American Naturalist, 166, S31–S41.

[61] Delph, L. F., Galloway, L. F., and Stanton, M. L. (1996). Sexual dimorphism in flower size. American Naturalist, 148(2), 299-320.

[62] Diller, C., Castañeda-Zárate, M., and Johnson, S. D. (2022). Why honeybees are poor pollinators of a mass-flowering plant: Experimental support for the low pollen quality hypothesis. American Journal of Botany, 109(8), 1305-1312.

[63] Diniz, U. M., Lima, S. A., and Machado, I. C. (2019). Short-distance pollen dispersal by bats in an urban setting: monitoring the movement of a vertebrate pollinator through fluorescent dyes. Urban Ecosystems, 22, 281-291.

[64] Dong, X., Hong, Z., Sivaramakrishnan, M., Mahfouz, M., and Verma, D. P. S. (2005). Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in Arabidopsis. Plant Journal, 42, 315–328.

[65] Dötterl, S., Glück, U., Jürgens, A., Woodring, J., and Aas, G. (2014). Floral reward, advertisement and attractiveness to honey bees in dioecious *Salix caprea*. PLoS ONE, 9, e93421.

[66] Dudash, M. R., Hassler, C., Stevens, P. M., and Fenster, C. B. (2011). Experimental floral and inflorescence trait manipulations affect pollinator preference and function in a hummingbird-pollinated plant. American Journal of Botany, 98(2), 275–282.

[67] Dufay, M., and Billard, E. (2011). How much better are females? The occurrence of female advantage, its proximal causes and its variation within and among gynodioecious species. Annals of Botany, 109(3), 505–519.

[68] Dufaÿ, M., Champelovier, P., Käfer, J., Henry, J. P., and Mousset, S., Marais, G. A. B. (2014). An angiosperm-wide analysis of the gynodioecy–dioecy pathway. Annals of Botany, 114(3), 539–548.

[69] Eckhart, V. M. (1991). The effects of floral display on pollinator visitation vary among populations of *Phacelia linearis* (Hydrophyllaceae). Evolutionary Ecology, 5(4), 370–384.

[70] Fahrig, L. (2002). Effect of habitat fragmentation on the extinction threshold: A synthesis. Ecological Applications, 12(2), 346–353.

[71] Felker, P., and Bunch, R. (2016). The importance of native bees, especially cactus bees (*Diadasia spp*) in the pollination of cactus pears. Journal of the Professional Association for Cactus Development, 18, 15–24.

[72] Fenster, C. B., Dudash, M. R., and Hassler, C. L. (1996). Fluorescent dye particles are good pollen analogs for hummingbird-pollinated *Silene virginica* (Caryophyllaceae). Canadian Journal of Botany, 74(2), 189–193.

[73] Ferguson, D. J. (1989). Revision of the US members of the *Echinocereus triglochidiatus* group. Cactus Succulent Journal, 61, 217–224.

[74] Field, D. L., Pickup, M., and Barrett, S. C. (2013). Comparative analyses of sex-ratio variation in dioecious flowering plants. Evolution: International Journal of Organic Evolution, 67(3), 661–672.

[75] Fleming, T. H., Maurice, S., and Hamrick, J. L. (1998). Geographic variation in the breeding system and the evolutionary stability of trioecy in *Pachycereus pringlei* (Cactaceae). Evolutionary Ecology, 12(3), 279–289.

[76] Flores-Rentería, L., Orozco-Arroyo, G., Cruz-García, F., García-Campusano, F., Alfaro, I., and Vázquez-Santana, S. (2013). Programmed cell death promotes male sterility in the functional dioecious *Opuntia stenopetala* (Cactaceae). Annals of Botany, 112, 789–800.

[77] Fong, R., Johnson, L., and Saucedo, J. (2019). Patterns of vegetative reproduction and distribution of *Cylindropuntia*. Natural Reserve System.

[78] Fotinos, T. D. (2013). Genetic Structure of the Florida Key Tree Cactus, *Pilosocereus robinii*, using Restriction Site associated DNA (RAD) markers.

[79] Freeman, D. C., Harper, K. T., and Ostler, W. K. (1979). Ecology of plant dioecy in

the intermountain region of western North America and California. Oecologia, 44(3), 410–417.

[80] Gandía-Herrero, F., García-Carmona, F., and Escribano, J. (2005). Floral fluorescence effect. Nature, 437, 334.

[81] García-Plazaola, J. I., Fernández-Marín, B., Duke, S. O., Hernández, A., López-Arbeloa, F., and Becerrila, J. M. (2015). Autofluorescence: biological functions and technical applications. Plant Science, 236, 136–145.

[82] Gaudreau, M. M., and Hardin, J. W. (1974). The use of neutron activation analysis in pollination ecology. Brittonia, 26, 316–320.

[83] Geraldes, A., Askelson, K. K., Nikelski, E., Doyle, F. I., Harrower, W. L., Winker, K., and Irwin, D. E. (2019). Population genomic analyses reveal a highly differentiated and endangered genetic cluster of northern goshawks (*Accipiter gentilis laingi*) in Haida Gwaii. Evolutionary Applications, 12(4), 757–772.

[84] Gibson, A. C., and Nobel, P. S. (1986). The cactus primer. Harvard University Press.

[85] Glaettli, M., and Barrett, S. C. (2008). Pollinator responses to variation in floral display and flower size in dioecious *Sagittaria latifolia* (Alismataceae). New Phytologist, 179(4), 1193–1201.

[86] Glick, L., Sabath, N., Ashman, T. L., Goldberg, E., and Mayrose, I. (2016). Polyploidy and sexual system in angiosperms: Is there an association? American Journal of Botany, 103(7), 1223–1235.

[87] Goettsch, B., Hilton-Taylor, C., Cruz-Piñón, G., Duffy, J. P., Frances, A., Hernández, H. M., ... Gaston, K. J. (2015). High proportion of cactus species threatened with extinction. Nature Plants, 1(10), 1–7.

[88] Goldblatt, P., Bernhardt, P., and Manning, J. C. (1998). Pollination of Petaloid Geophytes by Monkey Beetles (Scarabaeidae: Rutelinae: *Hopliini*) in Southern Africa. Annals of the Missouri Botanical Garden, 85(2), 215–230.

[89] Griffith, M. P. (2001). Experimental hybridization of northern Chihuahuan Desert region *Opuntia* (Cactaceae). Aliso: A Journal of Systematic and Evolutionary Botany, 20(1), 37–42.

[90] Gruber, B., Unmack, P., Berry, O., and Georges, A. (2019). Introduction to dartR. Retrieved from https://rdrr.io/cran/dartR/f/inst/doc/IntroTutorial_dartR.pdf

[91] Guerrero, P. C., Majure, L. C., Cornejo-Romero, A., and Hernández-Hernández, T. (2019). Phylogenetic relationships and evolutionary trends in the Cactus family. Journal of Heredity, 110(1), 4–21.

[92] Hamadeh, B., Chalak, L., d'Eeckenbrugge, G. C., Benoit, L., and Joly, H. I. (2018). Evolution of almond genetic diversity and farmer practices in Lebanon: impacts of the diffusion of a graft-propagated cultivar in a traditional system based on seed-propagation. BMC Plant Biology, 18(1), 155.

[93] Hamann, E., Denney, D., Day, S., Lombardi, E., Jameel, M. I., MacTavish, R., and Anderson, J. T. (2021). Plant eco-evolutionary responses to climate change: Emerging directions. Plant Science, 304, 110737.

[94] Hantson, S., Huxman, T. E., Kimball, S., Randerson, J. T., and Goulden, M. L. (2021). Warming as a driver of vegetation loss in the Sonoran Desert of California. Journal of Geophysical Research: Biogeosciences, 126(6), e2020JG005942.

[95] Harder, L. D., Barrett, S. C., Lloyd, D. G., and Barrett, S. C. (1996). Pollen dispersal and mating patterns in animal-pollinated plants. In Floral biology: Studies on floral evolution in animal-pollinated plants (pp. 140-190). New York: Chapman and Hall.

[96] Harris, M. S., and Pannell, J. R. (2008). Roots, shoots and reproduction: sexual dimorphism in size and costs of reproductive allocation in an annual herb. Proceedings of the Royal Society B: Biological Sciences, 275(1651), 2595-2602.

[97] Hartfield, M. (2016). On the origin of asexual species by means of hybridization and drift. Molecular Ecology, 25(14), 3264–3265.

[98] Heilbuth, J. C. (2000). Lower species richness in dioecious clades. The American Naturalist, 156(3), 221–241.

[99] Heilbuth, J. C., Ilves, K. L., and Otto, S. P. (2001). The consequences of dioecy for seed dispersal: modeling the seed-shadow handicap. Evolution, 55(5), 880–888.

[100] Helsen, P., Verdyck, P., and Van Dongen, S. (2011). The influence of historical gene flow, bathymetry and distribution patterns on the population genetics of morphologically diverse Galápagos' *Opuntia echios*. Journal of Molecular Evolution, 72, 315–325.

[101] Hempel de Ibarra, N., and Vorobyev, M. (2009). Flower patterns are adapted for detection by bees. Journal of Comparative Physiology A, 195(3), 319–323.

[102] Hernández-Cruz, R., Barrón-Pacheco, F., Sánchez, D., Arias, S., and Vázquez-Santana, S. (2018). Functional dioecy in *Echinocereus*: ontogenetic patterns, programmed cell death and evolutionary significance. International Journal of Plant Science, 179, 257–274.

[103] Hernández-Cruz, R., Silva-Martínez, J., García-Campusano, F., Cruz-García, F., Orozco-Arroyo, G., Alfaro, I., and Vázquez-Santana, S. (2019). Comparative development of staminate and pistillate flowers in the dioecious cactus *Opuntia robusta*. Plant Reproduction, 32(3), 257–273.

[104] Hoffman, M. T. (1992). Functional dioecy in *Echinocereus coccineus* (Cactaceae): breeding system, sex ratios and geographic range of floral dimorphism. American Journal of Botany, 79, 1382–1388.

[105] Hoffmann, E. O., Flores, T. R., Coover, J., and Garrett, H. B. (1983). Polychrome stains for high resolution light microscopy. Laboratory Medicine, 14(12), 779–781.

[106] Hossaert-McKey, M., Proffit, M., Soler, C. C. L., Chen, C., Bessière, J. M., Schatz, B., and Borges, R. M. (2016). How to be a dioecious fig: chemical mimicry between sexes matters only when both sexes flower synchronously. Scientific Reports, 6(1), 1-11.

[107] Huais, P. Y., Grilli, G., and Galetto, L. (2022). Forest connectivity boosts pollen flow among populations of the oil-producing *Nierembergia linariifolia*. Landscape Ecology, 37, 2435–2450.

[108] Iriel, A., and Lagorio, M. G. (2010). Is the flower fluorescence relevant in biocommu-

nication? Naturwissenschaften, 97, 915–924.

[109] Irish, E. E., and Nelson, T. (1989). Sex determination in monoecious and dioecious plants. Plant Cell, 1(8), 737.

[110] Jaccoud, D., Peng, K., Feinstein, D., and Kilian, A. (2001). Diversity arrays: A solid-state technology for sequence information independent genotyping. Nucleic Acids Research, 29(4), e25.

[111] Janzen, D. H. (1971). Seed predation by animals. Annual Review of Ecology and Systematics, 2(1), 465-492.

[112] Jombart, T. (2008). adegenet: A R package for the multivariate analysis of genetic markers. Bioinformatics, 24(11), 1403-1405.

[113] Jombart, T., and Collins, C. (2015). A tutorial for discriminant analysis of principal components (DAPC) using adegenet 2.0. Retrieved from http://adegenet.r-forge.r-project.org/files/tutorial-dapc.pdf

[114] Jombart, T., Devillard, S., and Balloux, F. (2010). Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. BMC Genetics, 11(1), 1-15.

[115] Joseph, N., and Siril, E. A. (2013). Floral color polymorphism and reproductive success in annatto (*Bixa orellana* L.). Tropical Plant Biology, 6(4), 217-227.

[116] Kevan, P., Giurfa, M., and Chittka, L. (1996). Why are there so many and so few white flowers? Trends in Plant Science, 1(8), 252.

[117] Klomberg, Y., Dywou Kouede, R., Bartoš, M., Mertens, J. E., Tropek, R., Fokam, E. B., and Janeček, Š. (2019). The role of ultraviolet reflectance and pattern in the pollination system of *Hypoxis camerooniana* (Hypoxidaceae). AoB Plants, 11(5), 057.

[118] Kodric-Brown, A. (1998). Sexual dichromatism and temporary color changes in the reproduction of fishes. American Zoologist, 31, 70-81.

[119] Krenn, H. W., Plant, J. D., and Szucsich, N. U. (2005). Mouthparts of flower-visiting insects. Arthropod Structure and Development, 34, 1–40.

[120] Lagorio, M. G., Cordon, G. B., and Iriel, A. (2015). Reviewing the relevance of fluorescence in biological systems. Photochemical and Photobiological Sciences, 14, 1538–1559.

[121] Lázaro, A., Jakobsson, A., and Totland, Ø. (2013). How do pollinator visitation rate and seed set relate to species' floral traits and community context? Oecologia, 173(3), 881–893.

[122] Lebel-Hardenack, S., and Grant, S. R. (1997). Genetics of sex determination in flowering plants. Trends in Plant Science, 2(4), 130–136.

[123] Leong, J. M., and Thorp, R. W. (1999). Colour-coded sampling: the pan trap colour preferences of oligolectic and nonoligolectic bees associated with a vernal pool plant. Ecological Entomology, 24(3), 329-335.

[124] Livia, D. J., Jose, R. F., Alfredo, A. C., Carlos, A. D., and Fernanda, V. D. (2015). Use of aniline blue stain to observing pollen tubes development in different Manihot Mill. species. African Journal of Agricultural Research, 10(15), 1805–1809.

[125] Lloyd, D. G., and Webb, C. J. (1977). Secondary sex characters in plants. Botanical Reviews, 43, 177–216.

[126] Lloyd, D. G. (1980). Sexual strategies in plants III. A quantitative method for describing the gender of plants. New Zealand Journal of Botany, 18(1), 103–108.

[127] Lloyd, D. G. (1982). Selection of combined versus separate sexes in seed plants. American Naturalist, 120, 571–585.

[128] Lunau, K., Konzmann, S., Winter, L., Kamphausen, V., and Ren, Z. X. (2017). Pollen and stamen mimicry: the alpine flora as a case study. Arthropod-Plant Interactions, 11, 427–447.

[129] Majure, L. C., Puente, R., Griffith, M. P., Judd, W. S., Soltis, P. S., and Soltis, D. E. (2012). Phylogeny of *Opuntia* s.s. (Cactaceae): clade delineation, geographic origins, and reticulate evolution. American Journal of Botany, 99(5), 847–864.

[130] Majure, L. C., Baker, M. A., Cloud-Hughes, M., Salywon, A., and Neubig, K. M. (2019). Phylogenomics in Cactaceae: A case study using the chollas sensu lato (Cylindropuntieae, Opuntioideae) reveals a common pattern out of the Chihuahuan and Sonoran deserts. American Journal of Botany, 106(10), 1327–1345.

[131] Maki, M. (1992). Fixation indices and genetic diversity in hermaphroditic and gynodioecious populations of Japanese *Chionographis* (Liliaceae). Heredity, 68(4), 329–336.

[132] Mark Welch, D. B., and Meselson, M. (2000). Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science, 288(5469), 1211–1215.

[133] Martins, A. E., Gélvez-Zúniga, I., Queiroz, S. N. P., and Paiva, B. (2018). Flower attractiness traits are affected differently by florivores in Tibouchina clavata (Pers.) Wurdack. Annals of the XII International Pollination Course, 23-30.

[134] Matsuki, Y., Isagi, Y., and Suyama, Y. (2007). The determination of multiple microsatellite genotypes and DNA sequences from a single pollen grain. Molecular Ecology Notes, 7(2), 194–198.

[135] Mayer, M. S., Gromova, A., Hasenstab-Lehman, K., Lippitt, M., Barnett, M., and Rebman, J. P. (2011). Is Cylindropuntia x fosbergii (Cactaceae) a hybrid? Madroño, 58(2), 106–112.

[136] Meagher, T. R. (1992). The quantitative genetics of sexual dimorphism in Silene latifolia(Caryophyllaceae) .1. Genetic variation. Evolution, 46(2), 445–457.

[137] Meirmans, P. G., and Van Tienderen, P. H. (2004). GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes, 4(4), 792–794.

[138] Meloni, M., Reid, A., Caujapé-Castells, J., Marrero, A., Fernández-Palacios, J. M., Mesa-Coelo, R. A., and Conti, E. (2013). Effects of clonality on the genetic variability of rare, insular species: the case of Ruta microcarpa from the Canary Islands. Ecology and Evolution, 3(6), 1569–1579.

[139] Messinger, O. J. (2013). The role of visual and olfactory cues in host recognition for the specialist bee genus *Diadasia*, and implications for the evolution of host choice. Southern Illinois University at Carbondale.

[140] Menzel, R., and Backhaus, W. (1991). Colour vision in insects. Vision and visual dysfunction, 6, 262-293.

[141] Minnaar, C., and Anderson, B. (2019). Using quantum dots as pollen labels to track the fates of individual pollen grains. Methods in Ecology and Evolution, 10(5), 604–614.

[142] Minnaar, C., Anderson, B., de Jager, M. L., and Karron, J. D. (2019). Plant–pollinator interactions along the pathway to paternity. Annals of Botany, 123(2), 225–245.

[143] Mitchell, C. H., and Diggle, P. K. (2005). The evolution of unisexual flowers: morphological and functional convergence results from diverse developmental transitions. American Journal of Botany, 92, 1068–1076.

[144] Montalvão, A. P. L., Kersten, B., Fladung, M., and Müller, N. A. (2020). The diversity and dynamics of sex determination in dioecious plants. Frontiers in Plant Science, 11.

[145] Moore, J. C., and Pannell, J. R. (2011). Sexual selection in plants. Current Biology, 21(5), R176–R182.

[146] Mori, S., Fukui, H., Oishi, M., Sakuma, M., Kawakami, M., Tsukioka, J., ... Hirai, N. (2018). Biocommunication between Plants and Pollinating Insects through Fluorescence of Pollen and Anthers. Journal of Chemical Ecology, 44(6), 591–600.

[147] Mõtlep, M., Ilves, A., Tali, K., Sild, E., and Kull, T. (2021). Artificial crossing and pollen tracking reveal new evidence of hybridization between sympatric Platanthera species. Plant Systematics and Evolution, 307(2), 25.

[148] Muyle, A., Martin, H., Zemp, N., Mollion, M., Gallina, S., Tavares, R., ... Marais, G. A. (2018). Dioecy in plants: an evolutionary dead end? Insights from a population genomics study in the *Silene* genus. bioRxiv, 414771.

[149] Nabutanyi, P., and Wittmann, M. J. (2022). Modeling minimum viable population size with multiple genetic problems of small populations. Conservation Biology, 36(5), e13940.

[150] Nakashima, H., Horner, H. T., and Palmer, R. G. (1984). Histological features of anthers from normal and ms3 mutant soybean 1. Crop Science, 24(4), 735–739.

[151] Nassar, J. M., Hamrick, J. L., and Fleming, T. H. (2002). Allozyme diversity and genetic structure of the leafy cactus (*Pereskia guamacho* [Cactaceae]). Journal of Heredity, 93, 193–200.

[152] Negrón-Ortiz, V., and Strittmatter, L. I. (2004). Embryology of floral dimorphism and gender system in *Consolea corallicola* (Cactaceae), a rare species of the Florida Keys. Haseltonia, 10, 16–25.

[153] Nei, M. (1986). Definition and estimation of fixation indices. Evolution, 40(3), 643-645.

[154] Niovi Jones, K., and Reithel, J. S. (2001). Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). American Journal of Botany, 88(3), 447-454.

[155] Olsson, M., Stuart-Fox, D., and Ballen, C. (2013). Genetics and evolution of colour

patterns in reptiles. In Seminars in cell and developmental biology (Vol. 24, No. 6-7), Academic Press, pp 133–150.

[156] Ômura, H., and Honda, K. (2005). Priority of color over scent during flower visitation by adult Vanessa indica butterflies. Oecologia, 142, 588–596.

[157] Orozco-Arroyo, G., Vázquez-Santana, S., Camacho, A., Dubrovsky, J. G., and Cruz-García, F. (2012). Inception of maleness: auxin contribution to flower masculinization in the dioecious cactus *Opuntia stenopetala*. Planta, 236, 225–238.

[158] Parachnowitsch, A. L., and Kessler, A. (2010). Pollinators exert natural selection on flower size and floral display in *Penstemon digitalis*. New Phytologist, 188, 393–402.

[159] Pearse, D. E., and Crandall, K. A. (2004). Beyond F ST: analysis of population genetic data for conservation. Conservation Genetics, 5, 585–602.

[160] Penagos Zuluaga, J. C., van der Werff, H., Park, B., Eaton, D. A., Comita, L. S., Queenborough, S. A., and Donoghue, M. J. (2021). Resolved phylogenetic relationships in the Ocotea complex (Supraocotea) facilitate phylogenetic classification and studies of character evolution. American Journal of Botany, 108(4), 664–679.

[161] Perkins, M. D. C. (1977). Dynamics of hummingbird-mediated pollen flow in a subalpine meadow. Thesis. University of British Columbia, Vancouver, British Columbia, Canada.

[162] Pinkava, D. J., and McLeod, M. G. (1971). Chromosome numbers in some cacti of western North America. Brittonia, 23(2), 171–176.

[163] Pinkava, D. J., Parfitt, B. D., Baker, M. A., and Worthington, R. D. (1992). Chromosome numbers in some cacti of western North America-VI, with nomenclatural changes. Madroño, 39(2), 98–113.

[164] Pyke, G. H. (1981). Optimal nectar production in a hummingbird-pollinated plant. Theoretical Population Biology, 20, 326-343.

[165] Queller, D. (1987). Sexual selection in flowering plants. Sexual selection: testing the alternative. In J. W. Bradbury, and M. B. Andersson (Eds.), Sexual selection: testing the alternative (pp. 165-179). Chichester: Wiley.

[166] Rae, J. M., and Vamosi, J. C. (2013). Ultraviolet reflectance mediates pollinator visitation in *Mimulus guttatus*. Plant Species Biology, 28, 177–184.

[167] Raj, A., Stephens, M., and Pritchard, J. K. (2014). fastSTRUCTURE: Variational inference of population structure in large SNP data sets. Genetics, 197, 573–589.

[168] Ramadoss, N., Orduño-Baez, A., Portillo, C., Steele, S., Rebman, J., and Flores-Rentería, L. (2022). Unraveling the development behind unisexual flowers in *Cylindropuntia wolfii* (Cactaceae). BMC Plant Biology, 22(1), 94.

[169] Ramadoss, N., Steele, S., and Flores-Rentería, L. (2023). Influence of sexual dimorphism and dichromatism on reproductive success in a rare native cactus. Oecologia, 203(3), 383-394.

[170] Rao, S., and Ostroverkhova, O. (2015). Visual outdoor response of multiple wild bee species: highly selective stimulation of a single photoreceptor type by sunlight-induced fluorescence. J Comp Physiol A, 201, 705–716.

[171] Rasmussen, K. K., and Kollmann, J. (2008). Low genetic diversity in small peripheral populations of a rare European tree (*Sorbus torminalis*) dominated by clonal reproduction. Conservation Genetics, 9, 1533–1539.

[172] Rebman, J. P., and Pinkava, D. (2001). Opuntia cacti of North America- an overview. Florida Entomologist, 84, 474–483.

[173] Rebman, J. P. (1998). A new cholla (Cactaceae) from Baja California. Haseltonia, 6, 17–21.

[174] Rebman, J. P. (2003). The genus *Echinocereus* in Lower California, Mexico: taxonomy, rarity and reproductive biology. Cactus Succulent Journal, 75, 194–196.

[175] Reed, D. H., and Frankham, R. (2003). Correlation between fitness and genetic diversity. Conservation Biology, 17(1), 230-237.

[176] Reincke, D. C., and Bloom, W. L. (1979). Pollen dispersal in natural populations: a method for tracking individual grains. Systematic Botany, 4, 223–229.

[177] Renner, S. S. (2006). Rewardless flowers in the angiosperms and the role of insect cognition in their evolution. In Plant-pollinator interactions: from specialization to generalization (pp. 123-144).

[178] Renner, S. S. (2014). The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online database. American Journal of Botany, 101, 1588–1596.

[179] Reverté, S., Retana, J., Gómez, J. M., and Bosch, J. (2016). Pollinators show flower

colour preferences but flowers with similar colours do not attract similar pollinators. Annals of Botany, 118(2), 249-257.

[180] Reynes, L., Thibaut, T., Mauger, S., Blanfuné, A., Holon, F., Cruaud, C., ... and Aurelle, D. (2021). Genomic signatures of clonality in the deep water kelp *Laminaria rodriguezii*. Molecular Ecology, 30(8), 1806-1822.

[181] Richards, A. J. (1997). Plant breeding systems (2nd ed.). Chapman and Hall.

[182] Rojas-Araya, D., Alto, B. W., Burkett-Cadena, N. D., and Cummings, D. A. (2020). Impacts of fluorescent powders on survival of different age cohorts, blood-feeding success, and tethered flight speed of *Aedes aegypti* (Diptera: Culicidae) females. Acta Tropica, 207, 105491.

[183] Ruiz, S. E. (1999). Plant Microtechnique and Microscopy. Oxford University Press.

[184] Sánchez, D., and Vázquez-Santana, S. (2018). Embryology of Mammillaria dioica (Cactaceae) reveals a new male sterility phenotype. Flora, 241, 16-26.

[185] Sansaloni, C., Franco, J., Santos, B., et al. (2020). Diversity analysis of 80,000 wheat accessions reveals consequences and opportunities of selection footprints. Nature Communications, 11, 4572.

[186] Schaal, B. A. (1975). Population structure and local differentiation in *Liatris cylindracea*. American Naturalist, 109, 511-528.

[187] Schlising, R. A., and Turpin, R. A. (1971). Hummingbird dispersal of *Delphinium cardinale* pollen treated with radioactive iodine. American Journal of Botany, 58,

401-406.

[188] Schmitt, J. (1980). Pollinator foraging behavior and gene dispersal in *Senecio* (Compositae). Evolution, 34, 934-943.

[189] Segura, S., Scheinvar, L., Olalde, G., Leblanc, O., Filardo, S., Muratalla, A., ... and Flores, C. (2007). Genome sizes and ploidy levels in Mexican cactus pear species *Opuntia* (Tourn.) Mill. series Streptacanthae Britton et Rose, Leucotrichae DC. Genetic Resources and Crop Evolution, 54(5), 1033-1041.

[190] Shams, F., Dyer, F., Thompson, R., Duncan, R. P., Thiem, J. D., Kilian, A., and Ezaz, T. (2019). Application of DArT seq derived SNP tags for comparative genome analysis in fishes; An alternative pipeline using sequence data from a non-traditional model species, *Macquaria ambigua*. PLoS One, 14(12), e0226365.

[191] Shykoff, J. A., and Bucheli, E. (1995). Pollinator visitation patterns, floral rewards and the probability of transmission of *Microbotryum violaceum*, a venereal disease of plants. Journal of Ecology, 83(2), 189-198.

[192] Silvertown, J. (2008). The evolutionary maintenance of sexual reproduction: evidence from the ecological distribution of asexual reproduction in clonal plants. International Journal of Plant Sciences, 169(1), 157-168.

[193] Sinclair, J. P., Emlen, J., and Freeman, D. C. (2012). Biased sex ratios in plants: theory and trends. The Botanical Review, 78, 63-86.

[194] Sletvold, N., Trunschke, J., Smit, M., Verbeek, J., and Ågren, J. (2016). Strong

pollinator-mediated selection for increased flower brightness and contrast in a deceptive orchid. Evolution, 70(3), 716-724.

[195] Sobral, R., Silva, H. G., Morais-Cecílio, L., and Costa, M. M. (2016). The quest for molecular regulation underlying unisexual flower development. Frontiers in Plant Science, 7, 160.

[196] Sohail, Q., Manickavelu, A., and Ban, T. (2015). Genetic Diversity Analysis of Afghan Wheat Landraces (*Triticum aestivum*) Using DArT Markers. Genetic Resources and Crop Evolution, 62, 1147–1157.

[197] Spielman, D., Brook, B. W., and Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. Proceedings of the National Academy of Sciences, 101(42), 15261-15264.

[198] Spigler, R. B., and Ashman, T.-L. (2012). Gynodioecy to dioecy: are we there yet? Annals of Botany, 109(3), 531-543.

[199] Stanton, M. L. (1994). Male-male competition during pollination in plant populations. American Naturalist, 144(Supplement), S40-S68.

[200] Stearns, S. C. (1992). The Evolution of Life Histories. Oxford University Press, Oxford.

[201] Stebbins, G. L. (1950). Chapter VIII. Polyploidy I. Occurrence and Nature of Polyploid Types. In Variation and evolution in plants. Columbia University Press, pp. 298–341.

[202] Steiner, K. E. (1985). Functional dioecism in the Malpighiaceae: the breeding system of *Spachea membranacea* Cuatr. American journal of botany, 72(10), 1537-1543.

[203] Stephenson, A. G. (1981). Flower and fruit abortion: proximate causes and ultimate functions. Annual review of ecology and systematics, 12, 253-279.

[204] Stephenson, A. G., and Bertin, R. I. (1983). Male competition, female choice, and sexual selection in plants. In Pollination Biology (pp. 109-149). Academic Press.

[205] Stockhouse, R. E. (1976). A new method for studying pollen dispersal using micronized fluorescent dusts. The American Midland Naturalist, 96, 241-245.

[206] Stoeckel, S., Grange, J., Fernández-Manjarres, J. F., Bilger, I., Frascaria-Lacoste, N., and Mariette, S. (2006). Heterozygote excess in a self-incompatible and partially clonal forest tree species—*Prunus avium* L. Molecular Ecology, 15(8), 2109-2118.

[207] Strittmatter, L. I., Negrón-Ortiz, V., and Hickey, R. J. (2006). Comparative microsporangium development in male-fertile and male sterile flowers of *Consolea* (Cactaceae): when and how does pollen abortion occur? Grana, 45, 81-100.

[208] Strittmatter, L. I., Negrón-Ortiz, V., and Hickey, R. J. (2002). Subdioecy in *Consolea spinosissima* (Cactaceae): breeding system and embryological studies. American Journal of Botany, 89, 1373–1387.

[209] Strittmatter, L. I., Hickey, R. J., and Negron-Ortiz, V. (2008). Heterochrony and its role in sex determination of cryptically dioecious *Consolea* (Cactaceae) staminate flowers. Botanical Journal of the Linnean Society, 156(2), 305-326.

[210] Teukeng, F. F. D., Blin, M., Bech, N., Gomez, M. R., Zein-Eddine, R., Simo, A. M. K., ... and Boissier, J. (2022). Hybridization increases genetic diversity in *Schistosoma haematobium* populations infecting humans in Cameroon. Infectious Diseases of Poverty, 11(1), 37.

[211] Thomson, J. D., and Plowright, R. C. (1980). Pollen carry-over, nectar rewards, and pollinator behavior, with special reference to *Diervilla lonicera*. Oecologia, 46, 68-74.

[212] Thomson, J. D., and Brunet, J. (1990). Hypotheses for the evolution of dioecy in seed plants. Trends in Ecology and Evolution, 5(1), 11-16.

[213] Townsend, P. A., and Levey, D. J. (2010). An experimental test of whether habitat corridors affect pollen transfer. Ecology, 86(2), 466–475.

[214] Tree of Sex Consortium. (2014). Tree of Sex: a database of sexual systems. Scientific data, 1, 140015.

[215] Tsuji, K., and Fukami, T. (2018). Community-wide consequences of sexual dimorphism: evidence from nectar microbes in dioecious plants. Ecology, 99(11), 2476-2484.

[216] Tsukui, T., and Sugawara, T. (1992). Dioecy in *Honckenya peploides var. major* (Caryophyllaceae). The botanical magazine= Shokubutsu-gaku-zasshi, 105(4), 615-624.

[217] Vamosi, J. C., and Vamosi, S. M. (2005). Present day risk of extinction may exacerbate the lower species richness of dioecious clades. Diversity and Distributions, 11(1), 25-32.

[218] Van Geert, A., Van Rossum, F., and Triest, L. (2010). Do linear landscape elements in farmland act as biological corridors for pollen dispersal? Journal of Ecology, 98(1), 178–187.

[219] Vanbergen, A. J., Insect Pollinators Initiative. (2013). Threats to an ecosystem service: Pressures on pollinators. Frontiers in Ecology and the Environment, 11, 251–259.

[220] Verhoeven, C., Ren, Z-X., and Lunau, K. (2018). False-colour photography: a novel digital approach to visualize the bee view of flowers. Journal of Pollination Ecology, 23, 102–118.

[221] Vrdoljak, S. M., and Samways, M. J. (2012). Optimising coloured pan traps to survey flower visiting insects. Journal of Insect Conservation, 16, 345-354.

[222] Waelti, M. O., Page, P. A., Widmer, A., and Schiestl, F. P. (2009). How to be an attractive male: floral dimorphism and attractiveness to pollinators in a dioecious plant. BMC Evolutionary Biology, 9(1), 1-7.

[223] Wang, S., Jin, C. F., Li, Z. Q., Li, Y., and Xie, Q. (2017). Breeding system and parental effect on fruit characters of *Idesia polycarpa* (Flacourtiaceae), a promising plant for biodiesel, in northwest China. Pakistan Journal of Botany, 49(5), 1885-1890.

[224] Waser, N. M., and Price, M. V. (1982). A comparison of pollen and fluorescent dye carry-over by natural pollinators of *Ipomopsis aggregata* (Polemoniaceae). Ecology, 1168-1172.

[225] Weiss, M. R. (1991). Floral color changes as cues for pollinators. Nature, 354(1991), 227-229.

[226] Welch, D. B., and Meselson, M. (2000). Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. Science, 288(5469), 1211-1215.

[227] Welsford, M. R., and Johnson, S. D. (2012). Solitary and social bees as pollinators of *Wahlenbergia* (Campanulaceae): single-visit effectiveness, overnight sheltering and responses to flower color. Arthropod-Plant Interactions, 6, 1–14.

[228] Wenzl, P., Carling, J., Kudrna, D., Jaccoud, D., Huttner, E., Kleinhofs, A., and Kilian, A. (2004). Diversity Arrays Technology (DArT) for Whole-Genome Profiling of Barley. Proceedings of the National Academy of Sciences USA, 101.

[229] Wessinger, C. A. (2021). From pollen dispersal to plant diversification: genetic consequences of pollination mode. New Phytologist, 229(6), 3125-3132.

[230] Westergaard, M. (1958). The mechanism of sex determination in dioecious flowering plants. Advances in Genetics, 9, 217–81.

[231] Wetzstein, B. (2022). Genetic Analysis of the Rare, Colorado Endemic Rocky Mountain Monkeyflower, *Mimulus gemmiparus*. Master's Thesis. University of Northern Colorado, USA.

[232] Whitney, H. M., Milne, G., Rands, S. A., Vignolini, S., Martin, C., and Glover, B. J. (2013). The influence of pigmentation patterning on bumblebee foraging from flowers of *Antirrhinum majus*. Naturwissenschaften, 100, 249-256.

[233] Williams, J. H., and Mazer, S. J. (2016). Pollen—Tiny and ephemeral but not forgotten: New ideas on their ecology and evolution. American Journal of Botany, 103, 365–374.

[234] Wilson, J. S., Griswold, T., and Messinger, O. J. (2008). Sampling bee communities (Hymenoptera: Apiformes) in a desert landscape: are pan traps sufficient?. Journal of the Kansas Entomological Society, 81(3), 288-300.

[235] Wu, H. M., and Cheung, A. Y. (2000). Programmed cell death in plant reproduction. Programmed Cell Death in Higher Plants, 23–37.

[236] Xia, Z., He, Y., Korpelainen, H., Niinemets, Ü., and Li, C. (2022). Sex-specific interactions shape root phenolics and rhizosphere microbial communities in *Populus cathayana*. Forest Ecology and Management, 504, 119857.

[237] Xu, J., Ding, Z., Vizcay-Barrena, G., Shi, J., Liang, W., Yuan, Z., ... Werck-Reichhart, D. (2014). Aborted microspores: acts as a master regulator of pollen wall formation in *Arabidopsis*. Plant Cell, 26(4), 1544–1556.

[238] Yampolsky, C., and Yampolsky, H. (1922). Distribution of the sex forms in the phanerogamic flora. Bibliography of Genetics, 3, 1-62.

[239] Zhang, D., Lv, X., Wang, Y., Xun, Z., Liu, Z., Li, F., and Lu, H. (2014). The Cysteine Protease CEP1, a key executor involved in tapetal programmed cell death, regulates pollen development in *Arabidopsis*. Plant Cell, 26(7), 2939–2961.

[240] Zhou, Y., Li, L., and Song, Z. (2019). Plasticity in sexual dimorphism enhances

adaptation of dioecious *Vallisneria natans* plants to water depth change. Frontiers in Plant Science, 10, 826.

[241] Zscheischler, J., Martius, O., Westra, S., Bevacqua, E., Raymond, C., Horton, R. M., and Vignotto, E. (2020). A typology of compound weather and climate events. Nature reviews earth and environment, 1(7), 333-347.